Articles

Unique High-Affinity Synthetic μ-Opioid Receptor Agonists with Central- and Systemic-Mediated Analgesia

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Unique opioid mimetic substances containing identical N-terminal aromatic residues separated by an unbranched alkyl chain containing two to eight methylene groups were developed. Regardless of the length of interposing alkyl chain, the bis-Tyr and bis-Phe compounds were inactive; however, replacement by a single Dmt (2′,6′-dimethyl-L-tyrosine) residue enhanced activity by orders of magnitude. Moreover, the bis-Dmt compounds were another 10-fold more potent with an optimum intra-aromatic ring distance of about four to six methylene units. 1,4-Bis(Dmt-NH)butane (**7**) had high μ-opioid receptor affinity (K_i = 0.041 nM) and functional μ-opioid agonist bioactivity (IC_{50} = 5.3 nM) with in vivo central (intracerebroventricular) and systemic (subcutaneous) analgesia in mice (1.5- to 2.5-fold greater than and 10−12% relative to morphine, respectively); these activities were reversed by naloxone to the same degree. It appears that the bis-Dmt compounds indiscriminately act as both message and address domains.

Introduction[1]

Opioid receptors play a role in relaying information for a variety of physiological events,[2] the effects of which are transmitted by a variety of peptides for δ-, μ-, and κ-opioid receptors.[3] Despite the structural diversity of opioid peptides, an N-terminal Tyr is a common structural element except in the nociceptin/orphanin ligand for ORL receptors, which has an N-terminal Phe residue.[4] Although replacement of Tyr by 2′,6′-dimethyl-L-tyrosine (Dmt) initially yielded a weakly active μ-receptor peptide,[5] incorporation of Dmt subsequently and dramatically altered the activity of numerous unrelated opioids by elevating affinities, affecting receptor selectivity, and changing the spectrum of their bioactivity.[6−11] The utilization of the Dmt-Tic pharmacophore[7] in lieu of the TIP(P) series of pseudopeptides[12] produced opioid ligand families consisting of highly potent δ-receptor antagonists, δ-receptor agonists, and bifunctional analogues exhibiting δ-receptor antagonism or δ-receptor agonism with μ-receptor agonism depending on the C-terminal constituents.[13−15]

The concept of message and address domains,[16] as applied to opioid peptide agonists[17] and non-peptide opiates,[18−20] stipulated that structurally distinct regions of the molecule exert different functions. In other words, the "message domain" in opioid peptides was considered to include the N-terminal Tyr with its free amine and hydroxyl groups, and a spacer consisting of one or two amino acids (D-Ala, D-Met, Pro, or Gly-Gly). On the other hand, the "address domain" would contain the subsequent C-terminal residues beginning with the second aromatic residue (usually Phe or Trp[3] in the case of endomorphin-1[21] and hemorphin,[22] respectively) that trigger the biological response. Our data, however, demonstrate that symmetric opioid mimetic substances, which contain two identical dimethylated tyrosylamides separated by a simple unbranched alkyl chain (Table 1), are able to serve as *both* the message and address domains when binding within the μ-opioid receptor to yield high-affinity compounds to produce morphine-like analgesia that was naloxone-reversible. In this report, we present opioid receptor affinity values (K_i), classical functional bioassays in vitro, biological activity in vivo with mice, and ^{1}H NMR analysis results that permit us to present a new interpretation or variation on the message−address domain hypothesis concerning opioid ligand−receptor interaction using these unique bis-Dmt substances.

Rationale

The design of a simple opioid mimetic containing minimal functional groups for receptor recognition, namely Dmt,[5−7] enabled the investigation of the role of

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Table 1. Opioid Receptor Binding and Functional Biological Activity of Opioid Mimetics Containing an Alkyl Spacer[a]

		receptor binding, K_i (nM)			functional bioactivity		
compd	peptide	δ	μ	δ/μ	GPI IC_{50} (nM)	MVD IC_{50} (μM)	$pA_2(\delta)$
1	Tyr-NH-$(CH_2)_2$-NH-Tyr	8290 ± 433 (3)	648 ± 95 (5)	13	nd	nd	
2	Dmt-NH-$(CH_2)_2$-NH-Dmt	115.7 ± 10.6 (6)	1.43 ± 0.01 (3)	81	2844 ± 517	>10	5.5
3	Tyr-NH-$(CH_2)_4$-NH-Tyr	6500 ± 278 (3)	309 ± 104 (5)	21	nd	nd	
4	Phe-NH-$(CH_2)_4$-NH-Phe	46190 ± 458 (3)	1530 ± 122 (3)	30	nd	nd	
5	Dmt-NH-$(CH_2)_4$-NH-Phe	62.0 ± 5.7 (3)	0.52 ± 0.089 (4)	119	181 ± 36	>10	5.5
6	Dmt-NH-$(CH_2)_4$-NH-Tyr	133 ± 18 (3)	0.38 ± 0.02 (3)	349	255 ± 11	>10	5.3
7	Dmt-NH-$(CH_2)_4$-NH-Dmt	53.4 ± 14.8 (5)	0.041 ± 0.003 (4)	1302	5.33 ± 0.65	>10	5.8
8	Tyr-NH-$(CH_2)_6$-NH-Tyr	21880 ± 4020 (3)	410 ± 85 (5)	53	nd	nd	
9	Dmt-NH-$(CH_2)_6$-NH-Dmt	46.1 ± 8.8 (5)	0.053 ± 0.01 (6)	870	3.08 ± 0.53	>10	6.1
10	Tyr-NH-$(CH_2)_8$-NH-Tyr	6150 ± 525 (3)	399 ± 41 (7)	34	nd	nd	
11	Dmt-NH-$(CH_2)_8$-NH-Dmt	14.8 ± 3.0 (7)	0.19 ± 0.024 (4)	78	53.7 ± 7	>10	6.4

[a] Opioid receptor binding data of the bis-amide compounds are listed as the mean ± SE and determined using rat brain P_2 synaptosomal preparations with [^{3}H]DPDPE for δ-opioid receptors and [^{3}H]DAGO for μ-opioid receptors as detailed elsewhere.[7] The number of independent repetitions is noted in parentheses (n). Receptor selectivity is the ratio (δ/μ) of the affinity constants (K_i), determined according to Cheng and Prusoff.[39] μ-Opioid agonism used GPI (guinea pig ileum), while MVD (mouse vas deferens) defines δ bioactivity; $n = 5-6$ for bioassays. The pA_2 is the negative log of the molar concentration required to double the agonist concentration to achieve the original response and defines antagonism. nd = not determined.

alkane linkers on selectivity for the μ-opioid receptor subtype. These symmetrical compounds suggest interaction at both of the hypothesized message and address regions in the receptor and supply prototypes for the design of opioid mimetics with novel and distinct linkers.[15,23]

Chemistry

All the opioid mimetic compounds in this study were synthesized by standard solution methodology for peptide synthesis using Fmoc- and Boc-amino protection groups and using PyBop as the coupling reagent.[23,24] Symmetrical ligands (**1**−**4**, **7**−**11**) were prepared by coupling Boc-amino acids with a diamine followed by TFA (1.0 mL, 13 mmol) containing anisole (0.10 mL, 0.90 mmol) for 1 h at room temperature. The compounds were then precipitated with diethyl ether, filtered, and purified by HPLC (vide infra). Asymmetric compounds (**5**, **6**) were prepared using mono-Boc-protected diamine[25] and Fmoc- and Boc-amino acids. The Fmoc group was removed by 20% piperidine, and the crude compounds were precipitated with ether and purified using RP-HPLC on a COSMOSIL C18 column (4.6 mm × 250 mm) or a YMC R & D R-ODS-5 Å column (4.6 mm × 250 mm) using a Waters model 600E for analytical and preparative HPLC. The compounds were eluted from the columns using linear gradients starting from 10% acetonitrile in 0.05% TFA with a 1% increase in acetonitrile concentration per minute at a flow rate of 1 mL/min; detection was at 220 nm. Retention times were recorded as either t_R(C) or t_R(Y), respectively. Purity was greater than 98%. ^{1}H, ^{13}C, and 2D NMR spectra were measured using a 30 mg sample of each compound dissolved in 0.5 mL of DMSO-d_6 and measured on a Bruker ARX-500 spectrometer at 25 °C.

