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Cyclic Enkephalins with a Diversely Substituted Guanidine Bridge or a Thiourea Bridge: Synthesis, Biological and Structural Evaluations

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Abstract

Two series of 22 and 15 atom cyclic enkephalins incorporating a diversely substituted guanidine bridge have been prepared to assess the potential effect of the bridge substitutions on their opioid activity profile. The most notable results were obtained with the shortest cyclic analogues, which showed a significant variation of their binding affinity toward μ and δ opioid receptors in relation to bridge substitution. NMR studies were performed to rationalize these data. Some small analogues were found to exist as at least one major and one minor stable forms, which could be separated by chromatography. In particular, the compounds **13** and **14** with a cyclic substituent were separated in three isomers and the basis of this multiplicity was explored by 2D NMR spectroscopy. All compounds were agonists with slight selectivity for the μ opioid receptor.

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Supporting Information

Guanidine bridge orientation, cyclization in the presence of LiCl, characterization data for compounds **1–14**, LC-MS analyses of crude **10** and **14**, experimental protocol for the internalization of hMOR-EGFP receptors, κ receptor binding affinities of selected compounds, chemical shifts for compounds **7a**, **9**, **10a**, **12a**, **13a–c**, effect of temperature on the spectra of **7a** and **10a**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

Compounds **7a** (thiourea bridge) and **10a** (*N*-Me-guanidine bridge) showed nanomolar affinity toward μ receptor, the latter being the more selective for this receptor (40-fold).

INTRODUCTION

We recently described a new kind of cyclic peptide incorporating a diversely substituted guanidine bridge formed between the side chains of two diaminoacyl residues.¹ One nitrogen of the guanidine group is extracyclic and can be non-, mono-, or disubstituted. A solid phase synthetic methodology was developed to prepare diversely substituted analogues of a single peptide sequence from a thiourea bridged intermediate via a *S*-methylation step. Assuming the quasipianarity of the guanidine group, we anticipated that the bridge could adopt four different orientations inside the peptide cycle named trans/trans (tt), trans/cis (tc), cis/trans (ct), and cis/cis (cc) (Figure 1S, Supporting Information). With the help of experimental and theoretical structural studies, which were performed on a model peptide, we found that the degree of guanidine bridge substitution could influence its orientation, mainly because of possible steric clashes between substituents, leading to a very significant impact on peptide conformation. Therefore, incorporation of a diversely substituted guanidine bridge into cyclic peptides could allow modulation of conformational flexibility and impact biological activity. Enkephalins appeared to us as a very pertinent model to explore this possibility.

Leu- and Met-enkephalins are major endogenous opioid agonists with high affinity for δ opioid receptors and somewhat lower affinity for μ opioid receptors. Since their discovery in 1975,² a considerable amount of analogues have been produced to develop potent and bioavailable analgesic drugs devoid of the highly undesirable side effects caused by opiates. Because the enkephalins show only slight preference for δ opioid receptors over μ opioid receptors, the main goal of these studies was the design of μ or δ receptor-selective analogues exhibiting agonist or antagonist activity as pharmacological tools or as potential therapeutic agents. Both Tyr¹ and Phe⁴ residues are required for opioid activity mediated by both receptors, but specific spatial arrangements of the aromatic ring of Phe⁴, the free *N*-terminal amine, and the phenolic group of Tyr¹ are critical in defining selectivity between the μ and δ receptors. Highly potent and selective ligands have been identified for each receptor, which most often are conformationally constrained analogues of the structurally flexible enkephalins. Their conformational analysis and the development of three-dimensional models of the opioid receptors allowed better understanding of the structural basis for receptor selectivity.³⁻⁵ The different strategies followed in the design of selective ligands included: (i) global conformational restriction through peptide cyclization, (ii) incorporation of conformationally constrained residues, (iii) the presence of a *C*-terminal carboxylate or carboxamide group, and (iv) steric limitations. One important approach was to limit peptide flexibility by cyclization. In most reported cyclic analogues, the cycle was formed between the side chains of residues 2 and 5, the residue in position 2 having the *D*-configuration to retain activity. The first cyclic enkephalin analogue H-Tyr-c[Dab-Gly-Phe-Leu-] (14-membered ring size) was prepared by Schiller's team and exhibited higher activity than the parent enkephalin with marked μ receptor selectivity.⁶ A similar analogue described by Goodman's team, H-Tyr-c[N β CH₃-Dap-Gly-Phe-NHCH₂CH₂-] (MABE, 13-membered ring size), exhibited high potency but no selectivity.⁷ The first cystine-containing cyclic enkephalin analogues H-Tyr-c[D-Cys-Gly-Phe-(*D*- or *L*-)Cys]-NH₂ (14-membered ring size) were synthesized independently by Sarantakis and Schiller's group and had high binding affinity for both receptors.^{8,9} A more striking result was obtained by Henry Mosberg's group when the two Cys residues were replaced with penicillamine (Pen) residues, as the resulting compounds H-Tyr-c[D-Pen-Gly-Phe-(*D*- or *L*-)Pen]-OH (DPDPE or DPLPE) were shown to be highly selective for the δ receptor.¹⁰ While the high binding

