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# Synthesis of Mixed Opioid Affinity Cyclic Endomorphin-2 Analogues with Fluorinated Phenylalanines

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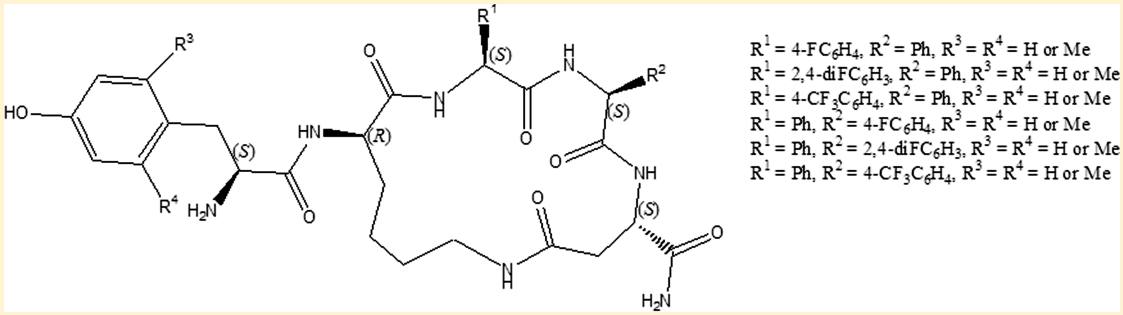
Supporting Information




**ABSTRACT:** As part of our continuing studies on the structure−activity relationships of cyclic pentapeptides based on the structure of endomorphin-2 (EM-2), we report here the synthesis and biological activities of a new series of analogues of a general sequence Tyr/Dmt-c[D-Lys-Phe-Phe-Asp]$NH_2$ (where Dmt = 2′,6′-dimethyltyrosine), incorporating fluorinated amino acids: 4-fluorophenylalanine (4-F-Phe), 2,4-difluorophenylalanine (2,4-F-Phe), or 4-trifluoromethylphenylalanine (4-$CF_3$-Phe) instead of the Phe residue in position 3 or 4. Depending on the fluorinated amino acid residue and its position in the sequence, analogues were mixed, high affinity MOP/KOP receptor agonists, MOP/DOP/KOP agonists, or selective KOP agonists. The *in vitro* potencies and efficacies of all novel analogues were assessed in calcium mobilization assay. The most potent analogues, Dmt-c[D-Lys-Phe-4-F-Phe-Asp]$NH_2$ and Dmt-c[D-Lys-Phe-2,4-F-Phe-Asp]$NH_2$, were tested *in vivo* in the mouse hot-plate test. They produced strong antinociceptive effect not only after intracerebroventricular but also after intraperitoneal injection, indicating that they were able to cross the blood−brain barrier.

**KEYWORDS:** *Opioid receptor affinity, calcium mobilization assay, antinociceptive activity, blood−brain barrier*

Endomorphins (EMs), Tyr-Pro-Trp-Phe-$NH_2$ (EM-1) and Tyr-Pro-Phe-Phe-$NH_2$ (EM-2), are selective ligands of the MOP receptor and the most effective endogenous analgesics being released in response to pain stimuli.[1] EMs, when centrally administered, display high antinociceptive activity in several animal models of acute, inflammatory, and neuropathic pain.[2] However, peripheral administration of EMs meets with failure due to their inability to penetrate the blood−brain barrier (BBB)[3] and to their rapid degradation by peptidases.[4]

Numerous chemical modifications have been developed to increase stability and bioavailability of EM analogues.[5,6] So far, only a few have been reported to be able to gain access to the central nervous system (CNS) and produce analgesia after

