Synthesis and Evaluation of the Affinity toward μ-Opioid Receptors of Atypical, Lipophilic Ligands Based on the Sequence *c*[-Tyr-Pro-Trp-Phe-Gly-]

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An ultimate and general model describing the interaction between opioid ligands and μ-opioid receptors is not available yet, so the mode of action of atypical peptide analogues or peptidomimetics is worthy of investigation. In this context, the peptide *c*[-Tyr-D-Pro-D-Trp-Phe-Gly-] was observed to act as an agonist toward μ-opioid receptors with appreciable potency, albeit deprived of a protonable nitrogen. This compound was synthesized as a member of a library of diastereo- or enantiomeric cyclic peptides based on the sequence of endomorphin-1, aiming to obtain lipophilic peptide ligands active at the μ-opioid receptors, having good performances in terms of resistance to enzymatic degradation and permeation of biological barriers.

Introduction

Due to their role as endogenous analgesics in mammals, opioid peptides have been conjectured as secondary effect-free pharmacological tools[1−4] to be used in place of morphine.[5−7] Among them, endomorphins are considered to be the endogenous ligands for μ-opioid receptors and are regarded as the most effective ones, being released in response to pain stimuli. Endomorphin-1, H-Tyr-Pro-Trp-Phe-NH_2, and endomorphin-2,[8,9] H-Tyr-Pro-Phe-Phe-NH_2, are more potent and longer acting than the majority of the other opioid peptides against neuropathic pain even at low doses.[10] They also show a strong antinociceptive effect in acute pain similar to that of morphine, whereas they seem to be less effective than morphine in inflammatory pain. Recent evidence has suggested that the analgesic efficacy of endomorphin-1 can be dissociated from the immunomodulatory,[11] cardiovascular, and respiratory effects.[12,13]

However, natural opioid peptides show in general a poor bioavailability, mainly due to their inability to penetrate the gut−blood or the blood−brain barriers,[14,15] and rapid degradation in vivo by several peptidases, such as aminopeptidases and dipeptidyl peptidase IV.[16−18]

To circumvent these problems, diverse strategies have been adopted to modify opioid peptide properties.[19] In several cases, peptide analogues or peptidomimetics have been found to possess much higher biological activity than that expected on the basis of simple binding studies or tissue bioassays. In this context, we have recently reported the synthesis and pharmacological characterization of a series of endomorphin-1 analogues in which each amino acid was replaced by its β-isomer, having the amino group shifted to the β-position,[20,21] or by the corresponding amino acid homologue.[22] Some of the β-analogues maintained a certain affinity toward the μ-opioid receptors, albeit slightly lower than that of endomorphin-1, but they showed a higher stability in vitro and in vivo. In particular, the peripheral inoculation of H-Tyr-(*S*)-homo-Pro-Trp-PheNH_2 produced analgesia in mice (tail-flick and acetic acid-induced abdominal constriction tests), whereas endomorphin-1 was completely ineffective.[23]

The main synthetic strategies described in the literature to ameliorate opioid peptide features, such as the insertion of unnatural amino acids, the introduction of conformational constraints, the design of bivalent peptide ligands, the modification of peptide bonds, and the cyclization of linear peptides, have been exhaustively reviewed.[24−28]

Lipophilicity is considered to be one of the most important features connected to penetration across membranes, because the modified peptide can cross in a passive fashion. The presence of hydrogen bonds tends to reduce the lipid solubility of the compound and would be expected to lower permeability. Many of the native opioid peptides, including endomorphins, contain polar residues; therefore, several chemical modifications have been explored to increase the overall lipophilicity.[29] For instance, terminal amino groups can be simply transformed into amides; recently, a couple of *N*-acyl-DADLE derivatives have been reported to show good activity and improved permeability.[30,31]

Among the different strategies, cyclization represents a useful method to provide a remedy for the abovementioned obstacles to the use of opioid peptides as pharmacological tools. Indeed, cyclization generally increases the molecular lipophilicity, increases the metabolic stability, and, in addition, reduces the molecular conformational freedom, which can be responsible for the simultaneous or concurrent activation of different receptors.