Results and Discussion

Opioid Receptor Affinity. The opioid mimetics containing bis-Tyr (**1**, **3**, **8**, **10**) and bis-Phe (**4**) interacted poorly with δ- and μ-opioid receptors (Table 1). Substitution by a single Dmt residue (**5**, **6**) enhanced μ-opioid receptor affinities several-hundred-fold ($K_i = 0.38-0.52$ nM), supporting observations with a large variety of opioid substances containing N-terminal Dmt.[6−10] The bis-Dmt-containing ligands **7** and **9** increased μ-opioid receptor affinity another 10-fold ($K_i = 0.041-0.053$ nM) with high receptor selectivity (δ/μ = 1302 and 870, respectively). Thus, the optimal distance between the Dmt residues for maximum μ-opioid receptor binding appeared to be butyl (**7**) ≅ hexyl (**9**) > octyl (**11**) ≫ ethyl (**2**), i.e., an unbranched aliphatic chain < ethyl < octyl in length. As suggested elsewhere,[7−10] the key residue in this transformation toward increased opioid receptor interaction was due solely to the presence of Dmt.

Function Bioactivity in Vitro. The high μ-receptor agonism of **7** and **9** (IC_{50} = 5.3 and 3.1 nM, respectively) and undetectable δ agonism coupled with their low δ-receptor antagonism ($pA_2 = 6.4-5.3$) and remarkable opioid receptor affinities (Table 1) support the following conclusions: (i) The compounds predominately act at μ-opioid receptor sites. (ii) The bioactivities of **2**, **7**, **9**, and **11** suggest that the optimum distance for μ-receptor agonism between the Dmt amides was greater than two but less than eight methylene groups. The effect of a spacer between aromatic centers was observed with other opioids containing N-terminal Dmt[14,15] or Tyr.[26] (iii) The increase in both μ- and δ-opioid receptor affinities with Dmt suggests a degree of structural similarity in their binding sites. The asymmetric analogues (**5**, **6**), particularly H-Dmt-NH-$(CH_2)_4$-NH-Phe-H (**5**), could be considered an analogue of known opioid peptides containing a single D-amino acid,[21,27,28] a dipeptide spacer,[29,30] or a heteroaromatic residue (e.g., a Tic residue[12]) between aromatic amino acids.[26] (iv) The weak opioid receptor binding affinities of the bis-Tyr and bis-Phe cognates (**1**, **3**, **4**, **8**, **10**) demonstrate an inability of these residues to promote ligand interaction. Whereas the methyl groups at the 2′ and 6′ positions of tyrosine might affect the χ_2 angle of Dmt, comparative modeling data suggest that they might reduce rotational freedom of the aromatic ring.[31−34] We hypothesize that these methyl groups stabilize the interaction with the pharmacophoric groups (hydroxyl, N-terminal amine, and the aromatic ring through hydrophobic forces, ring stacking, or $\pi-\pi$ interactions) within the receptor that could enhance its bioactivity. While this mechanism is not yet fully understood, our data as well as those in the literature[9,11,26,30,31,33,34] point in this direction.

In Vivo Biological Activity. In mice, **7** rapidly produced centrally mediated (icv) analgesia induced by

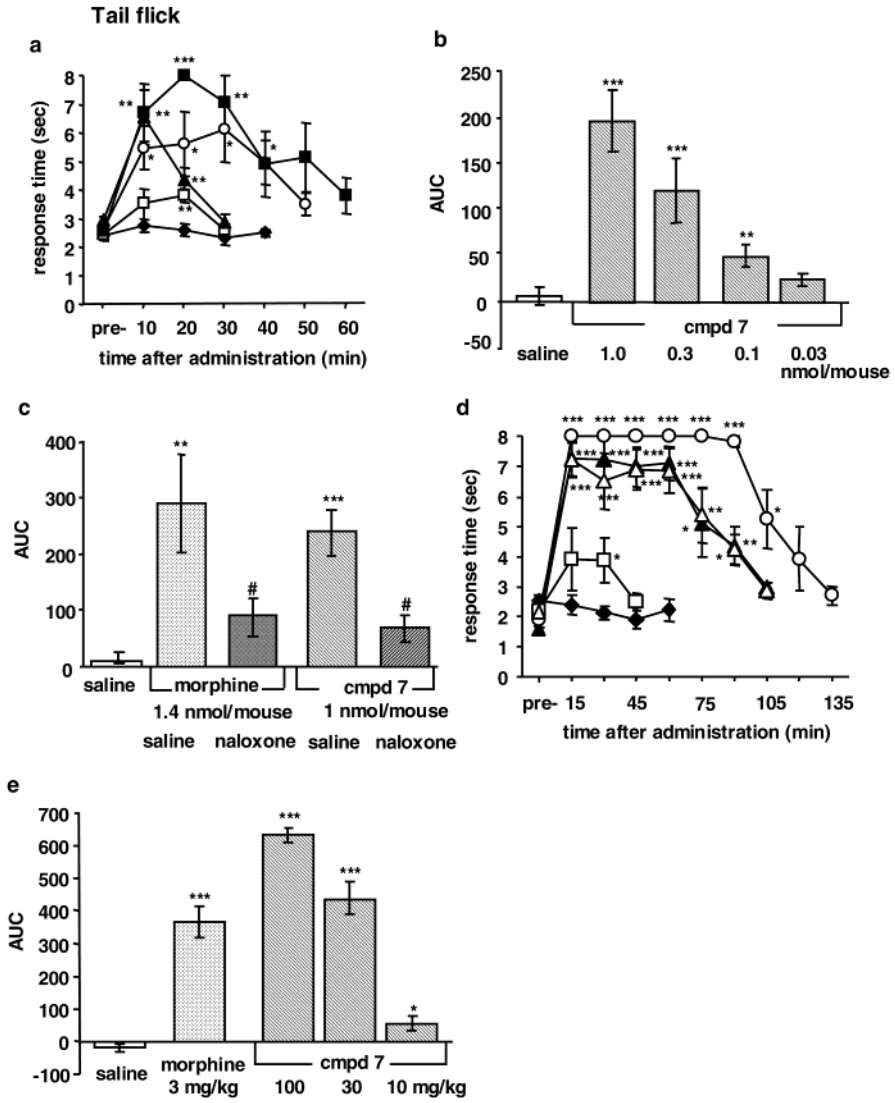


Figure 1. Analgesia of H-Dmt-NH-$(CH_2)_4$-NH-Dmt-H (**7**) in mice. Dose-dependent appearance of analgesia by the tail-flick test is expressed as a function of time (a, d) and area under the curve (AUC) (b, c, e). Intracerebroventricular (icv) injections (a−c): saline (◆) or **7** at 0.03 nmol (□), 0.1 nmol (▲), 0.3 nmol (○), or 1 nmol (■) in 4 μL of saline per mouse. (c) Naloxone (2 mg/kg) was injected sc 30 min before **7**. (d, e) Subcutaneous (sc) injections: saline (◆) or **7** at 10 mg/kg (□), 30 mg/kg (▲), 100 mg/kg (○) in saline, or 3 mg/kg morphine (△). Data are the mean ± SE with $n = 5-7$ animals per time point. Statistical significance used Student's *t*-test. Asterisks denote significant differences between mice treated with saline and **7** (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$). The symbol # indicates a significant difference ($p < 0.05$) between the effect of **7** and treatment with naloxone or that between morphine and the naloxone-treated animals.

