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The *cis*-4-Amino-L-proline Residue as a Scaffold for the Synthesis of Cyclic and Linear Endomorphin-2 Analogues

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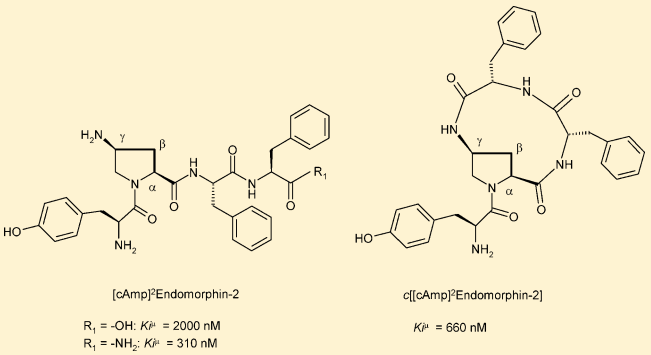
Supporting Information

ABSTRACT: Endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2) is an endogenous tetrapeptide that combines potency and efficacy with high affinity and selectivity toward the μ opioid receptor, the most responsible for analgesic effects in the central nervous system. The presence of the Pro^2 represents a crucial factor for the ligand structural and conformational properties. Proline is in fact an efficient stereochemical spacer, capable of inducing favorable spatial orientation of aromatic rings, a key factor for ligand recognition and interaction with receptors. Here the Pro^2 has been replaced by 4(*S*)-NH_2-2(*S*)-proline (cAmp), a proline/GABA cis-chimera residue. This bivalent amino acid maintains the capacity to influenc the tetrapeptide conformation and offers the opportunity to generate new linear models and unusually constrained cyclic analogues characterized by an N-terminal Tyr bearing a free α-amino group. The results indicate that the new analogues do not show affinity for both δ and κ opioid receptors and bind only poorly to the μ receptors (for cyclopeptide **9**: K_i^{μ} = 660 nM; GPI (IC_{50}) = 1.4% at 1 μM; for linear tetrapeptide acid **13**: K_i^{μ} = 2000 nM; GPI (IC_{50}) = 0% at 1 μM; for linear tetrapeptide amide **15**: K_i^{μ} = 310 nM; GPI (IC_{50}) = 894 nM).



INTRODUCTION

The two tetrapeptide amides endomorphin **1** (H-Tyr-Pro-Trp-Phe-NH_2; EM-1) and endomorphin **2** (H-Tyr-Pro-Phe-Phe-NH_2; EM-2) are endogenous opioid tetrapeptides isolated from bovine and human brain.[1] The high interest for these two ligands is mainly based on their high selectivity and affinity for μ-opioid receptors. Furthermore, EMs exhibit a strong antinociceptive effect on acute pain similar to that of morphine and are devoid of some undesirable side effects typical of morphine. The neuropathic pains are also strongly relieved with efficacy higher than that shown by the majority of the other opioid peptides. Thus, both the simple chemical structure and the beneficial effects shown by EMs in various pathological conditions continue to stimulate design and synthesis of analogues to improve the biological activity, the conformational heterogeneity, and the other limitations typical of peptides used as drugs.

As found in the case of enkephalins and many other opioid peptides, EMs contain a free and protonable Tyr NH_2 which is a key element for the μ-receptor recognition. However, a distinctive structural characteristic of endomorphins is the presence of the Pro residue in position 2 of the backbone.[2] The cis/trans isomerization around the Tyr^1-Pro^2 peptide bond associated with the $\pi-\pi$ interactions involving the three aromatic residues represents a relevant factor in controlling the preferred conformations of the tetrapeptide ligand. As previously noted,[3,4] the effect of this structural feature, together with the presence of the free Tyr NH_2, is enhanced by the location of the Tyr^1-Pro^2 moiety within the biologically relevant N-terminal tripeptide message sequence (Tyr-Pro-Phe) of this class of opioid tetrapeptides.

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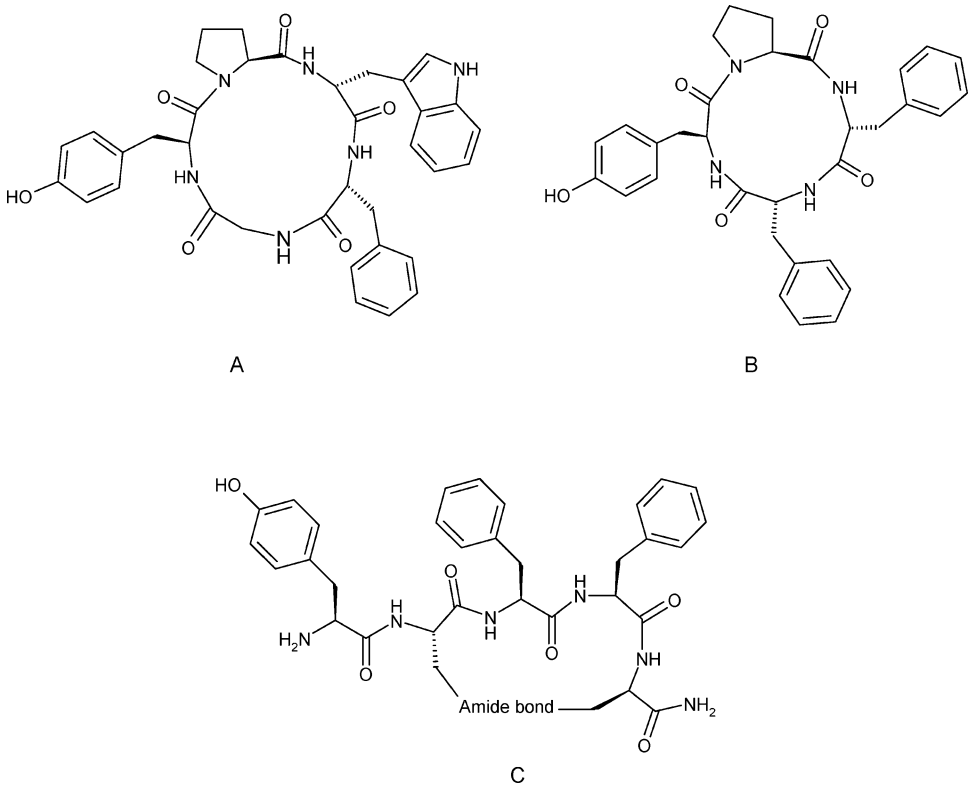

Figure 1. Previously reported endomorphin-1 and endomorphin-2 cyclic analogues obtained by adopting the "head-to-tail strategy" (compounds A and B)[31,28] and the "side chain-to-side chain" strategy (compound C).[32] Only the latter analogue maintains a free, biologically relevant, N-terminal Tyr primary amino group.

