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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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Abstract

Endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH₂) 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 Pro² 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 Here the Pro² has been replaced by 4(*S*)-NH₂-2(*S*)-proline (cAmp), a proline/GABA *cis*-chimera residue. This bivalent amino acid maintains the capacity to influence 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_d^μ = 660 nM; GPI (IC₅₀) = 1.4% at 1 μ M; for linear tetrapeptide acid **13**: K_d^μ = 2000 nM; GPI (IC₅₀) = 0% at 1 μ M; for linear tetrapeptide amide **15**: K_d^μ = 310 nM; GPI

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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>.

The authors declare no competing financial interest.

Graphical abstract

INTRODUCTION

The two tetrapeptide amides endomorphin **1** (H-Tyr-Pro-Trp-Phe-NH₂; EM-1) and endomorphin **2** (H-Tyr-Pro-Phe-Phe-NH₂; 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 NH₂ 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 Tyr¹-Pro² peptide bond associated with the π - π 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 NH₂, is enhanced by the location of the Tyr¹-Pro² moiety within the biologically relevant N-terminal tripeptide message sequence (Tyr-Pro-Phe) of this class of opioid tetrapeptides.

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 ϵ -[Tyr-Pro-Phe-Phe-]²⁸ or ϵ -[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 NH₂. In a second strategy a side chain-to-side chain approach is adopted and models of the type Tyr- ϵ -[Lys-Phe-Phe-Asp-] or Tyr- ϵ -[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 NH₂ or the Proresidue 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 NH₂ 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 Pro² (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- γ NH₂ and the Phe⁴ C-terminal carboxyl (see Figure 2). The obtained cyclic system gives rise to an 11-membered cyclotriptide 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-NH₂) (Scheme 2) possessing 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 $K_e = 1800 \pm 220$ (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 CONH₂ 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 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 stereoisomeric 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 N₂ 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 (C₁₈-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 (C₁₈-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% CH₃CN/H₂O 0.1% TFA, monitored with a Waters 2996 Photodiode Array Detector, was found to be >95%.

¹H NMR spectra were performed in CDCl₃ or DMSO-*d*₆ solution on a Varian Inova operating at the ¹H frequency of 300 MHz and on a Bruker AVANCE AQS600 operating at the ¹H frequency of 600.13 MHz. Chemical shifts were referred to TMS as internal standard in the case of CDCl₃ 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 ¹H–¹H TOCSY and ¹H–¹H 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-NH₂-Phe-Phe-OMe (2)

