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cis-4-Amino-L-proline Residue As a Scaffold for the Synthesis of Cyclic and Linear Endomorphin-2 Analogues: Part 2

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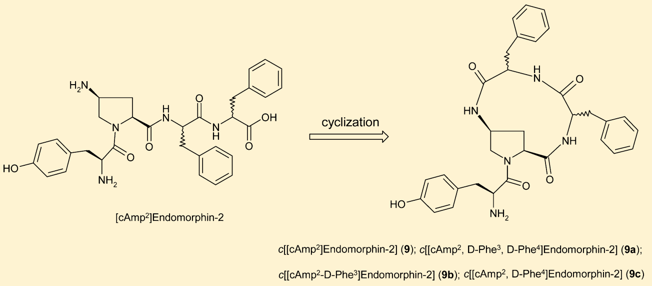
Ⓢ *Supporting Information*

ABSTRACT: Recently, we reported synthesis and activity of a constrained cyclic analogue of endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2) and related linear models containing the *cis*-4-amino-L-proline (cAmp) in place of native Pro^2. In the present article, the adopted rationale is the possible modulation of the receptor affinity of the cAmp containing EM-2 analogues by assigning a different stereochemistry to the Phe^3 and Phe^4 residues present in the ring. Thus, eight more analogues with different absolute configuration at the chiral center of the aromatic residues in positions 3 and 4 have been synthesized and their opioid activity examined. The stereochemical change at the α-carbon atoms leads to a meaningful enhancement of the affinity and activity toward μ opioid receptors with respect to the prototype compound **9**: e.g., **9a**, K_i^μ = 63 nM, GPI (IC_{50}) = 480 nM; **9b**, K_i^μ = 38 nM, GPI (IC_{50}) = 330 nM.



[cAmp^2]Endomorphin-2

c[[cAmp^2]Endomorphin-2] (**9**); *c*[[cAmp^2, D-Phe^3, D-Phe^4]Endomorphin-2] (**9a**); *c*[[cAmp^2-D-Phe^3]Endomorphin-2] (**9b**); *c*[[cAmp^2, D-Phe^4]Endomorphin-2] (**9c**)

INTRODUCTION

In the light of the intrinsic limitations of native opioid peptides as therapeutic agents,[1−3] the design of cyclic analogues appears particularly appealing.[4−7] In a recent article,[8] our research team reported the design and synthesis of a cyclic model and related linear structures, based on the sequence of endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2 ; EM-2)[9,10] containing a modification at the Pro^2 (see Figure 1). The relevant structural feature of those models was based on the utilization of a *cis*-4-amino-L-proline (cAmp) residue to replace the native proline. This synthetic amino acid[11] combines the conformational rigidity of the pyrrolidine Pro ring with the presence at position 4 of the heterocyclic ring of a *cis*-oriented primary amino group, thus realizing an arrangement corresponding to a proline/γ-amino-*n*-butyric acid chimera,[12,13] namely, proline/GABA *cis*-chimera.[11] The presence of the cAmp at position 2 of the tetrapeptide backbone should 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.

As a consequence of the structural properties of the poly functional cAmp residue, its insertion into a peptide backbone gives rise, in addition to usual linear analogues, to structurally interesting cyclic models.

In the specific field of EM-2, the study of linear analogues containing cAmp at position 2 can give information on the biological consequences of the presence of an additional free primary amino group adjacent to that of the native N-terminal tyrosine. Furthermore, an intramolecular side chain-to-tail coupling reaction between the cAmp-γ-NH_2 and the Phe^4 C-terminal carboxyl group gives rise to an 11-membered cyclic system further constrained by the presence of the cAmp inside the backbone[8] (see Figure 1).

A series of selected cyclic pentapeptide EM-1 analogues has been previously reported.[14−16] These cyclopeptides maintain the amino acid sequence of the parent and are characterized by an amino acidic bridge of different length and chirality inserted between the N-terminal Tyr^1 and the C-terminal Phe^4. Notable structural feature of these analogues is the D absolute configuration at position 2 and 3 of the backbone as well as the absence of the N-terminal free amino group, which is intramolecularly bound to the fifth amino acid used as a bridge.