In order to study structure−activity relationships and develop potent and selective analgesics, a great number of opioid peptide analogues have been synthesized and evaluated in the past decade. Native opioid peptides show poor bioavailability mainly due to the inability to penetrate the blood−brain barrier[5,6] and to the rapid degradation by peptidases such as aminopeptidases and dipeptidyl peptidase IV.[7−9] To overcome these limitations, different strategies have been developed and exhaustively reviewed.[10−20] Results from these studies suggest that the stabilization of folded structures may enhance endomorphin activity and underscore at the same time the relevant role that the spatial orientation of the three aromatic side chains may play.[3,21−27] In light of these observations and taking into account the intrinsic limitations of peptides as therapeutic agents, the synthesis of cyclic analogues appears particularly appealing. Although the adopted synthetic strategies may greatly differ, the most common methods are based on the introduction of bivalent amino acids so as to close lactam or disulfide bridges[21,26−29] or, more recently, on the adoption of ring closing metathesis.[30]

As compared with enkephalins, only few cyclic analogues of endomorphins have been reported so far.[28,31,32] A first approach adopts the head-to tail type of cyclization and leads to *c*[-Tyr-Pro-Phe-Phe-][28] or *c*[-Tyr-Pro-Trp-Phe-Gly-][31] models (Figure 1) characterized by a lactam bridge between a C-terminal carboxyl group and the N-terminal Tyr NH_2. In a second strategy a side chain-to-side chain approach is adopted and models of the type Tyr-*c*[-Lys-Phe-Phe-Asp-] or Tyr-*c*[-Asp-Phe-Phe-Lys-] are obtained through incorporation of two bivalent amino acids in positions 2 and 5 of a pentapeptide backbone.[32,33] In both cases some of the obtained cyclic EM analogues show sensibly improved activity and selectivity and this, despite the lack of elements, is considered relevant for the activity, such as the protonable N-terminal Tyr NH_2 or the Pro-residue in position 2. These results strongly suggest to apply the cyclization approach to EM analogues characterized by the presence of a free N-terminal Tyr NH_2 together with the rigid skeleton of the Pro residue at position 2.

On the basis of the above considerations, we report here synthesis and activity of new linear and cyclic models based on the sequence of EM-2 and containing modification at the Pro^2 (See Schemes 1 and 2). The relevant structural feature of the new models resides on the utilization of *cis*-4-amino-L-proline (cAmp) to replace the native Pro. This synthetic residue[34,35] combines the conformational rigidity of the pyrrolidine ring structure of the native Pro with the primary amino group of a γ-amino-*n*-butyric acid skeleton, thus realizing a chimera amino acid,[36] namely proline/GABA cis-chimera. The presence of the cAmp at position 2 of the tetrapeptide backbone does not alter significantly the EM-2 amino acid sequence and offers at the same time, as compared with the native Pro residue, an additional amino group available for a cyclization reaction. This leads to a "side-chain to tail" type of cyclization, namely an amide bond between the cAmp-γNH_2 and the Phe^4 C-terminal carboxyl (see Figure 2). The obtained cyclic system gives rise to an 11-membered cyclotripeptide further constrained by the presence of the pyrrolidine inside the ring. This is a very unusual structure which resembles already reported small ring cyclic tripeptides obtained by using β-amino acids, γ-amino acids, or mixed systems of α- and β-residues[15,37−43] as building blocks.

The biological consequences of the insertion of a cAmp residue in the linear EM analogues **13** (Tyr-cAmp-Phe-Phe-OH) and **15** (Tyr-cAmp-Phe-Phe-NH_2) (Scheme 2) possess-

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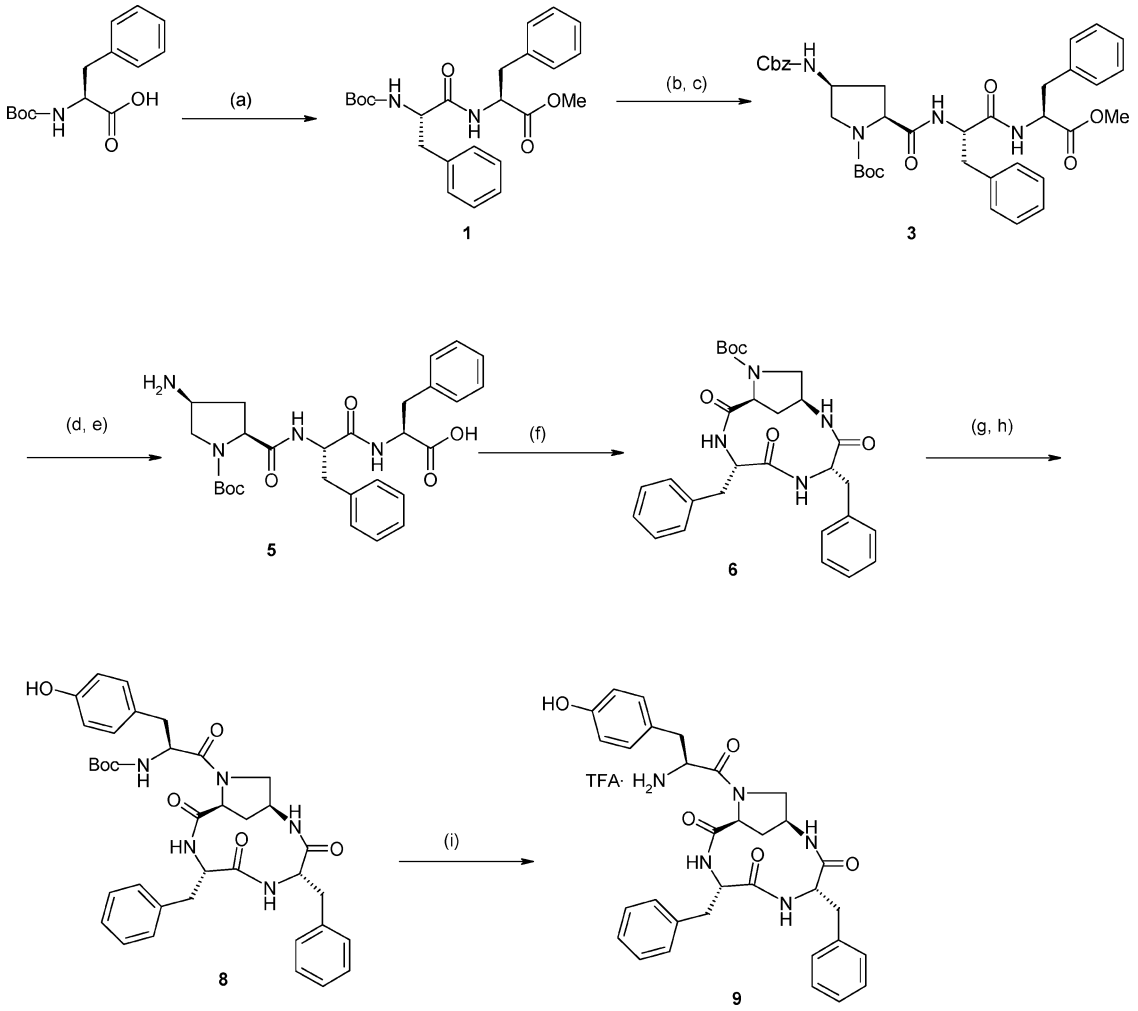
Scheme 1. Chemical Synthesis of Intermediates 1−8 and Final Product 9[a]



