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Structural Studies of [2′,6′-Dimethyl-L-tyrosine[1]]endomorphin-2 Analogues: Enhanced Activity and *cis* Orientation of the Dmt-Pro Amide Bond

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Abstract—Analogues of endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2) (**1**) were designed to examine the importance of each residue on μ-opioid receptor interaction. Replacement of Tyr[1] by 2′,6′-dimethyl-L-tyrosine (Dmt) (**9**–**12**) exerted profound effects: [Dmt[1]]EM-2 (**9**) elevated μ-opioid affinity 4.6-fold ($K_i\mu = 0.15$ nM) yet selectivity fell 330-fold as δ-affinity rose ($K_i\delta = 28.2$ nM). This simultaneous increased μ- and δ-receptor bioactivities resulted in dual agonism ($IC_{50} = 0.07$ and 1.87 nM, respectively). While substitution of Phe[4] by a phenethyl group (**4**) decreased μ affinity ($K_i\mu = 13.3$ nM), the same derivative containing Dmt (**12**) was comparable to EM-2 but also acquired weak δ antagonism ($pA_2 = 7.05$). ^{1}H NMR spectroscopy revealed a *trans* configuration (1:2 to 1:3, *cis*/*trans*) in the Tyr-Pro amide bond, but a *cis* configuration (5:3 to 13:7, *cis*/*trans*) with Dmt-Pro analogues.

Introduction

The G-protein coupled δ-, κ- and μ-opioid receptors are located throughout the central nervous system and peripheral tissues of various mammalian species, such as in mouse vas deferens, guinea pig ileum, rabbit jejunum, as well as in brain tissue of all vertebrates.[1,2] These receptors and their endogenous ligands, the enkephalins,[3] endorphins,[4,5] dynorphins,[6] and endomorphins[7,8] appear to be involved in the modulation and perception of pain. The endogenous neuropeptides, endomorphin-1 (EM-1: Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2), were initially isolated from bovine brain[7] and later from human brain cortex.[8] These endomorphins exhibited the highest affinity for the μ-opioid receptor and extraordinarily high selectivity relative to the δ- and κ-opioid receptor systems of all known opioid substances.[7] Furthermore, these tetrapeptides differ structurally from previously known endogenous opioid peptides, which contain the Tyr[1]-Gly[2] N-terminal sequence, by the presence of Pro[2] comparable to the weakly active μ-opioid casomorphin.[9] Since Pro residues tend to form β bends in protein, this characteristic may contribute to the unusual spectrum of activity we report with these endomorphin derivatives.

In order to develop novel analgesics mimicking the endomorphins in lieu of morphine or other opioids, we directed our attention to study the structure–activity relationships of specific EM-2 analogues. One aspect involved the influence of 2′,6′-dimethyl-L-tyrosine (Dmt) in lieu of the natural N-terminal residue Tyr, since it is known that Dmt markedly increases binding affinity and bioactivity of numerous opioid peptide agonists and antagonists.[10–12] Therefore, we synthesized a series of [Dmt[1]]EM-2 analogues and examined their binding affinity, functional bioactivity, and solution conformation by ^{1}H NMR spectroscopy and circular dichroism (CD).

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Results and Discussion

Optically pure 2′,6′-dimethyl-L-tyrosine (Dmt) was prepared as previously described.[13] Optical purity (98%) was ascertained both by HPLC using a chiral column [CROWNPAC CR(+)] and by the reaction with D-amino acid and L-amino acid oxidases, followed by amino acid analysis.[14] Boc-Dmt-OH was prepared in the usual manner. Peptides, except for **1** and **2**, were synthesized by a solution method. All final products of the peptide analogues were purified by semi-preparative reverse-phase HPLC. Table 1 summarizes the analytical data of compounds **3**–**12**. Each compound exhibited a single peak on analytical HPLC with a unique retention time. Analysis by MALDI-TOF mass spectrometry (MS) and by HPLC revealed that all compounds were the desired product with greater than 98% purity.

Alanine scan

The binding affinity for each peptide analogue was examined for δ- and μ-opioid receptors using rat brain synaptosomes (see Experimental) and summarized in Table 2. In compounds **5**–**8**, each constituent amino acid was replaced with an Ala residue, which decreased the binding affinity toward the μ-opioid receptor. The loss of Tyr in [Ala[1]]EM-2 (**5**) leads to a completely inactive analogue substantiating the importance of Tyr[1]; the OH function is considered an important component in anchoring the ligand within opioid receptors although it should be noted that an enkephalin analogue lacking tyrosyl exhibits high μ-receptor affinity.[15] Furthermore, the μ receptor prefers L-Tyr, since [D-Tyr[1]]EM-2 had moderate μ affinity ($K_i = 32.1$ nM).[16] Substitution of Pro[2] by Ala (**6**) was equally deleterious; however, D-Pro[2] partially improved μ affinity.[16] In addition, [D-Pro[2]]EM-2 was more potent than EM-2 in increasing tail flick latencies with a longer duration of action,[17] apparently because it resisted enzymatic degradation by dipeptidyl peptidase IV.[18–22] In contrast to EM-1, but similar to [D-Pro[2]]EM-2, [β-(*R*)-Pro[2]]EM-1 was 34-fold more potent than EM-1.[23] In this case, β-(*R*)-Pro might maintain the same spatial disposition as natural Pro and offer greater stability as well.

Table 1. Analytical data of synthetic peptides

Compd		TOF-MS		HPLC t_R (min)	$[\alpha]_D^{20}$	
		Calcd	Found		H_2O	
1	YPFF-NH_2[16]				−34.2°	*c* 1.0
2	YPF-NH_2[16]				−20.6°	*c* 0.67
3	YP-NH_2	$[M+H]^+$	278.3	278.1	7.7[a]	−21.8°	*c* 3.22
4	YPF-NH-C_2H_4-Ph	$[M+H]^+$	529.5	529.1	20.2[b]	−21.6°	*c* 1.0
5	APFF-NH_2	$[M+H]^+$	479.6	480.7	19.2[b]	−51.3°	*c* 1.0
6	YAFF-NH_2	$[M+H]^+$	546.6	546.9	29.7[c]	−21.3°	*c* 1.0
7	YPAF-NH_2	$[M+H]^+$	497.6	497.0	22.5[c]	−44.0°	*c* 1.0
8	YPFA-NH_2	$[M+H]^+$	497.6	498.1	25.1[b]	−23.2°	*c* 1.0
9	Dmt-PFF-NH_2	$[M+H]^+$	600.7	600.5	19.1[b]	+13.3°	*c* 0.87
10	Dmt-PF-NH_2	$[M+H]^+$	453.5	453.1	14.6[b]	+28.9°	*c* 0.93
11	Dmt-Pro-NH_2	$[M+H]^+$	306.4	306.5	8.4[d]	+78.7°	*c* 0.38
12	Dmt-PF-NH-C_2H_4-Ph	$[M+H]^+$	556.9	556.9	22.5[b]	−39.6°	*c* 1.0

Y, tyrosine; P, proline; F, phenylalanine; A, alanine.
[a]HPLC A/B = 90:10 to A/B = 90:10 for 5 min, A/B = 90:10 to A/B = 50:50 for 20 min, A/B = 50:50 to A/B = 10:90 for 5 min.
[b]A/B = 90:10 to A/B = 40:60 for 25 min, A/B = 40:60 to A/B = 10:90 for 5 min.
[c]A/B = 90:10 to A/B = 50:50 for 40 min, A/B = 50:50 to A/B = 10:90 for 5 min.
[d]A/B = 90:10 to A/B = 90:10 for 5 min, A/B = 90:10 to A/B = 60:40 for 15 min, A/B = 60:40 to A/B = 10:90 for 5 min.

Table 2. Receptor binding data of endomorphin-2 analogues

Compd			[^{3}H]DAMGO			[^{3}H]DPDPE			Binding selectivity
			$K_i\mu$ (nM)		R.P.	$K_i\delta$ (nM)		R.P.	$K_i\delta/K_i\mu$
C-term deletion analogues	**1**	YPFF-NH_2[7]	0.69 ± 0.16		1	9230 ± 200		1	13,400
	2	YPF-NH_2[16]	46.3 ± 3.8		0.015	15,900 ± 2300		0.58	343
	3	YP-NH_2	26107 ± 1339	(3)	2.6×10^{-6}	40,126 ± 1115	(3)	0.23	1.5
	4	YPF-NH-C_2H_4-Ph	13.3 ± 2.6	(3)	0.052	4310 ± 635	(3)	2.1	324
Ala scanning mutants	**5**	APFF-NH_2	16750 ± 6242	(3)	4.1×10^{-6}	4722 ± 650	(4)	2.0	0.28
	6	YAFF-NH_2	3272 ± 283	(3)	2.1×10^{-5}	3466 ± 710	(4)	2.7	1.06
	7	YPAF-NH_2	257.4 ± 9.7	(3)	0.0027	2658 ± 738	(4)	3.5	10
	8	YPFA-NH_2	65.7 ± 23	(3)	0.011	3740 ± 662	(4)	2.5	57
Dmt containing analogues	**9**	Dmt-PFF-NH_2	0.15 ± 0.04	(3)	4.6	28.2 ± 8.1	(3)	330	188
	10	Dmt-PF-NH_2	0.12 ± 0.09	(3)	5.8	53.2 ± 6.1	(3)	170	443
	11	Dmt-Pro-NH_2	41.7 ± 1.2	(3)	0.017	734.9 ± 97	(4)	12.6	17.6
	12	Dmt-PF-NH-C_2H_4-Ph	0.51 ± 0.15	(3)	1.4	18.0 ± 2.5	(3)	510	35

Y, tyrosine; P, proline; F, phenylalanine; A, alanine.
Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DPDPE (δ-selective) from rat brain membrane synaptosomes. K_i values are the mean ± SE. The potency is relative to that of EM-2.

C-Terminal deletion analogues

As shown in Figure 1, endomorphin-2 analogues were designed and synthesized. The loss of Phe[4] (**2**) and Phe[3]–Phe[4] (**3**) drastically reduced binding to μ-opioid receptors by orders of magnitude (Table 2). Replacement of Phe[4] by a phenethyl group (**4**) yielded a peptide with about 5% of the μ-receptor affinity of EM-2, demonstrating that not only the second Phe residue is critical, but also that the physical separation between Phe[3] and the third aromatic center appears important for receptor interaction.