As compared with these models, the here reported cyclic system, containing the cAmp residue, presents two main differences: (i) a more constrained peptide skeleton with lower

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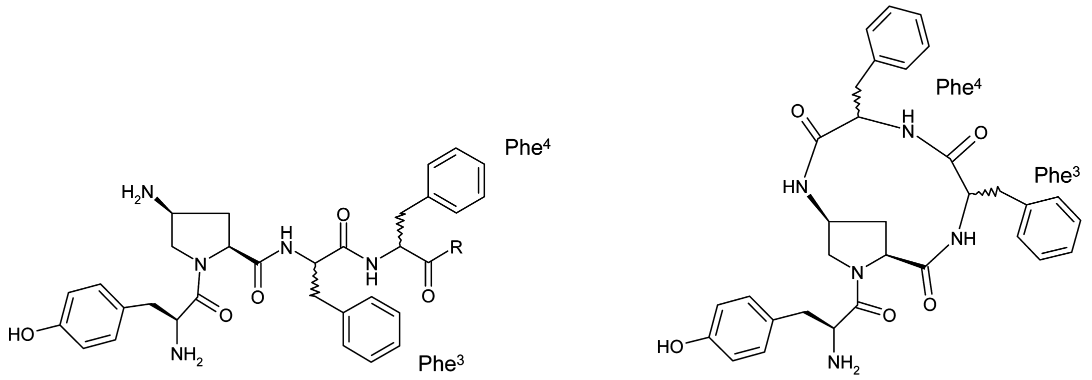


[cAmp^2]Endomorphin-2 (R = OH: **13**; R = NH_2: **15**)
[cAmp^2, D-Phe^3, D-Phe^4]Endomorphin-2 (R = OH: **13a**; R = NH_2: **15a**)
[cAmp^2, D-Phe^3]Endomorphin-2 (R = OH: **13b**; R = NH_2: **15b**)
[cAmp-D-Phe^4]Endomorphin-2 (R = OH: **13c**; R = NH_2: **15c**)

c[[cAmp^2]Endomorphin-2] (**9**)
c[[cAmp^2, D-Phe^3, D-Phe^4]Endomorphin-2] (**9a**)
c[[cAmp^2-D-Phe^3]Endomorphin-2] (**9b**)
c[[cAmp^2, D-Phe^4]Endomorphin-2] (**9c**)

Figure 1. Here reported EM-2 linear (**13a**−**c** and **15 a-c**) and cyclic (**9a**−**b**) analogues obtained by adopting the side chain-to-tail strategy. For the sake of clarity, the structures of the previously reported analogue (**9**) and its precursors (**13**−**15**) are also indicated. Product **9c** was not synthesized as reported in the text.

Table 1. Binding Affinity and in Vitro Activity for Compounds 9a-b, 13a-c and 15a-c.