[a]Reagents and conditions: (a) **1**, HCl·Phe-OMe, EDC, HOBt·H_2O, NMM, DMF, 0 °C, 20 min, then room temperature, 24 h, 90−95%. (b) **2**, TFA/DCM 1:1, room temperature, 3 h, quantitative. (c) **3**, Boc-cAmp (Cbz)-OH, EDC, HOBt·H_2O, NMM, DMF, 0 °C, 20 min, then room temperature, 24 h, 77−88%. (d) **4**, 1 N NaOH, MeOH, room temperature, 3 h, quantitative. (e) **5**, 10% Pd/C, MeOH, H_2, room temperature, 2−6 h, 73−81%. (f) **6**, PyBop, DIPEA, DMF, room temperature, 12 h, 58−60%. (g) **7**, TFA/DCM 1:1, room temperature, 3 h, quantitative. (h) **8**, Boc-Tyr-OH, EDC, HOBt·H_2O, NMM, DMF, 0 °C, 20 min, then room temperature, 24 h, 80−83%. (i) **9**, TFA/DCM 1:1, room temperature, 3 h, 76−93%.

ing a C-terminal free carboxyl or amide group, respectively, have also been examined. The receptorial affinity of all cyclic and linear analogues was determined by means of displacement binding and in vitro bioactivity assays (Table 1).

RESULTS

Biological tests (Table 1) performed on the linear tetrapeptide containing a C-terminal free carboxylic group **13** showed that the product was inactive at μ, δ, and κ opioid receptors. The corresponding linear amide derivative **15** showed some activity for μ opioid receptors. Also, the cyclic compound **9** showed a certain affinity for μ receptors and no affinity for δ and κ receptors. Furthermore, the second cationic center in position 4 of the cAmp moiety in products **13** and **15**, in general, seems to be less favorable for binding to μ, δ, and κ opioid receptors, probably due to repulsive interactions with another cationic center present in the binding pocket. The products were also tested for μ and δ opioid antagonism; in particular, product **15** was the only one of this series of compounds with any antagonist activity: **15** at 10 mM showed K_e = 1800 ± 220 (the concentration needed to shift the dose−response curve of PL-017 2-fold to the right).

DISCUSSION AND CONCLUSIONS

In this study, we focused attention on the design of a new class of linear and cyclic EM-2; namely, the 11-membered cyclic peptide **9** and the two corresponding linear COOH terminal and $CONH_2$ terminal tripeptides **13** and **15** are reported. As mentioned previously, attempts to synthesize cyclotripeptides made up of α-amino acids (i.e., nine-membered cyclic systems) are not successful because dimerization leading to cyclohexapeptides or formation of more complex systems (azacyclols) deriving from endoannular CO−NH interaction is, with the only exception being the cyclization of N-substituted α-amino acids such as proline, the unique result. In the present case, due to the presence of *cis*-4-amino-L-proline, a new, unusual, and stable 11-membered cyclotripeptide system has been isolated and tested for its bioactivity as an EM analogue.

Data reported in Table 1 indicate that cyclization associated with high restriction of the backbone flexibility produces

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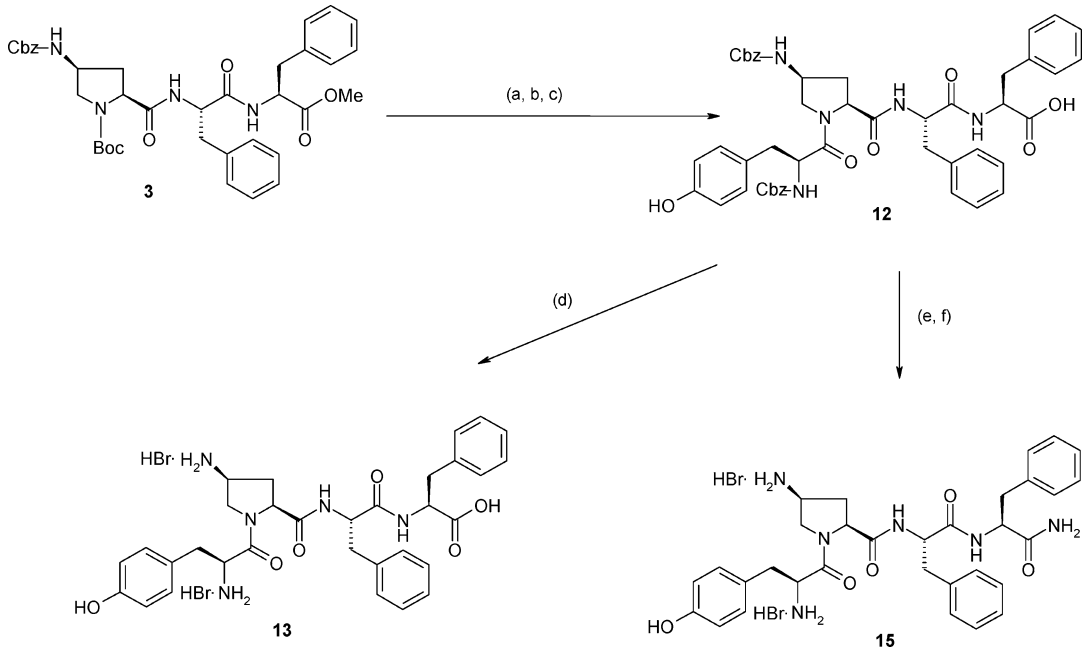
Scheme 2. Chemical Synthesis of Intermediates 10−12, 14 and Final Products 13 and 15[a]



[a]Reagents and conditions: (a) **10**, TFA/DCM 1:1, room temperature, 3 h, quantitative. (b) **11**, Cbz-Tyr-OH, EDC, HOBt·H_2O, NMM, DMF, 0 °C, 20 min, then 24 h at room temperature, 80−89%. (c) **12**, 1 N NaOH, MeOH, room temperature, 3 h, quantitative. (d) **13**, HBr/AcOH 33%, room temperature, 3 h, 70−75%. (e) **14**, IBCF, NMM, THF, NH_4OH, 30 min, −10 °C, then 3 h at room temperature, 85−87%. (f) **15**, 33% HBr/AcOH, 3 h at room temperature, 65−73%.