Many cyclic analogues have been synthesized by the introduction of natural or unnatural amino acids carrying an amino function in the side chain, giving access

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to peptides containing lactam bridges.[25−27,32] In several enkephalin derivatives, cyclization has been achieved by means of a sulfide or disulfide bond.[25−27] Recent examples are the cyclic μ-opioid receptor-selective tetrapeptide Tyr-*c*[D-Cys-Phe-D-Pen]NH_2 (Et) (JOM-6), and its modified analogues,[33] and a cyclic lanthionine enkephalin analogue containing a monosulfide bridge.[34,35] The introduction of diamines allowed the synthesis of the potent methylamine-bridged enkephalins MABE(I)[36] and MABE(II).[34] In the 18-membered analogue cyclo(*Nε*,*Nβ*-carbonyl-D-Lys^2,Dap^5)-enkephalinamide,[37] or in analogues related to the sequence of dermorphin/deltorphin,[38] cyclization was achieved by means of a carbonyl bridge.

Apparently, in these examples the cyclopeptides have been designed to preserve the main feature considered to be necessary to manifest an agonist activity, that is, the presence of an N-terminal protonable amine.[39] Indeed, the amino group of opioid peptide ligands is generally believed to play a crucial role in signal transduction at μ- and δ-receptors.

However, some opioid analogues lacking a positive charge have been reported to maintain a good receptor affinity (see Discussion). Therefore, starting from the assumption that specific, atypical peptide ligands deprived of a cationizable N-terminal amino group could still interact with opioid receptors, we envisaged the possibility to prepare cyclic and lipophilic opioid peptides based on the sequence of endomorphin-1, containing all amide bonds and the different residues in L or D configuration.

To verify the possibility that some of these compounds could assume a bioactive conformation capable of interacting with μ-opioid receptors mainly through their hydrophobic groups, their affinity for the receptors was determined by means of displacement binding assays.

Results

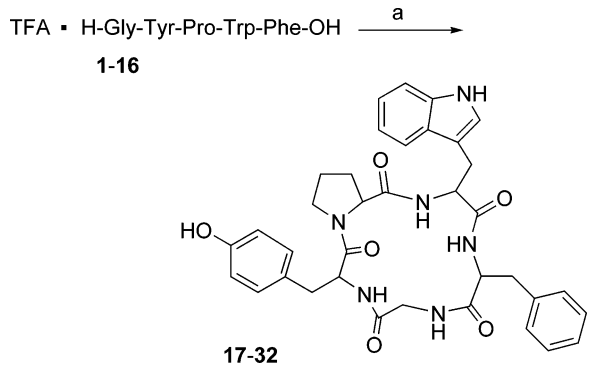
We planned to prepare a series of endomorphin-1 derivatives having the same amino acid sequence, Tyr-Pro-Trp-Phe, with the Tyr^1 and Phe^4 residues simply connected by a fifth amino acid through amide bonds. The residue chosen was Gly, because it bears no interfering side chain.

To define the best spatial disposition of the different aromatic side chains for an optimal ligand−receptor interaction, each residue has been introduced in D or L configuration, generating a selected library of stereoisomeric, three-dimensionally distinct cyclopeptides, having the general structure *c*[-L/D-Tyr-L/D-Pro-L/D-Trp-L/D-Phe-Gly-].

Moreover, the introduction of D-amino acids in the peptide sequence can give the opioid peptide further stability, because the enzymes known to effectively hydrolyze peptide bonds involving D-amino acids in multicellular organisms are few.[40]

The cyclopentapeptide analogues have been prepared by cyclization of the corresponding linear pentapeptide precursors **1**−**16**, H-Gly-L/D-Tyr-L/D-Pro-L/D-Trp-L/D-Phe-OH. These precursors have been obtained in turn by way of a standard SPPS, using a Wang resin, Fmoc-protected amino acids, and DCC/HOBt as coupling agents.[41] The Fmoc group cleavage was performed by means of 20% piperidine in DMF. After each coupling, efficacy was assayed by means of Kaiser[42] or chloranil tests.[43] The stereoisomeric linear pentapeptides **1**−**16** were obtained free after cleavage with a 95.0:2.5:2.5 mixture of TFA/PhOH/H_2O. After that, **1**−**16** were collected by precipitation from Et_2O and simply purified by crystallization from MeOH/Et_2O. Pentapeptides **1**−**16** were subjected to cyclization with diphenylphosphoryl azide (DPPA) in DMF in the presence of an excess of $NaHCO_3$, giving compounds **17**−**32** (Scheme 1).[41]

Scheme 1. Synthesis of Cycloendomorphins **17**−**32**[a]



[a] Reagents and conditions: (a) DPPA (2 equiv), $NaHCO_3$ (15 equiv), DMF, room temperature, 48 h.