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**Table 1. Physicochemical Data of the Cyclic Analogues**

| No. | sequence | $m/z$ [M + H]$^{+a}$ calcd M + H | $m/z$ [M + H]$^{+a}$ found M + H | RP-HPLC $t_R$[b] [min] | M/D ratio | area [%][c] 90 min |
|---|---|---|---|---|---|---|
| | Tyr-Pro-Phe-Phe-$NH_2$[d] (EM-2) | 571.70 | 571.85 | 17.25 | | 0.61 ± 0.04 |
| **1** | Tyr-c(D-Lys-pF-Phe-Phe-Asp)$NH_2$ | 718.336 | 718.339 | 15.0 | 2.16 | 96 ± 0.60* |
| **2** | Tyr-c(D-Lys-2,4F-Phe-Phe-Asp)$NH_2$ | 736.326 | 736.329 | 13.9 | 1.83 | 93 ± 1.00* |
| **3** | Tyr-c(D-Lys-p$CF_3$-Phe-Phe-Asp)$NH_2$ | 768.332 | 768.332 | 16.0 | 7.07 | 97 ± 0.84* |
| **4** | Tyr-c(D-Lys-Phe-pF-Phe-Asp)$NH_2$ | 718.336 | 718.335 | 14.5 | 2.41 | 93 ± 0.75* |
| **5** | Tyr-c(D-Lys-Phe-2,4F-Phe-Asp)$NH_2$ | 736.326 | 736.331 | 11.4 | 2.07 | 94 ± 1.15* |
| **6** | Tyr-c(D-Lys-Phe-p$CF_3$-Phe-Asp)$NH_2$ | 768.332 | 768.331 | 17.3 | 2.34 | 97 ± 0.90* |
| **7** | Dmt-c(D-Lys-pF-Phe-Phe-Asp)$NH_2$ | 746.367 | 746.368 | 15.2 | 3.38 | 96 ± 2.25* |
| **8** | Dmt-c(D-Lys-2,4F-Phe-Phe-Asp)$NH_2$ | 764.358 | 764.364 | 13.7 | 3.62 | 94 ± 1.40* |
| **9** | Dmt-c(D-Lys-p$CF_3$-Phe-Phe-Asp)$NH_2$ | 796.364 | 796.367 | 16.5 | 15.70 | 96 ± 0.95* |
| **10** | Dmt-c(D-Lys-Phe-pF-Phe-Asp)$NH_2$ | 746.367 | 746.366 | 15.4 | 3.14 | 93 ± 1.03* |
| **11** | Dmt-c(D-Lys-Phe-2,4F-Phe-Asp)$NH_2$ | 764.358 | 764.362 | 12.6 | 2.94 | 97 ± 1.25* |
| **12** | Dmt-c(D-Lys-Phe-p$CF_3$-Phe-Asp)$NH_2$ | 796.364 | 796.369 | 18.1 | 1.94 | 93 ± 0.71* |

[a]Monoizotopic mass observed by ESI $MS^+$ jonization [b]RP-HPLC $t_R$ values obtained on Vydac $C_{18}$ column (5 m, 4.6 × 250 mm) using the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0−100% solvent B over 25 min, with the flow rate 1 mL/min. [c]Amount of peptide remaining after 90 min incubation with rat brain homogenate. [d]Data from ref 16. *$p < 0.05$ as compared to EM-2 by using one-way ANOVA followed by the Student−Newman−Keuls test.

peripheral administration.[7−10] Among other strategies used in opioid peptide research, cyclization proved to be a powerful tool for generating analogues with increased chemical and enzymatic stability and improved pharmacodynamic properties.[11] Cyclization can be achieved through various bridging bonds between peptide ends or side-chains. Apart from disulfide bridges in peptides containing cysteine residues, the most often used bridging bonds between side-chains are amide bonds and urea bonds.[12−15]

For the past few years we have been focused on the synthesis of cyclic analogues based on the structure of EM-2 with incorporated bifunctional amino acids that facilitate ring closure.[16,17]