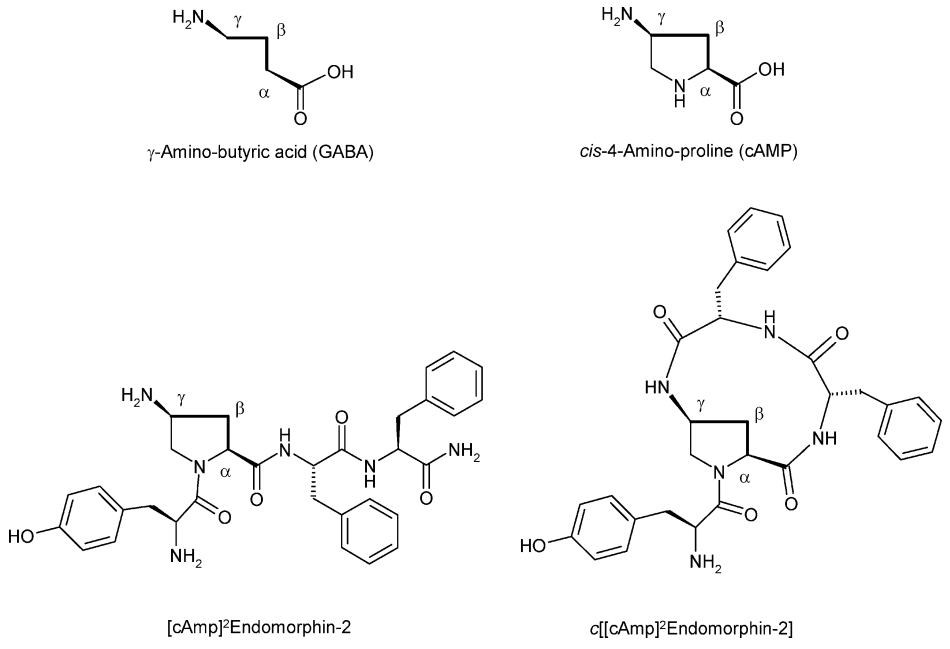


Figure 2. Structure of 4-amino-*n*-butyric acid (GABA), *cis*-4-amino-(*S*)-proline (cAMP), and the here-reported cAmp-containing endomorphin-2 analogues.

analogue **9**, which possesses a strongly altered biological profile. **9** displays potencies 70 times lower than that of the parent, thus indicating that the new hydrogen bond pattern and the 3D shape of the molecule is drastically modified and ultimately affects the receptor binding mode of action.

With regard to the linear analogues **13** and **15**, the replacement of the proline residue in position 2 with a more hydrophilic residue, namely cAmp, produces a drastic affinity loss at both μ and δ receptors. To the best of our knowledge, this is the first information about the influence exerted by an additional cationic center positioned at the second position of endomorphin-2. This result seems to indicate that the repulsion between two closely located electric charges (namely on Tyr1 and cAmp2 residues, respectively) significantly and negatively affects the properties bound to the backbone conformation and the receptor recognition.

Further studies are currently in progress to investigate the relationship between the conformation of different stereo-

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Table 1. Binding Affinity and in Vitro Activity for Compounds 9, 13, and 15

	rMor,[a] [^{3}H]DAMGO[c]				functional bioactivity	
compound	log IC_{50}	K_i[μg,f]	hDor,[a] [^{3}H]DPDPE,[d] K_i[δ]	hKor,[b] [^{3}H]U69593,[e] K_i[κ]	GPI[g] (IC_{50})	MVD[g] (IC_{50})
EM-2[h]		9.6 ± 0.98			15 ± 2	510 ± 35
9	−5.86	660 ± 79	nb[i]	nb	1.4% at 1 μM	0% at 1 μM
13	−5.38	2000 ± 224	nb	nb	0% at 1 μM	3.6% at 1 μM
15	−6.18	310 ± 81	nb	nb	894.50± 126	20% at 1 μM

[a]Competition analyses was carried out using membrane preparations from transfected HN9.10 cells that constitutively expressed the DOR and MOR, respectively. [b]Competition analyses for κ receptors were carried out using rat recombinant CHO cells. [c]K_d = 0.85 ± 0.2 nM. [d]K_d = 0.50 ± 0.1 nM. [e]K_d = 2.0 ± 0.05 nM. [f]The K_i values are calculated using the Cheng and Prusoff equation to correct for the concentration of the radioligand used in the assay. [g]±SEM. [h]Data from ref 3. [i]nb = no binding detected.

isomeric analogues of the cyclotripeptide **9** and receptor affinity, in particular concerning the relative spatial orientation of the three aromatic side chains.

EXPERIMENTAL SECTION

General. All solvents, reagents, and starting materials were obtained from commercial sources unless otherwise indicated. All reactions were performed under N_2 unless otherwise noted. Intermediate products **1**, **3**, **6**, and **8** were purified by silica gel chromatography. Products **9**, **13**, and **15** used for the biological assay were purified by RP-HPLC using a semipreparative Vydac (C_{18}-bonded, 300 Å) column and a gradient elution at a flow rate of 10 mL/min. The gradient used was 10−90% acetonitrile in 0.1% aqueous TFA over 40 min. Approximately 10 mg of crude peptide was injected each time, and the fractions containing the purified peptide were collected and lyophilized to dryness. The purity of the final products, determined by NMR analysis and by analytical RP-HPLC (C_{18}-bonded 4.6 × 150 mm) at a flow rate of 1 mL/min on a Waters Binary pump 1525 using an isocratic elution of 20% CH_3CN/H_2O 0.1% TFA, monitored with a Waters 2996 Photodiode Array Detector, was found to be >95%.

