Oral Bioavailability of a New Class of μ-Opioid Receptor Agonists Containing 3,6-Bis[Dmt-NH$(CH_2)_n$]-2(1*H*)-pyrazinone with Central-Mediated Analgesia

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The inability of opioid peptides to be transported through epithelial membranes in the gastrointestinal tract and pass the blood−brain barrier limits their effectiveness for oral application in an antinociceptive treatment regime. To overcome this limitation, we enhanced the hydrophobicity while maintaining the aqueous solubility properties in a class of opioid-mimetic substances by inclusion of two identical N-termini consisting of Dmt (2′,6′-dimethyl-L-tyrosine) coupled to a pyrazinone ring platform by means of alkyl chains to yield the class of 3,6-bis[Dmt-NH−$(CH_2)_n$]-2(1*H*)-pyrazinones. These compounds displayed high μ-opioid receptor affinity ($K_i\mu$ = 0.042−0.115 nM) and selectivity ($K_i\delta/K_i\mu$ = 204−307) and functional μ-opioid receptor agonism (guinea-pig ileum, IC_{50} = 1.3−1.9 nM) with little or undetectable bioactivity toward δ-opioid receptors (mouse vas deferens) and produced analgesia in mice in a naloxone reversible manner when administered centrally (intracerebroventricular, icv) or systemically (subcutaneously and orally). Furthermore, the most potent compound, 3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone (**7′**), lacked functional δ-opioid receptor bioactivity and was 50−63-fold and 18−21-fold more active than morphine by icv administration as measured analgesia using tail-flick (spinal involvement) and hot-plate (supraspinal effect) tests, respectively; the compound ranged from 16 to 63% as potent upon systemic injection. These analgesic effects are many times greater than unmodified opioid peptides. The data open new possibilities for the rational design of potential opioid-mimetic drugs that pass through the epithelium of the gastrointestinal tract and the blood−brain barrier to target brain receptors.

Introduction

A major concern in the application of opioid peptides as pharmacological drugs for clinical or therapeutic situations, such as the amelioration of pain associated with postsurgery procedures, cancer, or birth, involves their bioavailability.[1] The oral bioavailability of bioactive peptides requires not only a means to overcome physiological and metabolic barriers, but also to be able to transit the physical barriers encompassed in the epithelial membrane of the gastrointestinal tract[2] and at the microcapillary−brain junction, known as the blood−brain barrier (BBB).[3,4] In general, this involves the exclusionary properties of membrane tight junctions associated with cellular and tissue components.[2] Furthermore, passage through these barriers requires overcoming the physicochemical features of peptides, which includes their metabolic liability, charge at physiological pH, lipophilicity, molecular weight, and structure.[2,4] In regard to stability, peptides must survive the onslaught of a broad spectrum of proteolytic enzymes[2] and specific peptidases, termed the "blood−brain enzymatic barrier" that acts as another active deterrent in the cerebral microvasculature.[5]

In addition to the necessity for enzymic stability, the passage of peptides through membranes involves tissue and cellular transport mechanisms that also depend on the specific peptide in question.[3] Of the known transport systems by which peptides enter and exit membranes,[3] the most common include passive diffusion,[2] endocytotic mechanisms,[2] such as pinocytosis and transcytosis,[6] and carrier-mediated, energy dependent active transport systems[3] that involve peptide transporters,[7] particle transporters,[7] and lipid-absorption pathways.[2] Furthermore, the ability of a peptide to pass through the BBB appears to be mediated by an uptake mechanism that is correlated with its lipid solubility.[2,4] One characteristic of cerebral endothelium is that it acts as an effective barrier to penetration by hydrophilic molecules,[3] which is one rationale for the extensive covalent modification of opioid peptides in order to enhance their overall hydrophobic properities,[8−16] although intramolecular hydrogen bonding was also suggested to explain membrane transit by nonsaturable transmembrane diffusion.[17]

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Rationale

The inclusion of Dmt into a variety of opioid peptides in lieu of Tyr as the N-terminal residue brought about marked changes in their physical and biological properties.[18] Dmt was responsible for the change in peptide conformation, as indicated by low-energy conformational searching paradigms and supported by the X-ray diffraction analysis of the crystalline structures of three analogues with distinct bioactivities.[19] The passage of the bis[Dmt-NH]-alkyl compounds through the BBB appears to involve the increased hydrophobicity attributed to the two methyl groups in Dmt since the Tyr analogues were inactive,[20] and that apparently differs from effects observed with H-Dmt-Tic-OH.[21] An analgesic endpoint is crucial in identifying the biological effect elicited by opioid peptides to provide inferential and intuitive evidence on the composition of the compounds that enable them to transit the BBB. In this communication, we report the substitution of Dmt for Tyr in the series of symmetric 3,6-bis[Tyr-NH−$(CH_2)_n$]-2(1*H*)-pyrazinone substances (Figure 1) that not only increased receptor affinity and selectivity to the μ-opioid receptor by several orders of magnitude but also produced antinociception following a regime of central, systemic or oral administration.

Chemistry

Enantiomerically pure 2′,6′-dimethyl-L-tyrosine (Dmt) was prepared according to Dygos et al.[22] Enantiomeric purity (>98%) was ascertained both by HPLC using a chiral column [CROWNPAC CR(+)] and by the reaction with D- and L-amino acid oxidases followed by amino acid analysis. Boc-Dmt-OH and Boc-Dmt-$NHNH_2$ were prepared as described.[23] The 3,6-bis(aminoalkyl)-5-methyl-2(1*H*)-pyrazinones were synthesized by published procedures.[23] The Boc-X(Z)-OH intermediates were coupled with H-X(Z)-CH_2Cl by a mixed anhydride method to produce Boc-X(Z)-X(Z)-CH_2Cl [**2a**: X = Dap (2,3-diaminopropionic acid);[24] **2b**: X = Dab (2,4-diaminobutyric acid);[24] **2c**: X = Orn; **2d**: X = Lys]. After removal of Boc groups by HCl in dioxane, the corresponding amine hydrochloride in MeOH or THF was heated at a reflux for 1 h to produce the protected pyrazinone derivatives, which were then converted to the corresponding amine[23] by catalytic hydrogenation or by HBr/AcOH (Scheme 1). The resulting amines were coupled with Boc-Tyr-$NHNH_2$ or Boc-Dmt-$NHNH_2$ by use of azide,[25] or with Boc-Tyr-OH or Boc-Dmt-OH using Bop reagent,[26] to produce protected **1′**−**8′** (**5a**−**d** and **6a**−**d**). The products were treated with TFA to give crude **1′**−**8′** (Scheme 2). All final products were purified by semipreparative reverse-phase HPLC; each compound exhibited a single peak on analytical HPLC with a unique retention time.[20] Analysis by MALDI-TOF mass spectrometry (MS), ^{1}H and ^{13}C NMR, and by HPLC revealed that they were the desired compounds with greater than 98% purity. These data are summarized in Supporting Information (Table 1).

In the synthesis of the (1*H*)-pyrazinone derivatives, it was necessary to take specific precautions in the removal of the Z protecting groups. For example, to produce 3,6-bis[$NH_2(CH_2)_n$]-2(1*H*)-pyrazinones (Scheme 1: **4a** and **4b**: n = 1 and 2, respectively), the Z groups were removed by 25% HBr/AcOH; however, with the

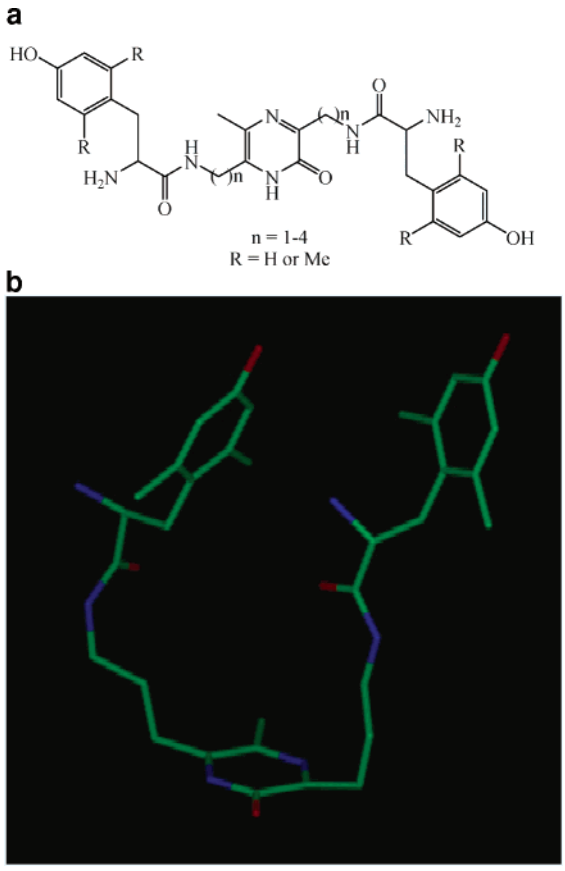
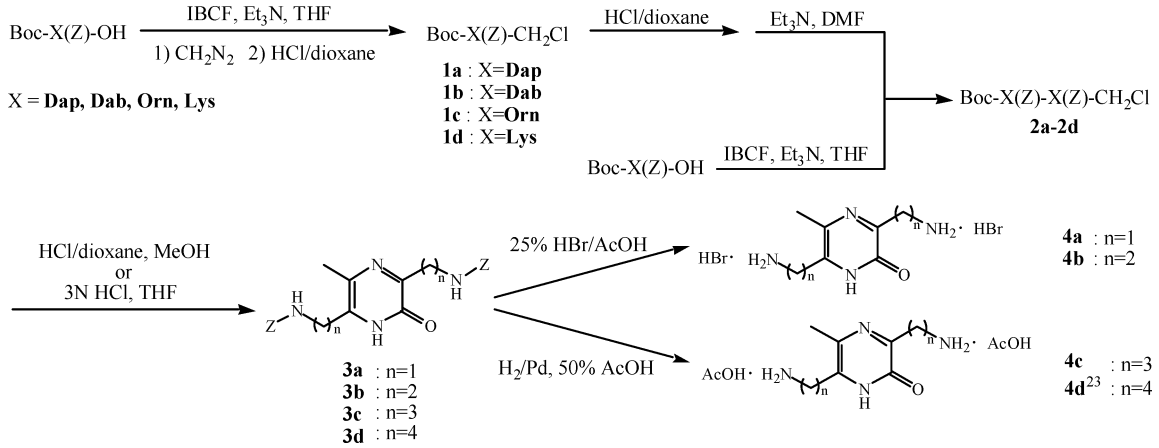