Dmt-containing analogues

The exchange of Tyr[1] in opioid peptides for Dmt resulted in a marked increase in receptor affinities and bioactivity.[10–12] Substitution of Dmt[1] in EM-2 and in C-terminal deletion analogues (**9**–**12**; see Fig. 1) profoundly affected all the subsequently measured parameters (Tables 2 and 3). In each case, Dmt[1] enhanced the affinities from about 5- to several hundred-fold relative to their Tyr cognates (**1**–**4**) for both μ and δ receptors. The functional bioactivity of [Dmt[1]]EM-2 (**9**) (Table 3) revealed an increase in μ- and δ-bioactivities by 82- and 184-fold greater than EM-2, respectively. While Dmt-Pro-Phe-NH_2 (**10**) was about twice as active as EM-2 (**1**), deletion of Phe[3] (**11**) yielded a marked drop in both bioactivities. In contrast, however, Dmt-Pro-Phe-NH-phenethyl (**12**) had an agonist potency equivalent to EM-2, but differs with the appearance of weak δ antagonist activity ($pA_2 = 7.05$). In keeping with the low affinity of Dmt-Pro-NH_2 (**11**), about 2% relative to EM-2 (Table 2), it was essentially biologically inactive (Table 3). The high affinity and pharmacological potency of Dmt in most analogues (except **11**) might be partly attributable to resistance towards proteolysis[24] at the N-terminus, or to enhanced receptivity by either influencing the bioactive or the molecular conformation of the peptide that interacts with the μ-receptor ligand-binding domain.

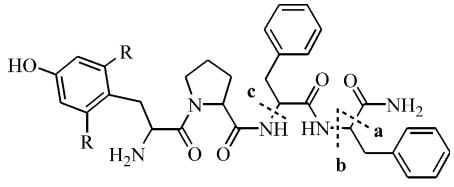

Figure 1. Structures of endomorphin 2 analogues.

^{1}H NMR spectrometry

Table 4 summarizes the results of analyses of the ^{1}H NMR spectra of EM-2 analogues in DMSO-d_6. The *cis*/*trans* ratios, derived from a conformational equilibrium around the Tyr-Pro amide bond and Dmt-Pro amide bond, and calculated by integration values of ^{1}H NMR, clearly demonstrated differences between the Tyr- and Dmt-containing analogues (Table 4). Published observations with EM-1[25] and EM-2[26] revealed that they contain a *trans* configuration with *cis*/*trans* ratios of 1:3 and 1:2, respectively. The *cis*/*trans* ratio of Tyr-Pro-Phe-NH_2 (**2**) was also 1:2 (Table 4). On the other hand, Dmt-containing peptides (**9**–**12**) existed predominantly in the *cis* configuration, including the C-terminally extended analogue (**12**) (Table 4). The *cis*/*trans* rotamers around the Dmt-Pro amide bond were further identified by NOESY observations. NOE cross peaks between Dmt CαH and Pro CαH, and Dmt-dimethyl H and Pro CαH, defining a *cis* conformation in the peptides are summarized in Figure 2. The Pro CαH and Dmt CαH of the *cis* isomer were observed at higher fields (3.6 and 2.9 ppm, respectively) than those of *trans* isomer (4.1 and 4.3 ppm, respectively). For the *trans* isomer, NOE cross peaks between Dmt-dimethyl H and Pro CδH, and Dmt CαH and Pro CδH were observed as shown in Figure 2. Pro $C\delta H_A$ of the *trans* isomer was measured at a higher field (2.3 ppm) than that of the *cis* isomer (3.2 ppm). The CH_3 groups of Dmt appear to sterically hinder the adoption of the *trans* configuration at the Dmt-Pro peptide bond, thereby pushing the conformational equilibrium towards a *cis* configuration (Table 4). In each *cis* and *trans* isomer, NOE cross peaks between the N- and C-terminal residues were not observed.

Based on NMR analyses, Podlogar et al.[25] proposed an extended conformation of *trans* EM-1 as the potential bioactive form. However, a study of Tyr-Xaa[Ψ^{CH_3,CH_3} Pro]EM-2 analogues by Keller et al.[27] found predominately a *cis* conformation (>98%). Even though both compounds were μ agonists with high μ affinities, the data indicate that *cis* and *trans* conformations can exist around the Tyr-Pro or Dmt-Pro peptide bond in EM-2. Our results, showing a preference for *cis* conformation in the Dmt-Pro sites of analogues, represent another variation to the data of Podlogar et al.[25] Furthermore, with the endomorphin-related morphiceptin analogues, Yamasaki et al.[28] demonstrated that [Val[4]]-morphiceptin (Tyr-Pro-Phe-Val-NH_2) and [D-Val[4]]morphiceptin containing *cis*-2-aminocyclopentane

Table 3. In vitro bioactivity data of [Dmt[1]]EM-2 and various analogues

Compd		GPI assay[a]		MVD assay[b]		pA_2 value
		IC_{50} (nM)	R.P.	IC_{50} (nM)	R.P.	versus Deltorphin-II
1	YPFF-NH_2	5.79±0.4	1	344±93	1	—
9	Dmt-PFF-NH_2	0.07±0.02	82.7	1.87±0.61	184	—
10	Dmt-PF-NH_2	2.33±0.49	2.5	113±35	3.0	—
11	Dmt-Pro-NH_2	>10,000	—	>10,000	—	—
12	Dmt-PF-NH-C_2H_4-Ph	5.03±0.99	1.2	>10,000	—	7.05

[a]Values are the mean of seven experiments ±SE.
[b]Values are the mean of six experiments±SE. The potency is related to that of EM-2.

Table 4. The *cis*/*trans* ratios of [Dmt[1]]EM-2 and various analogues in DMSO

Compd		*cis*/*trans*[a]
	EM-1	1:3[25]
1	EM-2	1:2[26]
2	YPF-NH_2	1:2
9	[Dmt[1]]EM-2	7:3
10	Dmt-PF-NH_2	13:7
11	Dmt-Pro-NH_2	5:3
12	Dmt-PF-NH-C_2H_4-Ph	13:7

[a]Determined by integration of ^{1}H NMR data (see Experimental methods).

carboxylic acid in place of Pro[2] interacted only with μ-receptors, while the *trans*-2-aminocyclopentane carboxylic acid substitution analogues were completely inactive at both μ- and δ-receptor sites.[28]

Circular dichroism

The CD spectra of the Tyr analogues of EM-2 (**1**, **2**, **4**, **8**) and their Dmt-derivatives (**9**, **10**, **12**) are shown in Figure 3A and B, respectively. The EM-2 analogues exhibited negative maxima (213.1–215.4 nm), while the Dmt-derivatives had negative maxima near 203 nm, which were stronger than those corresponding to the Tyr-containing peptides. These results confirm that the Dmt-containing analogues adopt a solution conformation different than other EM-2 analogues,[16] and further suggests that the Dmt residue affects the solution conformation of the peptide as suggested by the NOE data.

Conclusions

The data clearly indicate that Dmt markedly enhanced the biological properties of EM-2 analogues and as seen in its effects on the Dmt-Tic pharmacophore family of δ antagonists.[10–12] These changes in biological activity may be mediated by difference in the configuration about the Xaa-Pro amide bond, as both NMR and CD data of Dmt-containing peptides show the preference for *cis* conformation and different solution conformation than Tyr-containing peptides, respectively. Together, the bioassays and structural data suggest that conformational features of the peptide may be responsible for different activities and selectivities in peptide-receptor recognition. The methyl groups on the Tyr-amine ring of Dmt undoubtedly play a dominant role in the interaction within the opioid binding domain by either direct interaction with hydrophobic side-chains of receptor residues to align the critical OH group, or by stabilization of a favored *cis* conformer in solution prior to and during binding.[11,12] Even though the μ affinity of [Dmt[1]]EM-2 rose substantially, the interaction with the δ receptor was elevated to an even greater degree (Table 2), producing peptides with unusual and high biological effectiveness. These remarkable changes are comparable to those observed in unrelated opioid peptides, such as enkephalin[24] and an analogue of deltorphin,[29] dynorphin A (1-11),[30] as well as the Dmt-Tic-R,[31] and Dmt-Tic-R-R′ family of peptides.[32] Thus, these data clearly indicate that Dmt as an N-terminal residue in opioid peptides has the potential to readily contribute to the development of novel types of bioactive opioidmimetics for therapeutics and clinical applications.[11,12]

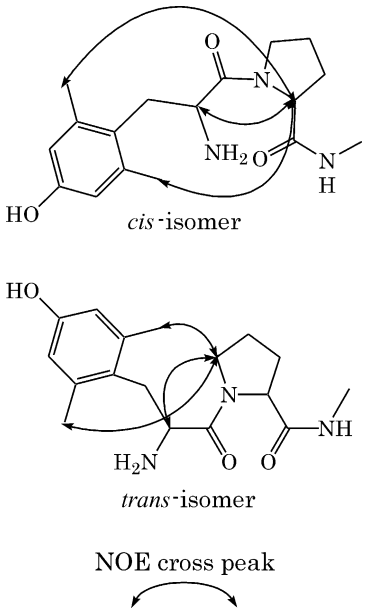

Figure 2. Characteristic NOE pairs of *cis*/*trans* configuration at the Dmt[1]-Pro[2] peptide bond.

Experimental

General procedures

Dmt was prepared according to the method of Dygos et al.,[13] and its chirality was assessed by HPLC and amino acid analysis. Peptides were synthesized using solution methods. Peptides were coupled by a mixed anhydride method using isobutyl chloroformate (IBCF), azide or using PyBop. The Boc group was used for N-terminal protection. Deprotection was performed using hydrogen chloride in dioxane (HCl/dioxane) or TFA. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Mass spectra were measured with MALDI-TOF mass spectrometry (KOMPACT MALDI IV mass spectra, Kratos Analytical). On TLC (Kieselgel G Merck), R_f^1 and R_f^2 values refer to the systems of $CHCl_3$, MeOH and AcOH (90:8:2) and $CHCl_3$, MeOH and H_2O (89:10:1), respectively. These peptides exhibited greater than 98% purity by analytical HPLC (Waters Model 600 E) using a Cosmosil 5C18-AR column (4.6×250 mm), absorbance monitored at 220 nm, and run in the following solvents: A, 0.05% TFA in H_2O; B, 0.05% TFA in MeCN. Purified peptides were characterized by mass spectrometry. Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry* **1996**, *5*, 2485; **1966**, *6*, 362l; **1972**, *11*, 1726. The customary L-configuration for amino acid residues is omitted. The following additional abbreviations are used: AcOEt, ethyl acetate; AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; CD, circular