affinity for the δ receptor could be attributed to the rigidifying effect of the *gem*-dimethyl groups of the penicillamine residues, adverse steric interactions between the D -Pen² methyl groups and the μ receptor binding sites were mainly responsible for the low μ receptor affinity.¹¹ Because the disulfide bond is sensitive to reduction resulting in ring-opening and incorporation of other types of bridges with different geometries could provide conformational diversity, several series of cyclic enkephalins with a stable bridge were developed. Dicarba analogues of H-Tyr-c[D -Cys-Gly-Phe- D -(or L -)Cys]-NH₂/-OH were reported.^{12,13} They were obtained by ring-closing metathesis between the side chains of two allylglycine residues. The methodology led to two isomers with a *cis* and a *trans* olefin bridge, which could be reduced to afford a bismethylene moiety. The olefinic peptides were found to be highly potent but only moderately selective. Several series of cyclic enkephalinamide analogues with a carbonyl (or urea) bridge formed between the side chains of two diaminoacyl residues and with various cycle sizes (17 atoms) were prepared by Izdebski's group and were moderately selective for the μ opioid receptor.¹⁴ Other modifications were introduced into some of these basic cyclic analogues to obtain more selective compounds, including Gly³ deletion¹⁵ or the replacement of specific residues (for instance, Tyr¹ by 2,6-dimethyl-Tyr¹⁶⁻¹⁸ or Gly³ by Phe¹⁹). While enkephalin cyclizations afforded analogues with very high potency, most of them were found to be only moderately selective for one receptor subtype, with the exception of Pen-containing compounds. This lack of selectivity is very probably related to a still too high flexibility of the backbone despite the cyclization. As a matter of fact, reducing the cycle size by deleting the Gly³ residue, and therefore further decreasing backbone mobility, allowed obtaining of highly selective analogues for μ or δ receptors.¹⁵

To explore the impact of our mode of cyclization on the biological activity profile of a peptide, we prepared and evaluated two series of cyclic enkephalins with diversely substituted guanidine bridges (Figure 1, Table 1).

The synthetic intermediates with a thiourea bridge and a *S*-methyl-isothioureia bridge were also isolated and evaluated. The first series (compounds **1–6**) possessed a large cycle composed of 22 atoms formed between the side chains of a D residue in position 2 and an L residue in position 6. The second series (compounds **7–14**) possessed a small cycle containing only 15 atoms and was directly comparable to some published cyclic enkephalin analogues. In particular, their cycle was larger by only one atom compared to H-Tyr-c[D -Cys-Gly-Phe- L -Cys]-NH₂ and DPLPE. All compounds were C-terminally amidated.

RESULTS AND DISCUSSION

Cyclic Peptide Synthesis

The synthesis of the two series followed a nearly identical pathway. The synthesis of the small cyclic analogues **7–14** is shown in Scheme 1.