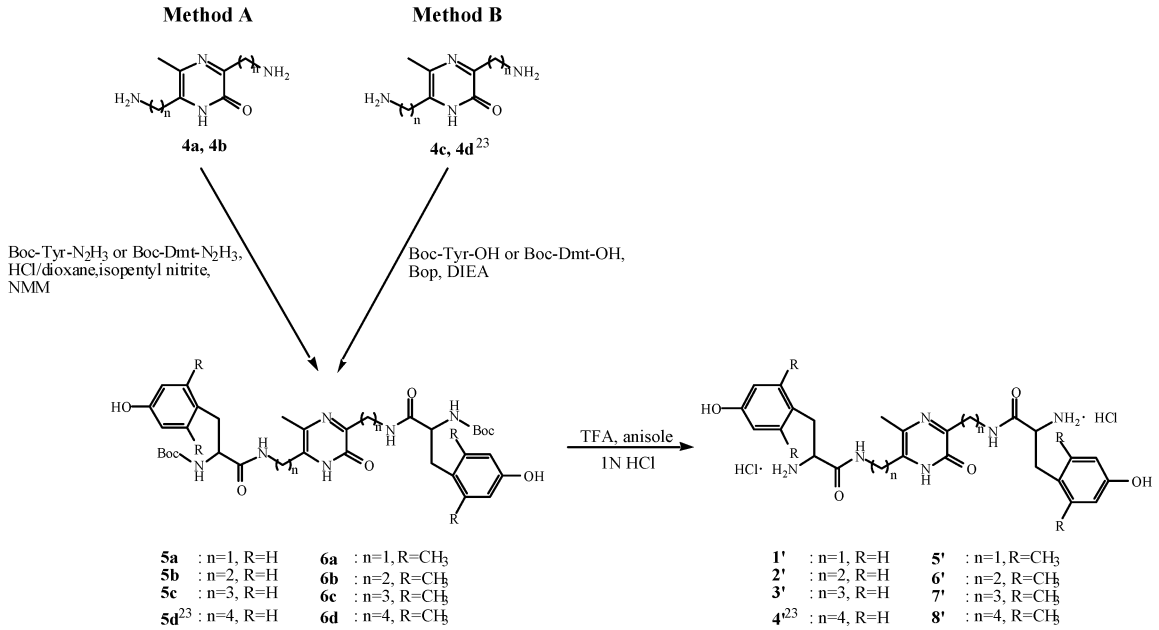
Figure 1. (a) Structure of 3,6-bis[Tyr-NH$(CH_2)_n$]-2(1*H*)-pyrazinone or 3,6-bis[Dmt-NH$(CH_2)_n$]-2(1*H*)-pyrazinone where n = 1−4 and R = H (Tyr) or CH_3 in Dmt. (b) A low energy representation of 3,6-bis[Dmt-NH−$(CH_2)_3$]-2(1*H*)-pyrazinone (**7′**). Carbon atoms are green, nitrogens blue and oxygens red; hydrogen atoms are not displayed. Only one possible conformation is shown since many low energy structures exist. In fact, due to the large number of number rotatable alkyl (C−C) bonds, the necessity to access g+, g-, and trans orientations associated with the alkyl groups, as well as to take into account the side chains of Dmt, selecting several low energy structures to serve as starting structures would be extremely difficult if not imprecise. Each of those structures in turn can generate thousands of conformations, such that both the starting and final structures will be based on assumptions about the best orientations of the alkyl bonds and not necessarily depict the conformation bound to the receptor.

3,6-bis[Z-NH$(CH_2)_n$]-2(1*H*)-pyrazinones, the Z groups were removed by catalytic hydrogenation over a Pd catalyst (Scheme 1: **4c** and **4d**;[23] n = 3 and 4, respectively). Studies on the hydrogenation of Z-protected pyrazinone derivatives revealed that deamination occurred at position 6 of compound **3a** [Scheme 1: 3,6-bis(Z-$NHCH_2$)-2(1*H*)-pyrazinone] due to the benzylic or allylic properties of the CN bond at position 6.[27] Because of the similarity in the CN bond at position 6 of **3b** [Scheme 1: 3,6-bis(Z-NHC_2H_4)-2(1*H*)-pyrazinone], which also has homobenzylic or homoallylic properties, catalytic hydrogenation was avoided and 25% HBr/AcOH was employed to remove Z groups. Furthermore, Boc-Tyr-N_2H_3 or Boc-Dmt-N_2H_3 was coupled with 3,6-bis[NH_2-$(CH_2)_n$]-2(1*H*)-pyrazinones (Scheme 2: **4a** and **4b**, n = 1 and 2, respectively) by an azide coupling procedure,[25] because **4a** and **4b** have a tendency to form pyrazinol-type rather than pyrazinone-type rings (data not shown); on the other hand, Boc-Tyr-OH or Boc-Dmt-OH was

Scheme 1



Scheme 2



coupled with **4c** and **4d**[23] by using Bop reagent.[26] These procedures provided high quality products: each of the compounds (**1′**−**8′**) exhibited single peak on reverse phase HPLC with greater than 98% purity.

Results

Opioid Receptor Affinity (K_i). The replacement of Tyr (**1′**-**4′**) by Dmt (**5′**−**8′**) to yield 3,6-bis[Dmt-NH−$(CH_2)_n$]-2(1*H*)-pyrazinone compounds dramatically increased the affinity to both δ- and μ-opioid receptors, but predominately the latter by several orders of magnitude (**6′**−**8′**, $K_i\mu = 0.042-0.115$ nM) and elevated the selectivity for μ-opioid receptors (**7′** and **8′**, $\delta/\mu =$ 307 and 204, respectively) (Table 1). Compound **7′** [3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone] exhibited the highest affinity ($K_i\mu = 0.042$ nM) and was ca. 3-fold greater than that of either **6′** [3,6-bis(2′-Dmt-aminoethyl)-5-methyl-2(1*H*)-pyrazinone] or **8′** [3,6-bis-(4′-Dmt-aminobutyl)-5-methyl-2(1*H*)-pyrazinone] and nearly 30 times that of **5′** [3,6-bis(Dmt-aminomethyl)-5-methyl-2(1*H*)-pyrazinone]. Thus, the length of the interposing alkyl chain determines efficacy of receptor binding: propyl > ethyl, butyl ≫ methyl.

Functional Bioactivity in Vitro. The in vitro functional bioactivity with isolated tissues from guinea-pig ileum (GPI) and mouse vas deferens (MVD) characterizes the action of a ligand as having agonist or antagonist activity. The data verify that the bis-Dmt-derivatives (**5′**−**8′**) are biologically active (Table 2) and generally reflect the values obtained for the affinity constants (Table 1). The weak K_i values of the Tyr

Table 1. Opioid Receptor Interactions of 3,6-Bis[Tyr-NH$(CH_2)_n$]-2(1*H*)-pyrazinone (**1′**−**4′**) and 3,6-Bis[Dmt-NH$(CH_2)_n$]-2(1*H*)-pyrazinone (**5′**−**8′**)[a]

no.	$(CH_2)_n$	$K_i\mu$ (nM)	*n*	$K_i\delta$ (nM)	*n*	$K_i\delta/K_i\mu$
1′	1	460 ± 14	3	1830 ± 520	3	4.0
2′	2	309 ± 11.2	3	2900 ± 590	4	9.4
3′	3	25.7 ± 3.0	5	435 ± 84	4	17
4′	4	70.2 ± 4.8	3	2190 ± 130	3	31
5′	1	1.16 ± 0.18	3	15.7 ± 2.1	3	13.5
6′	2	0.115 ± 0.009	3	7.26 ± 1.2	4	63
7′	3	0.042 ± 0.003	3	13.2 ± 1.7	3	307
8′	4	0.114 ± 0.008	3	23.2 ± 2.5	3	204

[a] Binding affinities are given as K_i values determined using [^{3}H]DPDPE for δ-opioid receptors and [^{3}H]DAGO for μ-opioid receptors (Experimental Section). the mean ± SE is based on three to five independent assays (*n*) conducted in duplicate using five to seven dosages of each compound. as seen in Figure 1a, $(CH_2)_n$ represents the *n* number of methylene units in the linkage between the pyrazinone ring and the N-terminal Dmt residues.

Table 2. Functional Bioactivity of 3,6-Bis[Dmt-NH$(CH_2)_n$]-2(1*H*)-pyrazinone Compounds **5′**−**8′**[a]

		IC_{50}, nM (mean ± SE)		
no.	$(CH_2)_n$	GPI (μ)	MVD (δ)	antagonism pA_2 (δ)
5′	1	1695 ± 365	>10000	6.47
6′	2	12.9 ± 2.4	>10000	6.56
7′	3	1.33 ± 0.2	>10000	none
8′	4	1.90 ± 0.67	41.5 ± 10.4	none

[a] In vitro bioactivity was determined as given in the Experimental Section. Repetitions are 5−7 for each bioassay. Agonists inhibited the electrically evoked twitch (IC_{50}); deltorphin C and dermorphin were used as the δ- and μ-opioid receptor agonist standard peptides, Respectively. the pA_2 defines antagonism and is the negative log(M concentration) required to double the concentration of a δ-opioid receptor agonist (deltorphin C) to achieve the original response. From Figure 1a, $(CH_2)_n$ is the number of *n* methylene units in the sequence.

cognates precluded any determination of their bioactivity. Not only was **7′** the most biologically active (GPI, IC_{50} = 1.33 nM) and also more potent than the bis[Dmt-NH]-alkyl compounds (GPI, IC_{50} = 3.08−5.33 nM),[20] but also it was a μ-opioid receptor agonist without measurable δ-opioid receptor bioactivity (Table 2). However, compound **8′**, which only had 30% less μ-opioid receptor agonism than **7′**, exhibited moderate δ-opioid receptor agonism (IC_{50} = 41.5 nM), while **5′** and **6′** exerted weak δ-opioid receptor antagonism (pA_2 = 6.56 and 6.47, respectively) similar to that of the bis[Dmt-NH]-alkyl analogues (pA_2 = 6.4−5.5).[20]

Antinociception in Mice. Compound **7′** produced analgesia in vivo following icv administration in mice as well as by subcutaneous (sc) injection and per oral (po) lavage (Figures 2−4). The effect was 50-fold and ca. 20-fold more potent than morphine in the tail-flick (Figure 2a,b) and hot-plate tests (Figure 3a,b), respectively, following icv administration. Naloxone (2 mg/kg) injected sc 30 min before **7′** suppressed the effect in both tests (Figure 2c,d and Figure 3c,d), suggesting the effect is mediated by opioid receptors. Injection of **7′** by sc and po routes exhibited a dose dependent analgesia in both tests (Figure 4). The bioactivity data are summarized in Table 3 relative to the action of morphine, which served as a positive control.

Discussion

Systemic and Oral Bioavailability of Opioid-Mimetic Compounds. Our results unequivocally demonstrated that an unmodified opioid-mimetic compound (**7′**) administered systemically and orally produced analgesia through a naloxone reversible mechanism (Figures 2−4). This implied that without chemical modification to enhance its physicochemical properties,[8−16] compound **7′** crossed epithelial membrane barriers in both the intestine and mirocapillaries in mouse brain. Furthermore, absorption on nanoparticles coated with polysorbate 80[7] was also unwarranted.

Passage of opioids through the BBB by a nonsaturable (passive diffusion) mechanism of the metabolic stable μ-opioid receptor agonist [D-Arg^2,Lys^4]dermorphin analogue (DALDA)[4] or N^{α}-1-iminoethyl-Tyr-D-MetO-Phe-MeβAla-OH[16] correlated with their lipid solubility, whereas the δ-opioid receptor agonist DPDPE appeared to be transmitted through the BBB by "an energy-dependent transcytotic mechanism".[28] The appearance of analgesia by systemically administered H-Dmt-Tic-OH[21] suggested this might involve the formation of a lipid-soluble diketopiperazine[29] analogous to TRH in the formation of the His-Pro diketopiperazine.[3] Owing to the high opioid receptor affinity and bioactivity of **7′** (Tables 1−3), it is equally plausible that only a small proportion of the administered compound might have crossed the luminal membrane and the BBB,[3] or they might be relatively more stable in vivo than natural opioid peptides, which have relatively short half-lives (in the range of a few minutes).[30,31] However, since a differential analgesic sensitivity exists in mice strains,[32] it may be also possible that bioactivity expressed by **7′** might exhibit similar differences. However, neither the mode of transit across membrane barriers nor its stability is addressed in this report.