In this study, cyclization was combined with introduction of fluorinated amino acids. It is well recognized that replacing a hydrogen atom or a functional group by fluorine can have a dramatic effect on biological activity by enhancing the stability of proteins against chemical and thermal degradation. In small molecules, hydrogen can be substituted by fluorine with little influence on their binding to receptors or enzymes.[18] Incorporating fluorine atoms increases fat solubility and therefore increases bioavailability.[19] The highly polarized C−F bond affects conformation of a molecule, which may better fit into the receptor binding pocket.[20] So far, only a few fluorinated amino acids have been introduced into the linear analogues of EMs.[21,22]

Fluorinated amino acids, 4-fluorophenylalanine (4-F-Phe), 2,4-difluorophenylalanine (2,4-F-Phe), or 4-trifluoromethylphenylalanine (4-$CF_3$-Phe), were incorporated into the sequence Tyr/Dmt-c[D-Lys-Phe-Phe-Asp]$NH_2$ (where Dmt = 2′,6′-dimethyltyrosine) instead of a Phe residue in position 3 or 4 (Table 1). Twelve new cyclic analogues were synthesized in two sets, with Tyr or Dmt in position 1. In each set the Phe residues in position 3 or 4 were successively replaced by 4-F-Phe, 2,4-F-Phe, or 4-$CF_3$-Phe. The syntheses were performed by an entirely solid-phase methodology using Fmoc/*t*Bu chemistry with the hyper-acid labile Mtt/O-2Ph*i*Pr groups for the selective amine/carboxyl side-chain protection. The LC−MS analysis of crude mixtures revealed in each case the presence of two main peaks. One was the expected cyclic peptide, which was always the main product, and the other was a cyclodimer. An interesting observation was that a monomer/dimer (M/D) ratio was higher in the case of Dmt-containing analogues and also when substitution of fluorinated Phe was in position 3. The highest M/D ratio was found for analogues with 4-$CF_3$-Phe in position 3 (Table 1).

**Table 2. Opioid Receptor Binding of the Cyclic Pentapeptides[a]**

| No. | sequence | $IC_{50}$[b] [nM] MOP | $IC_{50}$[b] [nM] DOP | $IC_{50}$[b] [nM] KOP |
|---|---|---|---|---|
| **EM-2** | Tyr-Pro-Phe-Phe-$NH_2$[c] | 0.79 ± 0.05 | >1000 | >1000 |
| **1** | Tyr-c(D-Lys-pF-Phe-Phe-Asp)$NH_2$ | 0.72 ± 0.09 | 348 ± 25.0 | 2.43 ± 0.16 |
| **2** | Tyr-c(D-Lys-2,4F-Phe-Phe-Asp)$NH_2$ | 0.69 ± 0.11 | >1000 | 3.26 ± 0.10 |
| **3** | Tyr-c(D-Lys-p$CF_3$-Phe-Phe-Asp)$NH_2$ | >1000 | >1000 | 24.5 ± 1.60 |
| **4** | Tyr-c(D-Lys-Phe-pF-Phe-Asp)$NH_2$ | 0.98 ± 0.06 | 286 ± 30 | 0.6 ± 0.07 |
| **5** | Tyr-c(D-Lys-Phe-2,4F-Phe-Asp)$NH_2$ | 0.42 ± 0.06 | 110 ± 9.0 | 0.9 ± 0.08 |
| **6** | Tyr-c(D-Lys-Phe-p$CF_3$-Phe-Asp)$NH_2$ | 1.6 ± 0.25 | 429 ± 28.4 | 1.5 ± 0.21 |
| **7** | Dmt-c(D-Lys-pF-Phe-Phe-Asp)$NH_2$ | 0.55 ± 0.04 | 280 ± 32.5 | 2.40 ± 0.42 |
| **8** | Dmt-c(D-Lys-2,4F-Phe-Phe-Asp)$NH_2$ | 0.54 ± 0.06 | 342 ± 38.0 | 0.60 ± 0.04 |
| **9** | Dmt-c(D-Lys-p$CF_3$-Phe-Phe-Asp)$NH_2$ | >1000 | >1000 | 6.40 ± 0.75 |
| **10** | Dmt-c(D-Lys-Phe-pF-Phe-Asp)$NH_2$ | 0.61 ± 0.07 | 168 ± 27.0 | 0.55 ± 0.03 |
| **11** | Dmt-c(D-Lys-Phe-2,4F-Phe-Asp)$NH_2$ | 0.49 ± 0.06 | 11.2 ± 5.2 | 0.30 ± 0.05 |
| **12** | Dmt-c(D-Lys-Phe-p$CF_3$-Phe-Asp)$NH_2$ | 0.92 ± 0.10 | >1000 | 1.15 ± 0.65 |