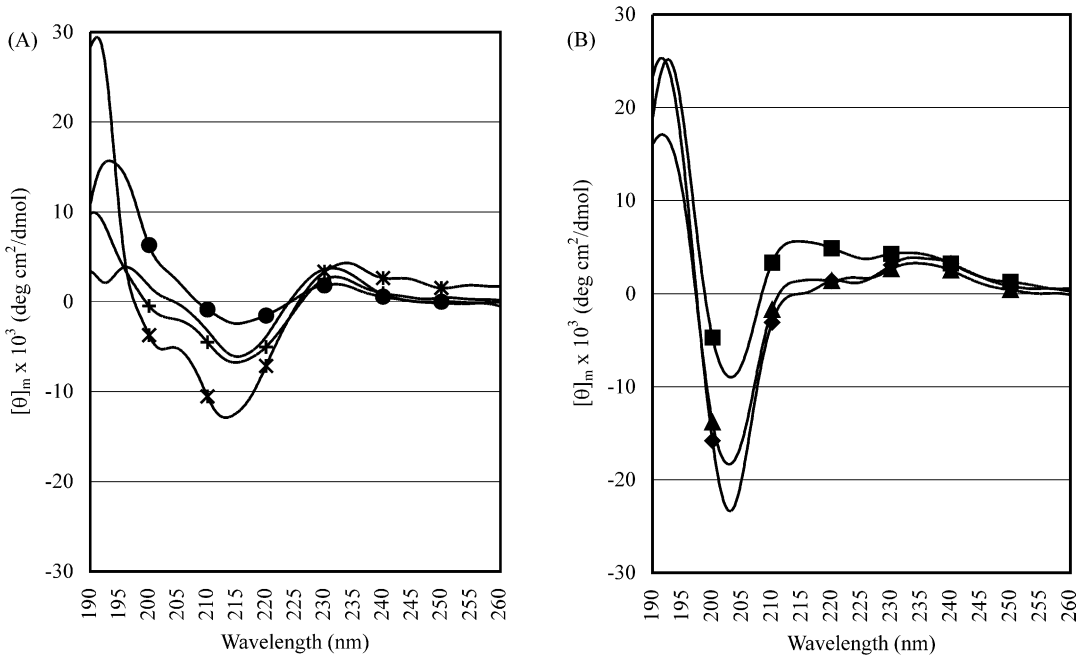

Figure 3. (A) CD spectra of EM-2 and its analogues in neat TFE. 1.31 mM EM-2 (**1**) (−), 1.47 mM Tyr-Pro-Phe-NH_2 (**2**) (●), 0.81 mM Tyr-Pro-Phe-NH-Phenethyl (**4**) (+). and 1.24 mM Tyr-Pro-Phe-Ala-NH_2 (**8**) (*). (B) 1.0 mM [Dmt^1]EM-2 (**9**) (▲), 1.45 mM Dmt-Pro-Phe-NH_2 (**10**) (■) and 0.79 mM Dmt-Pro-Phe-NH-Phenethyl (**12**) (◆).

dichroism; DAMGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, Tyr-*cyclo*(D-Pen-Gly-Phe-D-Pen); EDTA, ethylenediamine tetraacetic acid; EM-2, endomorphin-2; Et_3N, triethylamine; Et_2O, diethylether; GPI, guinea pig ileum; IBCF, isobutyl chloroformate; MeOH, methanol; MeCN, acetonitrile; MVD, mouse vas deference; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser and exchange spectroscopy; Ph, phenyl; PyBop, benzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TFE, 2,2,2,-trifluoroethanol; THF, tetrahydrofuran.

Boc-Tyr-Pro-NH_2. To a solution of H-Pro-NH_2·HCl [prepared from Boc-Pro-NH_2 (300 mg, 1.4 mmol) and 6.9 N HCl/dioxane (2.0 mL, 1.4 mmol) in the usual way] in DMF (30 mL) containing DIPEA (0.49 mL, 2.8 mmol), Boc-Tyr-OH (422 mg, 1.5 mmol) and PyBop (780 mg, 1.5 mmol) were added. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 462 mg (83.5%), mp 118–127°C, $R_f^1 = 0.48$, $[\alpha]_D^{20}$ −48.2 (*c* 1.0, MeOH), Anal. calcd for $C_{19}H_{27}N_3O_5 \cdot 1/3H_2O$: C, 59.9; H, 7.42; N, 10.1. Found: C, 59.5; H, 7.30; N, 10.0, ^{1}H NMR ($CDCl_3$) δ: 7.66 (1H, brs, NH), 6.89 (2H, d, Ar–H), 6.53 (2H, d, Ar–H), 5.77–6.02 (2H, m, $CONH_2$), 4.33–4.92 (2H, m, αCH of Tyr and Pro), 2.67–3.66 (4H, m, βCH_2 of Tyr, and δCH_2 of Pro), 1.39–2.25 (13H, m, Bu^t, γCH_2 and βCH_2 of Pro).

HCl·H-Tyr-Pro-NH_2 (3). Boc-Tyr-Pro-NH_2 (185 mg, 0.49 mmol) was treated with 8.0 N HCl/dioxane (0.6 mL, 4.9 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was collected by centrifugation, dried over KOH pellets and purified by semipreparative reverse-phase HPLC. The purified peptide was lyophilized from 1 N HCl to give amorphous powder: yield 120 mg (78.3%), MH^+ 278.1, $[\alpha]_D^{20}$ −21.8 (*c* 3.2, H_2O), Anal. calcd for $C_{14}H_{19}N_3O_3 \cdot HCl \cdot H_2O$: C, 50.7; H, 6.68; N, 12.2. Found: C, 50.5; H, 6.92; N, 12.1.

Boc-Phe-NH-Phenethyl. A mixed anhydride [prepared from Boc-Phe-OH (3.0 g, 11.4 mmol), IBCF (1.66 mL, 12.8 mmol) and NMM (1.38 mL, 12.8 mmol) in the usual way] in THF (30 mL) was added to a solution of phenethylamine (1.61 mL, 12.8 mmol) in DMF (30 mL) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 4.1 g (97.7%), mp 133–135 °C, $R_f^1 = 0.93$, $[\alpha]_D^{20}$ −0.052 (*c* 1.0, MeOH), Anal. calcd for $C_{22}H_{28}N_2O_3$: C, 71.2; H, 7.66; N, 7.60. Found: C, 71.6; H, 7.79; N, 7.66, ^{1}H NMR ($CDCl_3$) δ: 7.00–7.30 (10H, m, Ar–H), 5.91 (1H, br, NHC_2H_4), 5.11 (1H, br, 1H, αNH), 4.26 (1H, br q, $J = 7.0$ Hz, αCH), 3.31–3.50 (2H, m, CH_2CH_2NH), 3.01 (2H, d, $J = 6.5$ Hz, βCH_2), 2.53–2.75 (2H, m, CH_2CH_2NH), 1.38 (9H, s, Bu^t).

Boc-Pro-Phe-NH-Phenethyl. A mixed anhydride [prepared from Boc-Pro-OH (1.3 g, 5.98 mmol), IBCF (0.78 mL, 5.98 mmol) and Et_3N (0.65 mL, 5.98 mmol) in the

usual way] in THF (30 mL) was added to a solution of H-Phe-NH-phenethyl·HCl [prepared from Boc-Phe-NH-phenethyl (2.0 g, 5.43 mmol) and 6.9 N HCl/dioxane (7.3 mL, 54.3 mmol) in the usual way] in DMF (30 mL) containing NMM (0.65 mL, 5.98 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 1.3 g (51.1%), mp 157–160 °C, $R_f^1 = 0.66$, $[\alpha]_D^{20}$ −59.7 (*c* 1.0, MeOH), Anal. calcd for $C_{27}H_{35}N_3O_4$: C, 69.7; H, 7.58; N, 9.03. Found: C, 69.7; H, 7.62; N, 9.10, ^{1}H NMR ($CDCl_3$) δ: 7.10–7.30 (10H, m, Ar–H), 6.71 (1H, br, NH–C_2H_4), 6.48 (1H, br, NH of Phe), 4.68 (1H, br, αCH of Phe), 3.05–3.51 (6H, m, δCH_2 of Pro, and NH–C_2H_4), 4.14 (1H, br, αCH of Pro), 2.00–2.11 (2H, m, βCH_2 of Pro), 1.63–1.89 (2H, m, γCH_2 of Pro), 1.37 (9H, s, Bu^t). ^{13}C NMR ($CDCl_3$) δ: 171.6 (q), 170.4 (q), 155.7 (q), 139.0 (q), 136.6 (q), 129.3 (t, Ar), 128.7 (t, Ar), 128.6 (t, Ar), 128.5 (t, Ar), 127.1 (t, Ar), 126.4 (t, Ar), 80.8 (q, Bu^t), 60.9 (t, Pro α), 53.2 (t, Phe α), 47.2 (s, Pro δ), 41.1 (s, NH-CH_2–CH_2–), 35.6 (s, NH–CH_2–CH_2–), 37.3 (s, Phe β), 29.3 (s, Pro β), 28.3 (p, Bu^t), 24.5 (s, Pro γ).

Boc-Tyr-Pro-Phe-NH-Phenethyl. Boc-Tyr-$NHNH_2$ (320 mg, 1.07 mmol) was dissolved in DMF (10 mL). Under cooling with ice–NaCl, 6.9 N HCl/dioxane (0.29 mL, 2.14 mmol) and isoamylnitrite (0.16 mL, 1.18 mmol) were added. Stirring was continued for 10 min, when the hydrazine test became negative. The solution, after neutralization with NMM (0.24 mL, 2.14 mmol), was combinated with a solution of H-Pro-Phe-NH-phenethyl·HCl [prepared from Boc-Pro-Phe-NH-phenethyl (500 mg, 1.07 mmol) and 6.9 N HCl/dioxane (1.45 mL, 10.7 mmol) in the usual manner] and NMM (0.35 mL, 3.21 mmol) in DMF (20 mL). After the reaction mixture was stirred at 4 °C overnight, the solvent was evaporated and the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Ether was added to the residue to form crystals, which were collected by filtration to yield 381 mg (56.0%), mp 203–207 °C $R_f^1 = 0.39$, $[\alpha]_D^{20}$ −28.6 (*c* 1.0, DMSO), Anal. calcd for $C_{36}H_{44}N_4O_6 \cdot 1/2H_2O$: C, 67.8; H, 7.11; N, 8.78. Found: C, 67.7; H, 7.05; N, 9.30, ^{1}H NMR (DMSO-d_6) δ: 9.15 (1H, brs, Ar–OH), 7.85 (1H, br, NHC_2H_4), 7.76 (1H, d, $J = 8.1$ Hz, NH of Phe), 7.05–7.33 (12H, m, Ar–H), 6.98 (1H, d, $J = 8.3$ Hz, NH of Tyr), 6.65 (2H, d, $J = 8.2$ Hz, Ar–H), 4.41 (1H, br, αCH of Phe), 4.17 (2H, br, αCH of Tyr, and αCH of Pro), 3.59 (1H, br, δCH_B of Pro), 3.51 (1H, br, δCH_A of Pro), 3.08–3.41 (2H, m, NHCH_2CH_2), 3.01 (1H, dd, $J = 13.7$, 5.3 Hz, βCH_B of Phe), 2.60–2.90 (5H, m, βCH_2 of Tyr, βCH_A of Phe, and NHCH$_2$CH_2), 1.65-1.98 (4H, m, βCH_2, and γCH_2 of Pro), 1.30 (9H, s, Bu^t). ^{13}C NMR (DMSO-d_6) δ: 177.3 (q), 171.1 (q), 171.0 (q), 155.7 (q), 155.2 (q), 1139.2 (q), 137.8 (q), 130.1 (t, Ar), 129.0 (t, Ar), 128.5 (t, Ar), 128.2 (t, Ar), 127.9 (t, Ar), 126.1 (t, Ar), 126.0 (t, Ar), 114.8 (t, Ar), 77.9 (q, Bu^t), 59.8 (t, Pro α), 54.0 (t, Tyr α), 53.9 (t, Phe α), 46.7 (s, Pro δ), 40.2 (s, NH–CH_2–CH_2–), 37.2 (s, Phe β), 35.5 (s, Tyr β), 34.9 (s, NH–CH_2–CH_2–), 28.5 (s, Pro β), 28.1 (p, Bu^t), 24.4 (s, Pro γ).