a spinal nociceptive mechanism (tail-flick test) that was 1.5- to 2.2-fold greater than morphine and was naloxone-reversible; naloxone inhibited analgesia by **7** and morphine to the same degree (68% and 71%, respectively) (Figure 1). The supraspinal nociceptive pathway (hot plate test) revealed equivalent analgesia to morphine, and both substances were completely blocked by naloxone (Figure 2). Subcutaneous (sc) injection of **7** produced an analgesia in a dose-dependent mechanism that was 10−12% as potent as morphine (Figure 1), verifying that **7** indeed crossed the blood−brain barrier. While the receptor binding affinity and functional bioactivity are essentially similar for **7** and **9** (Table 1), the icv and sc administration of **7** and **9** in rats indicated, however, that **7** was about twice as potent as **9** in both experimental paradigms and statistically significant above the saline controls ($p < 0.05$) (data not shown).

^{1}H NMR Analysis. Solution structures of **5** and **6** determined by 2D ^{1}H and ^{13}C NMR spectroscopy (the molecular symmetry of **7** precluded its use for NMR analysis) revealed an absence of cross-peaks between N- and C-terminal protons indicative of an extended, open structure (Figure 3). The strongest cross-peaks detected occurred between the protons of the 2′ and 6′ methyl groups and those at 3′ and 5′ positions of the tyramine ring of Dmt. The nuclear Overhauser effect (NOE) cross-peak intensities became progressively weaker thereafter (Figure 3).

Molecular Modeling. The lowest energy structure of **7** was derived using systematic conformational searching and energy minimization based on the *J* coupling values and NOE cross-peaks of **6** (Figure 4). That structure was among 8323 stable conformations that fit the criteria defined by the NMR data reflecting the flexible nature of these compounds. The length of the alkyl chain maintains the distance and positioning of the two aromatic centers, the putative message and address regions; the flexible nature of the chain facili-

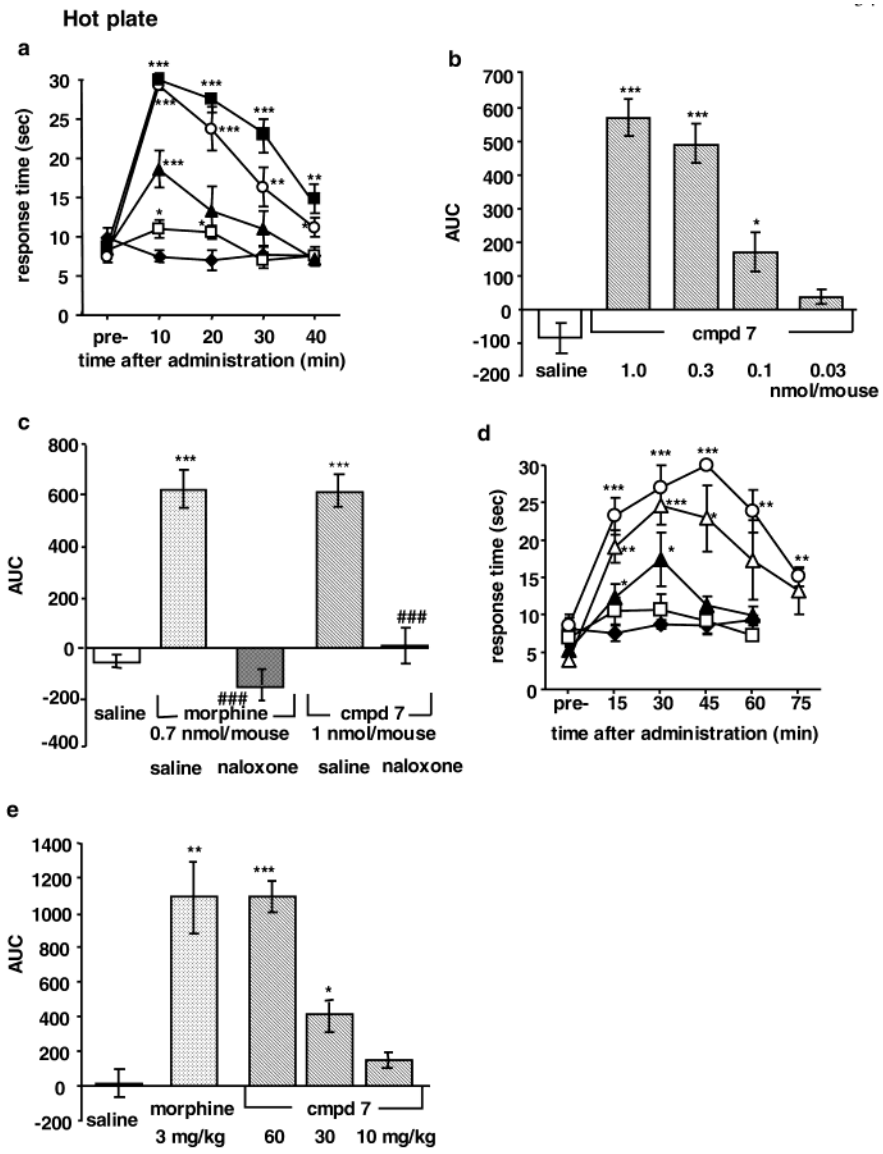


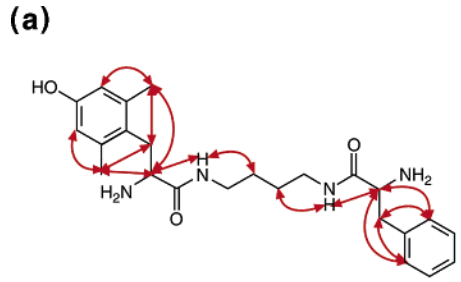
Figure 2. Supraspinal analgesia of H-Dmt-NH-$(CH_2)_4$-NH-Dmt-H (**7**) using the hot plate test in mice. See the caption to Figure 1 for details. Mice were injected intracerebroventricularly (icv), and the response was measured identically without (a, b) and with naloxone (c) and after subcutaneous (sc) injections (d, e). Statistical analyses are given in the caption to Figure 1.

tates the ability of the compound to conform to the topography of the receptor cleft. In general, the extended conformations of **7** were similar to extended structures reported for δ- and μ-opioid tetra-, penta-,[32] and heptapeptide agonists[32,33] and differed substantially from the X-ray derived structures of three Dmt-Tic analogues.[34]

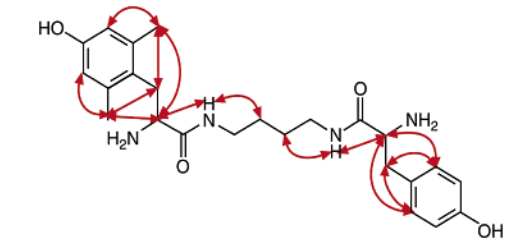
Conclusions

Dmt enhanced the receptor affinities of opioid ligands for both δ-and μ-opioid receptors.[8,13−15,35] The agonist activity measured in vitro and analgesic effects in vivo of the bis-Dmt compounds (**7**, **9**) appear to be mediated through μ-opioid receptors. Furthermore, the symmetry of these compounds verified that these molecules contain identical "message" and "address" domains. Our results suggest the following: the effectiveness of Dmt may involve not only hydrophobic forces that interact within a lipid milieu but also its ability to align and stabilize the tyrosyl ring, permitting more effective H bonding to occur between the required hydroxyl group as well as the N-terminal amine of the ligand and functional groups of the receptor. Perhaps a single Dmt residue interacts at one site of the receptor through combined hydrophobic/aromatic ring associations as well as hydrogen bonding, while Phe (**5**), Tyr (**6**), or the second Dmt (**7**, **9**) associates with another similar region of the opioid receptor.