[a]All values are expressed as mean ± SEM of three independent experiments performed in duplicate. [b]Binding affinity values determined by competitive displacement of the selective radioligands, [$^3$H]DAMGO, [$^3$H][Ile$^{5,6}$]deltorphin-2, and [$^3$H]nor-BNI for MOP, DOP, and KOP respectively. All values expressed as mean ± SEM of three independent experiments performed in duplicate. [c]Data from ref 16.

The receptor-binding affinities toward MOP, DOP, and KOP receptors determined against [$^3$H]DAMGO, [$^3$H][Ile$^{5,6}$]-deltorphin-2, and [$^3$H]nor-BNI, respectively, are summarized in Table 2. Almost all analogues showed MOP affinity in the



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**Table 3. Effects of Cyclic Pentapeptides at Human Recombinant Opioid Receptors Coupled with Calcium Signaling via Chimeric G Proteins[a]**

| | | MOP | | DOP | | KOP | |
|---|---|---|---|---|---|---|---|
| No. | sequence | $pEC_{50}$[b] ($CL_{95\%}$) | $\alpha \pm$ SEM[c] | $pEC_{50}$ ($CL_{95\%}$) | $\alpha \pm$ SEM | $pEC_{50}$ ($CL_{95\%}$) | $\alpha \pm$ SEM |
| | EM-2 | 7.86 (7.66−8.06) | 1.00 | inactive | inactive | inactive | inactive |
| | DPDPE | inactive | inactive | 7.80 (7.64−7.96) | 1.00 | inactive | inactive |
| | dynorphin A | 6.67[d] (6.17−7.17) | 0.83 ± 0.10[d] | 7.73[d] (7.46−8.00) | 0.99 ± 0.04[d] | 8.75 (8.59−8.92) | 1.00 |
| 1 | Tyr-c(D-Lys-pF-Phe-Phe-Asp)$NH_2$ | 8.59 (8.31−8.88) | 0.94 ± 0.04 | 6.34 (5.80−6.87) | 0.14 ± 0.06[e] | 8.00 (7.59−8.41) | 0.89 ± 0.08 |
| 2 | Tyr-c(D-Lys-2,4F-Phe-Phe-Asp)$NH_2$ | 8.39 (7.92−8.87) | 0.89 ± 0.06 | inactive | inactive | 8.12 (7.84−8.40) | 0.78 ± 0.05[e] |
| 3 | Tyr-c(D-Lys-p$CF_3$-Phe-Phe-Asp)$NH_2$ | inactive | inactive | inactive | inactive | 7.14 (6.66−7.61) | 0.70 ± 0.02[e] |
| 4 | Tyr-c(D-Lys-Phe-pF-Phe-Asp)$NH_2$ | 8.49 (8.05−8.92) | 0.95 ± 0.06 | 7.29 (6.64−7.94) | 0.20 ± 0.05[e] | 8.98 (8.74−9.22) | 0.88 ± 0.06 |
| 5 | Tyr-c(D-Lys-Phe-2,4F-Phe-Asp)$NH_2$ | 8.20 (7.90−8.50) | 1.13 ± 0.08 | 6.90 (6.26−7.53) | 0.50 ± 0.04[e] | 8.31 (7.92−8.71) | 0.78 ± 0.00[e] |
| 6 | Tyr-c(D-Lys-Phe-p$CF_3$-Phe-Asp)$NH_2$ | 8.28 (8.19−8.38) | 1.03 ± 0.04 | 6.79 (5.54−8.03) | 0.27 ± 0.06[e] | 7.93 (7.75−8.12) | 0.78 ± 0.05[e] |
| 7 | Dmt-c(D-Lys-pF-Phe-Phe-Asp)$NH_2$ | 8.60 (8.30−8.89) | 0.98 ± 0.08 | inactive | inactive | 8.32 (7.97−8.66) | 0.79 ± 0.05 |
| 8 | Dmt-c(D-Lys-2,4F-Phe-Phe-Asp)$NH_2$ | 8.29 (8.10−8.48) | 1.05 ± 0.13 | crc incomplete | crc incomplete | 8.55 (8.31−8.78) | 0.65 ± 0.06[e] |
| 9 | Dmt-c(D-Lys-p$CF_3$-Phe-Phe-Asp)$NH_2$ | inactive | inactive | 8.03 (7.98−8.07) | 0.28 ± 0.05[e] | | |
| 10 | Dmt-c(D-Lys-Phe-pF-Phe-Asp)$NH_2$ | 8.56 (8.18−8.95) | 1.03 ± 0.06 | 7.47 (5.93−9.01) | 0.21 ± 0.07[e] | 8.65 (8.28−9.02) | 0.71 ± 0.09[e] |
| 11 | Dmt-c(D-Lys-Phe-2,4F-Phe-Asp)$NH_2$ | 8.78 (8.55−9.00) | 1.03 ± 0.12 | 8.06 (7.35−8.77) | 0.37 ± 0.07[e] | 8.87 (8.74−9.00) | 0.82 ± 0.06 |
| 12 | Dmt-c(D-Lys-Phe-p$CF_3$-Phe-Asp)$NH_2$ | 8.17 (7.88−8.46) | 0.87 ± 0.08 | inactive | 8.44 (8.23−8.66) | 0.60 ± 0.03[e] | |