HCl·H-Tyr-Pro-Phe-NH-Phenethyl (4). Boc-Tyr-Pro-Phe-NH-phenethyl (242 mg, 0.38 mmol) was treated with 8.0 N HCl/dioxane (0.48 mL, 3.8 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 220 mg (92%), MH^+ 529.1, $[\alpha]_D^{20}$ +43.0 (*c* 1.0, H_2O), Anal. calcd for $C_{31}H_{36}N_4O_4 \cdot HCl \cdot 1/2H_2O$: C, 64.9; H, 6.67; N, 9.75. Found: C, 64.7; H, 6.72; N, 9.97.

Boc-Phe-Phe-NH_2. A mixed anhydride [prepared from Boc-Phe-OH (1.1 g, 4.17 mmol), IBCF (0.52 mL, 4.17 mmol) and Et_3N (0.58 mL, 4.17 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Phe-NH_2·HCl [prepared from Boc-Phe-NH_2 (1.0 g, 3.79 mmol) and 7.2 N HCl/dioxane (5.1 mL, 37.89 mmol) in the usual way] in DMF (30 mL) containing Et_3N (0.58 mL, 4.17 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 1.10 g (70.5%), mp 218–220 °C, $R_f^1 = 0.59$, $[\alpha]_D^{20}$ −18.8 (*c* 1.0, MeOH), Anal. calcd for $C_{23}H_{29}N_3O_4$: C, 67.1; H, 7.10; N, 10.2. Found: C, 67.3; H, 7.21; N, 10.5, ^{1}H NMR (DMSO-d_6) δ: 8.01 (1H, d, $J = 8.3$ Hz, NH of Phe^2), 7.44 (1H, brs, $CONH_B$), 7.13–7.29 (10H, m, Ar–H), 7.10 (1H, brs, $CONH_A$), 6.93 (1H, d, $J = 8.5$ Hz, NH of Phe^1), 4.46 (1H, br, αCH of Phe^2), 4.10 (1H, br, αCH of Phe^1), 3.03 (1H, dd, $J = 13.8$, 5.0 Hz, βCH_B of Phe^2), 2.83–2.80 (2H, m, βCH_A of Phe^1 and βCH_B of Phe^2), 2.68 (1H, dd, $J = 13.5$, 10.2 Hz, βCH_A of Phe^1), 1.26 (9H, s, Bu^t). ^{13}C NMR (DMSO-d_6) δ: 173.2 (q), 171.7 (q), 155.6 (q), 138.6 (q), 138.3 (q), 129.8 (t, Ar), 129.6 (t, Ar), 128.5 (t, Ar), 128.4 (t, Ar), 126.7 (t, Ar), 126.6 (t, Ar), 78.6 (q, Bu^t), 56.6 (t, Phe^1 α), 54.1 (t, Phe^2 α), 38.2 (s, Phe^2 β), 37.9 (s, Phe^1 β), 28.6 (p, Bu^t).

Boc-Pro-Phe-Phe-NH_2. A mixed anhydride [prepared from Boc-Pro-OH (440 mg, 2.24 mmol), IBCF (0.27 mL, 2.24 mmol) and NMM (0.44 mL, 2.24 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Phe-Phe-NH_2·HCl [prepared from Boc-Phe-Phe-NH_2 (840 mg, 2.04 mmol) and 7.2 N HCl/dioxane (2.7 mL, 20.4 mmol) in the usual way] in DMF (30 mL) containing NMM (0.24 mL, 2.24 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 810 mg (78.0%), mp 177–183 °C, $R_f^1 = 0.55$, $[\alpha]_D^{20}$ −24.2 (*c* 1.0, DMSO), Anal. calcd for $C_{28}H_{36}N_4O_5 \cdot 2H_2O$: C, 61.8; H, 7.40; N, 10.3. Found: C, 61.4; H, 7.18; N, 10.8, ^{1}H NMR (DMSO-d_6) δ: 7.75–8.05 (2H, m, NH of Phe^{2,3}), 7.06–7.29 (12H, m,

Ar–H and $CONH_2$), 4.39–4.55 (2H, m, αCH of Phe[2,3]), 4.03 (1H, br, αCH of Pro), 3.19–3.32 (2H, m, δCH_2 of Pro), 2.75–3.05 (4H, m, βCH_2 of Phe[2,3]), 1.98 (1H, br, βCH_B of Pro), 1.58–1.73 (3H, m, γCH_2 and βCH_A of Pro), 1.38 (3H, s, *trans* Bu^t), 1.13 (6H, s, *cis* Bu^t). ^{13}C NMR (DMSO-d_6) δ: 172.5 (q), 172.1 (q), 170.8 (q), 170.5 (q), 153.9 (q), 153.2 (q), 137.6 (q), 129.0 (t, Ar), 128.0 (t, Ar), 127.9 (t, Ar), 126.1 (t, Ar), 78.7 (q, *trans* Bu^t), 78.3 (q, *cis* Bu^t), 59.4 (t, Pro α), 53.8 (t, *cis*-Phe α), 53.5 (t, *trans*-Phe α), 46.5 (s, *trans*-Pro δ), 46.3 (s, *cis*-Pro δ), 37.5 (s, *cis*-Phe β), 37.1 (s, *trans*-Phe β), 30.6 (s, *cis*-Pro β), 29.3 (s, *trans*-Pro β), 28.1 (p, *trans* Bu^t), 27.7 (p, *cis* Bu^t), 23.7 (s, *trans*-Pro γ), 22.8 (s, *cis*-Pro γ).

Boc-Ala-Pro-Phe-Phe-NH_2. A mixed anhydride [prepared from Boc-Ala-OH (760 mg, 4.0 mmol), IBCF (0.52 mL, 4.0 mmol) and NMM (0.44 mL, 4.0 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Pro-Phe-Phe-NH_2·TFA [prepared from Boc-Pro-Phe-Phe-NH_2 (2.4 g, 4.0 mmol) and TFA (4.47 mL, 60 mmol) in the usual way] in DMF (30 mL) containing NMM (0.44 mL, 4.0 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 1.3 g (56.5%), mp 180–184°C $R_f^2 = 0.12$, $[\alpha]_D^{20}$ −54.2 (*c* 1.0, MeOH), Anal. calcd for $C_{31}H_{41}N_5O_6 \cdot 1/4H_2O$: C, 63.7; H, 7.16; N, 12.0. Found: C, 63.7; H, 7.00; N, 11.8.

HCl·H-Ala-Pro-Phe-Phe-NH_2 (5). Boc-Ala-Pro-Phe-Phe-NH_2 (1.0 g, 1.5 mmol) was treated with TFA (2.24 mL, 30 mmol) and anisole (0.22 g, 2 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 500 mg (83.5%), MH^+ 480.9, $[\alpha]_D^{20}$ −51.3 (*c* 1.0, H_2O), Anal. calcd for $C_{26}H_{33}N_5O_4 \cdot HCl \cdot 3/2H_2O$: C, 57.1; H, 6.87; N, 12.8. Found: C, 57.4; H, 6.44; N, 12.8, amino acid anal. Ala/Pro/Phe = 0.80:1.15:1.96 (average recovery 83.3%).

Boc-Ala-Phe-Phe-NH_2. A mixed anhydride [prepared from Boc-Ala-OH (1.9 g, 10 mmol), IBCF (1.31 mL, 10 mmol) and NMM (1.1 mL, 10 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Phe-Phe-NH_2·TFA [prepared from Boc-Phe-Phe-NH_2 (4.1 g, 10 mmol) and TFA (11.2 mL, 150 mmol) in the usual way] in DMF (30 mL) containing NMM (1.1 mL, 10 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 2.5 g (51.3%), mp 204–205 °C $R_f^2 = 0.20$, $[\alpha]_D^{20}$ −37.3 (*c* 1.0, MeOH), Anal. calcd for $C_{26}H_{34}N_4O_5$: C, 64.1; H, 7.13; N, 11.50. Found: C, 64.0; H, 7.07; N, 11.4.

Boc-Tyr(Bu^t)-Ala-Phe-Phe-NH_2. A mixed anhydride [prepared from Boc-Tyr(Bu^t)-OH (1.42 g, 4.2 mmol), IBCF (0.55 mL, 4.2 mmol) and NMM (0.46 mL, 4.2 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Ala-Phe-Phe-NH_2·TFA [prepared from Boc-Ala-Phe-Phe-NH_2 (2.0 g, 4.2 mmol) and TFA (4.62 mL, 62 mmol) in the usual way] in DMF (30 mL) containing NMM (0.46 mL, 4.2 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 2.4 g (81.1%), mp 251–253 °C $R_f^1 = 0.44$, $[\alpha]_D^{20}$ −14.3 (*c* 1.0, DMF), Anal. calcd for $C_{39}H_{51}N_5O_7$: C, 66.4; H, 7.34; N, 9.92. Found: C, 66.2; H, 7.31; N, 9.84.

HCl·H-Tyr-Ala-Phe-Phe-NH_2 (6). Boc-Tyr(Bu^t)-Ala-Phe-Phe-NH_2 (1.0 g, 1.4 mmol) was treated with TFA (2.66 mL, 36 mmol) and anisole (0.27 mL, 2.5 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 800 mg (98.3%), MH^+ 546.9, $[\alpha]_D^{20}$ −21.3 (*c* 1.0, H_2O), Anal. calcd for $C_{30}H_{35}N_5O_5 \cdot HCl \cdot 5/2H_2O$: C, 57.5; H, 6.59; N, 11.2. Found: C, 57.7; H, 6.21; N, 11.1, amino acid anal. Tyr/Ala/Phe = 0.94:0.90:2.15 (average recovery 80.0%).

Boc-Ala-Phe-NH_2. A mixed anhydride [prepared from Boc-Ala-OH (2.84 g, 15 mmol), IBCF (2.0 mL, 15 mmol) and NMM (1.65 mL, 15 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Phe-NH_2·TFA [prepared from Boc-Phe-NH_2 (4.0 g, 15 mmol) and TFA (17.1 mL, 0.23 mol) in the usual way] in DMF (30 mL) containing NMM (1.65 mL, 15 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 4.0 g (77.3%), mp 154–155 °C $R_f^2 = 0.14$, $[\alpha]_D^{20}$ −34.7 (*c* 1.0, MeOH), Anal. calcd for $C_{17}H_{25}N_3O_4$: C, 59.3; H, 7.60; N, 12.2. Found: C, 58.8; H, 7.26; N, 12.2.