Interestingly, hydrophobic analogues of the Dmt-Tic pharmacophore, which should be readily transmitted through the BBB, selectively inhibited the action of the membrane-bound hMDR-1 P-glycoprotein in vitro.[33]

Pyrazinone-Containing Opioid-Mimetic Compounds. The 3,6-bis[Tyr-NH-[$(CH_2)_n$]-2(1*H*)-pyrazinone compounds weakly interact with opioid receptors,[24,34] while the bis[Dmt-NH]-alkyl compounds provided evidence that a substance with two Dmt residues at the N-termini exhibited high μ-opioid receptor affinity and agonism.[20] In comparison to the 3,6-bis[Tyr-NH−$(CH_2)_n$]-2(1*H*)-pyrazinone derivatives (**1′**−**4′**) (Table 1), the inclusion of Dmt (**5′**−**8′**) enhanced affinity to μ-opioid receptors by factors of 400-, 2,700-, 612- and 616-fold relative to the Tyr cognates (**1′**−**4′**), respectively. The Dmt-containing compounds were μ-opioid receptor agonists with minimal δ-opioid activity except **8′**, which had modest δ-opioid receptor agonism. Comparable to the data on the bis[Dmt-NH]-alkyl compounds,[20] the length of the alkyl linker, in this case between the pyrazinone ring and the aromatic residue, markedly affects receptor interaction. Furthermore, the difference of a single methylene group between these opioid-mimetic compounds substantially changes their bioactivity profile, which is similar to the observations with the H-Dmt-Tic-NHCH(R)-R′ substances.[35]

Bifunctional Opioid-Mimetic Peptides. Earlier studies on opioid peptides with two identical N-termini were derived by tail-to-tail dimerization of enkephalin[36] and dermorphin;[37] in both cases, they exhibited increased activity toward opioid receptors. Generally, the increase in affinity and biological activity of bis-dermorphin[37] as well as bis[D-Ala^2,Leu^5]-enkephalin,[36] bis-[D-Ala^2-des-Leu^5]-enkephalin,[36] and a cystamine-enkephalin dimer[38] was slightly more potent for both μ− and δ-opioid receptors. The enhanced activity might be accounted for by their interaction with two opioid receptors in close proximity or they induced receptor aggregation due to their increased length.[36] On the other hand, the relatively small dimensions of **7′** or the bis-[Dmt-NH]-alkyl compounds[20] suggest that they bind within a single μ-opioid receptor pocket (unpublished observations).

Conclusions

Changes in the number of methylene units (n = 1−4) on either side of the pyrazinone ring between the Dmt N-termini produced opioid-mimetic substances with

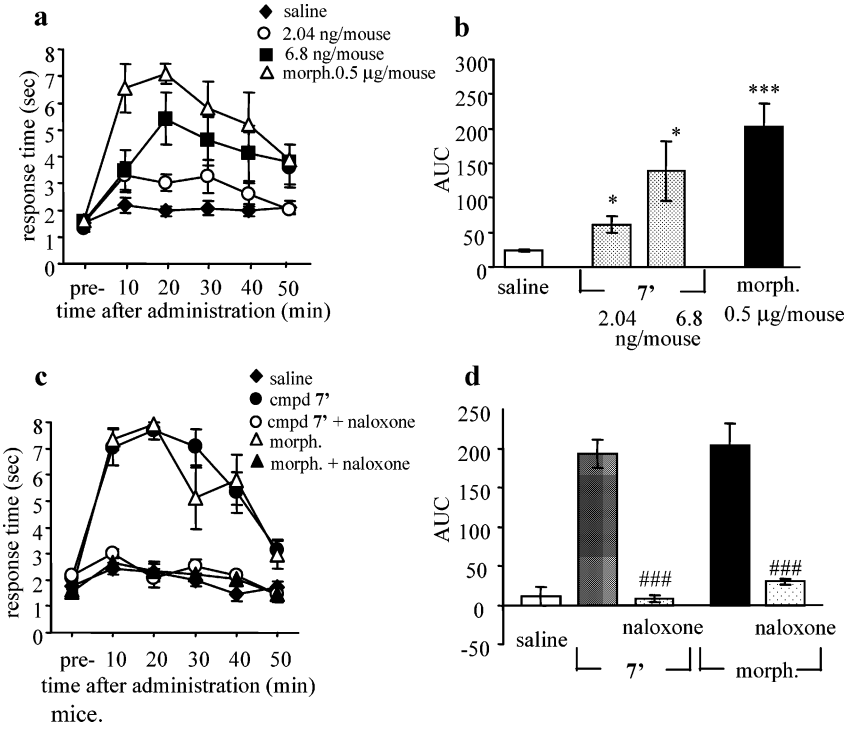


Figure 2. Effect of intracerebroventricular administrated 3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone (**7′**) and morphine on tail flick (spinal analgesia) latency in mice. Compounds dissolved in physiological saline (pH 7) were injected icv into male Swiss-Webster mice (20−25 g). Morphine (morph.) was used as a positive control; the antagonist naloxone was applied to counter the effect and to determine if analgesia involved opioid receptors.[20] The area under the curve (AUC) is derived from data based on the response (mean ± SE) of five to seven mice per time point. The dose response curve is seen in (a) [♦ = saline; ○ = 2.04 ng **7′**; ■ = 6.8 ng **7′**; ● = 20.4 ng **7′**; △ = morphine (0.5 μg)] with the AUC (b). Asterisks (*) denote values that are significantly different (ANOVA) from the control mice (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Dose−response curves with or without naloxone are in (c) [♦ = saline; ● = 20.4 ng **7′**; ○ = **7′**+ 2 mg naloxone injected sc 30 min before **7′**; △ = morphine (0.5 μg); ▲ = morphine + naloxone] and with the AUC (d). The symbol ### denotes the statistical differences between the effect of **7′** or morphine treatment relative to naloxone-treated mice (### $p < 0.001$).

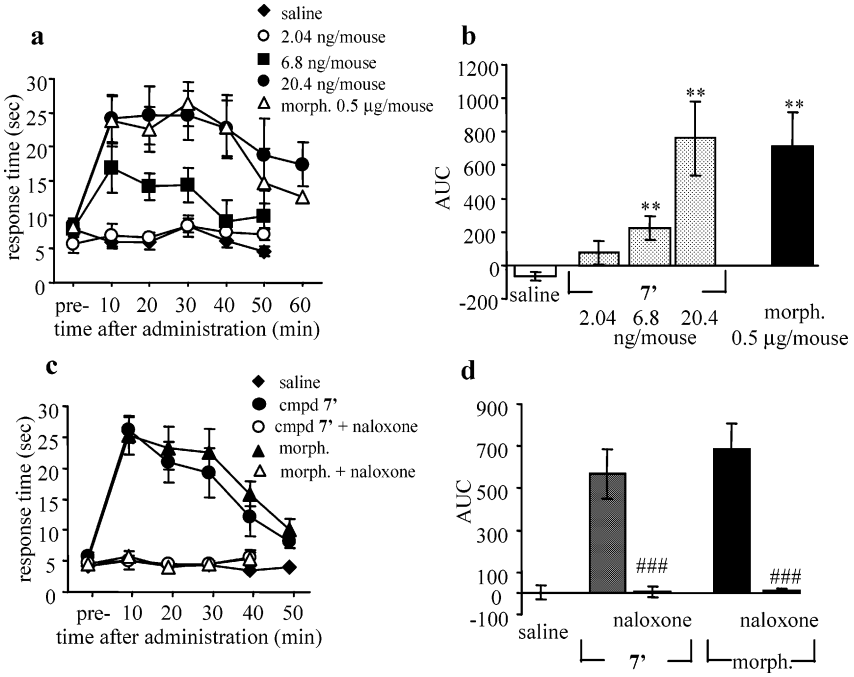


Figure 3. Effect of intracerebroventricularly injected 3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone (**7′**) and morphine on hot-plate (supraspinal analgesia) latency. Complete details are given in the legend to Figure 2. Statistical significances: (b) **, $p < 0.01$; (d) ###, $p < 0.001$.

distinct binding affinities to μ- and δ-opioid receptors, different bioactivity profiles, and analgesia following icv, sc, and po administration. Compound **7′** traversed the epithelial tissue in the gastrointestinal tract, transported by the blood plasma to the brain to where it passed through the BBB to interact with brain μ-opioid

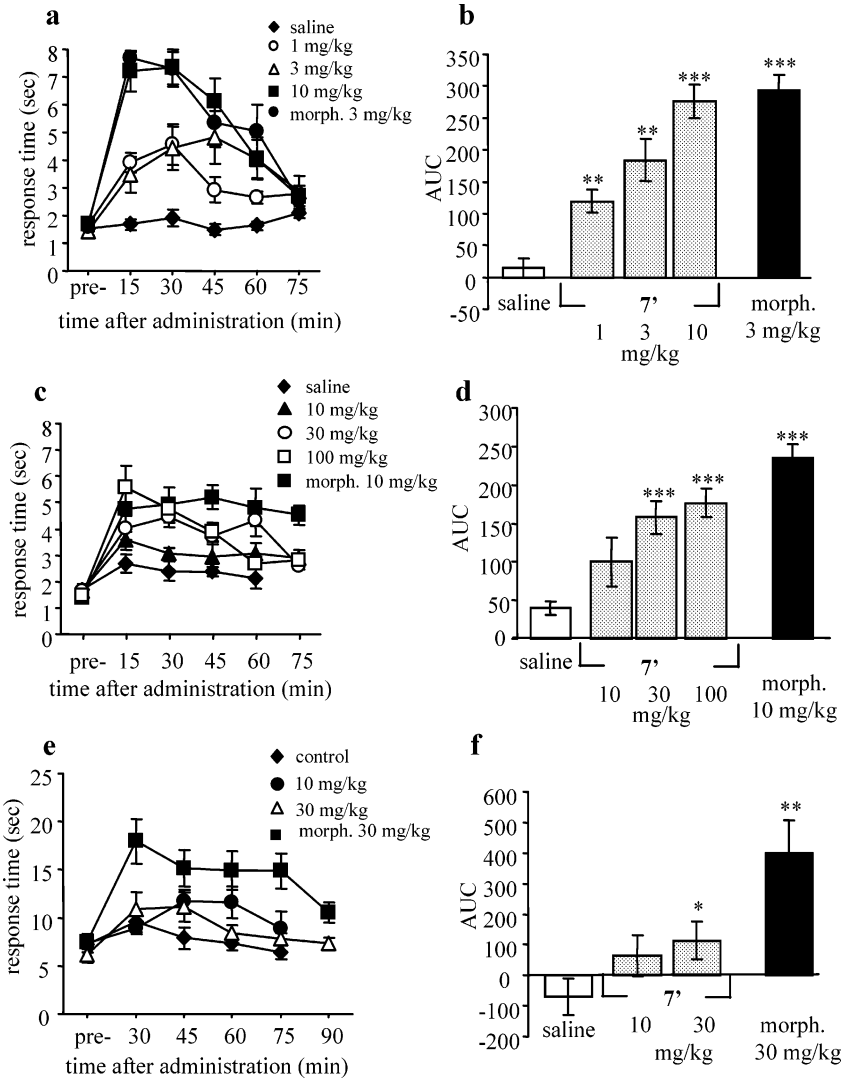


Figure 4. Effect of subcutaneously injected and orally administrated 3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone (**7′**) and morphine in tail-flick and hot-plate tests. The dose−response curves for sc injection determined in the tail-flick test is in (a) [♦ = saline; ○ = 1 mg/kg **7′**; △ = 3 mg/kg **7′**; ■ = 10 mg/kg **7′**; ● = 3 mg/kg morphine] and with the AUC (b). Per oral dose−response curves (c) [♦ = saline; ▲ = 10 mg/kg **7′**; ○ = 30 mg/kg **7′**; □ = 100 mg/kg **7′**; ■ = 10 mg/kg morphine] and AUC (d) (***, $p < 0.001$). The analgesia represented by the hot-plate tests is in parts e and f. Complete details are given in the legend to Figure 2. Statistical significance: (b, d, and f) *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Table 3. Biological Potency of 3,6-Bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone (**7′**) Relative to Morphine in Mice[a]

mode of delivery	tail flick[a]	hot plate[a]
intracerebroventricular	50−63	18−21
subcutaneous	0.63	0.55
per oral	0.42	0.16−0.24

[a] These values were derived from concentrations of **7′** that produced an equivalent analgesia as morphine.