As anticipated, the resulting cyclopeptides were much less polar than parent endomorphin; therefore, they were easily purified by flash chromatography over silica gel and were characterized by HPLC, ES-MS, and NMR. Yields after purification, RP-HPLC retention factors in two different analytical chromatographic systems (see Experimental Section), average purities, and mass characterization are reported in Table 1.

Although cyclopeptide purities were in general adequate, yields were quite sensitive to the peptide sequence, ranging from 12 to 65% after purification. Yield and purity of enantiomeric compounds are comparable. In some cases, the reactions were attempted also in the presence of DCC instead of DPPA, but this modification did not increase the yield. Disappointingly, compounds **27** and **28** were obtained only in low yield, and with scarce purity; therefore, they were not subjected to biological tests.

To determine the affinities toward the μ-opioid receptors, the diastereoisomeric or enantiomeric cyclopeptides were incubated with rat brain membrane homogenates containing the receptors, using [^{3}H]DAMGO as a μ-specific radioligand. The displacement binding assays were performed for DAMGO, endomorphin-1, and cyclopeptides **17**−**26** and **29**−**32**, to calculate K_I, IC_{50}, and Hill factors, as reported in Table 2.

The affinity values for DAMGO and endomorphin-1 (**1**) are in agreement with the literature (K_i = 1.9 and 0.36 nM, respectively).[8,44,45] In all cases, the peptides displayed a concentration-dependent displacement of [^{3}H]DAMGO, with a lower affinity for μ-opioid receptors in comparison to DAMGO and endomorphin-1. However, whereas most of the peptides had very poor affinities, with K_I values in the micromolar range, compound **32** *c*[-Tyr-D-Pro-D-Trp-Phe-Gly-] was the most potent in displacing [^{3}H]DAMGO, showing a K_I value of 34 nM. The Hill slopes (n_H) of these compounds were generally less than unity; several explanations are possible, including negative cooperativity (where the

Table 1. Synthesis and Analytical Characterization of Cyclopeptides **17**−**32** from Linear Precursors **1**−**16**

compd	sequence	yield (%)	R_f[a] (min)	R_f[b] (min)	purity[c] (%)	ES-MS [M + H][d]	ES-MS [M + Na][e]
17	*c*[-Tyr-Pro-Trp-Phe-Gly-]	60	19.3	13.8	96	651.1	673.2
18	*c*[-D-Tyr-D-Pro-D-Trp-D-Phe-Gly-]	55	19.3	13.8	94	651.1	673.2
19	*c*[-D-Tyr-Pro-Trp-Phe-Gly-]	35	19.2	13.7	93		673.0
20	*c*[-Tyr-D-Pro-D-Trp-D-Phe-Gly-]	40	19.2	13.7	93	651.2	673.1
21	*c*[-Tyr-D-Pro-Trp-Phe-Gly-]	65	20.1	14.4	96	651.2	673.2
22	*c*[-D-Tyr-Pro-D-Trp-D-Phe-Gly-]	60	20.1	14.4	96		673.2
23	*c*[-Tyr-Pro-D-Trp-Phe-Gly-]	45	19.1	13.4	95	651.2	673.2
24	*c*[-D-Tyr-D-Pro-Trp-D-Phe-Gly-]	48	19.1	13.4	93	651.2	
25	*c*[-Tyr-Pro-Trp-D-Phe-Gly-]	35	19.1	13.8	98	651.2	673.2
26	*c*[-D-Tyr-D-Pro-D-Trp-Phe-Gly-]	39	19.1	13.8	99	651.1	
27	*c*[-D-Tyr-D-Pro-Trp-Phe-Gly-]	14	19.6	14.0	75	651.2	
28	*c*[-Tyr-Pro-D-Trp-D-Phe-Gly-]	12	19.6	14.0	70	651.0	673.1
29	*c*[-D-Tyr-Pro-D-Trp-Phe-Gly-]	43	19.5	13.9	92		673.1
30	*c*[-Tyr-D-Pro-Trp-D-Phe-Gly-]	37	19.5	13.9	94	651.2	
31	*c*[-D-Tyr-Pro-Trp-D-Phe-Gly-]	64	19.2	13.9	97	651.6	673.6
32	*c*[-Tyr-D-Pro-D-Trp-Phe-Gly-]	62	19.2	13.9	96	651.2	673.3