Boc-Pro-Ala-Phe-NH_2. A mixed anhydride [prepared from Boc-Pro-OH (2.1 g, 9.0 mmol), IBCF (1.2 mL, 9.0 mmol) and NMM (1.0 mL, 9.0 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Ala-Phe-NH_2·TFA [prepared from Boc-Ala-Phe-NH_2 (3.0 g, 9.0 mmol) and TFA (10.1 mL, 0.14 mol) in usual way] in DMF (30 mL) containing NMM (1.0 mL, 9.0 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 2.0 g (52.5.%), mp 199–200 °C $R_f^2 = 0.11$, $[\alpha]_D^{20}$ −73.7 (*c* 1.0, MeOH), Anal. calcd for $C_{22}H_{32}N_4O_5$: C, 60.9; H, 7.51; N, 12.5. Found: C, 60.9; H, 7.46; N, 12.5.

Boc-Tyr(Bu^t)-Pro-Ala-Phe-NH$_2$. A mixed anhydride [prepared from Boc-Tyr(Bu^t)-OH (1.0 g, 2.9 mmol), IBCF (0.38 mL, 2.9 mmol) and NMM (0.32 mL, 2.9 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Pro-Ala-Phe-NH$_2$·TFA [prepared from Boc-Pro-Ala-Phe-NH$_2$ (1.30 g, 2.9 mmol) and TFA (3.2 mL, 0.04 mol) in the usual way] in DMF (30 mL) containing NMM (0.32 mL, 2.9 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 1.8 g (93.8%), mp 143–145 °C $R_f^1 = 0.44$, $[\alpha]_D^{20}$ −34.2 (*c* 1.0, DMF), Anal. calcd for $C_{35}H_{49}N_5O_7$: C, 63.6; H, 7.62; N, 10.6. Found: C, 63.3; H, 7.48; N, 10.8.

HCl·H-Tyr-Pro-Ala-Phe-NH$_2$ (7). Boc-Tyr(Bu^t)-Pro-Ala-Phe-NH$_2$ (650 mg, 1.0 mmol) was treated with TFA (1.86 mL, 25 mmol) and anisole (0.19 mL, 1.7 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 410 mg (83.1%), MH^+ 479.0, $[\alpha]_D^{20}$ −44.0 (*c* 1.0, H_2O), Anal. calcd for $C_{26}H_{33}N_5O_5$·HCl·2H_2O: C, 55.0; H, 6.74; N, 12.3; Found: C, 55.0; H, 6.32; N, 12.5, amino acid anal. Tyr/Ala/Pro/Phe = 0.90:1.09:0.92:1.02 (average recovery 77.1%).

Boc-Phe-Ala-NH$_2$. A mixed anhydride [prepared from Boc-Phe-OH (5.3 g, 20 mmol), IBCF (2.62 mL, 20 mmol) and NMM (2.2 mL, 20 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Ala-NH$_2$·TFA [prepared from Boc-Ala-NH$_2$ (3.76 g, 20 mmol) and TFA (22.4 mL, 0.3 mol) in the usual way] in DMF (30 mL) containing NMM (2.2 mL, 20 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 3.9 g (58.0%), mp 173–177 °C $R_f^2 = 0.13$, $[\alpha]_D^{20}$ −68.8 (*c* 1.0, MeOH), Anal. calcd for $C_{17}H_{25}N_3O_4$: C, 60.9; H, 7.51; N, 12.5. Found: C, 60.9; H, 7.46; N, 12.5.

Boc-Pro-Phe-Ala-NH$_2$. A mixed anhydride [prepared from Boc-Pro-OH (1.5 g, 7.0 mmol), IBCF (0.92 mL, 7.0 mmol) and NMM (0.77 mL, 7.0 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Phe-Ala-NH$_2$·TFA [prepared from Boc-Phe-Ala-NH$_2$ (2.3 g, 7.0 mmol) and TFA (7.8 mL, 105 mmol) in the usual way] in DMF (30 mL) containing NMM (0.77 mL, 7.0 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 2.8 g (93.3%), mp 215–217 °C $R_f^2 = 0.06$, $[\alpha]_D^{20}$ −59.0 (*c* 1.0, MeOH), Anal. calcd for $C_{22}H_{32}N_4O_5$: C, 61.1; H, 7.41; N, 13.0. Found: C, 60.8; H, 7.38; N, 13.0.

Boc-Tyr(Bu^t)-Pro-Phe-Ala-NH$_2$. A mixed anhydride [prepared from Boc-Tyr(Bu^t)-OH (648 mg, 1.92 mmol), IBCF (0.25 mL, 1.92 mmol) and NMM (0.20 mL, 1.92 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Pro-Phe-Ala-NH$_2$·TFA [prepare from Boc-Pro-Phe-Ala-NH$_2$ (830 mg, 1.92 mmol) and TFA (2.85 mL, 38.4 mmol) in the usual way] in DMF (30 mL) containing NMM (0.20 mL, 1.92 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 0.7 g (56.0%), mp 120–124 °C $R_f^2 = 0.44$, $[\alpha]_D^{20}$ −39.0 (*c* 1.0, MeOH), Anal. calcd for $C_{35}H_{49}N_5O_7$: C, 64.5; H, 7.58; N, 10.8. Found: C, 64.5; H, 7.52; N, 11.0.

HCl·H-Tyr-Pro-Phe-Ala-NH$_2$ (8). Boc-Tyr(Bu^t)-Pro-Phe-Ala-NH$_2$ (400 mg, 0.6 mmol) was treated with TFA (0.89 mL, 12 mmol) and anisole (0.09 g, 0.8 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (3): yield 300 mg (58.0%), MH^+ 496.5, $[\alpha]_D^{20}$ −23.2 (*c* 1.0, H_2O), Anal. calcd for $C_{26}H_{33}N_5O_5$·HCl·3/2H_2O: C, 55.9; H, 6.67; N, 12.5. Found: C, 55.7; H, 6.32; N, 12.4, amino acid anal. Tyr/Pro/Phe/Ala = 0.96:1.05:1.01:0.97 (average recovery 87.5%).

Boc-Dmt-OH. HCl H-Dmt-OH (4.7 g, 17.8 mmol) was dissolved in H_2O/dioxane and Et_3N (4.9 mL, 35.6 mmol) and $(Boc)_2O$ (4.27 g, 19.6 mmol) were added. The mixture was stirred at room temperature over night. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ester was added to the residue to form crystals, which were collected by filtration: 5.4 g (97%) yield; mp 178–181 °C, $R_f^2 = 0.33$, $[\alpha]_D^{20}$ −11.7 (*c* 1.0, MeOH), Anal. calcd for $C_{38}H_{42}N_4O_8$·1/10H_2O: C, 61.8; H, 7.53; N, 4.50. Found: C, 61.8; H, 7.40; N, 4.43, ^{1}H NMR (DMSO-d_6) δ: 8.95 (1H, brs, Ar–OH), 7.09 (1H, d, $J = 8.5$ Hz, NH), 6.38 (1H, s, Ar–H), 3.98 (1H, brq, $J = 8.0$ Hz, αCH), 2.95 (1H, dd, $J = 14.3$, 6.6 Hz, βCH_B), 2.78 (1H, dd, $J = 14.1$, 8.3 Hz, βCH_A), 2.18 (6H, s, Me), 1.32 (9H, s, Bu^t).

Boc-Dmt-Pro-Phe-Phe-NH$_2$. To a solution of H-Pro-Phe-Phe-NH$_2$·HCl [prepared from Boc-Pro-Phe-Phe-NH$_2$ (329 mg, 0.65 mmol) and 6.9 N HCl/dioxane (0.9 mL, 6.47 mmol) in the usual way] in DMF (30 mL) containing DIPEA (0.25 mL, 1.30 mmol), Boc-Dmt-OH (200 mg, 0.65 mmol) and PyBop (370 mg, 0.65 mmol) were added. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with

10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 276 mg (61.0%), mp 235–237 °C, $R_f^1 = 0.40$, $[\alpha]_D^{20}$ −38.5 (*c* 1.0, $CHCl_3$), Anal. calcd for $C_{39}H_{49}N_5O_7$: C, 66.9; H, 7.06; N, 10.0, Found: C, 66.7; H, 7.13; N, 9.84, ^{1}H NMR (DMSO-d_6) δ: 9.05 (0.4H, s, *cis* $CONH_B$), 8.86 (0.4H, s, *cis* $CONH_A$), 8.25 (0.6H, d, $J = 8.0$ Hz, *trans* NH), 7.89 (0.4H, d, 0.4H, $J = 8.4$ Hz, *cis* NH), 7.84 (0.4H, d, $J = 7.6$ Hz, *cis* NH), 7.75 (0.6H, d, $J = 8.2$ Hz, *trans* NH), 7.01–7.30 (11.8H, m, *trans* $CONH_2$, *trans* NH, and Ar–H of Phe[3,4]), 6.70 (0.4H, d, $J = 8.3$ Hz, *cis* NH), 6.38 (1.2H, s, Ar–H of *trans* Dmt), 6.36 (0.8H, s, Ar–H of *cis* Dmt), 4.25–4.48 (3H, m, αCH of Phe[3,4], αCH of *cis* Dmt, and αCH of *trans* Pro), 4.12 (0.6H, br, αCH of *trans* Dmt), 3.47 (0.4H, br, δCH_B of *cis* Pro), 2.65–3.18 (8.0H, m, αCH and δCH_A of *cis* Pro, βCH_2 of Phe[3,4], δCH_2 of *trans* Pro, and βCH_2 of Dmt), 2.15 (2.4H, s, Me of *cis* Dmt), 2.08 (3.6H, s, Me of *trans* Dmt), 1.62–1.93 (2.8H, m, γCH_2 and βCH_2 of *trans* Pro, and βCH_B of *cis* Pro), 1.35–1.44 (5.8H, m, *trans* Bu^t, and γCH_B of *cis* Pro), 1.25 (3.6H, s, *cis* Bu^t), 0.97 (0.4H, br, βCH_A of *cis* Pro), 0.77 (0.4H, br, γCH_A of *cis* Pro). ^{13}C NMR (DMSO-d_6) δ: 173.0 (q), 172.8 (q), 171.9 (q), 171.8 (q), 171.6 (q), 171.2 (q), 170.9 (q), 170.7 (q), 170.6 (q), 156.1 (q), 156.0 (q), 155.5 (q), 155.2 (q), 138.4 (q), 138.3 (q), 138.2 (q), 138.1 (q), 138.0 (q), 129.7 (t, Ar), 129.6 (t, Ar), 129.5 (t, Ar), 129.3 (t, Ar), 128.5 (t, Ar), 126.8 (t, Ar), 126.7 (t, Ar), 124.9 (q), 123.6 (q), 115.4 (t, Ar), 115.2 (t, Ar), 79.1 (q, *cis* Bu^t), 78.6 (q, *trans* Bu^t), 60.3 (t, *trans*-Pro α), 59.7 (t, *cis*-Pro α), 55.3 (t, *trans*-Phe α), 54.7 (t, *cis*-Phe α), 54.3 (t, *trans*-Phe α), 54.1 (t, *cis*-Phe α), 52.1 (t, *trans*-Dmt α), 51.9 (t, *cis*-Dmt α), 46.5 (s, *trans*-Pro δ), 46.3 (s, *cis*-Pro δ), 38.5 (s, *cis*-Phe β), 37.9 (s, *trans*-Phe β), 37.4 (s, *trans*-Phe β), 36.7 (s, *cis*-Phe β), 31.8 (s, *trans*-Dmt β), 31.5 (s, *cis*-Dmt β), 30.6 (s, *cis*-Pro β), 29.2 (s, *trans*-Pro β), 28.8 (p, *trans* Bu^t), 228.6 (p, *cis* Bu^t), 4.8 (s, *trans*-Pro γ), 21.6 (s, *cis*-Pro γ), 20.7 (p, *cis*-Dmt Me), 20.0 (p, *trans*-Dmt Me).