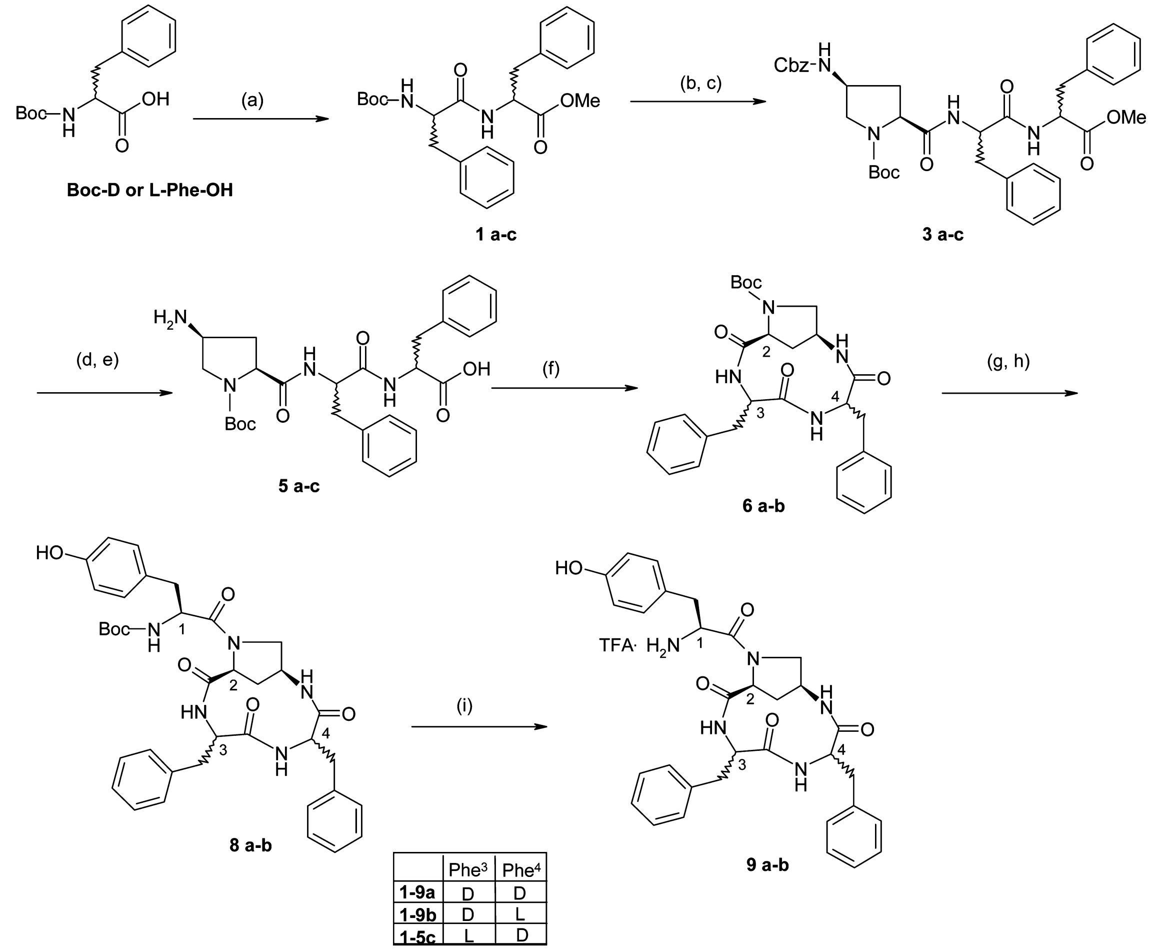
compound	receptor affinity^{a,b} (nM)			functional bioactivity (nM)	
	$K_i^{\mu c,f,g}$	$K_i^{\delta d,f,i}$	$K_i^{\kappa e,f,i}$	GPI^g (IC_{50})	MVD^g (IC_{50})
EM-2^{h,i}	9.6 ± 90.98	>500	>500	15 ± 2	510 ± 35
9^h	660 ± 79	nb^j	nb	1.4% at 1 μM	0% at 1 μM
9a	63 ± 13	1010 ± 150	>10000	480 ± 95	1400 ± 170
9b	38 ± 9	860 ± 110	>10000	330 ± 65	950 ± 120
13^h	1960 ± 2254	nb	nb	0% at 1 μM	3.6% at 1 μM
13a	>10000	>10000	nb	2% at 1 μM	3.6% at 1 μM
13b	1010 ± 200	nb	nb	11% at 1 μM	3.6% at 1 μM
13c	760 ± 120	nb	nb	3.2% at 1 μM	1.3% at 1 μM
15^h	315 ± 81	nb	nb	890± 130	20% at 1 μM
15a	3700 ± 340	nb	nb	0.9% at 1 μM	0% at 1 μM
15b	760 ± 90	nb	nb	12% at 1 μM	14% at 1 μM
15c	>10000	nb	nb	2300 ± 370	5.4% 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 8. [i]Data from ref 17. [j]nb = no binding detected.

conformational flexibility and more defined spatial orientation of the aromatic side chains; (ii) the presence of the positively charged Tyr N-terminal amino group, an element which, despite the structural diversity, is characteristic of practically all the endogenous opioid ligands including EMs and enkephalins. The first example of cyclic EM-2 analogue incorporating the cAmp residue in place of the native Pro^2 has been described by us in a previous paper and its structure, together with those of its related linear derivatives, is reported in Figure 1 (compounds **9**, **13**, and **15**, respectively). Both **9** and its linear derivatives showed, as compared with the parent EM-2, a sensible decrease of binding affinity toward the three (μ, δ, and κ) examined opioid receptors and only modest in vitro functional bioactivity (Table 1). As indicated in Figure 1, the three previously reported peptides (**9**, **13**, and **15**) maintain the homochiral all-L sequence found in the parent EM-2.