To a solution of *N*-Boc-Phe-OH (1 mmol) in DMF were added EDC (1.1 equiv), HOBt-H₂O (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**: ¹H NMR (CDCl_3) δ : 1.39 (s, 9H, $\text{C}(\text{CH}_3)_3$), 2.98–3.08 (m, 4H, βCH_2 Phe), 3.68 (s, 3H, OCH_3), 4.22–4.39 (m, 1H, αCH Phe¹), 4.70–4.83 (m, 1H, αCH Phe²), 4.85 (d, 1H, Boc-NH), 6.24 (d, 1H, NH Phe²), 6.91–7.01 (m, 10H, Ar). ESI-HRMS for $\text{C}_{24}\text{H}_{31}\text{N}_2\text{O}_5$ [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 $\text{C}_{19}\text{H}_{23}\text{N}_2\text{O}_3$ [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-H₂O (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%): ¹H NMR (CDCl_3) δ : 1.39 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.97–2.19 (m, 2H, Pro C^3H_2), 2.80–3.22 (m, 4H, $\beta\text{CH}_2\text{Phe}$), 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 $\text{C}_{37}\text{H}_{45}\text{N}_4\text{O}_8$ [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 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 Na₂SO₄, 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 C₃₆H₄₃N₄O₈ [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 H₂ 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 C₂₈H₃₇N₄O₆ [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, NaHCO₃ s.s., and brine. The organic layers were combined and dried under Na₂SO₄, filtered, and evaporated under reduced pressure to give the crude product **6**, which was purified by silica gel column chromatography (60%): ¹H NMR (CDCl₃) δ: 1.29 (s, 9H, C(CH₃)₃), 2.04–2.27 (m, 2H, Pro C³H₂), 2.95–3.25 (m, 4H, βCH₂Phe), 3.44 (m, 1H, Pro C⁵H_a), 3.60 (m, 1H, Pro C⁵H_b), 3.78 (m, 1H, αCHPhe³), 4.18 (m, 1H, Pro C⁴H), 4.45 (m, 1H, αCHPhe²), 4.56 (m, 1H, αCHPro), 6.00–7.30 (m, 13H, Ar and NH Phe² and NH Phe³ and γNHPro). ESI-HRMS for C₂₈H₃₅N₄O₅ [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 N₂ 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 C₂₃H₂₇N₄O₃ [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·H₂O (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%): ¹H NMR ((CD₃)₂SO) δ: 1.29 (s, 9H, C(CH₃)₃), 2.09 (m, 2H, Pro C³H₂), 2.60–2.94 (m, 2H, βCH₂ Tyr¹), 2.86–3.05 (m, 2H, βCH₂ Phe³), 3.08–3.20 (m, 2H, βCH₂ Phe⁴), 3.48–4.00 (m, 2H, Pro C⁵H₂), 3.77 (m, 1H, αCH Phe⁴), 4.16 (m, 1H, αCH Tyr¹), 4.34 (m, 1H, αCH Phe³), 4.40 (m, 1H, Pro C⁴H), 4.64 (m, 1H, αCH Pro), 6.59 (d, 1H, NH Pro), 6.65 (d, 2H, C^{3,5}H Tyr¹), 6.99 (d, 1H, NH Tyr¹), 7.10 (d, 2H, C^{2,6}H Tyr¹), 7.72 (d, 1H, NH Phe⁴), 8.27 (d, 1H, NH Phe³). ESI-HRMS for C₃₇H₄₄N₅O₇ [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 N₂ 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%): ¹H NMR ((CD₃)₂SO) δ : 2.04–2.19 (m, 2H, Pro C³H₂), 2.79–3.14 (m, 2H, β CH₂ Tyr¹), 2.89–3.07 (m, 2H, β CH₂ Phe³), 3.06–3.18 (m, 2H, β CH₂ Phe⁴), 3.24–3.97 (m, 2H, Pro C⁵H₂), 3.71 (m, 1H, α CH Phe⁴), 4.12 (m, 1H, α CH Tyr¹), 4.38 (m, 1H, Pro C⁴H), 4.40 (m, 1H, α CH Phe³), 4.69 (m, 1H, α CH Pro), 6.44 (d, 1H, NH Pro), 6.72 (d, 2H, C^{3,5}H Tyr¹), 7.18 (d, 2H, C^{2,6}H Tyr¹), 7.87 (d, 1H, NH Phe⁴), 8.04 (d, 1H, NH Tyr¹), 8.27 (d, 1H, NH Phe³). ESI-HRMS for C₃₂H₃₆N₅O₅ [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 N₂ 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 C₃₂H₃₇N₄O₆ [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·H₂O (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, NaHCO₃ s.s., and brine. The organic layers were combined and dried under Na₂SO₄, filtered, and evaporated under reduced pressure to give the crude product, purified with silica gel column chromatography (83%): ¹H NMR ((CD₃)₂SO) δ : 1.68–2.33 (m, 2H, Pro C³H₂), 2.61–2.77 (m, 2H, β CH₂ Tyr¹), 2.85–2.97 (m, 2H, β CH₂ Phe³), 2.93–2.99 (m, 2H, β CH₂ Phe⁴), 3.30–3.99 (m, 2H, Pro C⁵H₂), 3.54 (s, 3H, OCH₃), 4.11 (m, 1H, Pro C⁴H), 4.29 (m, 1H, α CH Tyr¹), 4.35 (m, 1H, α CH Pro), 4.45 (m, 1H, α CH Phe⁴), 4.47 (m, 1H, α CH Phe³), 5.03 (m, 2H, CH₂ Cbz), 6.64 (d, 2H, C^{3,5}H Tyr¹), 7.04 (d, 2H, C^{2,6}H Tyr¹), 7.47 (d, 1H, NH Pro), 7.55 (d, 1H, NH Tyr¹), 8.10 (d, 1H, NH Phe³), 8.36 (d, 1H, NH Phe⁴). ESI-HRMS for C₄₉H₅₂N₅O₁₀ [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 Na₂SO₄, filtered, and evaporated under reduced pressure to give the free C-terminal acid, used in the next step without further purification. **12**: ¹H NMR ((CD₃)₂SO) δ : 1.68–2.32 (m, 2H, Pro C³H₂), 2.61–2.75 (m, 2H, β CH₂ Tyr¹), 2.84–2.99 (m,