receptors. Interestingly, **7′** was systemically 5- to 6-fold more potent than the bis[Dmt-NH]-alkyl class of opioid compounds,[20] which suggests that the pyrazinone ring might confer enhanced structural and chemical properties permitting greater absorption through tissues. We can only conjecture at this point whether the binding of the pyrazinone derivatives occurs in an analogous manner to the bis[Dmt-NH]-alkyl compounds[20] or other opioid substances for that matter since the presence of the pyrazinone ring in the molecule introduces an unknown factor. Nonetheless, we observed that the presence of Dmt and different length alkyl chains (**5′**−**8′**) greatly affected opioid receptor binding affinities and in vitro bioactivity profiles, which might provide clues on the design of new opioid agonists. In light of the oral bioavailability of **7′**, further studies are underway to evaluate its therapeutic advantages and differences relative to morphine in terms of tolerance required to determine its potential use in clinical approaches in the treatment of postoperative or cancer pain in humans, pain associated with birthing, or as a possible veterinary drug. Our results open the way for the rational design of a new class of synthetic opioid-mimetic substances that target brain opioid receptors.[39]

Experimental Section

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-1000 (Japan Spectroscopic Co.). Mass spectra were measured with a KRATOS-MALDI TOF-MS. ^{1}H (400 MHz or 500 MHz) and ^{13}C (100 MHz or 125 MHz) NMR spectra were recorded on a Bruker DPX-400 or ARX-500 spectrometers, respectively. Purified compounds (30 mg) were dissolved in 1.0 mL pyridine-

d_5 (99.9% isotopic purity). Chemical shift values are expressed as ppm downfield from tetramethylsilane, used as an internal standard (δ-value), and the *J* values are given in hertz. The ^{13}C signals were assigned with the aid of distortionless enhancement by polarization transfer (DEPT) and two-dimensional experiments, and multiplicities are indicated by p (primary), s (secondary), t (tertiary), or q (quaternary). Reversed phase HPLC analysis used Waters model 600E (A) or Waters model delta 600 (B) with a COSMOSIL C18-AR-II (4.6 × 250 mm) column in the following solvent systems: A, 0.05% TFA in water; B, 0.05% TFA in CH_3CN. The retention time was reported as t_R (min). On TLC (Kieselgel G 60, Merck), R_f values refer to the following systems: (A) AcOEt−hexane (1:1), (B) $CHCl_3$−MeOH−AcOH (90:8:2), (C) $CHCl_3$−AcOEt−EtOH (6:4:1), (D) *n*-BuOH−AcOH−pyridine−water (4:1:1:2), (E) *n*-BuOH−AcOH−pyridine−water (1:1:1:1), and (F) $CHCl_3$−MeOH−water (8:3:1). Open column chromatography was run on silica gel 60 (70−230 mesh, YMC).

Synthesis of Boc-X(Z)-CH_2Cl (1a−c, X: a = Dap, b = Dab, or c = Orn). To a solution of mixed anhydride [prepared from Boc-X(Z)-OH (13.6 mmol), isobutyl chloroformate (16.4 mmol) and Et_3N (16.4 mmol)] in THF (50 mL) was added diazomethane in ether (60 mL) [prepared from *p*-toluensulfonyl-*N*-methyl-*N*-nitrosoamide (40.9 mmol)]. The reaction mixture was stirred at 4 °C overnight and 6.9 N HCl/dioxane (34.1 mmol) added to the solution under cooling with an ice−salt solution. The reaction mixture was stirred at −15 °C for 15 min before water and ice were added to the reaction mixture, and the product was extracted with AcOEt. The extract was washed with water, 5% $NaHCO_3$, and saturated sodium chloride solution, dried over Na_2SO_4, and evaporated. The residue was crystallized from petroleum ether, and the crystals were collected by filtration. Yield, mp, $[\alpha]^{25}_D$, R_f and elemental analyses of **1a−c** are summarized in Supporting Information (Table 2).

Boc-Dap(Z)-CH_2Cl (1a). ^1H NMR ($CDCl_3$) δ 1.44 (s, 9H, *tert*-butyl), 3.59 (m, 1H, β-CH_2), 3.70 (br, 1H, β-CH_2), 4.30 and 4.42 (AB-q, *J* = 15.6 Hz, 2H, $COCH_2Cl$), 4.59 (br, 1H, α-CH), 5.07 (s, 2H, benzyl), 5.22 (br, 1H, β-NH), 5.69 (d, *J* = 6.0 Hz, 1H, α-NH), 7.31−7.37 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 28.3 (p, *tert*-butyl), 41.9 (s, β-CH_2), 46.5 (s, COCH_2Cl), 58.2 (t, α-CH), 67.3 (s, benzyl), 80.7 (q, *tert*-butyl), 128.1, 128.3 and 128.6 (t, aryl C), 136.9 (q, aryl C), 155.5 and 157.1 (q × 2), 202.3 (q, *C*OCH_2Cl).

Boc-Dab(Z)-CH_2Cl (1b). ^1H NMR ($CDCl_3$) δ 1.44 (s, 9H, *tert*-butyl), 1.67 (br, 1H, β-CH_2), 2.09 (m, 1H, β-CH_2), 3.11 (br, 1H, γ-CH_2), 3.47 (br, 1H, γ-CH_2), 4.23 (s, 2H, $COCH_2Cl$), 4.57 (br, 1H, α-CH), 5.10 (s, 2H, benzyl), 5.45 (br, 2H, γ-NH and α-NH), 7.31−7.36 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 28.4 (p, *tert*-butyl), 31.7 (s, β-CH_2), 37.1 (s, γ-CH_2), 46.5 (s, COCH_2Cl), 55.0 (t, α-CH), 66.9 (s, benzyl), 80.7 (q, *tert*-butyl), 128.3−128.6 (t, aryl C), 136.5 (q, aryl C), 157.7 and 159.1 (q × 2), 202.1 (q, *C*OCH_2Cl).

Boc-Orn(Z)-CH_2Cl (1c). ^1H NMR ($CDCl_3$) δ 1.44 (s, 9H, *tert*-butyl), 1.48−1.62 (m, 3H, γ-CH_2 and β-CH_2), 1.87 (br, 1H, β-CH_2), 3.23 (m, 2H, δ-CH_2), 4.24 (s, 2H, $COCH_2Cl$), 4.51 (br, 1H, α-CH), 4.94 (br, 1H, δ-NH), 5.09 (s, 2H, benzyl), 5.18 (br, 1H, α-NH), 7.30−7.36 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 26.1 (s, γ-CH_2), 28.3 (p, *tert*-butyl), 28.5 (s, β-CH_2), 40.5 (s, δ-CH_2), 46.5 (s, COCH_2Cl), 57.0 (t, α-CH), 66.8 (s, benzyl), 80.5 (q, *tert*-butyl), 128.1−128.6 (t, aryl C), 136.5 (q, aryl C), 156.6 and 156.6 (q × 2), 201.6 (q, *C*OCH_2Cl).

Synthesis of Boc-X(Z)-X(Z)-CH_2Cl (2a−c, X: a = Dap, b = Dab, or c = Orn). To a solution of Boc-X(Z)-OH (3.37 mmol) in THF (30 mL) were added Et_3N (3.37 mmol) and isobutyl chloroformate (3.37 mmol) under cooling to −15 °C. After 10 min, a cold solution of HCl·H-X(Z)-CH_2Cl [prepared from Boc-X(Z)-CH_2Cl (4.04 mmol) and 7.2 N HCl/dioxane (12.1 mmol)] in DMF (30 mL) containing Et_3N (4.04 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. After removal of the solvent, the residue was dissolved in AcOEt, which was washed with water, 10% citric acid, 5% $NaHCO_3$, and saturated sodium chloride solution, dried over Na_2SO_4, and evaporated. The residue was crystallized from ether, and the crystals were collected by filtration. The crude product was purified by column chromatography. Yield, mp, $[\alpha]^{25}_D$, R_f, and elemental analyses of **2a−c** are summarized in Supporting Information (Table 2).

Boc-Dap(Z)-Dap(Z)-CH_2Cl (2a). ^1H NMR (DMSO-d_6 containing 1% pyridine-d_5) δ 1.38 (s, 9H, *tert*-butyl), 3.24−3.58 (m, 4H, β-CH_2), 4.03 (br, 1H, α-CH), 4.40 (br, 1H, α-CH), 4.53 and 4.60 (AB-q, 2H, *J* = 16.8 Hz, $COCH_2Cl$), 5.02 (s, 2H, benzyl), 5.03 (s, 2H, benzyl), 6.90 (d, *J* = 6.7 Hz, 1H, α-NH), 7.14 (br, 1H, β-NH), 7.23 (br, 1H, β-NH), 7.29−7.37 (m, 10H, ArH), 8.54 (d, *J* = 7.1 Hz, 1H, α-NH); ^{13}C NMR ($CDCl_3$) δ 28.0 (p, *tert*-butyl), 40.1 (s, β-CH_2), 41.6 (s, β-CH_2), 47.9 (s, COCH_2-Cl), 54.9 (t, α-CH), 56.7 (t, α-CH), 65.5 (s, benzyl), 78.6 (q, *tert*-butyl), 127.6−128.3 (t, aryl C), 136.8 (q, aryl C), 136.9 (q, aryl C), 155.3, 156.3 and 170.9 (q × 3), 199.2 (q, *C*OCH_2Cl).

Boc-Dab(Z)-Dab(Z)-CH_2Cl (2b). ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H, *tert*-butyl), 1.77−2.21 (m, 4H, β-CH_2), 3.11 (br, 1H, γ-CH_2), 3.28 (m, 1H, γ-CH_2), 3.52 (br, 1H, γ-CH_2), 3.63 (d, *J* = 10.8 Hz, 1H, $COCH_2Cl$), 3.73 (m, 1H, γ-CH_2), 4.09−4.30 (m, 2H, $COCH_2Cl$ and α-CH), 4.73 (br, 1H, α-CH), 5.08−5.16 (m, 4H, 2 × benzyl), 5.40 (br, 2H, γ-NH and α-NH), 6.89 (br, 1H, α-NH), 7.29−7.37 (m, 10H, ArH); ^{13}C NMR ($CDCl_3$) δ 27.9 (s, β-CH_2), 28.3 (p, *tert*-butyl), 33.9 (s, β-CH_2), 37.9 (s, γ-CH_2), 44.8 (s, $COCH_2Cl$), 45.5 (s, γ-CH_2), 52.2 (t, α-CH), 52.6 (t, α-CH), 67.1 (s, benzyl), 67.3 (s, benzyl), 80.3 (q, *tert*-butyl), 127.8−128.7 (t, aryl C), 136.0 (q, aryl C), 136.3 (q, aryl C), 154.5, 156.9 and 172.0, (q × 3), 199.0 (q, *C*OCH_2Cl).

Boc-Orn(Z)-Orn(Z)-CH_2Cl (2c). ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H, *tert*-butyl), 1.55 (br, 6H, β-CH_2 and 2 × γ-CH_2), 1.78−1.85 (m, 2H, β-CH_2), 3.17 (br, 3H, δ-CH_2), 3.33 (br, 1H, δ-CH_2), 4.21 (s, 2H, $COCH_2Cl$), 4.27 (br, 1H, α-CH), 4.69 (br, 1H, α-CH), 5.06 (s, 4H, 2 × benzyl), 5.22 (br, 2H, 2 × δ-NH), 5.30 (br, 1H, α-NH), 7.29−7.32 (m, 11H, ArH and α-NH); ^{13}C NMR ($CDCl_3$) δ 26.2 (s, 2 × γ-CH_2), 28.3 (s, β-CH_2), 28.4 (p, *tert*-butyl), 29.8 (s, β-CH_2), 39.7 (s, δ-CH_2), 40.2 (s, δ-CH_2), 46.6 (s, COCH_2Cl), 53.1 (t, α-CH), 56.0 (t, α-CH), 66.8 (s, 2 × benzyl), 80.1 (q, *tert*-butyl), 127.8−128.5 (t, aryl C), 136.5 (q × 2, aryl C), 155.9, 156.8, 157.1 and 172.9 (q × 4), 200.8 (q, *C*OCH_2Cl).