^{1}H NMR spectra were performed in $CDCl_3$ or DMSO-d_6 solution on a Varian Inova operating at the ^{1}H frequency of 300 MHz and on a Bruker AVANCE AQS600 operating at the ^{1}H frequency of 600.13 MHz. Chemical shifts were referred to TMS as internal standard in the case of $CDCl_3$ solution and to the residual proton signal of DMSO at 2.5 ppm in the case of DMSO-d_6 solution. Peptide structures were determined by means of 2D NMR experiments namely ^{1}H−^{1}H TOCSY and ^{1}H−^{1}H NOESY. Peptide structures were also confirmed by high resolution-mass spectra (HR-MS) ± 2 ppm. For the final products **9**, **13**, and **15** elemental analyses (within ±0.4% of the theoretical values) were performed.

Synthesis. N^{α}-Boc-cAmp(Cbz)-OH was synthesized based on a well-defined protocol as previously reported.[35,36] All couplings of the linear intermediates and linear products were performed using the standard coupling method of carbodiimide (EDC/HOBt/NMM) in DMF as described below. The synthesis of the cyclic peptide **9** begins with the coupling between Boc-Phe-OH and HCl·H-Phe-OMe (see Scheme 1) to obtain a dipeptide **1**. The dipeptide obtained was deprotected at the N-terminal moiety by TFA 1:1 DCM. The resulting TFA salts **2** were then coupled with N^{α} -Boc-cAmp(Cbz)-OH to yield the tripeptide **3**. This peptide was used in two different synthetic pathways: to give the cyclic product **9** (see Scheme 1) and to give the linear products **13** and **15** (see Scheme 2). The cyclic product **9** (see Scheme 1) was obtained by deprotecting the tripeptide **3** at the COOMe terminal by hydrolysis with 1 N NaOH in MeOH followed by deprotection of the cAmp 4-amino group by hydrogenolysis with 10% Pd/C in MeOH to give **5**. The deprotected tripeptide **5** was cyclized using PyBop coupling reagent in a highly diluted DMF solution (10^{-3} M). The reaction provided the cyclic products **6** in good yields. Then **6** was N-terminal deprotected in TFA/DCM mixture and the resulting TFA salt **7** coupled to Boc-Tyr-OH to give **8**. Product **8** was N-terminal deprotected in TFA/DCM mixture to give the resulting TFA salt **9**.

The linear products **13** and **15** were obtained from product **3** (see Scheme 2). Product **3** was deprotected at the terminal *N*-Boc by treatment with TFA in DCM 1:1 to give the TFA salt **10** that was coupled to Cbz-Tyr-OH to give the tetrapeptide **11**. This was deprotected at the C-terminal by hydrolysis of the methyl ester group to obtain the intermediate **12** as a common precursor of both **13** and **15**. Compound **13** was obtained as hydrobromide salt by treatment with HBr in glacial acetic while carboxy activation via mixed anhydride followed by treatment with NH_4OH gave the fully protected tetrapeptide amide **14**. This was deprotected with HBr in glacial acetic acid to give the final amide **15** as hydrobromide salt.

TFA·NH_2-Phe-Phe-OMe (2). To a solution of *N*-Boc-Phe-OH (1 mmol) in DMF were added EDC (1.1 equiv), HOBt·H_2O (1.1 equiv), and one portion of NMM (2.2 equiv) at 0 °C under stirring. After 10 min, HCl·Phe-OMe (1 equiv) and a second portion of NMM (1.1 equiv) were added, the mixture was warmed to room temperature overnight, DMF was evaporated under reduced pressure, the oily residue was dissolved in EtOAc, and the organic layer was washed with 5% citric acid, $NaHCO_3$ saturated solution (s.s.), and brine. The organic layers were combined, dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the crude product, which was purified with silica gel column chromatography (90−95%). **1**: ^{1}H NMR ($CDCl_3$) δ: 1.39 (s, 9H, $C(CH_3)_3$), 2.98−3.08 (m, 4H, βCH$_2$ Phe), 3.68 (s, 3H, OCH_3), 4.22−4.39 (m, 1H, αCH Phe^1), 4.70−4.83 (m, 1H, αCH Phe^2), 4.85 (d, 1H, Boc-NH), 6.24 (d, 1H, NH Phe^2), 6.91−7.01 (m, 10H, Ar). ESI-HRMS for $C_{24}H_{31}N_2O_5$ [MH^+], calcd 427.2254; found 427.2251. LRMS (ESI) m/z = 427.2.

Compound **1** was dissolved in a 1:1 TFA/DCM mixture for 3 h at r.t. under N_2 atmosphere. Then the mixture was evaporated under reduced pressure until complete elimination of TFA to give the crude intermediate product **2** that was used in the next step without further purification (88%): ESI-HRMS for $C_{19}H_{23}N_2O_3$ [MH^+], calcd 327.1735; found 327.1733. LRMS (ESI) m/z = 327.2.

***N*-Boc-cAmp(Cbz)-Phe-Phe-OMe (3).** *N*-Boc-cAmp(Cbz)-OH (1.2 equiv) was dissolved in DMF, and EDC (1.35 equiv) and HOBt·H_2O (1.35 equiv) were added to the mixture. Then TFA·Phe-Phe-OMe (**2**) (1.30 equiv) and NMM (0.56 mL) were added. The mixture was kept 12 h at room temperature, DMF was evaporated under reduced pressure, the oily residue was dissolved in EtOAc, and the organic layer was washed with 5% citric acid, $NaHCO_3$ s.s., and brine. The organic layers were combined, dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the crude product that was purified with silica gel column chromatography (84%): ^{1}H NMR ($CDCl_3$) δ: 1.39 (s, 9H, $C(CH_3)_3$), 1.97−2.19 (m, 2H, Pro C^3H_2), 2.80−3.22 (m, 4H, βCH_2Phe), 3.39−3.44 (m, 2H, Pro C^5H_2), 3.60 (s, 3H, OCH_3), 4.15 (m, 1H, αCHPro), 4.24 (m, 1H, Pro C^4H), 4.53 (m, 1H, αCHPhe^2), 4.72 (m, 1H, αCHPhe^3), 5.02 (m, 2H, CH_2 Cbz), 6.22−7.72 (m, 18H, Ar and NH Cbz and NH Phe^{2,3}). ESI-HRMS for $C_{37}H_{45}N_4O_8$ [MH^+], calcd 673.3225; found 673.3222. LRMS (ESI) m/z = 673.3.