[a] HPLC system 1. [b] HPLC system 2. [c] Average from HPLC systems 1 and 2. [d] Calculated value for M + H: 651.3. [e] Calculated value for M + Na: 673.3

Table 2. Affinities and Hill Slopes of DAMGO, Endomorphin-1, and Cyclopeptides **17**−**26**/**29**−**32** for [^{3}H]DAMGO Binding Sites in Rat Brain Membranes

compd	sequence	K_I[a] (μM)	IC_{50}[a] (μM)	n_H[a]
DAMGO	H-Tyr-D-Ala-Gly-*N*-Me-Phe-Glyol[b]	$(1.6 \pm 0.3) \times 10^{-3}$	$(9.9 \pm 0.6) \times 10^{-3}$	0.90 ± 0.05
endomorphin-1	H-Tyr-Pro-Trp-Phe-NH_2[b]	$(0.16 \pm 0.02) \times 10^{-3}$	0.5 ± 0.2^{-3}	0.74 ± 0.03
17	*c*[-Tyr-Pro-Trp-Phe-Gly-]	27 ± 1	29 ± 2	0.70 ± 0.03
18	*c*[-D-Tyr-D-Pro-D-Trp-D-Phe-Gly-]	18 ± 1	240 ± 15	0.78 ± 0.02
19	*c*[-D-Tyr-Pro-Trp-Phe-Gly-]	15 ± 1	19	1.2 ± 0.1
20	*c*[-Tyr-D-Pro-D-Trp-D-Phe-Gly-]	2.7 ± 0.1	3.2 ± 0.2	0.9 ± 0.1
21	*c*[-Tyr-D-Pro-Trp-Phe-Gly-]	2.9 ± 0.2	3.8 ± 0.1	0.40 ± 0.02
22	*c*[-D-Tyr-Pro-D-Trp-D-Phe-Gly-]	11 ± 1	14 ± 1	1.1 ± 0.1
23	*c*[-Tyr-Pro-D-Trp-Phe-Gly-]	36 ± 2	45 ± 2	0.33 ± 0.03
24	*c*[-D-Tyr-D-Pro-Trp-D-Phe-Gly-]	230 ± 20	102 ± 12	0.30 ± 0.02
25	*c*[-Tyr-Pro-Trp-D-Phe-Gly-]	30 ± 1	32 ± 1	0.65 ± 0.03
26	*c*[-D-Tyr-D-Pro-D-Trp-Phe-Gly-]	3.9 ± 0.1	5.2 ± 0.1	0.65 ± 0.02
29	*c*[-D-Tyr-Pro-D-Trp-Phe-Gly-]	23 ± 1	29 ± 1	0.70 ± 0.03
30	*c*[-Tyr-D-Pro-Trp-D-Phe-Gly-]	32 ± 1	39 ± 2	0.50 ± 0.04
31	*c*[-D-Tyr-Pro-Trp-D-Phe-Gly-]	6.6 ± 0.3	8.1 ± 0.2	0.42 ± 0.05
32	*c*[-Tyr-D-Pro-D-Trp-Phe-Gly-]	0.034 ± 0.002	0.044 ± 0.003	0.44 ± 0.05

[a] Means ± SE of three experiments. [b] TFA salts.