Synthesis of 3,6-Bis[Z-NH(CH_2)$_n$]-5-methyl-2(1*H*)-pyrazinone (3a: *n* = 1 or 3c: *n* = 3). A solution of HCl·H-X(Z)-X(Z)-CH_2Cl [prepared from Boc-X(Z)-X(Z)-CH_2Cl (1.35 mmol) and 7.2 N HCl/dioxane (27.1 mmol)] in MeOH (10 mL) was heated at a reflux for 1 h. After removal of the solvent, the residue was dissolved in $CHCl_3$, washed with water, 10% citric acid and water, dried over Na_2SO_4, and evaporated. The residue was crystallized from ether, and the crystals were collected by filtration and recrystallized from MeOH.

3,6-Bis(benzyloxycarbonylaminomethyl)-5-methyl-2(1*H*)-pyrazinone (3a). Yield 52.6%, mp 183−187 °C, R_f 0.58 (B). Anal. Calcd for $C_{23}H_{24}N_4O_5 \cdot 0.5H_2O$: C, 62.0; H, 5.66; N, 12.6. Found: C, 62.3; H, 5.47; N,12.7. ^1H NMR (pyridine-d_5) δ 2.42 (s, 3H, 5-CH_3), 4.56 (d, *J* = 4.9 Hz, 2H, CH_2NH-Z), 4.93 (d, *J* = 4.8 Hz, 2H, CH_2NH-Z), 5.32 and 5.34 (2s, 4H, benzyl), 7.23−7.48 (m, 10H, ArH), 8.03 (br, 1H, NH-Z), 8.49 (br, 1H, NH-Z); ^{13}C NMR (pyridine-d_5) δ 19.1 (p, 5-CH_3), 42.5 and 42.8 (s × 2, 2 × CH_2NH-Z), 66.5 (s, benzyl), 66.7 (s, benzyl), 128.1−128.8 (t, aryl C), 137.8, 138.1, 156.1, and 157.4 (q × 4).

3,6-Bis(2′-benzyloxycarbonylaminoethyl)-5-methyl-2(1*H*)-pyrazinone (3b). A solution of **2b** (198 mg, 0.32 mmol) and 3 N HCl (4 mL) in THF (2 mL) was heated at a reflux for 1 h. After removal of THF, the residue was dissolved in $CHCl_3$, washed with water, 5% $NaHCO_3$, and saturated sodium chloride solution, dried over Na_2SO_4, and evaporated. The residue was crystallized from ether, and the crystals were collected by filtration, and recrystallized from EtOH. Yield 54.8%, mp 183−185 °C, R_f 0.28 (C). Anal. Calcd for $C_{25}H_{28}N_4O_5$: C, 64.6; H, 6.08; N, 12.1. Found: C, 64.4; H, 5.87; N, 12.0. ^1H NMR (pyridine-d_5) δ 2.33 (s, 3H, 5-CH_3), 2.92 (t, *J* = 6.9 Hz, 2H, C$H_2$$CH_2$NH-Z), 3.32 (t, *J* = 6.9 Hz, 2H, C$H_2$$CH_2$-NH-Z), 3.72 (q, *J* = 6.5 Hz, 2H, CH_2CH_2NH-Z), 3.99 (q, *J* = 6.5 Hz, 2H, CH_2CH_2NH-Z), 5.29 and 5.32 (2s, 4H, benzyl), 7.24−7.45 (m, 10H, ArH), 8.04 (br, 1H, NH-Z), 8.34 (br, 1H, NH-Z); ^{13}C NMR (pyridine-d_5) δ 19.0 (p, 5-CH_3), 32.5 and 33.6

(s × 2, 2 × CH_2CH_2NH-Z), 39.5 and 40.2 (s × 2, 2 × CH_2CH_2-NH-Z), 66.2 and 66.3 (s × 2, 2 × benzyl), 128.1−128.8 (t, aryl C), 138.0, 138.2, 157.3, 157.3, and 157.2 (q × 5).

3,6-Bis(3′-benzyloxycarbonylaminopropyl)-5-methyl-2(1*H*)-pyrazinone (3c). Yield 52.3%, mp 133−135 °C, R_f 0.52 (B). Anal. Calcd for $C_{27}H_{32}N_4O_5 \cdot 0.3H_2O$: C, 65.1; H, 6.55; N, 11.3. Found: C, 65.2; H, 6.59; N, 11.3. ^{1}H NMR ($CDCl_3$) δ 1.79 (br, 4H, $CH_2CH_2CH_2NH$-Z), 2.25 (s, 3H, 5-CH_3), 2.53 (t, 2H, J = 7.0 Hz, $CH_2CH_2CH_2NH$-Z), 2.78 (t, J = 7.0 Hz, 2H, CH_2-CH_2CH_2NH-Z), 3.14−3.20 (m, 4H, 2 × $CH_2CH_2CH_2NH$-Z), 5.06 and 5.08 (2s, 4H, benzyl), 5.57 (br, 1H, NH-Z), 6.11 (br, 1H, NH-Z), 7.28−7.32 (m, 10H, ArH); ^{13}C NMR ($CDCl_3$) δ 18.4 (p, 5-CH_3), 26.7 (s, $CH_2CH_2CH_2NH$-Z), 27.0 and 28.5 (s × 2, 2 × $CH_2CH_2CH_2NH$-Z), 29.5 (s, $CH_2CH_2CH_2NH$-Z), 39.4 and 40.4 (s × 2, 2 × $CH_2CH_2CH_2NH$-Z), 66.5 and 66.8 (s × 2, 2 × benzyl), 127.7−128.5 (t, aryl C), 130.6, 134.4, 136.6, 136.8, 155.3, 156.5, 156.8, and 157.7 (q × 8).

Synthesis of 3,6-Bis[amino-$(CH_2)_n$]-5-methyl-2(1*H*)-pyrazinone (4a: *n* = 1 or 4b: *n* = 2). Compound **3a** or **3b** (0.41 mmol) was treated with 25% HBr/AcOH (4.12 mmol) for 2 h at room temperature. Dry ether was added to the solution until the product precipitated; the precipitate was collected by filtration and washed with ether. **4a**: R_f 0.49 (E), MS $[M + H]^+$ 169.2, $C_7H_{12}N_4O \cdot 2$ HBr; **4b**: R_f 0.26 (D), MS $[M + H]^+$ 197.3, $C_9H_{16}N_4O \cdot 2$HBr.

3,6-Bis(3′-aminopropyl)-5-methyl-2(1*H*)-pyrazinone·2AcOH (4c). A solution of **3c** (419 mg, 0.91 mmol) in 60% AcOH (20 mL) was hydrogenated for 1 h in the presence of Pd black. After removal of Pd and the solvent, ether was added to the residue resulting in crystals, which were collected by filtration and lyophilized from water. R_f 0.12 (D), MS $[M + H]^+$ 225.6, $C_{11}H_{20}N_4O \cdot 2$AcOH.

Synthesis of 3,6-Bis[N$^\alpha$-Boc-Tyr-NH−$(CH_2)_n$]-5-methyl-2(1*H*)-pyrazinone (5a: *n* = 1 or 5b: *n* = 2). To a solution of Boc-Tyr-N_2H_3 (0.65 mmol) in DMF (6 mL) were added 7.3 N HCl/dioxane (1.30 mmol) and isopentyl nitrite (0.78 mmol) under cooling to −15 °C; the solution was stirred for 10 min at the same temperature, and NMM (1.37 mmol) was added to adjust the pH to 8. This solution was added to a cooled solution of **4a** or **4b** (0.26 mmol) and NMM (0.52 mmol) in DMF (6 mL). The reaction mixture was stirred at 0 °C for 24 h, before adding an azide solution prepared from Boc-Tyr-N_2H_3 (69.6 μmol) in DMF (2 mL). The reaction mixture was stirred for another 24 h under the same conditions and the pH maintained at 8. After removal of DMF, the residue was dissolved in AcOEt and washed with water, 5% $NaHCO_3$, 10% citric acid, and saturated sodium chloride solution. The AcOEt layer was dried over Na_2SO_4 and evaporated. The product was crystallized from ether, and the crystals were collected by filtration.

3,6-Bis[(N$^\alpha$-Boc-Tyr)-aminomethyl]-5-methyl-2(1*H*)-pyrazinone (5a). Yield 53.6%, mp 143−144 °C, $[\alpha]^{25}_D$ −27.5° (c = 0.1, $CHCl_3$), R_f 0.57 (F), MS $[M + H]^+$ 696.2. Anal. Calcd for $C_{35}H_{46}N_6O_9 \cdot 1.25H_2O$: C, 58.6; H, 6.82; N, 11.7. Found: C, 58.8; H, 6.85; N, 11.3. ^{1}H NMR (pyridine-d_5) δ 1.44 (s, 18H, 2 × *tert*-butyl), 2.39 (s, 3H, 5-CH_3), 3.21 (m, 1H, β-CH_2), 3.31 (m, 1H, β-CH_2), 3.41 (m, 1H, β-CH_2), 3.59 (m, 1H, β-CH_2), 4.56 (d, J = 4.1 Hz, 2H, CH_2NH), 4.84 (bm, 1H, CH_2NH), 4.91 (bq, J = 7.3 Hz 1H, α-CH), 5.00 (bm, 1H, CH_2NH), 5.12 (bq, J = 7.1 Hz, 1H, α-CH), 7.02−7.41 (m, 8H, ArH), 8.08 (d, J = 9.6 Hz, 1H, NH of Tyr), 8.10 (d, J = 9.8 Hz, 1H, NH of Tyr), 8.88 (br, 1H, CH_2NH), 9.22 (br, 1H, CH_2NH); ^{13}C NMR (pyridine-d_5) δ 19.0 (p, 5-CH_3), 28.5 (p, 2 × *tert*-butyl), 38.6 and 38.7 (s × 2, 2 × β-CH_2), 40.7 and 41.2 (s × 2, 2 × CH_2NH), 57.2 and 57.3 (t × 2, 2 × α-CH), 78.7 and 78.8 (q × 2, 2 × *tert*-butyl), 116.1 (t, aryl C), 128.3 and 128.8 (q × 2), 131.0 and 131.3 (t × 2, aryl C), 156.1, 156.5, 157.6, 157.7, 172.9, and 173.3 (q × 7).

3,6-Bis[2′-(N$^\alpha$-Boc-Tyr)-aminoethyl]-5-methyl-2(1*H*)-pyrazinone (5b). Yield 85.2%, mp 148−150 °C, $[\alpha]^{25}_D$ +49.6° (c = 0.1, DMF), R_f 0.78 (F), MS $[M + H]^+$ 723.7. Anal. Calcd for $C_{37}H_{50}N_6O_9 \cdot 1.5H_2O$: C, 59.3; H, 7.12; N, 11.2. Found: C, 59.4; H, 6.76; N, 11.4. ^{1}H NMR (pyridine-d_5) δ 1.44 (s, 18H, 2 × *tert*-butyl), 2.33 (s, 3H, 5-CH_3), 3.02−2.75 (m, 2H, CH_2CH_2-NH), 3.32−3.11 (bm, 4H, β-CH_2 and CH_2CH_2NH), 3.50−3.40 (m, 2H, β-CH_2), 3.75 (bq, J = 5.1 Hz, 2H, CH_2CH_2NH), 3.96 (bm, 1H, CH_2CH_2NH), 4.05 (bm, 1H, CH_2CH_2NH), 4.95−4.88 (m, 2H, 2 × α-CH), 7.32−7.05 (m, 8H, 2 × ArH), 7.86 (d, J = 8.4 Hz, 1H, NH of Tyr), 7.99 (d, J = 8.4 Hz, 1H, NH of Tyr), 8.78 (br, 1H, C_2H_4NH), 9.08 (br, 1H, C_2H_4NH); ^{13}C NMR (pyridine-d_5) δ 18.8 (p, 5-CH_3), 28.2 (p, *tert*-butyl), 31.8 and 32.8 (s × 2, 2 × CH_2CH_2NH), 37.3 (s, CH_2CH_2NH), 38.3 (s, CH_2CH_2NH), 38.6 (s, 2 × β-CH_2), 57.0 (t, 2 × α-CH), 78.4 and 78.5 (q × 2, 2 × *tert*-butyl), 115.9 (t, aryl C), 128.4 and 128.6 (q × 2), 130.8 (t, aryl C), 156.1, 156.8, 157.3, 157.4, 172.1, 172.6, and 173.3 (q × 7).