HCl·[Dmt^1]EM-2 (9). Boc-Dmt-Pro-Phe-Phe-NH_2 (200 mg, 0.29 mmol) was treated with 8.0 N HCl/dioxane (0.38 mL, 3.0 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 159 mg (89.1%), MH^+ 600.5, $[\alpha]_D^{20}$ −5.60 (*c* 0.67, H_2O), Anal. calcd for $C_{34}H_{41}N_5O_6 \cdot HCl \cdot 3/2H_2O$: C, 60.1; H, 6.67; N, 10.3. Found: C, 60.3; H, 6.33; N, 10.3. ^{1}H NMR (DMSO-d_6) δ: 9.27 (0.7H, brs, *cis* Ar–OH), 9.13 (0.3H, s, *trans* Ar–OH), 8.55 (2.1H, br, NH_3^+ of *cis* Dmt), 8.29–8.36 (1.6H, m, NH of *cis* Phe[4], and NH_3^+ of *trans* Dmt), 8.15 (0.7H, d, $J = 8.4$ Hz, NH of *cis* Phe[3]), 8.00 (0.6H, d, $J = 7.7$ Hz, NH of *trans* Phe[3,4]), 7.60 (0.7H, brs, *cis* $CONH_B$), 7.10–7.32 (11H, m, Ar–H of Phe[3,4], *cis* $CONH_A$, and *trans* $CONH_B$), 7.08 (0.3H, brs, *trans* $CONH_A$), 6.43 (2H, s, Ar–H of Dmt), 4.32–4.52 (2.3H, m, αCH of *trans* Pro, and αCH of Phe[3,4]), 4.10 (0.3H, br, αCH of *trans* Dmt), 3.57 (0.7H, br, αCH of *cis* Dmt), 3.27–3.40 (1.0H, m, δCH_B of Pro), 3.17 (0.7H, br, δCH_A of *cis* Pro), 2.80–3.07 (6.7H, m, αCH of *cis* Pro, βCH_2 of Dmt, and βCH_2 of Phe[3,4]), 2.31 (0.3H, br, δCH_A of *trans* Pro), 2.16 (1.8H, s, Me of *trans* Dmt), 2.04 (4.2H, s, Me of *cis* Dmt), 1.81 (0.3H, br, βCH_B of *trans* Pro), 1.50–1.65 (1.6H, m, βCH_B of *cis* Pro, γCH_2 and βCH_A of *trans* Pro), 1.42 (0.7H, br, γCH_B of *cis* Pro), 1.21 (0.7H, br, βCH_A of *cis* Pro), 1.08 (0.7H, br, γCH_A of *cis* Pro). ^{13}C NMR (DMSO-d_6) δ: 173.62 (q), 173.06 (q), 171.34 (q), 170.88 (q), 170.54 (q), 168.32 (q), 167.93 (q), 156.50 (q), 156.24 (q), 139.02 (q), 138.73 (q), 138.39 (q), 138.22 (q), 138.07 (q), 129.83 (t, Ar), 129.70 (t, Ar), 129.63 (t, Ar), 128.58 (t, Ar), 128.52 (t, Ar), 128.51(t, Ar), 128.43 (t, Ar), 126.80 (t, Ar), 126.71 (t, Ar), 126.67 (t, Ar), 121.96 (q), 121.55 (q), 115.62 (t, Ar), 115.56 (t, Ar), 60.28 (t, *trans*-Pro α), 59.60 (t, *cis*-Pro α), 54.68 (t, *cis*-Phe α), 54.52 (t, *trans*-Phe α), 54.20 (t, *cis*-Phe α and *trans*-Phe α), 50.38 (t, *trans*-Dmt α), 50.30 (t, *cis*-Dmt α), 47.22 (s, *cis*-Pro δ), 46.82 (s, *trans*-Pro δ), 38.20 (s, *trans*-Phe β), 38.01 (s, *cis*-Phe β), 37.91 (s, *trans*-Phe β), 36.74 (s, *cis*-Phe β), 31.63 (s, *cis*-Pro β), 31.22 (s, *cis*-Pro β), 30.80 (s, *trans*-Dmt β), 29.40 (s, *trans*-Pro β), 24.67 (s, *trans*-Pro γ), 21.76 (s, *cis*-Pro γ), 20.61 (p, *trans*-Dmt Me), 19.83 (p, *cis*-Dmt Me).

Boc-Pro-Phe-NH_2. A mixed anhydride [prepared from Boc-Pro-OH (1.68 g, 7.58 mmol), IBCF (0.99 mL, 7.58 mmol) and NMM (1.63 mL, 15.16 mmol) in the usual way] in THF (30 mL) was added to a solution of H-Phe-NH_2·HCl [prepared from Boc-Phe-NH_2 (2.0 g, 7.58 mmol) and 7.2 N HCl/dioxane (10.0 mL, 75.8 mmol) in the usual way] in DMF (30 mL) containing NMM (0.90 mL, 8.34 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 2.29 g (90.5%), mp 71–76 °C, $R_f^1 = 0.47$, $[\alpha]_D^{20}$ −98.5 (*c* 1.0, DMSO), Anal. calcd for $C_{19}H_{27}N_3O_4$: C, 62.8; H, 7.34; N, 11.5. Found: C, 63.1; H, 7.53; N, 11.6, ^{1}H NMR ($CDCl_3$) δ: 7.15–7.33 (5H, m, Ar–H), 6.71 (1H, br, $CONH_B$), 6.39 (1H, br, $CONH_A$), 5.44 (1H, br, NH of Phe), 4.74 (1H, br, αCH of Phe), 4.18 (1H, dd, $J = 8.6$, 3.8 Hz, αCH of Pro), 3.21 (3H, m, βCH_B of Phe, and δCH_2 of Pro), 3.09 (1H, br, βCH_A of Phe), 1.93–2.14 (2H, m, βCH_2 of Pro), 1.82 (1H, br, γCH_B of Pro), 1.68 (1H, br, γCH_A of Pro), 1.35 (9H, s, Bu^t). ^{13}C NMR ($CDCl_3$) δ: 173.4 (q), 172.2 (q), 155.8 (q), 136.5 (q), 129.3 (t, Ar), 128.8 (t, Ar), 127.2 (t, Ar), 81.0 (q, Bu^t), 52.7 (t, Pro α), 47.2 (t, Phe α), 47.2 (s, Pro δ), 36.9 (s, Phe β), 29.4 (s, Pro γ), 28.3 (p, Bu^t), 24.5 (s, Pro β).

Boc-Dmt-Pro-Phe-NH_2. To a solution of H-Pro-Phe-NH_2·HCl [prepared from Boc-Pro-Phe-NH_2 (300 mg, 0.90 mol) and 6.9 N HCl/dioxane (1.3 mL, 8.99 mmol) in the usual way] in DMF (30 mL) containing DIPEA (0.33 mL, 1.89 mmol), Boc-Dmt-OH (277 mg, 0.90 mmol) and PyBop (467 mg, 0.90 mmol) were added. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to

the residue to form crystals, which were collected by filtration, yield 356 mg (71.7%), mp 211–213 °C, $R_f^1 = 0.42$, $[\alpha]_D^{20}$ −27.3 (*c* 1.0, $CHCl_3$), Anal. calcd for $C_{30}H_{40}N_4O_6$: C, 65.2; H, 7.30; N, 10.1. Found: C, 64.9; H, 7.20; N, 9.95, ^{1}H NMR ($CDCl_3$) δ: 7.50 (0.6H, d, $J = 7.5$ Hz, NH of *cis* Phe), 7.08–7.37 (5H, m, Ar–H of Phe), 6.80 (0.6H, br s, *cis* $CONH_B$), 6.62 (0.4H, brs, *trans* $CONH_B$), 6.59 (0.8H, s, Ar–H of *trans* Dmt), 6.52 (1.2H, s, Ar–H of *cis* Dmt), 6.13 (0.4H, d, $J = 8.0$ Hz, NH of *trans* Phe), 5.94 (0.4H, brs, *trans* $CONH_A$), 5.61 (0.6H, brs, *cis* $CONH_A$), 5.51 (0.6H, d, $J = 6.7$ Hz, NH of *cis* Dmt), 5.25 (0.4H, d, $J = 8.9$ Hz, NH of *trans* Dmt), 4.66–4.74 (0.8H, m, αCH of *trans* Phe, and αCH of *trans* Dmt), 4.40–4.50 (1H, m, αCH of *cis* Phe, and αCH of *trans* Pro), 4.15 (0.6H, br, αCH of *cis* Dmt), 3.60 (0.4H, m, δCH_B of *trans* Pro), 3.30 (0.4H, dd, $J = 14.0$, 4.3 Hz, βCH_B of *cis* Phe), 2.93–3.25 (4.8H, m, βCH_2 of *cis* Dmt, δCH_A of *trans* Pro, δCH_2 of *cis* Pro, βCH_2 of *trans* Phe, αCH of *cis* Pro, and βCH_A of *cis* Phe), 2.81–2.89 (1H, m, βCH_A of *cis* Dmt, and βCH_B of *trans* Dmt), 2.76 (0.4H, dd, $J = 13.9$, 7.0 Hz, βCH_A of *trans* Dmt), 2.30 (2.4H, s, Me of *trans* Dmt), 2.19 (3.6H, s, Me of *cis* Dmt), 1.63–1.94 (1.8H, m, βCH_B of *cis* Pro, and βCH_2 and γCH_B *trans* Pro), 1.47 (5.4H, s, *cis* Bu^t), 1.22–1.41 (4.6H, m, γCH_A of *trans* Pro, γCH_B of *cis* Pro, and *trans* Bu^t), 1.11–1.18 (0.6H, m, βCH_A of *cis* Pro), 0.70 (0.6H, br, γCH_A of *cis* Pro). ^{13}C NMR ($CDCl_3$) δ: 174.0 (q), 173.6 (q), 173.1 (q), 172.2 (q), 171.6 (q), 171.5 (q), 156.5 (q), 155.4 (q), 155.1 (q), 155.0 (q), 138.7 (q), 138.1 (q), 137.2 (q), 136.9 (q), 129.0 (t, Ar), 128.9 (t, Ar), 128.8 (t, Ar), 128.6 (t, Ar), 127.0 (t, Ar), 126.9 (t, Ar), 124.1 (q), 123.4 (q), 115.7 (t, Ar), 115.6 (t, Ar), 81.0 (q, *cis* Bu^t), 80.1 (q, *trans* Bu^t), 61.0 (t, *trans*-Pro α), 60.1 (t, *cis*-Pro α), 56.0 (t, *cis*-Phe α), 53.8 (t, *trans*-Phe α), 52.2 (t, *cis*-Dmt α), 51.3 (t, *trans*-Dmt α), 47.5 (s, *trans*-Pro δ), 47.0 (s, *cis*-Pro δ), 36.9 (s, *cis*-Phe β), 36.3 (s, *trans*-Phe β), 32.3 (s, *trans*-Dmt β), 31.9 (s, *cis*-Dmt β), 31.0 (s, *cis*-Pro β), 28.9 (s, *trans*-Pro β), 28.4 (p, *cis* Bu^t), 28.4 (p, *trans* Bu^t), 24.3 (s, *trans*-Pro γ), 21.4 (s, *cis*-Pro γ), 20.5(p, *trans*-Dmt Me), 19.9 (p, *cis*-Dmt Me).