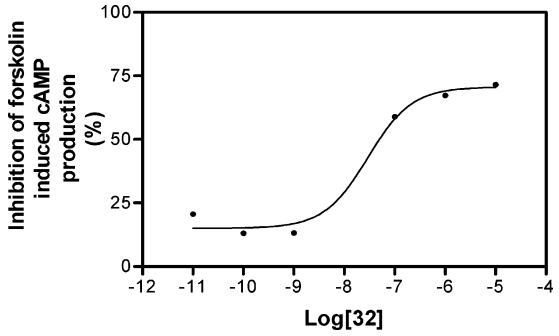


Figure 1. Effects of **32** on forskolin-stimulated cyclic AMP production in SH-SY5Y cells.

occupation of one site decreases the likehood that other sites on the same oligomeric molecule may bind the ligand) and/or multiple classes of non-interacting or non-interconvertable binding sites.

To test the agonist/antagonist behavior of **32**, we evaluated any effect on cyclic AMP production in intact SH-SY5Y cells,[45,46] in comparison to DAMGO. Both peptide **32** (Figure 1) and DAMGO produced an inhibition of forskolin (10 μM)-stimulated cyclic AMP formation in a concentration-dependent manner, thus behaving as μ-opioid agonists. The measured IC_{50} values of DAMGO and **32** were 1.10 ± 0.27 and 29.0 ± 9.2 nM, respectively. The potency of DAMGO was in the nanomolar range as previously reported.[45,46] The IC_{50} value of **32** was well correlated to the K_I value for the μ-opioid receptor obtained in binding studies.

Discussion

The good affinity for the μ-opioid receptor and the agonist behavior displayed by **32** seems to indicate that this lipophilic peptide can interact and activate the receptor even without the contribution of an H-bond involving a cationizable primary amine. The deletion of the positively charged N-terminal amino group or its replacement is commonly believed to be a general structural modification to convert opioid peptide agonists into antagonists.[47] For instance, the replacement of the N-terminal tyrosine residue with (2*S*)-2-methyl-3-(2′,6′-dimethyl-4′-hydroxyphenyl)propionic acid (Mdp) or 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) converted selective peptide agonists to antagonists, whose selectivity was often maintained or even improved.[47] In a similar way, the compound c-*N*,5-[Trp^3,Trp^4,Glu^5]Dyn A-(1−11)NH_2, an N-terminal-to-side chain cyclic dynorphin A analogue lacking the basic N terminus, showed κ-opioid receptor affinity and selectivity similar to those of the linear parent and an antagonist behavior.[48] Other examples are a cyclic peptide containing all amide bonds,[49] a β-casomorphin-

derived peptide in which the terminal amino group was eliminated or formylated,[49] the enkephalin analogue containing Mdp in place of tyrosine,[50] and a DynA analogue after replacement of Tyr[1] with Dhp or Mdp.[51] The absence of an N-terminal amino group has been considered to be primarily responsible for the antagonist behavior of these compounds. Very recently, an endomorphin-1 model lacking both the phenol group and the cationic amine, having the Tyr[1] substituted by a benzylcarbamate group, designed to increase peptide solubility in low polarity solvents, showed affinity for the μ-receptors in the nanomolar range, suggesting that the benzylcarbamate group could act at the receptor as a tyrosine mimic.[52]

At least one case has been reported of a peptidomimetic deprived of an N-terminal amino group displaying analgesic properties. Compounds of a library of lipophilic bicyclic systems designed as enkephalin or endomorphin models folded in a β-turn conformation were found to be μ-opioid receptor agonists with an initial level of analgesic activity similar to that of morphine, although the in vivo half-life was shorter than that of morphine.[53]

The efficacy of the overall ligand−receptor binding depends on a variety of factors.[54] Important partners are ion−ion and dipole−dipole interactions, charged-reinforced hydrogen bonding, and neutral−neutral hydrogen bonding. Another important contributor is the optimization of hydrophobic interaction resulting from the mutual complementarity of the ligand shape with the receptor cavity. The interaction between a ligand and a receptor depends on the overall energy involved in breaking and forming all of the hydrogen bonds,[55] plus the contribution of all the hydrophobic interactions. The latter is generally favorable, because the lipophilic groups of the ligand move from a outer aqueous biological environment to an inner hydrophobic cavity. On the other hand, the formation of hydrogen bonds between the ligand and the receptor is an energetically favorable process, but similar bonds have to be broken between the ligand and water and between the receptor and water. Whereas the contribution to binding of charged H-bonds is important, the contribution from neutral−neutral H-bonds is much lower.[56] The contribution of a single hydrophobic group is generally modest,[57] but the overall effect of many hydrophobic groups becomes important, in particular when large portions of the ligand and the receptor are strongly complementary. For the highly lipophilic peptide analogue **32**, the hydrophobic contribution to binding energy at the receptor seems to be predominant.