***N*-Boc-cAmp(Cbz)-Phe-Phe-OH (4).** NaOH (1 N, 3 equiv) was added slowly to a mixture of methyl ester **3** (0.54 equiv) in MeOH at 0 °C, and then the mixture was stirred 3 h at room temperature. Then the organic solvent was evaporated, and distilled water was added to the residue and extracted with one portion of diethyl ether. Then the

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aqueous layer was acidified by 1 N HCl to pH = 3, the precipitated product was extracted with three portions of EtOAc. The organic layers were combined, dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the free C-terminal acid that was used into the next step without further purification. **4**: ESI-HRMS for $C_{36}H_{43}N_4O_8$ [MH^+], calcd 659.3127; found 659.3130. LRMS (ESI) m/z = 659.3.

***N*-Boc-cAmp-Phe-Phe-OH (5).** Product **4** was dissolved in MeOH, Pd/C 10% was added, and the mixture was kept under H_2 atmosphere for 2−6 h at room temperature stirring vigorously. The catalyzer was removed by paper filtration and the solvent evaporated under reduced pressure. The obtained crude product **5** was used in the next step without further purification. ESI-HRMS for $C_{28}H_{37}N_4O_6$ [MH^+], calcd 525.2789; found 525.2791. LRMS (ESI) m/z = 535.3.

***c*[-4-NH-Pro-Phe-Phe-] (6).** A mixture of the tetrapeptide N^{α}-Boc protected **5** (10^{-3} mol) in DMF and DIPEA (0.5 mL) was added dropwise to a solution of PyBop (1.5 equiv) in DMF and DIPEA (3 equiv) under stirring. The mixture was kept 12 h at room temperature, DMF was evaporated under reduced pressure, and the oily residue was dissolved in EtOAc. The organic layer was washed with 5% citric acid, $NaHCO_3$ s.s., and brine. The organic layers were combined and dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the crude product **6**, which was purified by silica gel column chromatography (60%): ^{1}H NMR ($CDCl_3$) δ: 1.29 (s, 9H, $C(CH_3)_3$), 2.04−2.27 (m, 2H, Pro C^3H_2), 2.95−3.25 (m, 4H, βCH_2Phe), 3.44 (m, 1H, Pro C^5H_a), 3.60 (m, 1H, Pro C^5H_b), 3.78 (m, 1H, αCHPhe^3), 4.18 (m, 1H, Pro C^4H), 4.45 (m, 1H, αCHPhe^2), 4.56 (m, 1H, αCHPro), 6.00−7.30 (m, 13H, Ar and NH Phe^2 and NH Phe^3 and γNHPro). ESI-HRMS for $C_{28}H_{35}N_4O_5$ [MH^+], calcd 507.2637; found 507.2638. LRMS (ESI) m/z = 507.3.

TFA·*c*[-4-NH-Pro-Phe-Phe] (7). **6** (0.27 equiv) was dissolved in a TFA/DCM 1:1 mixture for 3 h at r.t. under N_2 atmosphere. Then the mixture was evaporated under reduced pressure until complete elimination of TFA to give the crude product **7** which was used in the next step without further purification (89%): ESI-HRMS for $C_{23}H_{27}N_4O_3$ [MH^+], calcd 407.2158; found 407.2160. LRMS (ESI) m/z = 407.2.

Boc-Tyr-*c*[-4-NH-Pro-Phe-Phe] (8). To a solution of Boc-Tyr-OH (0.32 equiv) in DMF were added EDC (0.32 equiv), HOBt·H_2O (0.32 equiv), and NMM (0.075 mL) at 0 °C. Then TFA·*c*[4-NH-Pro-Phe-Phe] in DMF and NMM were added to the reaction mixture to give **8** (83%): ^{1}H NMR ($(CD_3)_2SO$) δ: 1.29 (s, 9H, $C(CH_3)_3$), 2.09 (m, 2H, Pro C^3H_2), 2.60−2.94 (m, 2H, βCH_2 Tyr^1), 2.86−3.05 (m, 2H, βCH_2 Phe^3), 3.08−3.20 (m, 2H, βCH_2 Phe^4), 3.48−4.00 (m, 2H, Pro C^5H_2), 3.77 (m, 1H, αCH Phe^4), 4.16 (m, 1H, αCH Tyr^1), 4.34 (m, 1H, αCH Phe^3), 4.40 (m, 1H, Pro C^4H), 4.64 (m, 1H, αCH Pro), 6.59 (d, 1H, NH Pro), 6.65 (d, 2H, $C^{3,5}H$ Tyr^1), 6.99 (d, 1H, NH Tyr^1), 7.10 (d, 2H, $C^{2,6}H$ Tyr^1), 7.72 (d, 1H, NH Phe^4), 8.27 (d, 1H, NH Phe^3). ESI-HRMS for $C_{37}H_{44}N_5O_7$ [MH^+], calcd 670.3228; found 670.3231. LRMS (ESI) m/z = 670.3.

TFA·Tyr-*c*[-4-NH-Pro-Phe-Phe] (9). **8** (0.22 equiv) was dissolved in a TFA/DCM 1:1 mixture for 3 h at r.t. under N_2 atmosphere. Then the mixture was evaporated under reduced pressure until complete elimination of TFA to give the crude intermediate product **9**, used in the next step without further purification (87%): ^{1}H NMR ($(CD_3)_2SO$) δ: 2.04−2.19 (m, 2H, Pro C^3H_2), 2.79−3.14 (m, 2H, βCH_2 Tyr^1), 2.89−3.07 (m, 2H, βCH_2 Phe^3), 3.06−3.18 (m, 2H, βCH_2 Phe^4), 3.24−3.97 (m, 2H, Pro C^5H_2), 3.71 (m, 1H, αCH Phe^4), 4.12 (m, 1H, αCH Tyr^1), 4.38 (m, 1H, Pro C^4H), 4.40 (m, 1H, αCH Phe^3), 4.69 (m, 1H, αCH Pro), 6.44 (d, 1H, NH Pro), 6.72 (d, 2H, $C^{3,5}H$ Tyr^1), 7.18 (d, 2H, $C^{2,6}H$ Tyr^1), 7.87 (d, 1H, NH Phe^4), 8.04 (d, 1H, NH Tyr^1), 8.27 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{36}N_5O_5$ [MH^+], calcd 570.2712; found 570.2710. LRMS (ESI) m/z = 570.3.