[a]Inactive means that the compound was inactive up to 1 $\mu$M. EM-2, DPDPE, and dynorphin A were used as reference agonists for calculating intrinsic activity at MOP, DOP, and KOP, respectively. [b]Agonist potency values ($pEC_{50}$). [c]Efficacy values ($\alpha$). [d]Data from ref 23. [e]*$p < 0.05$ according to one way ANOVA followed by the Dunnett test for multiple comparisons.

subnanomolar range. The exceptions were analogues **3** and **9**, with 4-$CF_3$-Phe in position 3, which were KOP receptor selective. All tested analogues showed high affinity to the KOP binding sites and rather weak to DOP. Only analogue **11** with Dmt and 2,4-F-Phe in position 4 displayed affinity to all three opioid receptors; in the subnanomolar range to the MOP and KOP and in nanomolar range to the DOP receptor. Analogues incorporating Dmt did not have significantly increased affinity to any of the three opioid receptors as compared with Tyr-containing analogues.

However, it is interesting to note that introduction of a more sterically hindered Dmt residue instead of Tyr is probably responsible for a decreased formation of cyclodimers in the Dmt-containing analogues. Further drastic restriction of cyclodimerization was observed when a more bulky $CF_3$ group was present on the Phe residue in position 3 but not 4.

The resistance of the cyclic analogues to enzymatic degradation was tested *in vitro* by incubation with rat brain homogenate for 90 min and analyzing the obtained mixtures by RP-HPLC to assess the amount of a remaining peptide (area %). All cyclic analogues displayed little degradation, not higher than 7%, while EM-2 was almost completely digested after 90 min (Table 1).