A preliminary consideration to explain the lower activity of **9**, as compared with the parent, may be attributed to the consequences of the cyclization on the conformation of short linear peptides. As compared with more extended systems, the resulting cyclic structures are characterized with reduced conformational heterogeneity as well as more defined topography of the attached side chains. This feature sensibly reduces the population of conformers available for a proper receptor−ligand interaction. In the case under study, the cyclization was designed so as to involve the Pro^2-Phe^3-Phe^4 sequence strongly influencing the spatial orientation of the attached benzylic side chains and to leave external to the cyclopeptide moiety the N-terminal Tyr^1 residue. This arrangement changes profoundly the shape of the electron-rich zone bound to interact with the counterpart located in the binding pocket of μ opioid receptors. However, results reported in Table 1 clearly show that the parent linear ligand EM-2 adopts sensibly different and more favorable side chain topography relative to the cyclic prototype **9**.

Scheme 1. Chemical Synthesis of Intermediates 1−5a−c, 6−8a−b, and Final Products 9a−b[a]



	Phe^3	Phe^4
1-9a	D	D
1-9b	D	L
1-5c	L	D

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

On the basis of the above considerations and being confident on the efficient role that the constrained cyclic system of **9** may exert in controlling the spatial orientation of the side chains at positions 3 and 4 of the backbone, we considered interesting to examine a new series of EM-2 cyclic analogues, which maintains the cAmp at position 2 but is characterized by a different stereochemistry of the phenylalanine residues. As shown in Figure 1, the new products (**9a−b**, **13a−c**, and **15a−c**), as compared with the previously reported,[8] contain, at positions 3 and 4, a combination of phenylalanine residues with different L and D absolute configuration. Synthesis of the new linear and cyclic compounds is reported in Schemes 1 and 2. Table 1 reports the biological properties of the three cyclopeptides **9** and **9a−b.** In order to evaluate the effects of the introduced stereochemical modidifications on the linear models, the activity of the linear tetrapeptides **13a−c** and **15a−c**, possessing a C-terminal free carboxyl or an amide group, respectively, is also reported in Table 1.