TFA·H-cAmp(Cbz)-Phe-Phe-OMe (10). **3** (0.27 equiv) was dissolved in a TFA/DCM 1:1 mixture for 3 h at r.t. under N_2 atmosphere. Then the mixture was evaporated under reduced pressure until complete elimination of TFA to give the crude intermediate product **10**, used in the next step without further purification (92%): ESI-HRMS for $C_{32}H_{37}N_4O_6$ [MH^+], calcd 573.2783; found 573.2780. LRMS (ESI) m/z = 573.3.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-OMe (11). To a solution of Cbz-Tyr-OH (0.32 equiv) in DMF were added EDC (0.32 equiv), HOBt·H_2O (0.32 equiv), and NMM (0.075 mL) at 0 °C. Then TFA·H-cAmp(Cbz)-Phe-Phe in DMF and NMM were added, and the reaction mixture was kept 12 h at room temperature. Then DMF was evaporated under reduced pressure, the oily residue was dissolved in EtOAc, and the organic layer was washed with 5% citric acid, $NaHCO_3$ s.s., and brine. The organic layers were combined and dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the crude product, purified with silica gel column chromatography (83%): ^{1}H NMR ($(CD_3)_2SO$) δ: 1.68−2.33 (m, 2H, Pro C^3H_2), 2.61−2.77 (m, 2H, βCH_2 Tyr^1), 2.85−2.97 (m, 2H, βCH_2 Phe^3), 2.93−2.99 (m, 2H, βCH_2 Phe^4), 3.30−3.99 (m, 2H, Pro C^5H_2), 3.54 (s, 3H, OCH_3), 4.11 (m, 1H, Pro C^4H), 4.29 (m, 1H, αCH Tyr^1), 4.35 (m, 1H, αCH Pro), 4.45 (m, 1H, αCH Phe^4), 4.47 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.64 (d, 2H, $C^{3,5}H$ Tyr^1), 7.04 (d, 2H, $C^{2,6}H$ Tyr^1), 7.47 (d, 1H, NH Pro), 7.55 (d, 1H, NH Tyr^1), 8.10 (d, 1H, NH Phe^3), 8.36 (d, 1H, NH Phe^4). ESI-HRMS for $C_{49}H_{52}N_5O_{10}$ [MH^+], calcd 870.3715; found 870.3718. LRMS (ESI) m/z = 870.4.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-OH (12). NaOH (1 N, 3 equiv) was added slowly to a mixture of methyl ester **11** (0.54 equiv) in MeOH at 0 °C and then stirred 3 h at room temperature. Then the organic solvent was evaporated, distilled water was added to the residue, and the mixture was extracted with one portion of diethyl ether. Then the aqueous layer was acidified with 1 N HCl to pH = 3, and the precipitated product was extracted with three portions of EtOAc. The organic layers were combined, dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the free C-terminal acid, used in the next step without further purification. **12**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.68−2.32 (m, 2H, Pro C^3H_2), 2.61−2.75 (m, 2H, βCH_2 Tyr^1), 2.84−2.99 (m, 2H, βCH_2 Phe^3), 2.91−3.03 (m, 2H, βCH_2 Phe^4), 3.28−3.98 (m, 2H, Pro C^5H_2), 4.10 (m, 1H, Pro C^4H), 4.28 (m, 1H, αCH Tyr^1), 4.36 (m, 1H, αCH Pro), 4.41 (m, 1H, αCH Phe^4), 4.47 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.64 (d, 2H, $C^{3,5}H$ Tyr^1), 7.04 (d, 2H, $C^{2,6}H$ Tyr^1), 7.46 (d, 1H, NH Pro), 7.55 (d, 1H, NH Tyr^1), 8.08 (d, 1H, NH Phe^3), 8.18 (d, 1H, NH Phe^4). ESI-HRMS for $C_{48}H_{50}N_5O_{10}$ [MH^+], calcd 856.3624; found 856.3627. LRMS (ESI) m/z = 856.4.

2HBr·Tyr-cAmp-Phe-Phe-OH (13). **12** was dissolved in 33% HBr/acetic acid mixture for 3 h at room temperature, and then the mixture was evaporated under reduced pressure. The crude HBr salt obtained was purified by HPLC C_{18} (H_2O/CH_3CN) (72%): ^{1}H NMR ($(CD_3)_2SO$) δ: 1.85−2.49 (m, 2H, Pro C^3H_2), 2.85−3.00 (m, 2H, βCH_2 Phe^3), 2.93−3.06 (m, 2H, βCH_2 Phe^4), 2.96−3.02 (m, 2H, βCH_2 Tyr^1), 3.34−3.95 (m, 2H, Pro C^5H_2), 3.85 (m, 1H, Pro C^4H), 4.38 (m, 1H, αCH Tyr^1), 4.44 (m, 1H, αCH Phe^4), 4.56 (m, 1H, αCH Phe^3), 4.62 (m, 1H, αCH Pro), 6.70 (d, 2H, $C^{3,5}H$ Tyr^1), 7.06 (d, 2H, $C^{2,6}H$ Tyr^1), 7.96 (d, 3H, NH Pro), 8.17 (d, 3H, NH Tyr^1), 8.45 (d, 1H, NH Phe^4), 8.68 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{38}N_5O_6$ [MH^+], calcd 588.2875; found 588.2873. LRMS (ESI) m/z = 588.3.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-NH$_2$ (14). To the mixture of tetrapeptide COOH terminal **12** (1 mmol) in THF at −10 °C were added NMM (2.2 mmol) and isobutyl chloroformate (IBCF) (1.1 mmol) under stirring. After 15 min, NH_4OH s.s. was added in excess, and the mixture was kept for 15 min at −10 °C and then at room temperature for 3 h. Then the solvent was evaporated under reduced pressure, and the oily residue was dissolved in EtOAc. The organic layer was washed with 5% citric acid, $NaHCO_3$ s.s., and brine. The organic layers were combined and dried under Na_2SO_4, filtered, and evaporated under reduced pressure to give the crude product **14**, which was purified with silica gel column chromatography. ^{1}H NMR ($(CD_3)_2SO$) δ: 1.68−2.32 (m, 2H, Pro C^3H_2), 2.61−2.76 (m, 2H, βCH_2 Tyr^1), 2.83−3.02 (m, 2H, βCH_2 Phe^3), 2.85−2.94 (m, 2H, βCH_2 Phe^4), 3.30−3.99 (m, 2H, Pro C^5H_2), 4.11 (m, 1H, Pro C^4H), 4.29 (m, 1H, αCH Tyr^1), 4.34 (m, 1H, αCH Pro), 4.39 (m, 1H, αCH Phe^3), 4.39 (m, 1H, αCH Phe^4), 5.03 (m, 2H, CH_2 Cbz), 6.64 (d, 2H, $C^{3,5}H$ Tyr^1), 7.04 (d, 2H, $C^{2,6}H$ Tyr^1), 7.50 (d, 1H, NH Pro), 7.58 (d, 1H, NH Tyr^1), 7.99 (d, 1H, NH Phe^3), 8.13 (d, 1H, NH Phe^4). ESI-HRMS for $C_{48}H_{51}N_6O_9$ [MH^+], calcd 855.3744; found 855.3743. LRMS (ESI) m/z = 855.4.