The pharmacological profiles of analogues **1**−**12** were evaluated *in vitro* at all three opioid receptors in the calcium mobilization assay. This functional assay has been previously used and validated for investigating the pharmacological profile of classical opioid and nociceptin/orphanin (NOP) receptor ligands.[23,24] In this assay, the Chinese hamster ovary (CHO) cells expressing human recombinant opioid receptors and chimeric G proteins were used to monitor calcium changes.[23] The concentration−response curves of all tested compounds were obtained and the calculated agonist potencies ($pEC_{50}$) and efficacies ($\alpha$) of the analogues are summarized in Table 3. At the MOP receptor, all compounds except for **3** and **9** (containing 4-$CF_3$-Phe residue in position 3) stimulated calcium release with high potency and efficacy. They mimicked the stimulatory effect of EM-2, showing similar maximal effects and higher values of potency (8.17−8.78). Peptide **11** was 10-fold more potent than EM-2. Compounds **3** and **9** showed very low efficacy ($\alpha < 0.1$). At the DOP receptor, compounds **2**, **3**, **7**, **9**, and **12** were completely inactive, **1** only weakly stimulated calcium mobilization ($\alpha \approx 0.14$ and $pEC_{50} \approx 6.3$), while for **8** an incomplete concentration response curve was obtained. Compounds **4** and **10** displayed lower maximal effects than DPDPE (Tyr-c(D-Pen-Gly-Phe-D-Pen)OH) (0.20 and 0.21, respectively) and similar potency (7.29 and 7.47, respectively). Compounds **5**, **6**, and **11** behaved as partial agonists ($\alpha \approx 0.50$, 0.27, and 0.37, respectively), with moderate (6.90 and 6.79 for **5** and **6**, respectively) or high (8.06 for **11**) potencies. At the KOP receptor, peptides **1**, **4**, **7**, and **11** stimulated calcium mobilization with slightly lower maximal effects as compared with dynorphin A, the other cyclopeptides showed a significant reduction of the maximal effects. In terms of potency, the lowest $pEC_{50}$ (~7.14) was obtained for analogue **3** (as compared with 8.75 for dynorphin A). All other compounds displayed high values of potency (7.93−8.98), with analogues **4** and **11** being exceptionally potent (8.98 and 8.87, respectively). Calcium mobilization assay results were well



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correlated with the results of the binding assay (see Supporting Information).

In vivo experiments were performed on male Swiss albino mice (CD1). Antinociceptive activity was assessed for two analogues, **10**, which displayed the highest affinity to MOP and KOP, and **11**, which had high affinity to all three opioid receptor types. The mouse hot-plate test was performed after i.c.v. and i.p. administration in the dose range 0.001−10 μg/animal and 0.3−10 mg/kg, respectively. Both analogues produced dose-dependent antinociceptive responses after i.c.v. injection (Figure 1). These peptides were also effective after

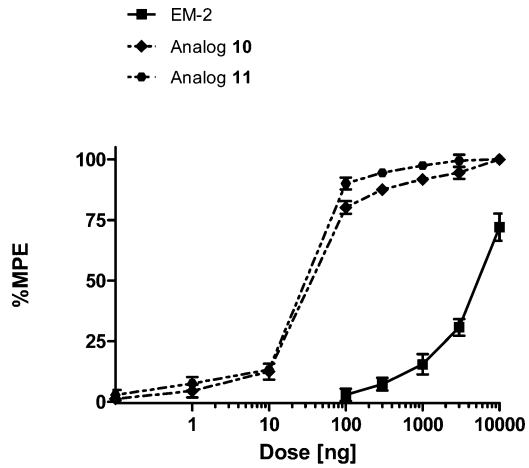


**Figure 1.** Dose−response curves determined in the hot-plate test in mice. Results are expressed as percentage (mean ± SEM) of the maximal possible effect (%MPE) for the inhibition if jumping induced by i.c.v. injection of a peptide. Number of animals in the group was 8.