HCl·H-Dmt-Pro-Phe-NH_2 (10). Boc-Dmt-Pro-Phe-NH_2 (563 mg, 1.07 mmol) was treated with 8.0 N HCl/dioxane (1.25 mL, 10 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 356 mg (71.7%), MH^+ 453.1, $[\alpha]_D^{20}$ −13.3 (*c* 0.87, H_2O), Anal. calcd for $C_{25}H_{32}N_4O_4 \cdot HCl \cdot H_2O$: C, 59.2; H, 6.96; N, 11.1. Found: C, 59.6; H, 6.82; N, 10.7. ^{1}H NMR (DMSO-d_6) δ: 9.30 (0.65H, br, Ar–OH of *cis* Dmt), 9.17 (0.35H, br, Ar–OH of *trans* Dmt), 8.55 (1.95H, br, NH_3^+ of *cis* Dmt), 8.42 (1.05H, br, NH_3^+ of *trans* Dmt), 7.98 (0.65H, d, $J = 8.7$ Hz, NH of *cis* Phe), 7.93 (0.35H, d, $J = 8.3$ Hz, NH of *trans* Phe), 7.77 (0.65H, brs, *cis* $CONH_B$), 7.37 (0.35H, brs, *trans* $CONH_B$), 7.15–7.30 (5.65H, m, Ar–H, and *cis* $CONH_A$), 7.04 (0.35H, brs, *trans* $CONH_A$), 6.44 (2H, s, Ar–H of Dmt), 4.39–4.47 (1H, m, αCH of Phe), 4.36 (0.35H, dd, $J = 8.2$, 3.6 Hz, αCH of *trans* Pro), 4.11 (0.35H, br, αCH of *trans* Dmt), 3.60 (0.65H, br, αCH of *cis* Dmt), 3.32-3.47 (1H, m, δCH_B of Pro), 3.15 (0.65H, dt, $J = 11.7$, 8.0 Hz, δCH_A of *cis* Pro), 3.10 (0.35H, br, βCH_B of *trans* Dmt), 3.08 (0.65H, dd, $J = 13.9$, 4.3 Hz, βCH_B of *cis* Phe), 3.02 (1.3H, d, $J = 4.7$ Hz, βCH_2 of *cis* Dmt), 2.99–3.01 (1.7H, m, βCH_A of *trans* Dmt, βCH_A of *cis* Phe, and βCH_2 of *trans* Phe), 2.87 (0.65H, dd, $J = 8.0$, 2.7 Hz, αCH of *cis* Pro), 2.30–2.39 (0.35H, m, δCH_A of *trans* Pro), 2.18 (2.1H, s, Me of *trans* Dmt), 2.07 (3.9H, s, Me of *cis* Dmt), 1.81–1.90 (0.35H, m, βCH_B of *trans* Pro), 1.40–1.68 (2.35H, m, γCH_B and βCH_B of *cis* Pro, γCH_2 and βCH_A of *trans* Pro), 1.13–1.31 (1.3H, m, γCH_A and βCH_A of *cis* Pro). ^{13}C NMR (DMSO-d_6) δ: 173.24 (q), 172.52 (q), 170.26 (q), 170.24 (q), 1167.67 (q), 167.49 (q), 155.95 (q), 155.68 (q), 138.45 (q), 138.13 (q), 138.09 (q), 137.79 (q), 129.14 (t, Ar), 128.85 (t, Ar), 128.10 (t, Ar), 127.95 (t, Ar), 1126.19 (t, Ar), 126.09 (t, Ar), 121.41 (q), 121.00 (q), 115.02 (t, Ar), 115.00 (t, Ar), 59.92 (t, *trans*-Pro α), 59.27 (t, *cis*-Pro α), 54.22 (t, *cis*-Phe α), 53.83 (t, *trans*-Phe α), 49.81 (t, *trans*-Dmt α), 49.72 (t, *cis*-Dmt α), 46.70 (s, *cis*-Pro δ), 46.29 (s, *trans*-Pro δ), 37.39 (s, *trans*-Phe β), 36.47 (s, *cis*-Phe β), 30.94 (s, *cis*-Pro β), 30.52 (s, *cis*-Pro β), 30.03 (s, *trans*-Dmt β), 28.82 (s, *trans*-Pro β), 24.15 (s, *trans*-Pro γ), 21.41 (s, *cis*-Pro γ), 20.09 (p, *trans*-Dmt Me), 19.32 (p, *cis*-Dmt Me).

Boc-Dmt-Pro-NH_2. To a solution of H-Pro-NH_2·HCl [prepared from Boc-Pro-NH_2 (139 mg, 0.65 mmol) and 6.9 N HCl/dioxane (0.9 mL, 6.47 mmol) in the usual way] in DMF (30 mL) containing DIPEA (0.25 mL, 1.30 mmol), Boc-Dmt-OH (200 mg, 0.65 mmol) and PyBop (370 mg, 0.65 mmol) were added. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 307 mg (74.2%), mp 129–132 °C, $R_f^1 = 0.42$, $[\alpha]_D^{20}$ −9.82 (*c* 1.0, $CHCl_3$), Anal. calcd for $C_{21}H_{31}N_3O_5$: C, 62.2; H, 7.71; N, 10.3. Found: C, 62.4; H, 7.89; N, 10.50, ^{1}H NMR ($CDCl_3$) δ: 7.38 (1H, brs, NH), 6.53 (2H, s, Ar–H), 5.51–5.86 (2H, m, $CONH_2$), 4.46–4.79 (2H, m, αCH of Dmt and Pro), 2.89–3.70 (4H, m, βCH_2 of Dmt, and δCH_2 of Pro), 2.32 (6H, s, Me), 1.62–2.20 (4H, m, γCH_2 and βCH_2 of Pro), 1.42 (9H, s, Bu^t).

HCl·H-Dmt-Pro-NH_2 (11). Boc-Dmt-Pro-NH_2 (142 mg, 0.35 mmol) was treated with 8.0 N HCl/dioxane (0.44 mL, 3.5 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 75 mg (63.2%), MH^+ 306.5, $[\alpha]_D^{20}$ +78.7 (*c* 0.38, H_2O), Anal. calcd for $C_{16}H_{23}N_3O_3 \cdot HCl \cdot 1/2H_2O$: C, 54.8; H, 7.18; N, 12.0. Found: C, 55.1; H, 7.32; N, 12.1. ^{1}H NMR (DMSO-d_6) δ: 9.25 (0.63H, s, *cis* $CONH_B$), 9.13 (0.37H, s, *trans* $CONH_B$), 8.34 (3H, br, NH_3^+ of Dmt), 7.38 (0.63H, s, *cis* $CONH_A$) 7.25 (0.37H, s, *trans* $CONH_A$), 6.45 (1.26H, s, Ar–H of *cis* Dmt), 6.40 (0.74H, s, Ar–H of *trans* Dmt), 4.23 (0.37H, dd, $J = 8.3$, 4.3 Hz, αCH of *trans* Pro), 4.16 (0.63H, dd, $J = 9.7$, 6.0 Hz, αCH of *cis* Pro), 2.88–3.63 (5H, m, αCH of Dmt, βCH_2 of Dmt, δCH_B of *trans* Pro, and δCH_2 of *cis* Pro), 2.41 (0.37H, m, δCH_A of *trans* Pro), 2.19 (2.22H, s, Me of *trans*

Dmt), 2.13 (3.78H, s, Me of *cis* Dmt), 1.36–1.90 (2H, m, βCH_2 and γCH_2 of Pro).