Preparation of cyclic peptides began with the manual solid phase assembly of the linear intermediates on a low-loaded Rink amide polystyrene resin to limit cyclodimerization, using conventional Fmoc chemistry and HBTU as a coupling agent. The pairs of orthogonally protected diaminoacyl residues were Fmoc- D -Orn(Mtt)-OH/Fmoc-Orn(Alloc)-OH and Fmoc- D -Dap(Alloc)-OH/Fmoc-Dap(Mtt)-OH for the synthesis of the large (**1–6**) and small (**7–14**) cyclic enkephalin series, respectively. Then the cyclization sequence was performed according to the previously published procedure.¹ Briefly, the Alloc protecting group was first removed and the free side chain amine group was converted into isothiocyanate using an excess of di-2-pyridylthionocarbonate (DPT). The Mtt protecting group was removed using a mixture of DCM/TFE/AcOH, and the free amino group was reacted overnight with the isothiocyanate group in THF and in the presence of TEA to afford

the cyclic peptide with a thiourea bridge. Because the two series contained a D-residue and a Gly residue, which could favor a turn conformation,^{20,21} and because of the pseudodilution effect due to low resin loading, we expected that intramolecular cyclization would be highly favored. In the case of the large cycle series, a significant side product was formed (about 30%) which possessed a molecular mass twice than expected, indicating cyclodimer formation. To try to decrease the extent of cyclodimerization in this series, we investigated the use of LiCl. Indeed, such salt was shown to facilitate peptide cyclization by inducing a turn structure, bringing the reacting groups in closer proximity through coordination of amide oxygen atoms along the peptide chain.^{22–24} In a dedicated assay, cyclization was performed on small resin samples as described above but in the presence of 0–1.25 M increasing concentrations of LiCl. Whatever LiCl concentration, cyclization was more than 95% completed and no significant side product, which could result from LiCl utilization, could be observed. The monomer/dimer ratio was determined by LC-MS analysis (Figure 2S, Supporting Information). The dimer proportion decreased with increasing LiCl concentration to a plateau at 10%. 0.4 M LiCl was the optimal concentration. In the case of the small cycle series, less dimer formation was observed (<10%) and LiCl addition was without effect. The easier formation of the monomeric cycle in the small series might be due to the shorter peptide length, which obviously brought the reacting groups closer together. After cyclization, a portion of the thiourea derivatives was cleaved off the resins to afford compounds **1** and **7**. S-Methylation was performed before guanidinylation. At this step, the solid supports were divided in several portions and one portion of each resin was cleaved to yield compounds **2** and **8** with a S-methyl-isothiourea bridge. Finally, the other portions were reacted with various amine compounds before final cleavage from the resin, affording compounds **3–6** and **9–14** with diversely substituted guanidine bridges (Table 1S, Supporting Information).

All crude peptides were analyzed by RP-HPLC and LC-MS. Compounds **1–6** showed a major peak (around 45–55% according to detection at 214 nm), the only one with the expected mass. The situation was more complex for some compounds of the small cycle series. Indeed, at least a second peak with the expected mass could be observed (Table 2).

Compounds **7** and **9–12** exhibited a major peak (named **7a** and **9a–12a**) and a smaller one (about 5–20% compared to the major peak, named **7b** and **9b–12b**) (Figure 3S, Supporting Information, for compound **10**). Such minor peak was hardly seen for compound **8**. Compounds **13** and **14** with a cyclic substituent even showed a more striking HPLC profile. Indeed, three forms with comparable abundance were observed at significantly different retention times (Table 2, Figure 4S, Supporting Information for LC-MS analysis of crude **14**). The high similarity of the two crude HPLC profiles indicated that analogues **13a/14a**, **13b/14b**, and **13c/14c** could possess close three-dimensional structures two by two. It was noteworthy that crude compound **12**, which contains a disubstituted guanidine bridge ($X = N(Me)_2$, not cyclic) as compounds **13** ($X = Pyr$) and **14** ($X = Pip$), gave a major and a minor chromatographic peaks as monosubstituted compounds **10** and **11**, suggesting a specific effect of the cyclic substituent Pyr or Pip. In all cases, all major and minor species were separated during HPLC purification. Surprisingly, no re-equilibration could be observed for any species, indicating that the origin of these isomers was not a slow conformational equilibrium.