The small dimensions of the bis-Dmt compounds appear to fit the μ-opioid receptor binding site (pocket) with ease and precludes binding between two adjacent receptors in the cell membrane, as suggested with the larger dimeric enkephalin[36] and dimeric dermorphin analogues.[37] Furthermore, the ability of bis-(Dmt-NH)-alkyl opioid mimetic compounds to transit the blood−brain barrier is an important characteristic of this class of compounds. In many ways, the simple design of our final products resembles the minimalist themes presented in music by Philip Glass, the early artwork by Frank Stella, or a single note played to perfection on the Japanese bamboo flute. Combination of Dmt and an alkyl chain might also enhance proteolytic stability



Dmt Me	Dmt Ar-H	S
	Dmt βCH$_2$	S
	Dmt αCH	M
H-Dmt-NH-CH$_2$-	Dmt αCH	M
	H-Dmt-NH-CH$_2$-CH$_2$-	W
Phe Ar-H (2,6)	Phe βCH$_2$	M
	Phe αCH	M
-CH$_2$-NH-Phe-H	Phe αCH	M
	-CH$_2$-CH$_2$-NH-Phe-H	W



Dmt Me	Dmt Ar-H	S
	Dmt βCH$_2$	M
	Dmt αCH	M
H-Dmt-NH-CH$_2$-	Dmt αCH	M
	H-Dmt-NH-CH$_2$-CH$_2$-	W
Tyr Ar-H (2,6)	Tyr βCH$_2$	M
	Tyr αCH	W
-CH$_2$-NH-Tyr-H	Tyr αCH	M
	-CH$_2$-CH$_2$-NH-Tyr-H	W

Figure 3. (a) NOE cross-peaks of H-Dmt-NH-(CH$_2$)$_4$-NH-Phe-H (**5**) measured in DMSO using ^{1}H NMR spectrometry. The cross-peaks are indicated by the arrows, and the cross-peak intensities are summarized as being strong (S) (<2.25 Å), medium (M) (2.25−3.0 Å), or weak (W) (3.0−4.0 Å). (b) ^{1}H NMR analysis of H-Dmt-NH-(CH$_2$)$_4$-NH-Tyr-H (**6**) in DMSO. ^{1}H, ^{13}C, and 2D NMR spectra were measured using a 30 mg sample of each compound dissolved in 0.5 mL of DMSO-d_6 and measured on a Bruker ARX-500 spectrometer at 25 °C.

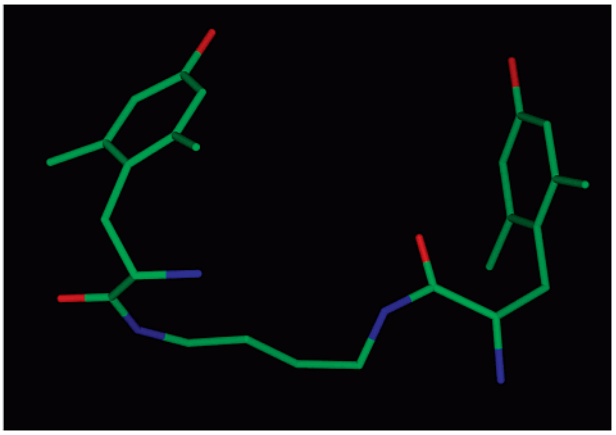


Figure 4. Low-energy conformation of H-Dmt-NH-(CH$_2$)$_4$-NH-Dmt-H (**7**). Molecular modeling was performed on a Silicon Graphics Octane2 computer system using software from Accelrys described previously.[34] Extensive conformational searching was performed utilizing conformational constraints based on ^{1}H NMR data for H-Dmt-NH-(CH$_2$)$_4$-NH-Tyr-H (**6**). Minimizations were performed in the AMBER force field, stopping with a root-mean-square gradient of 0.001 kcal/(mol·Å^2). A total of 8232 conformations were analyzed with relative energies ranging from 59 to 443 kcal/mol. The lowest energy conformer is displayed with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue. Hydrogen atoms are not displayed.

and increase the therapeutic potential of these derivatives for human or veterinary applications.

Experimental Section

Materials. The amino acid Dmt was synthesized according to the procedure of Dygos et al. in this laboratory.[38] [^{3}H]-DPDPE (32.0 Ci/mmol) was purchased from NEN-DuPont, Billerica, MA, and [^{3}H]DAGO (58.0 Ci/mmol) was obtained from Amersham, Arlington Heights, IL, for use in δ- and μ-opioid receptor binding assays, respectively.

1,4-Bis(*N*α-Boc-Dmt-amino)butane. BOP reagent (700 mg, 1.6 mmol) was added to a solution of *N*^{α}- Boc-Dmt-OH (500 mg, 1.6 mmol) and 1,4-diaminobutane (60 mg, 0.68 mmol) in DMF (15 mL) containing Et$_3$N (0.66 mL, 4.7 mmol) at room temperature. The reaction mixture was stirred for 18 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na$_2$CO$_3$, and water, dried over Na$_2$SO$_4$, and evaporated down. Petroleum ether was added to the residue to obtain a precipitate, which was collected by filtration. The crude product in CHCl$_3$ (5 mL) was applied to a silica gel column (BW-127ZH, 3 cm × 16 cm), which was equilibrated, and eluted with CHCl$_3$ (2100 mL). After removal of the solvent of the effluent (1500−2100 mL), petroleum ether was added to the residue to yield crystals, which were collected by filtration: yield 300 mg (58.4%); mp 214−217 °C; R_{f1} = 0.46, R_{f2} = 0.32. Anal. Calcd for C$_{36}$H$_{54}$N$_4$O$_8$·0.5H$_2$O: C, 63.6; H, 8.15; N, 8.24. Found: C, 63.8; H, 8.16; N, 8.04.

1,4-Bis(Dmt-amino)butane·2HCl (7). A solution of 1,4-bis-(*N*^{α}- Boc -Dmt-amino)butane (200 mg, 0.30 mmol) in TFA (1.0 mL, 13 mmol) containing anisole (0.10 mL, 0.90 mmol) was stirred for 1 h at room temperature. Ether was added to a solution to form a precipitate, which was collected by filtration and dried in vacuo. The solution of the product in 1 M HCl (0.6 mL) was lyophilized to give a fluffy powder: yield 110 mg (68%); R_{f4} = 0.19, R_{f5} = 0.32, t_R(C) = 14.97 min. TOF-MS *m/z*: calcd for C$_{26}$H$_{38}$N$_4$O$_4$ (M + H)$^+$ 471.6; found 471.9. ^{1}H NMR (DMSO-d_6) δ: 7.78 (2H, t, *J* = 5.5 Hz, −CO−NH−), 6.43 (4H, s, aromatic 3,5-H), 3.69 (2H, dd, *J* = 11.1, 4.6 Hz, α-H), [3.02 (2H, dd, *J* = 13.7, 11.1 Hz) and 2.89 (2H, dd, *J* = 13.7, 4.6 Hz), β-H$_2$], [2.94 (2H, dd, *J* = 12.7, 6.2 Hz) and 2.89 (2H, m), NH−C*H*$_2$−], 2.18 (12H, s, 3,6-CH$_3$), 0.95−0.85 (4H, m, NH−CH$_2$−C*H*$_2$−). ^{13}C NMR (DMSO-d_6) δ: 167.9 (q, >C=O), 155.3 (q, aromatic C-4), 138.3 (q, aromatic C-2,6), 122.1 (q, aromatic C-1), 114.9 (t, aromatic C-3,5), 51.8 (t, C-α), 38.1

(s, NH-CH_2−) 30.4 (s, C-β), 25.1 (s, NH−CH_2-CH_2−), 19.7 (p, 2,6-CH_3).