3,6-Bis[3′-(N$^\alpha$-Boc-Tyr)-aminopropyl]-5-methyl-2(1*H*)-pyrazinone (5c). To a solution of **4c** (74.3 mg, 0.33 mmol) in DMF (20 mL) were added Boc-Tyr-OH (205.3 mg, 0.73 mmol), Bop (336.6 mg, 0.76 mmol), and DIEA (0.29s mL, 1.71 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 4 h. After removal of DMF, the residue was dissolved in AcOEt and washed with water, 5% $NaHCO_3$, 10% citric acid, and saturated sodium chloride solution. The AcOEt layer was dried over Na_2SO_4 and evaporated. Ether was added to the residue to give crystals, which were collected by filtration. The crude product was purified by column chromatography. Yield 31.5%, mp 125−130 °C, $[\alpha]^{25}_D$ −2.57° (c = 0.2, $CHCl_3$), R_f 0.68 (F), MS $[M + H]^+$ 751.8. Anal. Calcd for $C_{39}H_{54}N_6O_9 \cdot H_2O$: C, 60.9; H, 7.34; N, 10.9. Found: C, 60.7; H, 7.24; N, 11.1. ^{1}H NMR (pyridine-d_5) δ 1.44 (s, 18H, 2 × *tert*-butyl), 1.90 (br, 2H, $CH_2CH_2CH_2NH$), 2.14−2.21 (m, 2H, $CH_2CH_2CH_2NH$), 2.28 (s, 3H, 5-CH_3), 2.59 (t, J = 7.5 Hz, 2H, $CH_2CH_2CH_2NH$), 3.06 (t, J = 7.3 Hz, 2H, $CH_2CH_2CH_2NH$), 3.15−3.32 (m, 2H, β-CH_2), 3.40−3.52 (m, 4H, $CH_2CH_2CH_2NH$ and β-CH_2), 3.56 (m, 1H, $CH_2CH_2CH_2NH$), 3.68 (m, 1H, $CH_2CH_2CH_2NH$), 4.89−4.96 (m, 2H, 2 × α-CH), 7.06−7.10 (m, 4H, ArH), 7.28−7.33 (m, 4H, ArH), 7.88 (d, J = 7.8 Hz, 1H, NH of Tyr), 8.09 (d, J = 7.9 Hz, 1H, NH of Tyr), 8.77 (br, 1H, C_3H_6NH), 8.90 (br, 1H, C_3H_6NH); ^{13}C NMR (pyridine-d_5) δ 18.9 (p, 5-CH_3), 27.4 (s, $CH_2CH_2CH_2$-NH), 28.5 (p, *tert*-butyl), 29.2 (s, $CH_2CH_2CH_2NH$), 30.4 (s, CH_2-CH_2CH_2NH), 38.7 (s, β-CH_2), 38.9 (s, $CH_2CH_2CH_2NH$), 39.0 (s, $CH_2CH_2CH_2NH$), 39.1 (s, β-CH_2), 39.6 (s × 2, 2 × CH_2-CH_2CH_2NH), 57.3 and 57.5 (t × 2, 2 × α-CH), 78.6 and 78.8 (q × 2, 2 × *tert*-butyl), 116.2 (t, aryl C), 128.6 (q), 131.0 and 131.1 (t × 2, aryl C), 156.5, 156.9, 157.7, 172.5, and 173.1 (q × 5).

Synthesis of 3,6-Bis[Tyr-NH−$(CH_2)_n$]-5-methyl-2(1*H*)-pyrazinone (1′: *n* = 1; 2′: *n* = 2; 3′: *n* = 3). Compounds **5a**, **5b**, or **5c** (0.13 mmol) were treated with TFA (3.75 mmol) containing anisole (0.38 mmol) for 1 h at room temperature. Dry ether was added to the solution until the product precipitated. The precipitate was collected by filtration, purified by reverse-phase HPLC, and lyophilized from water containing 1 N HCl to give an amorphous powder. Yield, $[\alpha]^{25}_D$ and mass spectrum analyses of **1′**−**3′** are summarized in Supporting Information (Table 1).

3,6-Bis(Tyr-aminomethyl)-5-methyl-2-(1*H*)-pyrazinone·2HCl (1′). Anal. Calcd for $C_{25}H_{30}N_6O_5 \cdot 2HCl \cdot 2H_2O$: C, 49.8; H, 6.01; N, 11.8. Found: C, 49.6; H, 6.15; N, 11.8. ^{1}H NMR (pyridine-d_5) δ 2.37 (3H, s, 5-CH_3), 3.77 (d, J = 5.0 Hz, 2H, β-CH_2), 3.84 (d, J = 6.2 Hz, 2H, β-CH_2), 4.61 (bm, 2H, CH_2-NH), 4.81 (bm, 1H, CH_2NH), 4.99 (bm, 1H, CH_2NH), 5.24 (br, 2H, 2 × α-CH), 7.00−7.57 (m, 8H, ArH), 9.62 (br, 1H, CH_2NH), 10.33 (br, 1H, CH_2NH).

3,6-Bis(2′-Tyr-aminoethyl)-5-methyl-2-(1*H*)-pyrazinone·2HCl (2′). Anal. Calcd for $C_{27}H_{34}N_6O_5 \cdot 2HCl \cdot 4H_2O$: C, 48.6; H, 6.64; N, 12.6. Found: C, 48.5; H, 6.38; N, 12.7. ^{1}H NMR (pyridine-d_5) δ 2.18 (s, 3H, 5-CH_3), 2.95−3.25 (m, 4H, 2 × CH_2-CH_2NH), 3.70−4.07 (m, 8H, 2 × CH_2CH_2NH and 2 × β-CH_2), 5.17 (br, 2H, 2 × α-CH), 7.00−7.67 (m, 8H, ArH), 9.64 (br, 1H, C_2H_4NH), 10.0 (br, 1H, C_2H_4NH); ^{13}C NMR (pyridine-d_5) δ 18.7 (p, 5-CH_3), 30.8 (s, CH_2CH_2NH), 33.5 (s, CH_2CH_2NH), 37.3, 37.4, 37.7 and 38.6 (s × 4, 2 × CH_2CH_2NH and 2 × β-CH_2), 56.0 (t, α-CH), 56.2 (t, α-CH), 116.4 and 116.5 (t × 2, aryl C), 126.0 and 126.5 (q × 2), 131.6 and 131.7 (t × 2, aryl C), 157.0, 158.2, 158.2, 169.7, and 169.8 (q × 5).

3,6-Bis(3′-Tyr-aminopropyl)-5-methyl-2-(1*H*)-pyrazinone·2HCl (3′). Anal. Calcd for $C_{29}H_{38}N_6O_5 \cdot 2HCl \cdot 4H_2O$ C,

50.1; H, 6.95; N, 12.1. Found: C, 50.1; H, 6.75; N, 12.2. ^{1}H NMR (pyridine-d_5) δ 1.94 (br, 2H, $CH_2CH_2CH_2NH$), 2.14−2.17 (m, 2H, $CH_2CH_2CH_2NH$), 2.24 (s, 3H, 5-CH_3), 2.62 (t, J = 7.8 Hz, 2H, $CH_2CH_2CH_2NH$), 3.00 (t, J = 7.4 Hz, 2H, CH_2CH_2-CH_2NH), 3.43−3.68 (m, 8H, 2 × $CH_2CH_2CH_2NH$- and 2 × β-CH_2), 4.85 (br, 1H, α-CH), 5.01 (br, 1H, α-CH), 7.02 (d, J = 8.4 Hz, 2H, ArH), 7.04 (d, J = 8.3 Hz, 2H, ArH), 7.40 (d, J = 7.7 Hz, 4H, ArH), 9.42 (br, 1H, C_3H_6NH), 9.75 (br, 1H, C_3H_6NH); ^{13}C NMR (pyridine-d_5) δ 18.8 (p, 5-CH_3), 27.0 (s, $CH_2CH_2CH_2NH$), 28.5 (s, $CH_2CH_2CH_2NH$), 28.6 (s, CH_2CH_2-CH_2NHH), 30.2 (s, $CH_2CH_2CH_2NH$), 37.9 (s, 2 × β-CH_2), 39.4 and 39.8 (s × 2, 2 × $CH_2CH_2CH_2NH$), 56.1 (t, 2 × α-CH), 116.5 (t, aryl C), 126.0 (q), 131.4 (t, aryl C), 156.9, 158.3, 169.7, and 170.2 (q × 4).

Synthesis of 3,6-Bis[N$^\alpha$-Boc-Dmt-NH(CH$_2$)$_n$]-5-methyl-2(1*H*)-pyrazinone (6a: *n* = 1 or 6b: *n* = 2). To a solution of Boc-Dmt-N_2H_3 (0.27 mmol) in DMF (5 mL) were added 7.3 N HCl/dioxane (0.54 mmol) and isopentyl nitrite (0.32 mmol) under cooling to −15 °C. After the solution was stirred for 10 min at the same temperature, NMM (0.57 mmol) was added to adjust the pH to 8. This was then added to a cold solution of **4a** or **4b** (0.11 mmol) and NMM (0.22 mmol) in DMF (3 mL). The reaction mixture was stirred at 0 °C for 24 h before adding the azide solution prepared from Boc-Dmt-N_2H_3 (95.6 μmol) in DMF (2 mL). The reaction mixture was stirred for another 24 h under the same conditions and the pH kept at 8. After removal of DMF, the residue was extracted with AcOEt and washed with water, 5% $NaHCO_3$, 10% citric acid, and saturated sodium chloride solution. The AcOEt layer was dried over Na_2SO_4 and evaporated. The product was crystallized from ether and collected by filtration.

3,6-Bis(N$^\alpha$-Boc-Dmt-aminomethyl)-5-methyl-2(1*H*)-pyrazinone (6a). Yield 49.6%, mp 175−178 °C, $[\alpha]^{25}_D$ −10.3° (c = 0.1, $CHCl_3$), R_f 0.78 (F), MS $[M + H]^+$ 752.0. Anal. Calcd for $C_{39}H_{54}N_6O_9 \cdot 1.25H_2O$: C, 60.6; H, 7.37; N, 10.9. Found: C, 60.6; H, 7.06; N, 11.2. ^{1}H NMR (pyridine-d_5) δ 1.46 and 1.47 (2s, 18H, 2 × *tert*-butyl), 2.32, 2.34 and 2.43 (3s, 15H, 4 × CH_3 of Dmt and 5-CH_3), 3.15−3.54 (m, 4H, 2 × β-CH_2), 4.50 (m, 2H, CH_2NH), 4.68−4.96 (m, 3H, CH_2NH- and α-CH), 5.01 (bq, J = 9.9 Hz, 1H, α-CH), 6.83 (bs, 4H, ArH), 7.93 (d, J = 8.2 Hz, 1H, NH of Dmt), 8.08 (d, J = 8.1 Hz, 1H, NH of Dmt), 8.51 (br, 1H, CH_2NH), 9.09 (br, 1H, CH_2NH); ^{13}C NMR (pyridine-d_5) δ 18.8, 20.4 and 20.6 (p × 3, 4 × CH_3 of Dmt and 5-CH_3), 28.5 (p, *tert*-butyl), 33.3 and 33.6 (s × 2, 2 × β-CH_2), 40.8 and 41.2 (s × 2, 2 × CH_2NH), 55.5 and 55.7 (t × 2, 2 × α-CH), 78.7 and 78.8 (q × 2, 2 × *tert*-butyl), 116.0 and 116.1 (t × 2, aryl C), 125.3, 125.8, 139.0, 155.9, 156.2, 156.8, 157.0, 172.8, and 173.2 (q × 9).