To check if the guanidinylation conditions could promote this re-equilibration, each separated form of compounds **13** and **14** was submitted to these conditions. Compounds **13a** and **14a** remained stable, while **13b** and **14b** were partially converted into **13c** and **14c**, respectively, and vice versa as shown by LC-MS analysis, suggesting these isomers were more or less stable conformers. Such behavior could result from the constraints contributed by the cyclization and the rigid guanidinium bridge, which could adopt various orientations.

However, this hypothesis still remains to be fully demonstrated. Indeed, although the presence of several isomers was not observed in the first series as well as in the small cycle series of our preceding study,¹ racemization occurring during synthesis of the small cycle series cannot be ruled out.

All species (excepted the minor species **9b**, **11b**, and **12b**) were isolated with purity greater than 95%. They were evaluated in binding experiments.

Biological Evaluations: Receptor Binding Affinity and Functional Activity

Cyclic peptides were evaluated for their binding affinity at μ and δ opioid receptors, and in some cases at κ opioid receptors, using displacement binding assays with radioligands (Tables 3, 4 and Table 2S, Supporting Information). Data in Tables 3 and 4 indicate that all new compounds had a higher affinity for μ opioid receptors (MOR) than for δ opioid receptors (DOR) ($K_i\delta/\mu$ ratios between 2.6 and 41.3) with nanomolar affinities toward MOR for the best compounds (0.4 nM for **7a**, 1.5 nM for **10a**).

In each series, the MOR affinity and $K_i\delta/\mu$ ratios were compared according to the nature of the bridge and the substitution degree of the guanidine bridge. In the first series (compounds **1–6**, Table 3), moderate variations could be observed. The thiourea bridge afforded the best compound (**1**) of the series with a K_i of 9.1 nM for MOR binding, while *S*-methylation of this bridge (compound **2**) decreased the affinity by a factor of 2. Compounds **3–5** with non-, mono- ($X = \text{Me}$), and disubstituted ($X = -\text{N}(\text{Me})_2$) guanidine bridge, respectively, showed similar affinities for MOR ($14 \text{ nM} < K_i < 19 \text{ nM}$). A bigger variation was observed for the disubstituted compound **6** ($X = \text{Pyr}$), which was up to 10-fold less potent than the other guanidine-bridged analogues **3–5**. The reason of the difference in affinity between **5** and **6** was not understood, but the pyrrolidine substituent might have specific effects on the peptide conformation because of its cyclic nature. In addition, its size might sterically hinder receptor binding. The highest δ/μ selectivity was about 10-fold in this series. A slight variation was observed between compounds **3** and **6** (K_i ratios of 3.6 to 10.3). Taken together, binding affinities were found to vary moderately according to the nature of the extracyclic substitution in this series.

More important variations were observed with compounds **7–14** (small cycle series, Table 4). For clarity, we first compared the receptor binding affinities of the major species of each compound (**7a**, **8**, **9a**, **10a**, **11a**, **12a**, **13a**, and **14a**), which were all found to have higher MOR and DOR affinity (Table 4).

A notable subnanomolar MOR affinity was measured for the thiourea compound **7a**, whereas the *S*-methylated analogue **8** had 10 times lower MOR affinity. Among all the guanidine-bridged cyclic analogues of this series, the monosubstituted **10a** ($X = \text{Me}$) showed the highest affinity for MOR (1.5 nM). All others were at least 10 times less potent with 12-, 14-, 38-, 26-, and 71-fold lower affinity observed for the non- (**9**), mono- (**11a**), and disubstituted (**12a–14a**) compounds, respectively. These results indicated a significant variation in MOR affinity depending on the degree of guanidine bridge substitution, the disubstitution being less favorable. δ/μ affinity ratios were generally in the same range (10–14-fold) with two exceptions, compounds **9** and **10a**, which showed a higher selectivity (about 40-fold). Compound **10a** was the most interesting of all analogues because of its affinity and selectivity toward MOR.