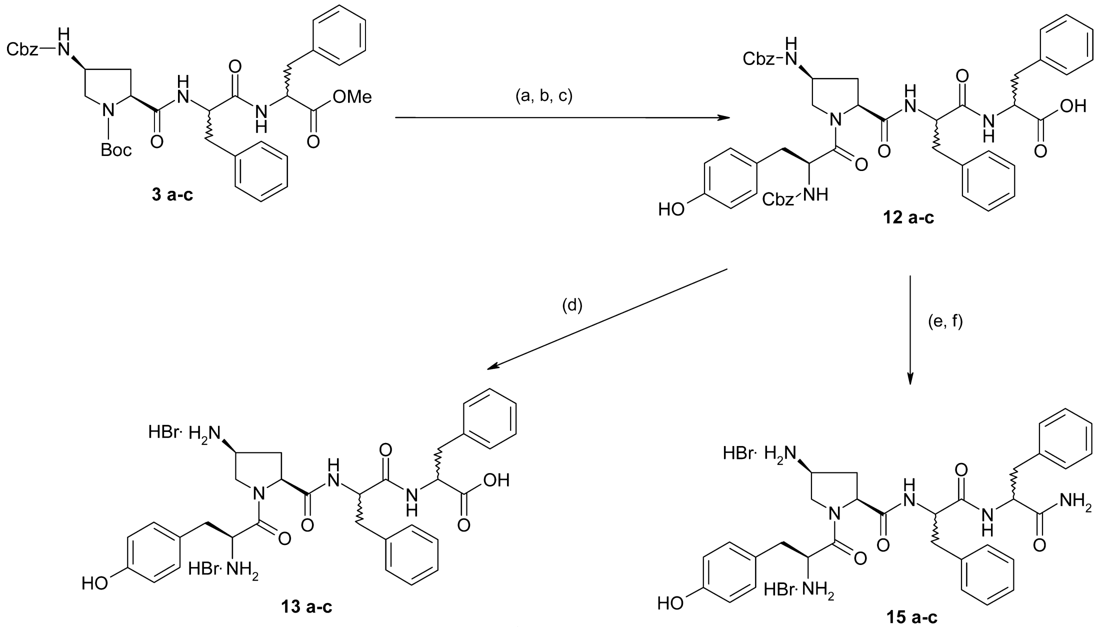
■ RESULTS AND DISCUSSION

Binding affinity and in vitro fuctional activity of both cyclic tetrapeptides **9a−b** and related linear analogues **13a−c** and **15a−c** are reported in Table 1. All compounds showed greater activity in the GPI than the MVD, defining their predominantly μ agonist character.

The data show that all the linear tetrapeptides **13a−c** containing a C-terminal free carboxyl are inactive at the μ, δ, and κ opioid receptors. Compounds **13a−c** and **15a−c** possess similar activity toward μ receptor; as already noted,[8,17] the cationic center in position 4 of the cAmp ring, which adds to that of the *N*-terminal Tyr, seems not to favor the binding. This could be related to repulsive interaction with an electropositive area present in the involved binding pocket. On the contrary, the cyclic compounds **9a−b** showed very good affinity for μ receptors and weak to scarce affinity for both δ and κ receptors. The highest activity is shown by the cyclic model **9b** with good binding values for μ receptors (K_i) and only modest activity at δ receptors with 20-fold selectivity. Product **9b**, with D-Phe^3 and L-Phe^4, show a K_i larger that **9a** but still in the nanomolar range.

As the data in Table 1 show, the affinity toward the μ opioid receptors of the analogues **13a−b**, linear precursors of **9a−b**, is 10 to 20 times lower as compared with the corresponding cyclic models. Thus, the cyclization, locking the peptide backbone

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



	Phe^3	Phe^4
10-15a	D	D
10-15b	D	L
10-15c	L	D

[a]Reagents and conditions: (a) **10a−c**, TFA/DCM 1:1, room temperature, 3 h, quantitative. (b) **11a−c**, Cbz-Tyr-OH, EDC, HOBt·H$_2$O, NMM, DMF, 0 °C, 20 min, then room temperature, 24 h, 80−89%. (c) **12a−c**, 1N NaOH, MeOH, room temperature, 3 h, quantitative. (d) **13a−c**, HBr/AcOH 33%, room temperature, 3 h, 70−75%. (e) **14a−c**, IBCF, NMM, THF, NH$_4$OH, 30 min, −10 °C, then room temperature, 3 h, 85−87%. (f) **15a−c**, HBr/AcOH 33%, room temperature, 3 h, 65−73%.

and the aromatic side chains in a semirigid conformation, strongly improves the binding. This puts in evidence how in the EM-2 molecule, which, differently from enkephalins, possesses an additional Phe residue in position 3, favorable and probable critical $\pi-\pi$ interactions between adjacent aromatic residues may take place.

Chemistry. Linear peptide analogues were prepared as previously described[1] by following usual peptide chemistry (see Schemes 1 and 2). The two cyclic products **9a−b** (Figure 1) were obtained as follows: deprotecting the tripeptide **3a−c** at the COOMe terminal by hydrolysis with 1 N NaOH in MeOH, followed by hydrogenolysis with Pd/C gave **5a−c**. The cyclization reaction was performed by using the pyBop coupling reagent in a highly diluted DMF (10^{-3} mol) solution. Both the precursor tripeptide acids containing D-Phe^3/D-Phe^4 (**5a**) and D-Phe^3/Phe^4 (**5b**) gave the corresponding *N*-Boc protected cyclotripeptide intermediates **6a**,**b** in good yields (60 and 58%, respectively). A different behavior was shown by the Phe^3/D-Phe^4 containing tripeptide (**5c**) which, differently from the other three studied diastereomeric tripeptides of this series, namely, the here reported D-Phe^3/D-Phe^4 (**5a**) and D-Phe^3/Phe^4 (**5b**) intermediates, as well as the previously examined precursor of (**9**) (i.e., *N*-Boc-cAmp-Phe-Phe-OH), failed to give, under the adopted experimental conditions, the corresponding cyclic derivatives. This result is not unexpected, e.g., refs 18−23, and puts in evidence that the cyclization reaction, which leads to a very small ring and low flexible cyclopeptide, is highly influenced by the spatial orientation of the three adjacent aromatic chains.