1-Boc-amino-4-(N^{α}-Fmoc-Phe-amino)butane. 1-(Boc-amino)-4-aminobutane[3] (2.0 g, 10 mmol), N^{α}-Fmoc-Phe-OH (4.6 g, 12 mmol), PyBOP (6.24 g, 10 mmol), and HOBt (1.6 g, 12 mmol) were dissolved in DMF (50 mL) containing DIEA (4.2 mL, 24 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, AcOEt was added to the residue to obtain a precipitate, which was collected by filtration and recrystallized from EtOH: yield 3.0 g (45%); mp 165−167 °C; $R_{f1} = 0.66$. Anal. Calcd for $C_{33}H_{39}N_3O_5 \cdot 0.25H_2O$: C, 70.5; H, 7.08; N, 7.47. Found: C, 70.5; H, 7.06; N, 7.64.

1-N^{α}-Boc-Dmt-amino-4-(N^{α}-Fmoc-Phe-amino)butane. N^{α}-Boc-Dmt-OH (460 mg, 1.50 mmol), 1-amino-4-(N^{α}-Fmoc-Phe-amino)butane·TFA [prepared from 1-Boc-amino-4-(N^{α}-Fmoc-Phe-amino)butane (830 mg, 1.50 mmol), anisole (0.25 mL, 2.3 mmol), and TFA (2.2 mL, 30 mmol) as usual], PyBOP (930 mg, 1.8 mmol), and HOBt (270 mg, 1.8 mmol) were dissolved in DMF (10 mL) containing DIEA (0.50 mL, 3.80 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na_2CO_3, and water, dried over Na_2SO_4, and evaporated down. Petroleum ether was added to the residue to yield a precipitate. The crude product in $CHCl_3$ (5 mL) was applied to a silica gel column (YMC 70−230 mesh, 3 cm × 16 cm), which was equilibrated, and eluted with $CHCl_3$. After removal of the solvent of the effluent (210 mL), petroleum ether was added to the residue to give crystals, which were collected by filtration: yield 600 mg (63%); mp 201−203.5 °C; $R_{f1} = 0.52$. Anal. Calcd for $C_{44}H_{52}N_4O_7 \cdot 0.3H_2O$: C, 70.0; H, 7.03; N, 7.42. Found: C, 70.0; H, 7.24; N, 7.48.

1-Dmt-amino-4-Phe-aminobutane·2HCl (5). 1-N^{α}-Boc-Dmt-amino-4-(N^{α}-Fmoc-Phe-amino)butane (500 mg, 0.78 mmol) was treated with 20% piperidine in DMF (11.5 mL) for 2 h at room temperature. After removal of the solvent, ether was added to the residue to obtain a precipitate, which was collected by filtration ($R_{f1} = 0.16$, $R_{f3} = 0.70$). This product (300 mg, 0.57 mmol) was dissolved in TFA (1.0 mL, 13 mmol) containing anisole (0.10 mL, 0.90 mmol), and the solution was stirred for 1 h at room temperature. Ether was added to the solution to give a precipitate, which was collected by filtration. The crude product was purified with HPLC and lyophilized from 1 M HCl to obtain a fluffy amorphous powder: yield 220 mg (56%); $R_{f4} = 0.30$, $R_{f5} = 0.46$, t_R(C) = 17.07 min. TOF-MS *m*/*z*: calcd for $C_{24}H_{34}N_4O_3$ $(M + H)^+$ 427.6; found 427.5. ^{1}H NMR (free compound, DMSO-d_6) δ: 8.648 (1H, t, $J = 5.5$ Hz, NH of Phe amide), 7.997 (1H, t, $J = 5.5$ Hz, NH of Dmt amide), 7.32−7.23 (5H, m, aromatic H of Phe), 6.441 (2H, s, aromatic H of Dmt), 4.043 (1H, t, $J = 7.0$ Hz, α-H of Phe), 3.711 (1H, dd, $J = 9.3$, 6.4 Hz, α-H of Dmt), 3.071 (2H, d, $J = 7.0$ Hz, β-CH_2 of Phe), 3.01−2.70 (6H, m, 1,4-CH_2 + β-CH_2 of Dmt), 2.187 (6H, s, diMe of Dmt), 1.16−1.01 (4H, m, 2,3-CH_2). ^{13}C NMR (DMSO-d_6) δ: 167.91 (q, >C═O of Dmt), 167.52 (q, >C═O of Tyr), 155.54 (q, >C═, 4 of Dmt), 138.14 (q, >C═, 2,6 of Dmt), 135.10 (t, >C═, 1 of Phe), 129.42 (t, H−C═, 2,6 of Phe), 128.25 (t, H−C═, 3,5 of Phe), 126.84 (t, H−C═, 4 of Phe), 122.08 (q, >C═, 1 of Dmt), 114.80 (t, H−C═, 3, 5 of Dmt), 53.39 (t, H−C<, α of Phe), 51.65 (t, H−C<, α of Dmt), 38.09 (s, $-CH_2-$, 1 or 4 CH_2), 38.05 (s, $-CH_2-$, 4 or 1 CH_2), 36.83 (s, β of Phe), 30.44 (s, β of Dmt), 25.52 (s, $-CH_2-$, 2 or 3 CH_2), 25.48 (s, $-CH_2-$, 3 or 2 CH_2), 19.90 (p, $-CH_3$, diMe of Dmt).

1-Boc-amino-4-N^{α}-Fmoc-Tyr(BrZ)-aminobutane. 1-Boc-amino-4-aminobutane (0.94 g, 5 mmol), N^{α}-Fmoc-Tyr(BrZ)-OH (3.1 g, 12 mmol), and PyBOP (6.24 g, 12 mmol) were dissolved in DMF (30 mL) containing DIEA (1.7 mL, 10 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, AcOEt and 5% Na_2CO_3 were added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH: yield 3.1 g (80%); mp 145−148 °C; $R_{f1} = 0.80$, $R_{f2} = 0.80$. Anal. Calcd for $C_{41}H_{44}N_3O_8Br$: C, 62.6; H, 5.63; N, 5.34. Found: C, 62.6; H, 5.65; N, 5.25.

1-N^{α}-Boc-Dmt-amino-4-N^{α}-Fmoc-Tyr(BrZ)-aminobutane. N^{α}-Boc-Dmt-OH (550 mg, 1.80 mmol), 1-amono-4-N^{α}-Fmoc-Tyr(BrZ)-aminobutane·TFA [prepared from Boc-amino-4-N^{α}-Fmoc-Tyr(BrZ)-aminobutane (1.2 g, 1.5 mmol), anisole (0.25 mL, 2.3 mmol), and TFA (2.2 mL, 30 mmol) as usual], and PyBOP (1.12 g, 2.2 mmol) were dissolved in DMF (20 mL) containing DIEA (0.88 mL, 5.1 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na_2CO_3, and water, dried over Na_2SO_4, and evaporated down. Ether was added to the residue to yield crystals, which were collected by filtration and recrystallized from EtOH: yield 1.1 g (63%); mp 186−190 °C; $R_{f1} = 0.77$. Anal. Calcd for $C_{52}H_{57}N_4O_{10}Br$: C, 63.7; H, 5.87; N, 5.73. Found: C, 63.6; N, 5.84; N, 5.50.