Considering the minor isomers **7b**, **10b**, **13b**, **14b**, **13c**, and **14c**, their binding affinities for MOR were found significantly lower than those of the corresponding isomers **7a**, **10a**, **13a**, and **14a** (K_i ratios of about 72, 12, 7, 9, 3, and 6 for **7b**, **10b**, **13b**, **13c**, **14b**, and **14c**, respectively). For all these **Xb** and **Xc** species, a more or less significant decrease in MOR

selectivity was observed when compared to the **Xa** isomers. Taken together, these results indicated that the most significant variations in binding affinity and selectivity were observed in the small cycle series, most likely because of their lower global conformational freedom, which resulted in a higher impact of guanidine bridge substitution on peptide conformation, as previously shown.¹ The smaller cycle size also afforded the more potent compounds.

Ten compounds (**3–5** of the large cycle series, **7a, 9a, 10a, 12a**, and **13a–c** of the small cycle series) were examined in the κ receptor binding assay (Table 2S, Supporting Information). None of them showed significant κ receptor binding affinity ($K_i > 300$ nM), except for the thiourea compound **7a**, which had moderate κ affinity ($K_i = 54.2 \pm 5.4$ nM).

Several functional assays were performed. Similarly to the competition binding experiments, G-protein stimulation studies were carried out in rat brain membrane preparations. This functional assay evaluated the agonist-induced receptor mediated G-protein activation that is the primary step in the G-protein coupled receptor signaling cascade. The maximal stimulation (% $E_{\max}$ or efficacy) and the potency (pEC_{50}) of the peptides are summarized in Tables 3 and 4. The linear pentapeptide Met-enkephalin was used as a reference agonist compound determining the full agonist stimulation level ($E_{\max}$ 205%). Efficacy above 170% indicated a full agonist compound (compounds **5, 7a**, and **8**). G-Protein activity was negligible for compounds **13b,c** and **14b,c**. The rest of the compounds showed moderate partial agonist activity with efficacy values ranging between 130% and 169%. Overall, the robustness of the G-protein activations was in good agreement with the efficacy values obtained from the isolated tissue experiments (see below). Furthermore, the relative potency of compounds determined in the [³⁵S]GTP γ S binding assay was in good agreement with their opioid receptor binding affinity.

The agonist activity was further confirmed for compounds **7a, 8, 9a, 10a**, and **14a** by receptor internalization experiments performed with HEK cells expressing human MOR fusioned to eGFP, using confocal fluorescence microscopy (see Figure 5S, Supporting Information for representative pictures).

Finally, compounds **7a, 8, 9a, 10a**, and **14a** were found to be agonists in both the functional guinea pig ileum (GPI) (MOR representative) and mouse vas deferens (MVD) (DOR-representative) bioassays, as expected from the previous experiments (Table 5). All compounds showed a higher activity in the GPI assay compared to Leu-enkephalin in accordance to their general MOR preference except compound **14a**. In general, there was a good correlation between the receptor affinity measured in the binding assays and the agonist potency determined in GPI and MVD assays. In particular, the three best μ binders (**7a, 8**, and **10a**) were also the best agonists in the GPI assay, compound **7a** showing potency in the nanomolar range.

NMR Study

The proton chemical shifts of peptides **7a, 9a, 10a, 12a**, and **13a–c** (Table 3S, Supporting Information) were assigned using COSY, TOCSY, and ROESY experiments. To facilitate the chemical shifts assignment and overcome resonances superimposition, 1D and 2D spectra were recorded at three temperatures (283, 298 and 313 K). Experiments were run at pH 4, the optimum pH to observe the narrowest line widths for amide protons. But due to fast solvent exchange or conformational flexibility, not all amide protons resonances were observable.