2H, β CH₂ Phe³), 2.91–3.03 (m, 2H, β CH₂ Phe⁴), 3.28–3.98 (m, 2H, Pro C⁵H₂), 4.10 (m, 1H, Pro C⁴H), 4.28 (m, 1H, α CH Tyr¹), 4.36 (m, 1H, α CH Pro), 4.41 (m, 1H, α CH Phe⁴), 4.47 (m, 1H, α CH Phe³), 5.03 (m, 2H, CH₂ Cbz), 6.64 (d, 2H, C^{3,5}H Tyr¹), 7.04 (d, 2H, C^{2,6}H Tyr¹), 7.46 (d, 1H, NH Pro), 7.55 (d, 1H, NH Tyr¹), 8.08 (d, 1H, NH Phe³), 8.18 (d, 1H, NH Phe⁴). ESI-HRMS for C₄₈H₅₀N₅O₁₀ [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 C₁₈ (H₂O/CH₃CN) (72%): ¹H NMR ((CD₃)₂SO) δ : 1.85–2.49 (m, 2H, Pro C³H₂), 2.85–3.00 (m, 2H, β CH₂ Phe³), 2.93–3.06 (m, 2H, β CH₂ Phe⁴), 2.96–3.02 (m, 2H, β CH₂ Tyr¹), 3.34–3.95 (m, 2H, Pro C⁵H₂), 3.85 (m, 1H, Pro C⁴H), 4.38 (m, 1H, α CH Tyr¹), 4.44 (m, 1H, α CH Phe⁴), 4.56 (m, 1H, α CH Phe³), 4.62 (m, 1H, α CH Pro), 6.70 (d, 2H, C^{3,5}H Tyr¹), 7.06 (d, 2H, C^{2,6}H Tyr¹), 7.96 (d, 3H, NH Pro), 8.17 (d, 3H, NH Tyr¹), 8.45 (d, 1H, NH Phe⁴), 8.68 (d, 1H, NH Phe³). ESI-HRMS for C₃₂H₃₈N₅O₆ [MH⁺], calcd 588.2875; found 588.2873. LRMS (ESI) m/z = 588.3.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-NH₂ (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, NH₄OH 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, NaHCO₃ s.s., and brine. The organic layers were combined and dried under Na₂SO₄, filtered, and evaporated under reduced pressure to give the crude product **14**, which was purified with silica gel column chromatography. ¹H NMR ((CD₃)₂SO) δ : 1.68–2.32 (m, 2H, Pro C³H₂), 2.61–2.76 (m, 2H, β CH₂ Tyr¹), 2.83–3.02 (m, 2H, β CH₂ Phe³), 2.85–2.94 (m, 2H, β CH₂ Phe⁴), 3.30–3.99 (m, 2H, Pro C⁵H₂), 4.11 (m, 1H, Pro C⁴H), 4.29 (m, 1H, α CH Tyr¹), 4.34 (m, 1H, α CH Pro), 4.39 (m, 1H, α CH Phe³), 4.39 (m, 1H, α CH Phe⁴), 5.03 (m, 2H, CH₂ Cbz), 6.64 (d, 2H, C^{3,5}H Tyr¹), 7.04 (d, 2H, C^{2,6}H Tyr¹), 7.50 (d, 1H, NH Pro), 7.58 (d, 1H, NH Tyr¹), 7.99 (d, 1H, NH Phe³), 8.13 (d, 1H, NH Phe⁴). ESI-HRMS for C₄₈H₅₁N₆O₉ [MH⁺], calcd 855.3744; found 855.3743. LRMS (ESI) m/z = 855.4.