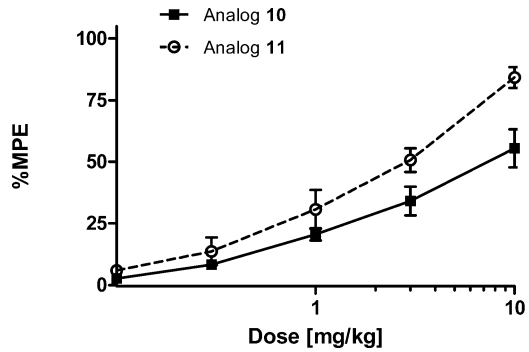


**Figure 2.** Dose−response curves for the hot-plate inhibition of jumping induced by i.p. injection of analogues **10** and **11** (male Swiss albino mice (CD1)). Number of animals in the group was 8.

peripheral administration (Figure 2), which indicated that they were able to cross the BBB. Peptide **11** was the more potent of the two and at 10 mg/kg produced about 84% of the maximal possible effect (Figure 2). To confirm that the analgesic action of **10** and **11** was mediated through the opioid system, selective MOP and KOP antagonists, β-funaltrexamine (β-FNA) and nor-binaltorphimine (nor-BNI), respectively, were used in the dose of 1 μg/animal i.c.v. Even though analogues **10** and **11** both showed high affinity for MOP and KOP, only β-FNA reversed the antinociceptive effect of these cyclopeptides (Figure 3). Such result is in agreement with a generally accepted fact that the antinociceptive effects are mainly mediated by the MOP.

Summing up, our results indicate that analogues containing mono- and difluorinated Phe residues were full MOP and

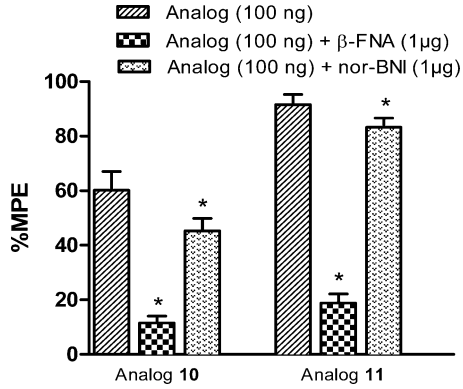


**Figure 3.** Antagonist effect of β-FNA (1 μg/animal, i.c.v) and nor-BNI (1 μg/animal, i.c.v) on the inhibition of jumping induced by analogues **10** and **11** (100 ng/animal, i.c.v.). Number of animals per group was 8. *$p < 0.05$ for analogue + β-FNA or analogue + nor-BNI versus analogue alone using one way ANOVA followed by the Student−Newman−Keuls test.

partial KOP agonists, showing high potency at the MOP and KOP receptors and much lower potency and efficacy at the DOP receptor.

Central action of KOP agonists produces analgesia accompanied by some dysphoric effects and that property has limited the therapeutic development of this class of ligands.[25,26] However, some evidence suggests that mixed MOP/KOP agonists display fewer side-effects. MOP/KOP agonists of the alkaloid structure, such as ethylketazocine (EKC), have been known and used to treat cocaine addiction.[25,27] Fighting pain is one of the oldest fields in medicinal chemistry, yet one where true success has proved illusive. Although in recent times a number of effective opiate derived analogues have been developed for clinical use, the ideal opiate analgesic lacking side-effect attributed to the use of morphine still remains out of reach. The search for a safe, orally active, and nonaddictive analgesic based on the opioid structure continues.

The demonstration that some cyclic peptide analogues of EMs can cross the BBB is an important advance toward the peptide-based analgesics. Peptides have several advantages as drugs, including high activity, high specificity, low toxicity, and minimal drug−drug interactions.

New cyclopeptides reported here that have mixed opioid affinity profile may represent yet another approach in the search for the safer EM-based analgesics, and further *in vivo* tests are now in progress.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Solid-phase peptide synthesis, metabolic stability determination, opioid receptor binding assays, calcium mobilization assay, and in vivo antinociception assessment. 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.

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