Superimpositions of the 1D ¹H spectra of the analyzed compounds are presented in Figures 2 (peptides **7a, 9a, 10a, 12a**, and **13a**) and 3 (peptides **13a–c**). Only one series of signals

was detected for each cyclic analogue, with the exception of compound **9a**, which showed two conformational states, named conformation 1 and conformation 2 (prime residues). On the basis of proton chemical shifts, we clearly observed that various peptide conformations were obtained depending on the degree and the nature of the guanidine bridge substitution. Chemical shift variations not only concerned the guanidine bridge, but the entire cycle, and also the tyrosine side chain to a lesser extent. The highest chemical shift variations corresponded to the dap^2 and Dap^5 methylene resonances in agreement with the idea that substituent variations might influence the guanidine bridge orientation. The Gly^3 amide proton ($\text{Gly}^3\text{-NH}$) also globally underwent significant shifts ($\Delta\delta = 0.64$ ppm between **9a/10a** and **13c**), a behavior that was probably related to the existence or not of hydrogen bonding. Indeed, $\text{Gly}^3\text{-NH}$ was particularly unshielded in the case of compounds **9a** conformation 2 ($\delta = 8.62$ ppm) and **10a** ($\delta = 8.62$ ppm) and may be implicated in hydrogen bonding in these cases. In contrast, the $\text{Gly}^3\text{-NH}$ resonance of **13c** was at 7.98 ppm. The $\text{Gly}^3\text{-H}\alpha$ resonances of the three most active compounds (**7a**, **10a**, and **9a** conformation 2) were non-equivalent (3.66–3.98 and 3.50–3.55 ppm), contrary to the other analogues (Table 3S, Supporting Information), the $\text{Gly}^3\text{-H}\alpha$ chemical shifts of the latter being similar ($\delta = 3.73$ –3.83 ppm for **9a** conformation 1, **12a**, **13a**, **13b**, and **13c**). Finally, concerning Tyr^1 and Phe^4 residues, both Phe^4 side chain and Tyr^1 $\text{H}\alpha$ and $\text{H}\beta$ signals were less affected by the different substitutions. More strikingly, the tyrosine aromatic protons seemed to rigorously alternate between the two positions indicated on Figures 2 and 3 by asterisks. In particular, compounds **7a**, **9a** conformation 2, **10a**, and **13b** exhibited Tyr^1 aromatic $\text{H}\delta/\text{H}\epsilon$ downfield resonances compared with the other products. Taken together, these observations indicated that the most active compounds shared similar characteristics.

Comparison of the 1D ^1H spectra of **7a**, **9a**, **10a**, **12a**, and **13a** (Figure 2) showed high similarities between compounds **12a** ($\text{X} = \text{N}(\text{Me})_2$), **13a** ($\text{X} = \text{pyrrolidine}$), and **9a** conformation 1 ($\text{X} = \text{NH}_2$). Because compounds **12a**, **13a**, and this conformation of **9a** had also the same ROESY spectra, they very likely adopted the same 3D conformation. This observation was in agreement with their similar chromatographic behavior together with their close μ binding affinity and selectivity, at least for compounds **12a** and **13a** (Table 4). In the case of **9a**, the slightly higher activity at μ receptors might be due to the other conformational state, “conformation 2”, the spectrum of which resembled that of compound **10a**. Spectra of the more active analogues **7a** ($\text{X} = \text{S}$) and **10a** ($\text{X} = \text{NHMe}$) were quite different from the others. Their Dap^5 amide protons were particularly shielded. The $\text{Gly}^3\text{-NHs}$ were moved downfield and may indicate their implication in a hydrogen bond, as mentioned above. The aliphatic region of **7a** and **10a** 1D spectra could be easily distinguished from that of the other peptides, which shared a quasisimilar pattern. In addition, specific broadening or narrowing of the Dap methylene line widths was observed for the more active compounds **7a** and **10a** when the temperature was decreased (Figure 6S, Supporting Information) underlining a notable conformational flexibility. This phenomenon was not observed with the other compounds. Taken together, these observations indicated that the more active compounds shared similar conformational features.