Conclusions

We synthesized a series of cyclic endomorphin analogues containing L or D residues by introduction of a Gly bridge between residues 1 and 4 by means of amide bonds. The resulting peptides showed different affinities toward μ-opioid receptors depending on the stereochemistry. The peptide *c*[-Tyr-D-Pro-D-Trp-Phe-Gly-] **32** showed an affinity in the nanomolar range and inhibited the forskolin-stimulated production of cyclic AMP in SH-SY5Y cells in a concentration-dependent manner. The absence of the tyrosine primary amine makes this agonist behavior noteworthy. The high lipophilicity displayed by **32**, the cyclic structure and the presence of residues in D-configuration render the peptide a promising candidate for the development of peptide ligands with increased bioavailability. Further studies are currently in progress in our laboratory to investigate the relationship between the conformation of the different stereoisomeric compounds and receptor affinity, in particular concerning the three-dimensional disposition of the aromatic side chains.

Experimental Section

General Methods. Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Flash chromatography was performed on Merck silica gel 60 (230−400 mesh), and solvents were simply distilled. NMR spectra were recorded with a Mercury spectrometer (Oxford magnet) at 400 (^{1}H NMR) and at 75 MHz (^{13}C NMR). Chemical shifts are reported as δ values relative to the solvent peak of $CDCl_3$ set at δ 7.27 (^{1}H NMR) or δ 77.0 (^{13}C NMR). Infrared absorptions were recorded with an FT-IR Nicolet 210 spectrophotometer. Optical activity measurements were performed with a Perkin-Elmer 343 polarimeter. ES-MS was performed with an HP 1100MSD. Homogenates were centrifuged in Beckman J6B and Beckman J2-21 centrifuges. Radioactivity was measured by liquid scintillation spectrometry using a Beckman apparatus. Analytical RP-HPLC was performed on an HP series 1100. Conditions for method 1 were as follows: column, Zorbax Eclipse XDB-C8 (5-μm particle size, 150 mm); solvent system, A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, gradient 100% A to 50% B in 20 min at a 1.0 mL/min flow, followed by 20 min at 50% B; DAD 210 nm. Conditions for method 2 were as follows: column, HP Hypersil ODS (4.6-μm particle size, 250 mm); solvent system, A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, gradient 100% A to 100% B in 20 min at 1.0 mL/min flow; DAD 220 nm.

Peptide Synthesis. Peptides were prepared by means of standard solid phase synthesis using Fmoc chemistry. Wang resin (0.5 g, 1 mmol/g) suspended in 9:1 DCM/DMF (6 mL) was treated with a solution of Fmoc-Phe (0.39 g, 1 mmol) and HOBt (0.14 g, 1 mmol) in DMF (4 mL), followed by DCC (0.21 g, 1 mmol) and cat. DMAP. The mixture was mechanically shaken for 6 h, and then the mixture was filtered and the resin washed twice with the sequence DMF (5 mL), CH_3OH (5 mL), DCM (5 mL). To end-cap unreacted OH groups, the resin was suspended in DCM (5 mL), treated with Ac_2O (0.5 mL) and pyridine (0.5 mL), and mechanically shaken. After 0.5 h, the mixture was filtered and the resin was washed twice with the sequence DMF (5 mL), CH_3OH (5 mL), DCM (5 mL).

The Fmoc group was cleaved by treatment of the resin with 3:1 DMF/piperidine (5 mL) under mechanical shaking. After 10 min, the mixture was filtered, and the resin was washed once with DCM (5 mL) and treated under mechanical shaking with a second portion of 3:1 DMF/piperidine. After 40 min, the mixture was filtered, and the resin was washed twice with the sequence DMF (5 mL), CH_3OH (5 mL), DCM (5 mL).