3,6-Bis(2′-N$^\alpha$-Boc-Dmt-aminoethyl)-5-methyl-2(1*H*)-pyrazinone (6b). Yield 53.3%, mp 148−158 °C, $[\alpha]^{25}_D$ +89.0° (c = 0.1, $CHCl_3$), R_f 0.78 (F), MS $[M + H]^+$ 780.0. Anal. Calcd for $C_{41}H_{58}N_6O_9 \cdot 1.75H_2O$: C, 60.8; H, 7.65; N, 10.4. Found: C, 60.5; H, 7.77; N, 10.2. ^{1}H NMR (pyridine-d_5) δ 1.46 and 1.48 (2s, 18H, 2 × *tert*-butyl), 2.30 (s, 3H, 5-CH_3) 2.40 (2s, 12H, 4 × CH_3 of Dmt), 2.68−3.02 (m, 2H, CH_2CH_2NH), 3.04−3.33 (m, 4H, β-CH_2 and CH_2CH_2NH), 3.42−3.57 (m, 2H, β-CH_2), 3.57−3.80 (m, 2H, CH_2CH_2NH), 3.81−4.17 (m, 2H, CH_2CH_2NH), 4.83 (br, 2H, 2 × α-CH), 6.86 and 6.84 (2s, 4H, ArH), 7.77 (d, J = 8.3 Hz, 1H, NH of Dmt), 7.94 (d, J = 8.0 Hz, 1H, NH of Dmt), 8.73 (br, 1H, C_2H_4NH), 9.03 (br, 1H, C_2H_4NH); ^{13}C NMR (pyridine-d_5) δ 19.0 (p, 5-CH_3), 20.5 and 20.6 (p × 2, 2 × CH_3 of Dmt), 28.5 (p, *tert*-butyl), 32.0 and 32.7 (s × 2, 2 × CH_2-CH_2NH), 33.5 and 33.7 (s × 2, 2 × β-CH_2), 37.5 and 38.6 (s × 2, 2 × CH_2CH_2NH), 55.4 and 55.6 (t × 2, 2 × α-CH), 78.6 and 78.7 (q × 2, 2 × *tert*-butyl), 109.2 (q), 116.1 (t, aryl C), 139.0, 139.1, 156.1, 156.9, 157.0, 172.3, and 173.0 (q × 7).

Synthesis of 3,6-*bis*-[N$^\alpha$-Boc-Dmt-NH(CH$_2$)$_n$]-5-methyl-2(1*H*)-pyrazinone (6c: *n* = 3 or 6d: *n* = 4). To a solution of **4c** or **4d** (0.81 mmol) in DMF (20 mL) were added Boc-Dmt-OH (1.79 mmol), Bop (1.88 mmol) and DIEA (4.10 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 4 h. After removal of DMF, the residue was dissolved in AcOEt and washed with water, 5% $NaHCO_3$, 10% citric acid, and saturated sodium chloride solution. The AcOEt layer was dried over Na_2SO_4 and evaporated. Ether was added to the residue to give crystals, which were collected by filtration and the crude product purified by column chromatography.

3,6-Bis(3′-N$^\alpha$-Boc-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone (6c). Yield 59.4%, mp 153−155 °C, $[\alpha]^{25}_D$ +52.1° (c = 0.1, $CHCl_3$), R_f 0.73 (F), MS $[M + H]^+$ 808.5. Anal. Calcd for $C_{43}H_{62}N_6O_9 \cdot H_2O$: C, 62.6; H, 7.77; N, 10.2. Found: C, 62.6; H, 7.80; N, 10.2. ^{1}H NMR (pyridine-d_5) δ 1.46 (s, 18H, 2 × *tert*-butyl), 1.85 (br, 2H, $CH_2CH_2CH_2NH$), 2.05−2.18 (m, 2H, $CH_2CH_2CH_2NH$), 2.26 (s, 3H, 5-CH_3), 2.38 and 2.43 (2s, 12H, 4 × CH_3 of Dmt), 2.53 (t, J = 7.6 Hz, 2H, $CH_2CH_2CH_2NH$), 2.96 (m, 2H, $CH_2CH_2CH_2NH$), 3.14−3.26 (m, 2H, β-CH_2), 3.33 (m, 1H, $CH_2CH_2CH_2NH$), 3.40−3.54 (m, 4H, $CH_2CH_2CH_2NH$- and β-CH_2), 3.61 (m, 1H, $CH_2CH_2CH_2NH$), 4.76−4.90 (m, 2H, 2 × α-CH), 6.86 and 6.88 (s, 4H, ArH), 7.81 (d, J = 8.5 Hz, 1H, NH of Dmt), 8.04 (d, J = 8.3 Hz, 1H, NH of Dmt), 8.71(br, 1H, C_3H_6NH), 8.83 (br, 1H, C_3H_6NH); ^{13}C NMR (pyridine-d_5) δ 18.9 (p, 5-CH_3), 20.5 and 20.6 (p, 4 × CH_3 of Dmt), 27.1 (s, $CH_2CH_2CH_2NH$), 28.5 (s, $CH_2CH_2CH_2NH$), 28.6 (p × 2, *tert*-butyl), 29.0 (s, $CH_2CH_2CH_2NH$), 30.3 (s, $CH_2CH_2CH_2NH$), 33.8 (s, β-CH_2), 39.2 (s, $CH_2CH_2CH_2NH$), 39.7 (s, $CH_2CH_2CH_2NH$), 55.6 and 55.7 (t × 2, 2 × α-CH), 78.6 and 78.7 (q × 2, 2 × *tert*-butyl), 116.2 and 123.0 (t × 2, aryl C), 125.6, 125.7, 139.1, 156.1, 156.2, 156.8, 157.0, 172.5, and 173.2 (q × 9).

3,6-Bis(4′-N$^\alpha$-Boc-Dmt-aminobutyl)-5-methyl-2(1*H*)-pyrazinone (6d). Yield 12.7%, mp 124−128 °C, $[\alpha]^{25}_D$ +25.3° (c = 0.5, MeOH), R_f 0.61 (F), MS $[M + H]^+$ 837.5. Anal. Calcd for $C_{45}H_{66}N_6O_9 \cdot H_2O$: C, 63.4; H, 7.98; N, 9.86. Found: C, 63.5; H, 7.99; N, 9.34. ^{1}H NMR (pyridine-d_5) δ 1.35−1.53 (m, 20H, $CH_2CH_2CH_2CH_2NH$- and 2 × *tert*-butyl), 1.56−1.72 (m, 4H, $CH_2CH_2CH_2CH_2NH$ and $CH_2CH_2CH_2CH_2NH$), 1.87 (br, 2H, $CH_2CH_2CH_2CH_2NH$), 2.30 (s, 3H, 5-CH_3), 2.38 and 2.41 (2s, 12H, 4 × CH_3 of Dmt), 2.53 (br, 2H, $CH_2CH_2CH_2CH_2NH$), 2.98 (t, J = 7.3 Hz, 2H, $CH_2CH_2CH_2CH_2NH$), 3.14−3.25 (m, 2H, β-CH_2), 3.25−3.39 (m, 3H, $CH_2CH_2CH_2CH_2NH$), 3.40−3.58 (m, 3H, β-CH_2 and $CH_2CH_2CH_2CH_2NH$), 4.71−4.88 (m, 2H, 2 × α-CH), 6.88 (s, 4H, 2 × ArH), 7.81 (d, J = 8.6 Hz, 1H, NH of Dmt), 7.90 (d, J = 8.5 Hz, 1H, NH of Dmt), 8.61 (br, 1H, C_4H_8NH), 8.66 (br, 1H, C_4H_8NH); ^{13}C NMR (pyridine-d_5) δ 18.7 (p, 5-CH_3), 20.3 (p, 4 × CH_3 of Dmt), 24.6 and 26.0 (s, 2 × $CH_2CH_2CH_2CH_2NH$), 28.1 (p, *tert*-butyl), 29.3 and 29.4 (s × 2, 2 × $CH_2CH_2CH_2CH_2NH$), 30.6 and 32.4 (s × 2, 2 × CH_2-$CH_2CH_2CH_2NH$), 33.3 and 33.4 (s × 2, 2 × β-CH_2), 39.1 and 39.5 (s × 2, 2 × $CH_2CH_2CH_2CH_2NH$), 55.3 (t, 2 × α-CH), 78.3 and 78.4 (q × 2, 2 × *tert*-butyl), 115.8 (t × 2, aryl C), 125.4, 138.8, 138.9, 155.9, 156.7, and 172.2 (q × 6).

Synthesis of 3,6-Bis[Dmt-NH(CH$_2$)$_n$]-5-methyl-2(1*H*)-pyrazinone (5′: *n* = 1; 6′: *n* = 2; 7′: *n* = 3; 8′: *n* = 4). Compounds **6a**, **6b**, **6c**, or **6d** (1.04 mmol) were treated with TFA (34.4 mmol) containing anisole (3.44 mmol) for 2 h at room temperature. Ether was added to the solution until the product precipitated. The precipitate was collected by filtration, purified by reverse-phase HPLC, and lyophilized from water containing 1 N HCl to give an amorphous powder. Yield, $[\alpha]^{25}_D$, and mass spectrum analyses of **5′**−**8′** are summarized in Supporting Information (Table 1).

3,6-Bis(Dmt-aminomethyl)-5-methyl-2(1*H*)-pyrazinone·2HCl (5′). Anal. Calcd for $C_{29}H_{38}N_6O_5 \cdot 2.5HCl \cdot 0.5H_2O$: C, 51.4; H, 6.61; N, 12.4. Found: C, 51.2; H, 6.34; N, 12.3. ^{1}H NMR (pyridine-d_5) δ 2.31, 2.34 and 2.37 (3s, 15H, 4 × CH_3 of Dmt and 5-CH_3), 3.56−3.94 (m, 4H, 2 × β-CH_2), 4.50 (bm, 2H, CH_2-NH), 4.73−4.93 (m, 4H, 2 × α-CH and CH_2NH), 6.80 (s, 2H, ArH), 6.84 (s, 2H, ArH), 8.89 (br, 1H, CH_2NH), 9.81 (br, 1H, CH_2NH); ^{13}C NMR (pyridine-d_5) δ 18.8 (p, 5-CH_3), 20.6 (p, 2 × CH_3), 31.9 and 32.1 (s × 2, 2 × β-CH_2), 40.1 and 41.4 (s × 2, 2 × CH_2NH), 53.9 (t, 2 × α-CH), 116.2 and 116.3 (t × 2, aryl C), 139.3, 155.8, 157.3, 157.4, 170.0, and 170.5 (q × 6).