As several stable peptide conformations might exist for a single substituent, we independently compared the three isomers of compound **13** carrying the pyrrolidine substituent to better understand the origin of these multiple species as well as their differences in biological activity. As a matter of fact, 1D ^1H spectra of **13a–c** (Figure 3) showed clear differences as expected. The first notable discrepancy concerned the amide proton resonances, with the apparition of the two Dap^5 amide resonances and the significant shielding of the C-terminal NH_2 protons of **13a** compared to **13b** and **13c**. In addition, we observed in the aliphatic region of the spectra that the pyrrolidine resonances of **13b/13c** were particularly split between 1.48 and 2.65 ppm while the signals were degenerated at

1.88 ppm for the CH₂₋₂ of **13a**. Then, differences between the two isomers **13b** and **13c** mainly resided in the strong shielding of Gly³-HN and the aromatic protons of Tyr¹ and Dap⁵ H β splitting for **13c**.

ROESY spectra were acquired typically at 298 and 283 K to decrease the peptide internal motion and promote the ROE effect. The few long-range ROE observed on the spectra are represented in Figure 4. A strong ROE appeared between the Dap⁵ HN γ and H α of Tyr¹ for compound **13a**. Compounds **13b** and **13c** had the same long-range ROE between protons of one side of the pyrrolidine ring and the H δ aromatic protons of Phe⁴, which underlined the proximity of the aromatic moiety and the pyrrolidine group. This observation was supported by the splitting of the pyrrolidine proton resonances that underwent a ring effect, in contrast to those of compound **13a**, which were degenerated. Finally, a small ROE cross peak was observed between Gly³ H α and Tyr¹ H δ only for **13c**, explaining the strong shielding of these signals in the 1D ¹H spectrum. Although only a few long-range ROE were observed for each isomer, clearly distinct structural arrangements could be inferred from them. In addition, these ROE involved the two aromatic residues, which are known to be of great importance for opioid activity.

CONCLUSION

In this study, we have synthesized several cyclic enkephalin analogues with a thiourea or a guanidine bridge. The most interesting results were obtained with the small cycle series, indicating that the influence of a diverse guanidine bridge substitution on biological activity and structure could only be observed in a sufficiently constrained environment. This observation had already been reported for RGD-related guanidine-bridged cyclic analogues.¹ The constrained nature of a diversely substituted guanidine group should be progressively counteracted by the higher flexibility of a longer peptide chain. Nevertheless, we showed that variation of the degree of guanidine bridge substitution in the small cycle series could indeed greatly impact the biological activity, whereas analogues shared the same peptide sequence and cycle size.

The lack of unambiguous NOEs restraints to converge toward a single set of conformations during the molecular modeling stage prevented us from proposing major conformations for each compound and explaining the different opioid receptor affinities from a topological point of view. In particular, no NMR data allowed establishment of a particular orientation for the guanidine bridge for any analogue. However, the careful analysis of the NMR data of selected analogues showed that variation of biological activity could be correlated to preferential conformations or behaviors in solution. For example, the most potent compounds **7a** and **10a** shared comparable NMR spectra but presented significantly different 1D spectra compared to others. In addition, compounds **12a** and **13a** possessing very similar 1D and ROESY spectra displayed similar μ receptor affinity and selectivity. Finally, the three isomers of compound **13** exhibited different activities and clear differences in their conformations. Having established that the degree of guanidine bridge substitution could influence the guanidine orientation, this feature could be at the origin of multiple forms.

Compound **7a** (thiourea bridge) was the best μ opioid receptor ligand of the present study and was much more potent than guanidine-bridged analogues, with the exception of **10a**. The guanidinium substitution could influence the bridge orientation, making the cycle somewhat less flexible than that with a thiourea bridge, may be leading to a less favorable conformation in most cases. In the case of compound **10a**, which is only 4-fold less potent than **7a** toward μ opioid receptor, the *N*-methyl substitution would allow a conformation better recognized by this receptor compared to other guanidine analogues. But the probable