Boc-Dmt-Pro-Phe-NH-Phenethyl. To a solution of H-Pro-Phe-NH-Phenethyl·HCl [prepared from Boc-Pro-Phe-NH-phenethyl (500 mg, 1.1 mmol) and 6.9 N HCl/dioxane (1.6 mL, 11.1 mmol) in the usual way] in DMF (30 mL) containing DIPEA (0.57 mL, 3.3 mmol), Boc-Dmt-OH (340 mg, 1.1 mmol) and PyBop (570 mg, 1.1 mmol) were added. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration, yield 430 mg (60.0%), mp 178–180 °C $R_f^1 = 0.29$, $[\alpha]_D^{20}$ −39.3 (*c* 1.0, MeOH), Anal. calcd for $C_{38}H_{48}N_4O_6$: C, 69.5; H, 7.37; N, 8.53. Found: C, 69.6; H, 7.25; N, 8.51, ^{1}H NMR (DMSO-d_6) δ: 9.05 (0.55H, s, Ar–OH of *trans* Dmt), 8.86 (0.45H, s, Ar–OH of *cis* Dmt), 8.15 (0.55H, d, $J = 8.3$ Hz, NH of *trans* Phe), 7.84 (1H, br, $-N\underline{H}-C_2H_4$), 7.77 (0.45H, d, $J = 7.9$ Hz, NH of *cis* Phe), 7.12–7.35 (10H, m, Ar–H), 7.07 (0.55H, d, $J = 5.5$ Hz, NH of *trans* Dmt), 6.74 (0.45H, d, $J = 7.7$ Hz, NH of *cis* Dmt), 6.40 (1.1H, s, Ar–H), 6.37 (0.9H, s, Ar–H), 4.12–4.48 (2,45H, m, αCH of Dmt, αCH of Phe, and αCH of *trans* Pro), 3.48 (0.45H, br, δCH_B of *cis* Pro), 3.25–3.33 (1H, m, –NH–$C\underline{H}_B$–CH_2–), 2.97–3.25 (4H, m, βCH_B of Phe, –NH–$C\underline{H}_A$–CH_2–, αCH and δCH_A of *cis* Pro, and δCH_2 of *trans* Pro), 2.63–2.95 (5H, m, βCH_2 of Dmt, βCH_A of Phe, and –NH–CH_2–$C\underline{H}_2$–Ph), 2.18 (2.7H, s, Me of *cis* Dmt), 2.11 (3.3H, s, Me of *trans* Dmt), 1.62–1.95 (2.65H, m, βCH_2 and γCH_2 of *trans* Pro, and βCH_B of *cis* Pro), 1.35–1.45 (5.4H, m, *trans* Bu^t, and γCH_B of *cis* Pro), 1.26 (4.05H, s, *cis* Bu^t), 1.03 (0.45H, br, βCH_A of *cis* Pro), 0.84 (0.45H, br, γCH_A of *cis* Pro). ^{13}C NMR (DMSO-d_6) δ: 171.10 (q), 170.9 (q), 170.8 (q), 170.4 (q), 170.2 (q), 170.1 (q), 155.5 (q), 155.0 (q), 154.1 (q), 139.2 (q), 138.1 (q), 138.0 (q), 137.9 (q), 129.0 (t, Ar), 128.8 (t, Ar), 128.4 (t, Ar), 128.4 (t, Ar), 128.3 (t, Ar), 128.2 (t, Ar), 127.9 (t, Ar), 126.2 (t, Ar), 126.1 (t, Ar), 126.0 (t, Ar), 125.9 (t, Ar), 124.3 (q), 123.0 (q), 114.8 (t, Ar), 114.6 (t, Ar), 78.5 (q, Bu^t), 77.9 (q, Bu^t), 59.9 (t, *trans*-Pro α), 59.2 (t, *cis*-Pro α), 54.4 (t, *trans*-Phe α), 53.9 (t, *cis*-Dmt α), 51.4 (t, *trans*-Dmt α), 46.6 (s, *cis*-Pro δ), 46.2 (s, *trans*-Pro δ), 40.2 (s, –NH–$C\underline{H}_2$–CH_2–), 37.0 (s, *trans*-Phe β), 36.6 (s, *cis*-Phe β), 35.0 (s, *trans*-CH_2–$C\underline{H}_2$–Ph), 34.9 (s, *cis*–CH_2–$C\underline{H}_2$–Ph), 31.4 (s, *trans*-Dmt β), 30.8 (s, *cis*-Dmt β), 30.1 (s, *cis*-Pro β), 28.6 (s, *trans*-Pro β), 28.2 (p, *trans* Bu^t), 27.9 (p, *cis* Bu^t), 24.3 (s, *trans*-Pro γ), 21.0 (s, *cis*-Pro γ), 20.1 (p, *cis*-Dmt Me), 19.4 (p, *trans*-Dmt Me).

HCl·H-Dmt-Pro-Phe-NH-Phenethyl (12). Boc-Dmt-Pro-Phe-NH-phenethyl (297 mg, 0.47 mmol) was treated with 8.0 N HCl/dioxane (0.59 mL, 4.7 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was prepared as described for the preparation of (**3**): yield 240 mg (90.2%), MH^+ 556.9, $[\alpha]_D^{20}$ +13.3 (*c* 1.0, H_2O), Anal. calcd for $C_{33}H_{40}N_4O_4$·HCl·1/2H_2O: C, 65.8; H, 6.98; N, 9.30. Found: C, 65.7; H, 6.91; N, 9.47. ^{1}H NMR (DMSO-d_6) δ: 8.39 (1.95H, br, NH_3^+ of *cis* Dmt), 8.22–8.31 (1.7H, m, *cis* $N\underline{H}$–C_2H_4, and NH_3^+ of *trans* Dmt), 7.97–8.04 (1.35H, m, NH of Phe, and *trans* $N\underline{H}$–C_2H_4), 7.15–7.33 (10H, m, Ar–H), 6.43 (2H, s, Ar–$\underline{H}$), 4.40–4.49 (1H, m, αCH of Phe), 4.37 (0.35H, dd, $J = 8.6$, 4.6 Hz, αCH of *trans* Pro), 4.17 (0.35H, br, αCH of *trans* Dmt), 3.56 (0.65H, br, αCH of *cis* Dmt), 3.12–3.48 (3.65H, m, δCH_B of *trans* Pro, NH–$C\underline{H}_2$–CH_2–, and δCH_2 of *cis* Pro), 2.81–3.00 (4.65H, m, α$\underline{C}$H of *cis* Pro, βCH_2 of Phe, and βCH_2 of Dmt), 2.71 (1.3H, t, $J = 7.2$ Hz, *cis*-CH_2–$C\underline{H}_2$–Ph), 2.64 (0.7H, t, $J = 7.2$ Hz, *trans*-CH_2–$C\underline{H}_2$–Ph), 2.30–2.37 (0.35H, m, δCH_A of *trans*-Pro), 2.18 (2.1H, s, Me of *trans* Dmt), 2.06 (3.9H, s, Me of *cis* Dmt), 1.80–1.90 (0.35H, m, βCH_B of *trans* Pro), 1.28–1.67 (3.65H, m, γCH_2 and βCH_2 of *cis* Pro, γCH_2 and βCH_A of *trans* Pro). ^{13}C NMR (DMSO-d_6) δ: 171.1 (q), 170.4 (q), 170.3 (q), 170.2 (q), 167.6 (q), 167.4 (q), 158.2 (q), 157.8 (q), 155.9 (q), 155.7 (q), 139.3 (q), 139.2 (q), 138.5 (q), 138.2 (q), 137.7 (q), 137.6 (q), 129.1 (t, Ar), 128.9 (t, Ar), 128.8 (t, Ar), 128.7 (t, Ar), 128.6 (t, Ar), 128.5 (t, Ar), 128.2 (t, Ar), 128.0 (t, Ar), 126.3 (t, Ar), 126.1 (t, Ar), 126.0 (t, Ar), 125.9 (t, Ar), 121.4 (q), 120.1 (q), 118.4 (q), 115.4 (q), 115.0 (t, Ar), 59.8 (t, *trans*-Pro α), 59.2 (t, *cis*-Pro α), 54.0 (t, Phe α), 49.9 (t, *trans*-Dmt α), 49.8 (t, *cis*-Dmt α), 46,9 (s, *cis*-Pro δ), 46.2 (s, *trans*-Pro δ), 40.3 (s, *cis*-NH–$C\underline{H}_2$–CH_2–), 40.0 (s, *trans*-NH–$C\underline{H}_2$–CH_2–), 37.8 (s, *trans*-Phe β), 36.9 (s, *cis*-Phe β), 34.9 (s, *trans*–CH_2–$C\underline{H}_2$–Ph), 34.8 (s, *cis*–CH_2–$C\underline{H}_2$–Ph), 31.1 (s, *cis*-Pro β), 30.6 (s, *cis*-Dmt β), 30.1 (s, *trans*-Dmt β), 28.9 (s, *trans*-Pro β), 24.2 (s, *cis*-Pro γ), 21.6 (s, *trans*-Pro γ), 20.0 (p, *trans*-Dmt Me), 19.2 (p, *cis*-Dmt Me).

Nuclear magnetic resonance spectroscopy

^{1}H (400 or 500 MHz) and ^{13}C (100 or 125 MHz) NMR spectra were recorded on a Bruker DPX-400 or ARX-500 spectrometers, respectively. Chemical shift values expressed as ppm downfield from tetramethylsilane, used as an internal standard (δ value). The *J* values are given in Hz. The ^{13}C signals were assigned with the aid of distortionless enhancement by polarization transfer (DEPT) and two-dimensional experiments, and multiplicities are indicated by p (primary), s (secondary), t (tertiary) or q (quaternary). Final compounds (30 mg) were dissolved in 1.0 mL DMSO-d_6 (99.9% isotopic purity).

Circular dichroism spectroscopy

CD spectra were recorded at room temperature using a JASCO J-725 spectrapolarimeter in 0.02 cm cylindrical cell. Two scans were collected for each sample over a wavelength range of 185–260 nm. The peptide solutions were prepared in neat TFE (2,2,2-trifuluoroethanol) with peptide concentration ranging from 0.79 to 1.45 mM. Band intensities are expressed as molar ellipticities, $[\theta]_m \times 10^3$ (deg cm^2/dmol).

Radioligand binding

Synaptosomal brain membrane P_2 preparations from Sprague–Dawley rats were prepared and used as the source for μ- and δ-receptors after the removal of

endogenous opioid.[33,34] The competitive displacement assay used 5.57 nM [^{3}H]-DPDPE (NEN-DuPont) and 3.5 nM [^{3}H]DAMGO (Amersham) for δ- and μ-sites, respectively as published.[33,34] Affinity constants (K_i) were determined as given earlier.[34]

In vitro bioactivity assay

For the GPI assay, the myenteric plexus-longitudinal muscle was obtained from male Hartley strain guinea-pig (250–300 g) ileum as described by Rang,[35] and the tissue was mounted in a 10-mL bath that contained aerated (95% O_2, 5% CO_2) Krebs–Henseleit solution at 35 °C. The tissue was stimulated transmurally between the platinum wire electrodes using pulses of 0.5 ms duration with a frequency of 0.1 Hz at the supramaximal voltage. Longitudinal contractions were recorded via an isometric transducer. For the MVD assay, the vas deferens of male ddY strain mouse (25–35 g) were prepared, as described by Hughes et al.[36] A pair of vasa was mounted in a 10-mL bath that contained aerated (95% CO_2, 5% O_2), modified Mg^{2+}-free Krebs solution containing ascorbic acid (0.1 mM) and EDTA·4Na (0.027 mM) at 35 °C. The tissue was stimulated transmurally with trains of rectilinear pulses of 1 ms. Stimulation trains were given at intervals of 20 s and consisted of seven stimuli of 1 ms duration with intervals of 10 ms.

In both assays, three to four washings were done with intervals of 15 min between each dose. Dose–response curves were constructed and IC_{50} values (concentration causing a 50% reduction of the electrically induced twitches) were calculated graphically. For antagonism experiments, the test sample was added to the bath 15 min prior to addition of deltorphin II as agonist. The concentration–response curves of the agonist (deltorphin II) were performed in the absence and in the presence of increasing concentrations of the sample, and the pA_2 values were calculated according to the method of Arunlakshana and Schild.[37]

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