2HBr-Tyr-cAmp-Phe-Phe-NH₂ (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 C₁₈ (H₂O/CH₃CN) (75%): ¹H NMR ((CD₃)₂SO) δ : 1.88–2.51 (m, 2H, Pro C³H₂), 2.80–2.97 (m, 2H, β CH₂ Tyr¹), 2.84–3.01 (m, 2H, β CH₂ Phe⁴), 2.88–2.96 (m, 2H, β CH₂ Phe³), 3.47–3.98 (m, 2H, Pro C⁵H₂), 3.87 (m, 1H, Pro C⁴H), 4.27 (m, 1H, α CH Tyr¹), 4.45 (m, 1H, α CH Phe⁴), 4.51 (m, 1H, α CH Phe³), 4.61 (m, 1H, α CH Pro), 6.70 (d, 2H, C^{3,5}H Tyr¹), 7.06 (d, 2H, C^{2,6}H Tyr¹), 8.06 (d, 3H, NH Tyr¹), 8.06 (d, 3H, NH Pro), 8.18 (d, 1H, NH Phe⁴), 8.62 (d, 1H, NH Phe³). ESI-HRMS for C₃₂H₃₉N₆O₅ [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% O₂, 5% CO₂) 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. IC₅₀ values represent the mean of not less than four tissues. IC₅₀ 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 [³H]DAMGO (0.02–6 nM, 47.2 Ci/mmol) or six concentrations of [³H]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 [³H]DAMGO (1.0 nM, 50 Ci/mmol) or of [³H]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 [³H]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 MgCl₂ 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 [³H]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 [³H]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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS USED

Boc	<i>tert</i> -butoxycarbonyl
BSA	bovine serum albumin
Cbz	carbobenzoxo
GPI	guinea pig ileum
[³H]DAMGO	[³ H]-[D-Ala (2), <i>N</i> -Me-Phe-(4), Gly-ol(5)]enkephalin
[³H]-U69593	[³ H]-(+)-(5 <i>α</i> , 7 <i>α</i> , 8 <i>β</i>)- <i>N</i> -methyl- <i>N</i> -[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide
DCM	dichloromethane
DIPEA	diisopropylethylamine
[³H]-DPDPE	[³ H]- <i>c</i> [2-D-penicillamine, 5-D-penicillamine]enkephalin
DMF	<i>N,N</i> 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	<i>N</i> -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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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.

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.

Scheme 1. Chemical Synthesis of Intermediates 1–8 and Final Product 9^a

^aReagents and conditions: (a) **1**, HCl-Phe-OMe, EDC, HOBT·H₂O, 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·H₂O, 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, H₂, 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·H₂O, 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%.

Scheme 2.

Chemical Synthesis of Intermediates 10–12, 14 and Final Products 13 and 15^a

^aReagents and conditions: (a) **10**, TFA/DCM 1:1, room temperature, 3 h, quantitative. (b) **11**, Cbz-Tyr-OH, EDC, HOBT·H₂O, 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, NH₄OH, 30 min, –10 °C, then 3 h at room temperature, 85–87%. (f) **15**, 33% HBr/AcOH, 3 h at room temperature, 65–73%.

Table 1

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

compound	rMor, ^a [³ H]DAMGO ^c			hDor, ^a [³ H]DPDPE, K_i , ^d	hKor, ^b [³ H]U69593, eK_i , ^e	functional bioactivity		
	log IC ₅₀	K_i , ^d	K_i , ^d			GPIg (IC ₅₀)	MVDg (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.