The following residues were introduced without side-chain protection by means of the same procedure described above for Fmoc-Phe, apart from the capping step with Ac_2O/Py. Coupling efficacy was determined by means of Kaiser or chloranil tests.[42,43]

Peptide Cleavage. The N-deprotected resin was suspended in a mixture of TFA (5.7 mL), H_2O (0.15 mL), and PhOH (0.15 mL) and agitated with a mechanical shaker at room temperature. After 2 h, the mixture was filtered, and the resin was washed twice with 10% TFA in Et_2O and twice with Et_2O; each filtrate was poured into 200 mL of ice-cold Et_2O. The resulting precipitate was filtered, and the crude solid peptide−TFA salt was purified by crystallization from MeOH/Et_2O.

Cyclization. The peptide (0.23 g, 0.3 mmol) was dissolved in dry DMF (20 mL) and treated while magnetically stirred with $NaHCO_3$ (0.38 g, 4.5 mmol) and diphenylphosphoryl azide (DPPA) (0.13 mL, 0.6 mmoli) at 0 °C. After 2 days, the mixture

was filtered, the solvent was distilled at reduced pressure, and the residue was transferred in a separating funnel. The residue was diluted with water (5 mL), and the mixture was extracted with EtOAc (4 × 20 mL). The collected organic layers were dried over Na_2SO_4, and solvent was evaporated at reduced pressure. The oily residue was purified by flash chromatography over silica gel (eluant: EtOAc/MeOH 97:3), affording the cyclopeptides **17**−**32** as powders.

Membrane Preparations, Determination of Protein Content, and Binding Assays. Rat brain, without cerebellum, was weighed and homogenized in 10 volumes of ice-cold 0.32 M sucrose/10 mM Tris-HCl (pH 7.4 at 4 °C). The homogenate was centrifuged at 2000 rpm, for 10 min, at 4 °C, and the surnatant was in turn centrifuged at 19000 rpm, for 20 min, at 4 °C. The resulting pellet was suspended in 10 volumes of 50 mM Tris-HCl/100 mM NaCl (pH 7.4 at 4 °C), as incubation buffer, and incubated for 1 h at room temperature (in a water bath at 37 °C) to remove any endogenous opioid ligands. After a final centrifugation at 19000 rpm, for 20 min, at 4 °C, the pellet was stored at −80 °C for up to 2 weeks.

Protein concentration was determined according to the method of Lowry et al.[58] [^{3}H]DAMGO was used as μ-selective radioligand (1 nM); specific activity was 64 Ci/mmol, $K_d = 4.85$ nM and $B_{max} = 48$ fmol/mg of protein; $n = 3$. Nonspecific binding was determined in the presence of 100 μM DAMGO. The incubation buffer consisted of 50 mM Tris-HCl, 0.1%BSA (pH 7.4 at 4 °C), and 2 mM EDTA. To prevent any peptidase degradation, the following protease inhibitors were added to the binding buffer: captopril, 25 μg/mL; bacitracin, 0.2 mg/mL; leupeptin, 10 μg/mL; phenylmethanesulfonyl fluoride, 0.19 mg/mL, aprotinin, 5 KIU/mL. δ- and κ-opioid receptors were blocked with 0.01 M DADLE and 0.01 M U50,488, respectively.

The mixture (1 mL) was incubated for 1 h at room temperature, then it was filtered under vacuum through glass fibers (GFB, Whatman, soaked for 1 h in 0.1% polyethyleneimine) and washed with ice-cold washing buffer (50 mM Tris-HCl, pH 7.4, at 4 °C). The ligand−receptor complex radioactivity retained in the filter was measured by liquid scintillation spectrometry using a scintillator after 12 h of incubation in scintillation cocktail. All assays were performed in triplicate and repeated at least three times. Stock solutions (10^{-2} M) comprised dimethyl sulfoxide or methanol/0.1 N HCl (1:1 v/v).