■ CONCLUSIONS

As previously mentioned, we have examined here a series of cyclic EM-2 analogues characterized by relevant new structural features specifically bound to the presence inside the backbone of the cAmp residue. The previously reported compound **9** as well as its here examined diastereisomers **9a−b** are the first cyclic EM analogues obtained without addition, in order to help the cyclization reaction, of extra residues to the native tetrapeptidic sequence. Here, the use of a proline/GABA chimera replacing the native Pro^2 as well as an effective cyclization step, leads to a highly constrained cyclic backbone surrounded by three aromatic rings pertaining to Tyr^1, Phe^3, and Phe^4 residues. Results confirm the high influence of the relative stereochemistry of the residues on both the output of the cyclization reaction and the proper fitting of the resulting cyclopeptide with the involved receptor area. Failure to obtain the cyclotripeptide **9c** and the different values of bioactivity shown by **9** and **9a−b** are clearly related to this effect.

However, with the identification of the two active compounds in the nanomolar range (**9a** and **9b**), a step further has been done after the design and synthesis of the prototype cyclotripeptide **9**.[8] Still other studies need to be done in order to better understand the stereospecific and topographic requirements of the opioid receptors and the rules governing the conformational preferences in here reported cyclic tripeptides.

■ EXPERIMENTAL SECTION

General Procedure for the Synthesis of 9a−b, 13a−c, and 15a−c. N^{α}-Boc-cAmp(Z)−OH was synthesized as previously reported.[11] All couplings of the linear intermediates and linear products were performed using the standard coupling method of carbodiimmide (EDC/HOBt/NMM) in DMF as described below. The synthesis of the cyclic peptides **9a−b** begins with the coupling between D or L Boc-Phe-OH and D or L HCl·H-Phe-OMe (Scheme 1) to obtain three diastereoisomeric dipeptides **1a−c**. The dipeptides obtained were N-terminal deprotected in TFA 1:1 DCM. The resulting TFA salts **2a−c** were coupled with N^{α}-Boc-cAmp(Z)−OH to yield the three diastereoisomeric tripeptides **3a−c**. Final peptides were prepared from **3a−c** by two different synthetic pathways; in the first way, to give the cyclic products **9a−b** (see Scheme 1) and in the second way, to give the linear products **13a−c** and **15a−c** (see Scheme 2). The two cyclic products **9a−b** (Scheme 1) were obtained by deprotecting the tripeptide **3a−b** at the COOMe terminal by hydrolysis with NaOH 1 N in MeOH, followed by deprotection of Z group in position 4 of the cAmp by hydrogenolysis with Pd/C 10% in MeOH to give **5a−c**. The deprotected tripeptides **5a−c** were cyclized using pyBop coupling reagent in a highly diluted DMF (10^{-3} mol) solution. As mentioned before, the reaction provided only the two cyclic products **6a−b** but not the **6c** diasteromer. Then, **6a−b** were N-terminal deprotected in TFA/DCM mixture, and the resulting TFA salts **7a−b** coupled to Boc-Tyr-OH give the tetrapeptides **8a−b**, which were deprotected by TFA/DCM to give the two final products **9a−b**. As shown in Scheme 2, the linear products were obtained by deprotection of the N-terminal Boc group of **3a−c** and coupling the resulting TFA salts **10a−c** with Cbz-Tyr-OH to obtain the three linear fully protected products **11a−c**. Then, the COOMe terminal ester groups were hydrolyzed by NaOH 1 N in MeOH to give the free acids **12a−c**. Free acids were transformed into an amide group by activation of the free carboxylic function by mixed anhydride and subsequent reaction with NH_4OH to give the terminal amides **14a−c**. Finally, the Cbz groups on Tyr and cAmp side chain were removed by HBr in glacial acetic acid to give the six final linear products **13a−c** and **15a−c** as HBr salts.