1-(Dmt-amino)-4-Tyr-aminobutane·2HCl (6). 1-N^{α}-Boc-Dmt-amino-4-N^{α}-Fmoc-Tyr(BrZ)-aminobutane (600 mg, 0.61 mmol) was prepared according to the procedure for **5** (vide supra) with a 100 mg (32%) yield of a white fluffy amorphous powder: $R_{f4} = 0.20$, $R_{f5} = 0.36$, t_R(C) = 15.78 min. TOF-MS *m*/*z*: calcd for $C_{24}H_{34}N_4O_4$ $(M + H)^+$ 443.5; found 443.8. ^{1}H NMR (free compound, DMSO-d_6) δ: 8.541 (1H, t, $J = 5.4$ Hz, NH of Tyr amide), 7.972 (1H, t, $J = 5.4$ Hz, NH of Dmt amide), 7.046 (2H, d-like, $J = 8.5$ Hz, 2,6-H of Tyr), 6.717 (2H, d-like, $J = 8$ 5 Hz, 3,5-H of Tyr), 6.434 (2H, s, 3,5-H of Dmt), 3.923 (1H, t, $J = 7.0$ Hz, α-H of Tyr), 3.701 (1H, dd, $J = 10.6$ Hz, α-H of Dmt), 3.02−2.79 (8H, m, 1,4-CH_2 + β-CH_2 of Tyr and Dmt), 2.184 (6H, s, diMe of Dmt), 1.18−1.03 (4H, m, 2,3-CH_2). ^{13}C NMR (DMSO-d_6) δ: 167.91 (q, >C═O of Dmt), 167.63 (q, >C═O of Tyr), 156.40 (q, >C═, 4 of Tyr), 155.52 (q, >C═, 4 of Dmt), 138.16 (q, >C═, 2,6 of Dmt), 130.31 (t, H−C═, 2,6 of Tyr), 124.89 (q, >C═, 1 of Tyr), 122.08 (q, 1 of Dmt), 115.15 (t, H−C═, 3,5 of Tyr), 114.81 (t, H−C═, 3,5 of Dmt), 53.69 (t, H−C<, α of Tyr), 51.67 (t, H−C<, α of Dmt), 38.11 (s, $-CH_2-$, 1 or 4 CH_2), 38.05 (s, $-CH_2-$, 4 or 1 CH_2), 36.12 (s, β of Tyr), 30.45 (s, β of Dmt), 25.56 (s, $-CH_2-$, 2 or 3 CH_2), 25.52 (s, $-CH_2-$, 3 or 2 CH_2), 19.88 (p, $-CH_3$, diMe of Dmt).

1,4-Bis(N^{α}-Boc-Phe-amino)butane. N^{α}-Boc-Phe-OH (1.1 g, 4.0 mmol), 1,4-diaminobutane (180 mg, 2.0 mmol), and PyBOP (2.5 g, 4.8 mmol) were dissolved in DMF (25 mL) containing Et_3N (0.67 mL, 4.8 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, AcOEt and water were added to the residue to obtain crystals, which were collected by filtration and recrystallized from EtOH: yield 0.75 g (32%); mp 186−189 °C; $R_{f1} = 0.65$, $R_{f2} = 0.45$. Anal. Calcd for $C_{32}H_{46}N_4O_6$: C, 66.0; H, 7.96; N, 9.62. Found: C, 65.9; H, 7.75; N, 9.61.

1,4-Bis(Phe-amino)butane·2HCl (4). A solution of 1,4-bis(N^{α}-Boc-Phe-amino)butane (580 mg, 1.0 mmol) and anisole (0.15 mL, 1.4 mmol) in TFA (1.5 mL, 20 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was collected by filtration. The crude product in 3% AcOH (3 mL) was applied to a Sephadex G-15 column (2.5 cm × 43 cm), which was equilibrated, and eluted with 3% AcOH. Fractions (8 g each) were collected, and the solvent of the effluent (nos. 8−11) was removed. The residue was lyophilized from 1 M HCl to yield an amorphous powder: yield 340 mg (75%), $R_{f4} = 0.27$, $R_{f5} = 0.47$, t_R(C) = 21.04 min. TOF-MS *m*/*z*: calcd for $C_{22}H_{30}N_4O_2$ $(M + H)^+$ 383.4; found 383.3.

1,4-Bis(N^{α}-Boc-Tyr-amino)butane. N^{α}-Boc-Tyr-OH (5.6 g, 20 mmol), 1,4-diaminobutane (880 mg, 10 mmol), BOP (10.6 g, 24 mmol), and HOBt (3.0 g, 20 mmol) were dissolved in DMF (50 mL) containing Et_3N (5.6 mL, 40 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4, and evaporated down. Ether was added to the residue to form a precipitate, which was collected by filtration: yield 4.2 g (68%); mp 123−126 °C; $R_{f1} = 0.40$. Anal. Calcd for $C_{32}H_{46}N_4O_8 \cdot 0.7H_2O$: C, 61.3; H, 7.56; N, 8.93. Found: C, 61.4. H, 7.81; N, 8.78.

1,4-Bis(Tyr-amino)butane·2HCl (3). A solution of 1,4-bis(N^{α}-Boc-Tyr-amino)butane (550 mg, 0.90 mmol) and anisole

(0.28 mL, 2.6 mmol) in TFA (2.75 mL, 36 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was collected by filtration and lyophilized from 1 M HCl: yield 340 mg (70%), R_{f4} = 0.27, t_R(C) = 7.1 min. TOF-MS *m/z*: calcd for $C_{22}H_{30}N_4O_4$ $(M + H)^+$ 414.4; found 414.1.

1,6-Bis(*N*α-Boc-Dmt-amino)hexane. *N*α-Boc-Dmt-OH (500 mg, 1.6 mmol), 1,6-diaminohexane (92 mg, 0.80 mmol), and PyBOP (1.0 g, 1.9 mmol) were dissolved in DMF (15 mL) containing Et_3N (0.27 mL, 1.9 mmol). The reaction mixture was stirred for 18 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na_2CO_3, and water, dried over Na_2SO_4, and evaporated down. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The crude product in AcOEt/*n*-hexane (1:1, 5 mL) was applied to a silica gel column (BW-127ZH, 3 cm × 16 cm), which was equilibrated, and eluted with AcOEt/*n*-hexane (1:1, 300 mL). After removal of the solvent of the effluent (1−200 mL), petroleum ether was added to the residue to form a precipitate, which was collected by filtration: yield 300 mg (53%); mp 183−186 °C; R_{f1} = 0.52, R_{f2} = 0.08. Anal. Calcd for $C_{38}H_{58}N_4O_8$·0.25H_2O: C, 64.9; H, 8.38; N, 7.96. Found: C, 65.0; H, 8.22; N, 7.78.

1,6-Bis(Dmt-amino)hexane·2HCl (9). A solution of 1,6-bis(*N*α-Boc-Dmt-amino)hexane (150 mg, 0.21 mmol) and anisole (0.10 mL, 0.90 mmol) in TFA (1.0 mL, 13 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was collected by filtration, dried in vacuo, and lyophilized from 1 M HCl: yield 90 mg (75%), R_{f4} = 0.21, R_{f5} = 0.42, t_R(C) = 18.15 min. TOF-MS *m/z*: calcd for $C_{28}H_{42}N_4O_4$ $(M + H)^+$ 499.6; found 499.2. ^{1}H NMR (DMSO-d_6) δ: 7.96 (2H, t, *J* = 5.5 Hz, CO−NH−), 6.40 (4H, s, aromatic 3,5-H), 3.70 (2H, dd, *J* = 10.7, 5.0 Hz, α-H), [3.00 (2H, m) and 2.81 (2H, m) NH−CH_2−], [2.98 (2H, dd, *J* = 13.9, 10.8 Hz) and 2.93 (2H, dd, *J* = 13.9, 5.0 Hz), β-H_2], 2.18 (12H, s, 2,6-CH_3), 1.13 (4H, m, NH−CH_2−CH_2−), 0.90 (4H, m, NH−CH_2−CH_2−CH_2−). ^{13}C NMR (DMSO-d_6) δ: 167.8 (q, >C=O), 155.5 (q, aromatic C-4), 138.1 (q, aromatic C-2,6), 122.0 (q, aromatic C-1), 114.8 (t, aromatic C-3,5), 51.6 (t, C-α), 38.5 (s, NH−CH_2−), 30.5 (s, C-β), 28.3 (s, NH−CH_2-CH_2−), 25.7 (s, NH−CH_2−CH_2−CH_2−), 19.9 (p, 2,6-CH_3).