Determination of Inhibition of Cyclic AMP Accumulation. Activity of endomorphin-1 analogues was determined by measuring potency for the inhibition of forskolin-stimulated cyclic AMP accumulation in SH-SY5Y cells (obtained from European Collection of Cell Cultures; passage no. 12). Cells were routinely grown as indicated by the purchaser. A 75 cm flask at 95−100% confluence was split into 24 wells and incubated overnight. When the confluence arrived at 85−95%, the medium was removed and the cells were washed three times with PBS. Then 240 μL of medium without serum [F12: DMEM 1:1 + 2 mM glutamine + 1% nonessential amino acids (NEAA)] with 0.5 mM 3-isobutyl-1-methylsanthine (IBMX, Sigma) and protease inhibitors (captopril, 25 μg/mL; bacitracin, 0.2 mg/mL; leupeptin, 10 μg/mL; phenylmethanesulfonyl fluoride, 0.19 mg/mL; and aprotinin, 5 IU/mL) and 0.01 M DADLE (δ-opioid receptors blocker) were added, and the cells were incubated at 37 °C for 10 min. Forskolin (10 μM) and the ligand of interest at the concentration range studied were added (30 μL of each, to a final volume of 300 μL) and incubated at 37 °C for 15 min. Cells were washed three times with PBS, and then 500 μL of ethanol 95% was added to each well, incubated at 37 °C for 5 min, then transferred to 1.5 mL tubes, and centrifuged at 6000 rpm/5 min at 4 °C; the resulting surnatant was transferred to a new tube. Pellets were washed with 500 μL of ethanol/water (2:1 v/v) and centrifuged again at 6000 rpm/5 min at 4 °C, and the surnatant was added to the first one. Then the samples were dried under vacuum by Speed Vac for 4−6 h until completely dried; cyclic AMP levels were then determined using a commercial kit (Amersham). Each well was determined individually, the triplicates were averaged, and IC_{50} values were determined.[59]

Spectroscopic Characterization of *c*[-Tyr-D-Pro-D-Trp-Phe-Gly-] (32): IR (Nujol) ν 3279, 1653, 1650, 1649, 1458, 1376, 1186 cm^{-1}; ^{1}H NMR ($CDCl_3$ + 3% DMSO-d_6) δ 8.86 (s, 1H), 8.54 (s, 1H), 7.57 (d, 2H, $J = 7.8$ Hz), 7.40 (d, 1H, $J = 8.1$ Hz), 7.30 (d, 1H, $J = 8.0$ Hz), 7.30 (d, 2H, $J = 8.0$ Hz), 6.90−7.20 (m, 5H), 6.90 (d, 2H, $J = 8.7$ Hz), 6.83 (s, 1H), 6.68 (d, 2H, $J = 8.7$ Hz), 6.52 (d, 1H, $J = 7.5$ Hz), 6.49 (d, 1H, $J = 8.7$ Hz), 4.64−4.71 (m, 1H), 4.54−4.59 (q, 1H, $J = 8.0$ Hz), 4.40−4.49 (q, 1H, $J = 8.2$ Hz), 4.28 (dd, 1H, $J = 14.4$, 9.6 Hz), 4.00−4.11 (m, 1H), 3.20−3.33 (m, 2H), 3.10−3.20 (m, 2H), 2.95 (dd, 1H, $J = 14.4$, 3.0 Hz), 2.70−2.85 (m, 3H), 2.30−2.45 (m, 1H), 1.70−1.82 (m, 1H), 1.40−1.55 (m, 1H), 1.25−1.40 (m, 2H); ^{13}C NMR ($CDCl_3$ + 3% DMSO-d_6) δ 24.3, 26.6, 26.7, 35.7, 37.7, 46.7, 46.8, 52.2, 52.3, 53.4, 53.9, 110.3, 111.5, 115.4, 118.7, 119.1, 119.8, 122.6, 125.6, 126.2, 128.2, 129.2, 129.8, 130.0, 136.1, 137.4, 156.3, 170.0, 170.7, 171.2, 171.5, 172.1; $[\alpha]^{20}_D$ +102 (*c* 1, MeOH); ES-MS, 651.2 [M + H], 673.3 [M + Na].

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