2HBr·Tyr-cAmp-Phe-Phe-NH_2 (15). **14** was dissolved in 33% HBr/acetic acid mixture for 3 h at room temperature, and then the mixture was evaporated under reduced pressure. The crude HBr salt obtained was purified by HPLC C_{18} (H_2O/CH_3CN) (75%): ^{1}H NMR ($(CD_3)_2SO$) δ: 1.88−2.51 (m, 2H, Pro C^3H_2), 2.80−2.97 (m, 2H, βCH_2 Tyr^1), 2.84−3.01 (m, 2H, βCH_2 Phe^4), 2.88−2.96 (m, 2H, βCH_2 Phe^3), 3.47−3.98 (m, 2H, Pro C^5H_2), 3.87 (m, 1H, Pro C^4H), 4.27 (m, 1H, αCH Tyr^1), 4.45 (m, 1H, αCH Phe^4), 4.51 (m, 1H, αCH Phe^3), 4.61 (m, 1H, αCH Pro), 6.70 (d, 2H, $C^{3,5}H$ Tyr^1), 7.06 (d, 2H, $C^{2,6}H$ Tyr^1), 8.06 (d, 3H, NH Tyr^1), 8.06 (d, 3H, NH Pro), 8.18 (d, 1H, NH Phe^4), 8.62 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{39}N_6O_5$ [MH^+], calcd 587.3047; found 587.3045. LRMS (ESI) m/z = 587.3.

Functional GPI and Mouse Vas Deferens (MVD) Assays. In vitro biological assays were performed on **9** as TFA salts, and **13**, **15** as hydrobromide salts. GPI and MVD in vitro bioassays were performed as described previously.[43−45] Electrically induced smooth muscle contractions of mouse vas deferens and guinea pig ileum longitudinal muscle-myenteric plexus were used as bioassays. Tissue came from male ICR mice weighing 25−30 g and from male Hartley guinea pigs weighing 150−400 g. The tissues were tied to gold chains with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium-free for MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g tension (0.5 g for MVD), allowed to equilibrate for 15 min, and stimulated transmurally between platinum plate electrodes at 0.1 Hz for 0.4 ms pulses (2.0 ms pulses for MVD) and supramaximal voltage. Drugs were added to the baths in 20−60 μL volumes. The agonists remained in contact with the tissue for 3 min, and the baths were then rinsed several times with fresh Krebs solution until tissues regained predrug contraction height. IC_{50} values represent the mean of not less than four tissues. IC_{50} estimates and relative potency estimates were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method. All biological data are summarized in Table 1.

Radioligand Labeled Binding Assays. *μ and δ Opioid Receptors.* Crude membranes were prepared as previously described[44,45] from transfected cells that express the MOR or the DOR. The protein concentration of the membrane preparations was determined by the Lowry method, and the membranes were stored at −80 °C until use. Membranes were resuspended in assay buffer [50 mM Tris, pH 7.4, containing 50 μg/mL bacitracin, 30 μM bestatin, 10 μM captopril, 100 μM PMSF, 1 mg/mL BSA]. For saturation analysis, six concentrations of [^{3}H]DAMGO (0.02−6 nM, 47.2 Ci/mmol) or six concentrations of [^{3}H]DPDPE (0.1−10 nM, 44 Ci/mmol) were each mixed with 200 μg of membranes from MOR- or DOR-expressing cells, respectively, in a final volume of 1 mL. For competition analysis, 10 concentrations of a test compound were each incubated with 50 μg of membranes from MOR- or DOR-expressing cells and a K_d concentration of [^{3}H]DAMGO (1.0 nM, 50 Ci/mmol) or of [^{3}H]DPDPE (1.0 nM, 44 Ci/mmol), respectively. Naloxone at 10 μM was used to define the nonspecific binding of the radioligands in all assays. All samples were carried out in duplicate. The samples were incubated in a shaking water bath at 25 °C for 3 h, followed by a rapid filtration through Whatman grade GF/B filter paper (Gaithersburg, MD) presoaked in 1% polyethyleneimine and washing four times each with 2 mL of cold saline, and the radioactivity was determined by liquid scintillation counting (Beckman LS5000 TD).

κ Opioid Receptors. κ opioid receptor (KOR) binding affinities were carried out by CEREP - Rue du Bois l'Eveque - BP 30001 - 86600 Celle l'Evescault (France) following a slightly modified procedure previously reported by Meng et al.[46] Chinese hamster ovary (CHO) cell lines that stably express human KOP were established as previously described.[46] The K_d values of [^{3}H]U69593 binding to KOP were 2.0 ± 0.5 nM (n = 3). Expressing cells were harvested after 65 h in culture, homogenized in 50 mM Tris buffer (pH 7.4) that contained 10 mM $MgCl_2$ and 1 mM EDTA, pelleted by centrifugation for 20 min at 30 000g, and resuspended in the same buffer. For the saturation binding assays, cell membrane suspensions were incubated for 60 min at 25 °C with various concentrations [^{3}H]U69593. Nonspecific binding was determined in the presence of 10 μM of naloxone. For the competitive binding assays, the cell membrane suspensions were incubated for 60 min at RT with 3 nM [^{3}H]U69593 in the presence of various concentrations of ligands. After incubation for 60 min, the membrane suspensions were rapidly filtered, and the radioactivity of each filter was then measured by liquid scintillation counting. The binding assay results are the mean ± SEM of four independent experiments, each performed in duplicate.

■ ASSOCIATED CONTENT

Supporting Information

Elemental analysis data for compounds **1**−**15** are reported. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Cbz, carbobenzoxy; GPI, guinea pig ileum; [^{3}H]DAMGO, [^{3}H]-[D-Ala(2),*N*-Me-Phe-(4),Gly-ol(5)]enkephalin; [^{3}H]-U69593, [^{3}H]-(+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; DCM, dichloromethane; DIPEA, diisopropylethylamine; [^{3}H]-DPDPE, [^{3}H]-*c*[2-D-penicillamine,5-D-penicillamine]enkephalin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DOR, δ opioid receptor; EDC, 1-ethyl-(3-dimethylaminopropyl)-carbodiimide; HOBt, 1-hydroxybenzotriazole; IBCF, isobutyl chloroformate; MOR, μ opioid receptor; MVD, mouse vas deferens; NMM, *N*-methylmorpholine; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMS, tetramethylsilane; PyBop, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate

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