1,6-Bis(*N*α-Boc-Tyr-amino)hexane. *N*α-Boc-Tyr-OH (5.6 g, 20 mmol), 1,6-diaminohexane (1.9 g, 10 mmol), BOP (11 g, 24 mmol), and HOBt (3.0 g, 20 mmol) were dissolved in DMF (50 mL) containing Et_3N (5.6 mL, 40 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4, and evaporated down. Ether was added to the residue to form a precipitate, which was collected by filtration: yield 5.0 g (79%); mp 146−151 °C; R_{f1} = 0.56. Anal. Calcd for $C_{34}H_{50}N_4O_8$·2.5H_2O: C, 59.4; H, 8.06; N, 8.15. Found: C, 59.2; H, 7.84; N, 8.11.

1,6-Bis(Tyr-amino)hexane·2TFA (8). A solution of 1,6-bis(*N*α-Boc-Tyr-amino)hexane (500 mg, 1.6 mmol) and anisole (0.12 mL, 1.1 mmol) in TFA (1.2 mL, 16 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was collected by filtration, dried in vacuo, and lyophilized from water: yield 250 mg (48%), R_{f4} = 0.31, t_R(Y) = 20.1 min. TOF-MS *m/z*: calcd for $C_{24}H_{34}N_4O_4$ $(M + H)^+$ 442.5; found 443.1.

1,2-Bis(*N*α-Boc-Dmt-amino)ethane. *N*α-Boc-Dmt-OH (500 mg, 1.6 mmol), 1,2-diaminoethane (50 μL, 0.80 mmol), BOP (840 mg, 1.9 mmol), and HOBt (260 mg, 1.9 mmol) were dissolved in DMF (15 mL) containing Et_3N (0.54 mL, 3.8 mmol). The reaction mixture was stirred for 18 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na_2CO_3, and water, dried over Na_2SO_4, and evaporated down. Petroleum ether was added to the residue to yield crystals, which were collected by filtration and recrystallized from EtOH: yield 300 mg (58%); mp 201−205 °C; R_{f1} = 0.48. Anal. Calcd for $C_{34}H_{50}N_4O_8$·0.5H_2O: C, 62.7; H, 7.88; N, 8.59. Found: C, 62.7; H, 7.80; N, 8.59.

1,2-Bis(Dmt-amino)ethane·2TFA (2). A solution of 1,2-bis(*N*α-Boc-Dmt-amino)ethane (200 mg, 0.30 mmol) and anisole (0.1 mL, 0.90 mmol) in TFA (1.0 mL, 13 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was collected by filtration, dried in vacuo, and lyophilized from water: yield 120 mg (60%), R_{f4} = 0.14, R_{f5} = 0.58, t_R(C) = 13.8 min. TOF-MS *m/z*: calcd for $C_{24}H_{34}N_4O_4$ $(M + H)^+$ 443.5; found 443.6.

1,2-Bis(*N*α-Boc-Tyr-amino)ethane. *N*α-Boc-Tyr-OH (4.1 g, 15 mmol), 1,2-diaminoethane (0.36 g, 6.0 mmol), and BOP (7.6 g, 17 mmol) were dissolved in DMF (40 mL) containing Et_3N (2.4 mL, 17 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4, and concentrated. Petroleum ether was added to the residue to form a precipitate. The crude product in $CHCl_3$ (5 mL) was applied to a silica gel column (BW-127ZH, 3 cm × 34 cm), which was equilibrated, and eluted with $CHCl_3$. After removal of the solvent of the effluent (300−2300 mL), petroleum ether was added to the residue to obtain a precipitate: yield 1.0 g (30%); mp 203−206 °C; R_{f1} = 0.49. Anal. Calcd for $C_{30}H_{42}N_4O_8$·0.7H_2O: C, 60.1; H, 7.29; N, 9.34. Found: C, 60.2; H, 6.96; N, 9.01.

1,2-Bis(Tyr-amino)ethane·2TFA (1). A solution of 1,2-bis(*N*α-Boc-Tyr-amino)ethane (500 mg, 0.90 mmol) and anisole (0.14 mL, 1.3 mmol) in TFA (1.4 mL, 18 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate. The precipitate was purified with HPLC and lyophilized from water: yield 350 mg (63%), R_{f4} = 0.30, t_R(C) = 5.5 min. TOF-MS *m/z*: calcd for $C_{20}H_{26}N_4O_4$ $(M + H)^+$ 387.4; found 387.7.

1,8-Bis(*N*α-Boc-Dmt-amino)octane. *N*α-Boc-Dmt-OH (500 mg, 1.6 mmol), 1,8-diaminooctane (120 mg, 1.80 mmol), BOP (840 mg, 1.9 mmol), and HOBt (260 mg, 1.9 mmol) were dissolved in DMF (15 mLl) containing Et_3N (0.54 mLl, 3.8 mmol). The reaction mixture was stirred for 18 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na_2CO_3, and water, dried over Na_2SO_4, and concentrated. Petroleum ether was added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt: yield 310 mg (53%); mp 142−144 °C; R_{f1} = 0.56. Anal. Calcd for $C_{40}H_{62}N_4O_8$·0.5H_2O: C, 65.3; 8.62; N, 7.61. Found: C, 65.2; H, 8.40; N, 7.68.

1,8-Bis(Dmt-amino)octane·2TFA (11). A solution of 1,8-bis(*N*α-Boc-Dmt-amino)octane (260 mg, 1.6 mmol) and anisole (0.1 mL, 0.90 mmol) in TFA (1.0 mL, 13 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was collected by filtration, dried in vacuo, and lyophilized from water: yield 120 mg (60%), R_{f4} = 0.56, R_{f5} = 0.68, t_R(C) = 20.7 min. TOF-MS *m/z*: calcd for $C_{30}H_{46}N_4O_4$ $(M + H)^+$ 527.7; found 527.7.

1,8-Bis(*N*α-Boc-Tyr-amino)octane. *N*α-Boc-Tyr-OH (5.6 g, 20 mmol), 1,8-diaminooctane (1.4 g, 10 mmol), and BOP (11 g, 24 mmol) were dissolved in DMF (50 mL) containing Et_3N (3.4 mL, 24 mmol). The reaction mixture was stirred for 15 h at room temperature. After removal of the solvent, the residue was extracted with AcOEt, which was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4, and concentrated. Petroleum ether was added to the residue to form a precipitate, which was collected by filtration: yield 6.1 g (91%); mp 159−160 °C; R_{f4} = 0.60. Anal. Calcd for $C_{36}H_{54}N_4O_8$·0.5H_2O: C, 63.6; H, 8.09; N, 8.23. Found: C, 63.9; H, 8.23; N, 7.97.