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 **1a−c**, **3a−c**, **6a-b**, and **8a−b** were purified by silica gel chromatography. Products **9a−b**, **13a−c**, and **15a−c** 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 a isocratic elution of 20% CH_3CN/H_2O 0.1% TFA, monitored with a Waters 2996 Photodiode Array Detector, was found to be >95%.

^1H NMR spectra were performed in $CDCl_3$ 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 $CDCl_3$ 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, $^1H-^1H$ TOCSY and $^1H-^1H$ NOESY. Peptide structures were also confirmed by high resolution-mass spectra (HR-MS) ± 2 ppm. For the final products, **9a−b**, **13a−c**, and **15a−c**, elementary analyses (within ±0.4% of the theoretical values) were performed.

TFA·Tyr-*c*[4-NH-Pro-Phe-Phe] (9a−b). Compound **8a−b** (1.0 equiv) was dissolved in 1:1 CH_2Cl_2/TFA mixture according to the general procedure to give **9a** (93%) and **9b** (87%). **9a**: ^1H NMR ($(CD_3)_2SO$) δ 1.39−2.38 (m, 2H, Pro C^3H_2), 2.76−2.93 (m, 2H, βCH_2 Phe^4), 2.84−3.09 (m, 2H, βCH_2 Phe^3), 2.89−2.97 (m, 2H, βCH_2 Tyr^1), 3.42−3.50 (m, 2H, Pro C^5H_2), 3.50 (m, 1H, αCH Pro), 3.86 (m, 1H, αCH Tyr^1), 3.89 (m, 1H, Pro C^4H), 4.41 (m, 1H, αCH Phe^4), 4.62 (m, 1H, αCH Phe^3), 6.15 (d, 1H, NH Pro), 6.74 (d, 2H, $C^{3,5}H$ Tyr^1), 6.98 (d, 2H, $C^{2,6}H$ Tyr^1), 7.65 (d, 1H, NH Phe^3), 7.94 (d, 1H, NH Phe^4), 8.36 (br, 3H, NH_3^+ Tyr^1). ESI-HRMS for $C_{32}H_{36}N_5O_5$ [MH$^+$], calcd 570.2712; found, 570.2715. LRMS (ESI) m/z = 570.3. Anal. Calcd for $C_{32}H_{35}N_5O_5$: C, 67.47; H, 6.19; N, 12.29; O, 14.04. Found: C, 67.45; H, 6.15; N, 12.32; O, 14.09. **9b**: ^1H 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 (br, 3H, NH_3^+ 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. Anal. Calcd for $C_{32}H_{35}N_5O_5$: C, 67.47; H, 6.19; N, 12.29; O, 14.04. Found: C, 67.51; H, 6.23; N, 12.25; O, 14.01.

Biological Activity and Binding Assays. *Functional Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays.* In vitro biological assays were performed on **9a−b** as TFA salts and **13a−c** and **15a−c** as hydrobromide salts. GPI and MVD in vitro bioassays were performed as described previously.[24,25] For a brief description, see Supporting Information.

Radioligand Labeled Binding Assays. *μ and δ Opioid Receptors.* Crude membranes were prepared as previously described[26] from transfected cells that express the MOR or the DOR. For a brief description, see Supporting Information.

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.[27] Experiments were performed on Chinese hamster ovary (CHO) cell lines that stably express human KOP, established as previously described.[27] For a brief description, see Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Details of syntheses, general procedures, compound characterization, biochemistry, and experimental section. 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; DAMGO[^3H], [^3H]-[D-Ala(2),*N*-Me-Phe-(4),Gly-ol(5)]enkephalin; [^3H]-U69593, [^3H]-(+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; DCM, dichloromethane; DIPEA, diisopropylethylamine; [^3H-]DPDPE, [^3H]-[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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