3,6-Bis(2′-Dmt-aminoethyl)-5-methyl-2(1*H*)-pyrazinone·2HCl (6′). Anal. Calcd for $C_{31}H_{42}N_6O_5 \cdot 2HCl \cdot 3.5H_2O$: C,52.1; H, 7.19; N, 11.8. Found: 52.3, H, 6.80; N, 12.0. ^{1}H NMR (pyridine-d_5) δ 2.19, 2.43 and 2.53 (3s, 15H, 4 × CH_3 of Dmt and 5-CH_3), 2.71 (br, 2H, CH_2CH_2NH), 2.92−3.14 (m, 2H, CH_2-CH_2NH), 3.21 (br, 1H, CH_2CH_2NH), 3.42 (br, 1H, CH_2CH_2NH), 3.56−3.75 (m, 3H, β-CH_2), 3.75−3.92 (m, 2H, β-CH_2 and

CH_2CH_2NH), 4.10 (br, 1H, CH_2CH_2NH), 4.73 (br, 1H, α-CH), 5.20 (br, 1H, α-CH), 6.76 (s, 2H, ArH), 6.85 (s, 2H, ArH), 8.61 (br, 1H, C_2H_4NH), 9.55 (br, 1H, C_2H_4NH); ^{13}C NMR (pyridine-d_5) δ 18.5 (p, 5-CH_3), 20.7 and 20.8 (p × 2, 4 × CH_3 of Dmt), 30.7 (s, CH_2CH_2NH), 31.3 and 31.4 (s × 2, β-CH_2 and CH_2CH_2-NH), 31.6 (s, β-CH_2), 32.2 (s, CH_2CH_2NH), 38.4 (s, CH_2CH_2-NH), 54.1 and 54.2 (t × 2, 2 × α-CH), 116.1 and 116.2 (t × 2, aryl C), 139.4, 157.3, 157.3, 169.8, and 169.9 (q × 5).

3,6-Bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone·2HCl (7′). Anal. Calcd for $C_{33}H_{46}N_6O_5$·2HCl·$3H_2O$: C, 54.0; H, 7.42; N, 11.5. Found: C, 54.3; H, 7.15; N, 11.8. ^{1}H NMR (pyridine-d_5) δ 1.76 (m, 1H, $CH_2CH_2CH_2NH$), 1.86−2.10 (m, 3H, $CH_2CH_2CH_2NH$), 2.14, 2.43 and 2.45 (3s, 15H, 4 × CH_3 of Dmt and 5-CH_3), 2.64 (m, 1H, $CH_2CH_2CH_2$-NH), 2.73−2.94 (m, 3H, $CH_2CH_2CH_2NH$), 3.16 (m, 1H, CH_2-CH_2CH_2NH), 3.42 (m, 1H, $CH_2CH_2CH_2NH$), 3.52 (m, 1H, $CH_2CH_2CH_2NH$), 3.57−3.73 (m, 3H, $CH_2CH_2CH_2NH$- and β-CH_2), 3.73−3.88 (m, 2H, β-CH_2), 4.76 (dd, J = 4.4 Hz and 6.4 Hz, 1H, α-CH), 4.89 (dd, J = 4.8 Hz and 5.7 Hz, 1H, α-CH), 6.88 (s, 2H, ArH), 6.90 (s, 2H, ArH), 9.20 (br, 1H, C_3H_6NH), 9.57 (br, 1H, C_3H_6NH); ^{13}C NMR (pyridine-d_5) δ 18.3 (p, 5-CH_3), 20.5 (p, 4 × CH_3 of Dmt), 26.2 (s, $CH_2CH_2CH_2NH$), 27.6 (s, $CH_2CH_2CH_2NH$), 28.2 (s, $CH_2CH_2CH_2NH$), 29.7 (s, $CH_2CH_2CH_2NH$), 31.5 and 31.6 (s × 2, 2 × β-CH_2), 38.9 and 39.5 (s × 2, 2 × $CH_2CH_2CH_2NH$), 53.9 (t, 2 × α-CH), 116.0 and 116.1 (t × 2, aryl C), 139.2, 139.3, 156.4, 157.2, 169.6, and 169.9 (q × 6).

3,6-Bis(4′-Dmt-aminobutyl)-5-methyl-2(1*H*)-pyrazinone·2HCl (8′). Anal. Calcd for $C_{35}H_{50}N_6O_5$·2HCl·$2H_2O$: C, 56.5; H, 7.59; N, 11.3. Found: C, 56.3; H, 7.45; N, 11.4. ^{1}H NMR (pyridine-d_5) δ 1.41−1.72 (m, 6H, $CH_2CH_2CH_2CH_2NH$- and 2 × $CH_2CH_2CH_2CH_2NH$), 1.73−1.83 (m, 2H, $CH_2CH_2CH_2CH_2$-NH), 2.25, 2.42 and 2.44 (3s, 15H, 4 × CH_3 of Dmt and 5-CH_3), 2.48−2.65 (m, 2H, $CH_2CH_2CH_2CH_2NH$), 2.86 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2CH_2NH$), 3.14−3.25 (m, 1H, $CH_2CH_2CH_2CH_2$-NH), 3.28−3.52 (m, 3H, $CH_2CH_2CH_2CH_2NH$), 3.63 (m, 2H, β-CH_2), 3.80 (m, 2H, β-CH_2), 4.74 (dd, J = 4.3, 6.9 Hz, 1H, α-CH), 4.86 (dd, J = 4.4, 6.6 Hz, 1H, α-CH), 6.88 (s, 2H, ArH), 6.90 (s, 2H, ArH), 9.14 (br, 1H, C_4H_8NH), 9.32 (br, 1H, C_4H_8NH); ^{13}C NMR (pyridine-d_5) δ 18.7 (p, 5-CH_3), 20.7 and 20.8 (p × 2, 4 × CH_3 of Dmt), 24.6 and 26.2 (s × 2, 2 × CH_2CH_2-CH_2CH_2NH), 28.9 and 29.0 (s × 2, 2 × $CH_2CH_2CH_2CH_2NH$), 29.8 (s, $CH_2CH_2CH_2CH_2NH$), 31.8 (s, 2 × β-CH_2), 32.5 (s, CH_2-$CH_2CH_2CH_2NH$), 39.2 (s, 2 × $CH_2CH_2CH_2CH_2NH$), 53.9 and 54.0 (t × 2, 2 × α-CH), 116.3 (t, aryl C), 139.5, 156.9, 157.4,, 169.8, and 169.9 (q × 5).

Animals. Swiss-Webster male mice (20−25 g) (Taconic, Germantown, NY) were housed under pathogen-free conditions by the Comparative Medicine Branch at NIEHS, an AALAC affiliated institution; food and sterile water were freely available in microisolator cages. Male rats (140−160 g) (Charles River, CD strain, Wilmington, MA) were euthanized with CO_2 and decapitated according to approved protocols by the ACUC at NIEHS. Guinea pigs and mice for use in functional bioassays in vitro were obtained by Tohoku Pharmaceutical University and treated humanely following prescribed protocols.

Determination of Analgesia in Vivo. Tail-flick and hot-plate latencies in mice measured the effect of icv, sc, and po administration of morphine or **7′** [3,6-bis(3′-Dmt-aminopropyl)-5-methyl-2(1*H*)-pyrazinone] as follows. Compounds dissolved in physiological saline (pH 7) were injected into mice icv or sc, or dissolved in water for po administration. Tail-flick tests (spinal analgesia) were performed by applying radiant heat to the dorsal surface of the tail (Columbus Instruments, Columbus, OH). The latency period for removal of the tail, defined as the tail-flick latency (TFL), was adjusted between 2 and 3 s (pre-response time), and a cutoff time was set at 8 s to avoid external heat-related damage. The analgesic response was measured beginning at 10, 15, or 30 min following icv, sc, or po administration of either morphine or **7′**, respectively, and testing was terminated when TFL approached the pre-response time. The AUC (area under the curve) was obtained by plotting the response time (s) versus time (min) after administration of the compound. Morphine was used as a positive control, and naloxone was used to antagonize the effect.[20]

For the hot-plate test (supraspinal analgesia), mice were set on an electrically heated plate at 55 ± 0.1 °C (IITC Inc., Woodland Hills, CA) following the same drug injection paradigm above. Hot-plate latency (HPL) was measured as the interval between the placement of the mice on the hot plate and observing movements consisting of either jumping, licking, or shaking their hind paws with a baseline latency of 15 s and maximal cut off time of 30 s. The area under the curve (AUC) is derived from data based on the response (mean ± SE) of five to seven mice per time point. The analgesic effect of morphine and **7′** are relative to saline or water.

Statistical Analysis (ANOVA). Asterisks denote values that are significantly different from the controls. Mice treated with morphine or **7′** are relative to saline or water controls (*, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$) or both substances relative naloxone treatment (###, $p < 0.001$).

Functional Bioassays in Isolated Tissue Preparations. The myenteric plexus longitudinal muscle preparations (2−3 cm segments) were surgically removed from the small intestine of guinea pigs (GPI) and used to measure μ-opioid receptor agonism. A single mouse vas deferens (MVD), containing primarily δ-opioid receptors, was used to determine agonism or antagonism for δ-opioid receptor activity. The isolated tissues were suspended in organ baths containing balanced salt solutions in a physiological buffer, pH 7.5. Agonists were tested for the inhibition of electrically evoked contraction and expressed as IC_{50} (nM) obtained from the concentration−response curves in comparison to dermorphin with GPI and deltorphin C for MVD. IC_{50} values represent the mean ± SE of five to seven separate tissue samples. Antagonism is listed as the pA_2, which is the negative log of the molar concentration required to double the agonist concentration to achieve the original response.

Competitive Opioid Receptor Binding Assays. Opioid receptor binding affinities were determined under equilibrium conditions (2.5 h at 22 °C) in a competition assay using rat brain P_2 synaptosomes membranes.[20,29,35] The synaptosomes were preincubated to remove endogenous opioids, extensively washed in ice cold buffer containing protease inhibitor, resuspended in buffered 20% glycerol, and stored at −80 °C.[37] δ- and μ-Opioid receptors were radiolabeled with [^{3}H]DPDPE (45 Ci/mmol, Dupont-NEN, Boston, MA) and [^{3}H]DAMGO (50 Ci/mmol, Amersham Biosciences, Arlington, IL), respectively. Excess unlabeled peptide (2 μM) established nonspecific binding background. Radiolabeled membranes were rapidly filtered on Whatman GF/C glass fiber filters presoaked in 0.1% polyethylenimine to enhance the signal-to-noise ratio, washed with ice-cold BSA buffer, and dried at 75 °C for 60 min, and radioactivity was determined using CytoScint (ICN, Costa Mesa, CA). The analogues were analyzed in duplicate assays using 5−8 dosages and 3−5 independent repetitions (n values noted in parentheses in Table 1) with different synaptosomal preparations to ensure statistical significance and listed as mean ± SE (Prism 3.03). The affinity constants (K_i) were calculated according to Cheng and Prusoff.[40]

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Supporting Information Available: Yield, $[\alpha]^{25}_D$, t_R and mass spectrum analysis of compounds 3,6-bis[Tyr-NH$(CH_2)_n$]-5-methyl-2(1*H*)pyrazinone (**1′**−**3′**) and 3,6-bis[Dmt-NH$(CH_2)_n$]-5-methyl-2(1*H*)pyrazinone (**5′**−**8′**) as well as the yield, mp, $[\alpha]^{25}_D$, R_f, and elemental analyses of Boc-X(Z)-CH_2Cl (**1a**−**c**) and Boc-X(Z)-X(Z)-CH_2Cl (**2a**−**c**). This material is available free of charge via the Internet at http://pubs.acs.org.

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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; AcOH, acetic acid; BBB, blood−brain barrier; Boc,

tert-butyloxycarbonyl; BOP, benzoltriazol-1-yloxytris(dimethylamino)phosphonium hexafluorphosphate; *n*-BuOH, *n*-butanol; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; DALDA, Tyr-D-Arg-Phe-Lys-NH_2; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, *cyclic*[D-Pen^{2,5}]enkephalin; EtOH, ethanol; GPI, guinea-pig ileum; HPLC, high performance liquid chromatography; IC_{50}, concentration required for 50% inhibition of the electrically induced contraction in muscle derived from a dose−response curve; MeOH, methanol; NMM, 4-methylmorpholine; ^{1}H NMR, proton nuclear magnetic resonance spectrometry; MVD, mouse vas deferens; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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