1,8-Bis(Tyr-amino)octane·2TFA (10). A solution of 1,8-bis(*N*α-Boc-Tyr-amino)octane (1.0 g, 1.5 mmol) and anisole (0.45 mL, 4.2 mmol) in TFA (4.5 mL, 60 mmol) was stirred for 1 h at room temperature. Ether was added to the solution to form a precipitate, which was purified with HPLC and lyophilized from water: yield 320 mg (30%), R_{f4} = 0.30, t_R(C) = 18.3 min. TOF-MS *m/z*: calcd for $C_{26}H_{38}N_4O_4$ $(M + H)^+$ 471.6; found 471.2.

Biological Activity in Isolated Tissue Preparations. Preparations of the myenteric plexus longitudinal muscle were obtained from the small intestine of guinea pigs (GPI) for μ-opioid agonism; mouse vas deferens (MVD contains δ−opioid receptors. Both tissues were used for field stimulation with bipolar rectangular pulses of supramaximal voltage. Agonists were tested for their inhibition of the electrically evoked twitch, and the results are expressed as the IC_{50} values obtained from concentration−response curves. The IC_{50} values represent the mean ± SE of five to six separate assays. [D-Ala^2]deltorphin I and dermorphin were used as internal standards with MVD and GPI, respectively.

Determination of Analgesia. Spinal analgesia of H-Dmt-NH-$(CH_2)_4$-NH-Dmt-H (**7**) was determined using the tail-flick test in mice (Figure 2). Radiant heat was applied on the dorsal surface of the tail using a tail-flick apparatus (Columbus Instruments, Columbus, OH). Baseline tail withdrawal latency was adjusted between 2 and 3 s, and a cutoff time was set at 8 s to avoid external heat-related damage. The analgesic response was measured after 10 or 15 min following intracerebroventricular (icv) or subcutaneous (sc) injections, respectively, and groups of five to seven animals were tested at each time point. The dose-dependent appearance of analgesia following icv administration of **7** is expressed as the time course of its appearance (a) and area under the curve (AUC) (b). The AUC was obtained by plotting the response time (s) on the ordinate and time (min) on the abscissa after administration of **7**. Mice were injected icv with either saline (◆) or 0.03 nmol (□), 0.1 nmol (▲), 0.3 nmol (○), or 1 nmol (■) of **7** in 4 μL of saline per mouse. (c) The reversal by the μ-opioid antagonist naloxone (2 mg/kg sc) on analgesia induced by **7** was observed by injection 30 min before **7**. (d) The sc injection of **7** is expressed as the time course of action and AUC (e). Mice were injected either with saline (◆) or with 10 mg/kg (□), 30 mg/kg (▲), 100 mg/kg (○) of **7** in saline, or 3 mg/kg morphine (□). Male Swiss−Webster mice weighing 20−25 g (Taconic, NC) were used throughtout. All data are reported as the mean ± SE. Statistical significance was determined by Student's *t*-test. Asterisks denote significant differences between mice treated with saline and **7** (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

Methods for the determination of analgesia reported in Figure 2 for the hot plate test, which is a measure of supraspinal analgesia of H-Dmt-NH-$(CH_2)_4$-NH-Dmt-H (**7**) in mice. The animals were placed on an electrically heated plate (IITC model 39D hot plate analgesia meter, IITC Inc., Woodland Hills, CA) at 55 ± 0.1 °C after 10 or 15 min following icv administration or sc injection, respectively. The response time was measured by observing movements consisting of jumping, licking, or shaking their hind paws with a baseline latency of 15 s and maximal cutoff time of 30 s. Groups of five to seven mice were tested at each time point. Analgesia of **7** after icv administration is expressed as a time course (a) and AUC (b). Mice were injected either with saline (◆) or with 0.03 nmol (□), 0.1 nmol (▲), 0.3 nmol (○), or 1 nmol (■) of **7** in 4 μL of saline per mouse. (c) Naloxone (2 mg/kg sc) was injected 30 min before icv administration of **7**. (d) The sc analgesic effect of **7** is expressed as a time course and AUC (e). Mice were injected either with saline (◆) or with 10 mg/kg (□), 30 mg/kg (▲), 60 mg/kg (○) of **7**, or 3 mg/kg morphine (□). The mice strain and statistical analyses used are given in the caption to Figure 2.

Opioid Receptor Binding Assays. Binding affinity was determined under equilibrium conditions (2 h at room temperature) in a competition assay using rat brain P_2 synaptosomes. The membranes were preincubated in 0.1 M NaCl, 0.4 mM GDP, 50 mM HEPES, pH 7.5, and 50 μg/mL soybean trypsin inhibitor for 60 min at room temperature to remove endogenous opioids. They were extensively washed in ice-cold buffer containing protease inhibitor and resuspended in HEPES, pH 7.5, containing 50 μg/mL soybean trypsin inhibitor and 20% glycerol, aliquoted into 4 mL lots and stored at −80 °C. The δ- and μ-opioid receptors were radiolabeled with [^{3}H]-DPDPE and [^{3}H]DAGO, respectively. Excess unlabeled peptide (2 μM) established nonspecific binding. Labeled membranes were rapidly filtered on Whatman GF/C glass fiber filters, washed three times with 2 mL amounts, and dried at 75 °C for 60 min. The radioactivity was determined using CytoScint (ICN). All analogues were analyzed in duplicate using five to eight dosages and three to five independent repetitions; different synaptosomal preparations were frequently used to ensure statistical significance (*n* values are listed in Table 1 in parentheses and results are mean ± SE) The affinity constants (K_i) were calculated according to Cheng and Prusoff.[39]

Molecular Modeling. Low-energy conformations of H-Dmt-NH-$(CH_2)_4$-NH-Dmt-H (**7**) were generated using a Silicon Graphics Octane2 computer system and Insight II software (version 98) from Accelrys. Twenty-four structures were generated on the basis of ^{1}H NMR data and NOE cross-peaks (Figure 3b) for H-Dmt-NH-$(CH_2)_4$-NH-Tyr-H (**6**) from which the values of 29.9° and 190.5° for ϕ_1 and ϕ_2, 47.9°, 120.1°, and 151.2° for $\chi_1^{(1)}$, and 21.9° and 139.4° for $\chi_1^{(2)}$ were derived. Extensive conformational searching was performed utilizing constraints on ϕ_1, ϕ_2, $\chi_1^{(1)}$, and $\chi_1^{(2)}$ and rotating the remaining amino acid and carbon-chain bonds in 60° and 180° increments for the peptide bonds within the range of 0−360°. The energy threshold was set at 10.0 kcal/mol with a derivative of 0.01 kcal/(mol·Å), the energy tolerance was set at 1.0 kcal/mol, and the rms was set at 1.0 Å. Minimizations were performed in the AMBER force field, stopping with a root-mean-square gradient of 0.001 kcal/(mol·Å^2). On the basis of these parameters and computations, 343 conformations were generated for each of the 24 starting structures and a total 8232 conformations were analyzed with relative energies ranging from 59 to 443 kcal/mol.

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(1) Abbreviations. In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14−42), this paper uses the following symbols and abbreviations: AcOEt, ethyl acetate; Boc, *tert*-butyloxycarbonyl; BOP, benzoltriazol-1-yloxytris(dimethylamino)phosphonium hexafluorphosphate; Bzl, CH_2−Ph; DAGO, [D-Ala^2, *N*-Me-Phe^4,Gly-ol^5]enkephalin; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Dmt, 2′,6′-dimethyl-L-tyrosine; DMSO, dimethylsulphoxide; DPDPE, cyclic [D-Pen^{2,5}]enkephalin; EtOH, ethanol; GPI, guinea pig ileum; HPLC, high-performance liquid chromatography; Me, methyl; MVD, mouse vas deferens; ^{1}H NMR, proton nuclear magnetic resonance; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; Ph, phenyl; PyBOP, benzoltriazol-1-yloxytrispyrrolidinophosphonium hexafluorphosphate; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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