more constrained nature of **10a** (due to guanidine substitution) compared to **7a** could be the reason of the 3-fold higher selectivity toward μ opioid receptor. With its 40-fold selectivity, compound **10a** compared favorably with previously reported and closely related cyclic enkephalins like the disulfide bridged H-Tyr-c[D-Cys-Gly-Phe-Cys]-NH₂⁹ or analogues with a dicarba bridge.¹² All have similar potency on μ opioid receptor but showed a much lower selectivity for this receptor, confirming the higher constraint displayed by a guanidine bridge compared to a disulfide or a dicarba bridge. Other closely related enkephalin analogues having a carbonyl (or urea) bridge but with a larger cycle size are also potent μ agonists, but their selectivity was assessed with the GPI/MVD assay, making difficult a direct comparison.¹⁴ Finally, compounds **7a** and **10a** were tested in vivo in mice and shown to display antinociceptive activity in the tail-immersion test after ip injection (Bart De Spiegeleer, personal communication, to be published elsewhere).

EXPERIMENTAL SECTION

General

Fmoc-protected amino acids, HBTU, DIEA, TFA, piperidine, solvents, and other reagents were purchased from Iris-Biotech, Novabiochem, Riedel-de Haën, Carlo Erba, or Acros Organics and used without further purification. Fmoc Rink amide polystyrene resin (100–200 mesh, 0.14 mmol/g) was purchased from Iris-Biotech. Solvents used for RP-HPLC and LC-MS were of HPLC grade. [³H]DAMGO ([D-Ala², NMePhe⁴, Gly⁵-ol]-enkephalin; 41 Ci/mmol) and [³H]DIDI ([Ile^{5,6}]-deltorphin-II, 48 Ci/mmol) were radiolabeled in the Isotope Laboratory of BRC, Szeged. Guanosine-5- γ -[³⁵S]-triphosphate (1204 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). MgCl₂, EGTA, Tris-(hydroxymethyl)aminomethane (Tris), protease-free bovine serum albumin (protease-free BSA, fraction V), guanosine 5'-diphosphate (GDP), guanosine-5'-O-(3-thiotriphosphate), GTP γ S, and all the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Peptide Synthesis

Peptide chain assembly was manually and stepwise accomplished following an Fmoc/tBu strategy. The protected diaminoacyl residues were Fmoc-D-Orn(Mtt)-OH/Fmoc-Orn(Alloc)-OH and Fmoc-D-Dap(Alloc)-OH/Fmoc-Dap(Mtt)-OH for the synthesis of the large and small cycle series, respectively. Boc-Tyr(tBu)-OH was used as the N-terminal residue. Fmoc-Rink amide-PS resin (0.14 mmol/g) was first conditioned for 15 min in NMP. The amino acid derivatives were then added by a succession of deprotection and coupling steps. Fmoc removal was performed through two consecutive treatments with piperidine/DMF (20/80) for 10 and 25 min. Coupling of Fmoc-protected amino acids (3 equiv) was carried out in NMP in the presence of HBTU (3 equiv) and DIEA (4 equiv) for 3 h. After each step, the resin was washed with DMF, MeOH, DCM, and DMF. Monitoring of the deprotection and coupling steps was performed using the TNBS test. A second coupling was performed when necessary.

After peptide assembly, the alloc group was removed by treatment of the resin with Pd[PPh₃]₄ (0.2 equiv) and phenylsilane (24 equiv) in anhydrous DCM. After 4 h stirring at rt, the resin was filtered and washed twice with DCM, DMF, and DCM and the treatment was repeated. The deprotected side chain amino group was then converted into isothiocyanate by reaction with di-2-pyridyl-thionocarbonate (DPT, 5 equiv) in DCM for 12 h. After washing with DCM and DMF, the completion of the reaction was assessed by the TNBS test. The Mtt protecting group of the second diaminoacyl residue was removed by six 20 min treatments of the resin with a DCM/TFE/AcOH mixture (7/2/1) followed by washings with DMF, MeOH, and DCM. After conditioning the resin in anhydrous THF, 4

equiv of TEA were added and the suspension was stirred 3 h under reflux and 12 h at rt to yield the cyclic peptide with a thiourea bridge. In the case of the large cycle series, 0.4 M LiCl was added to the cyclization mixture to limit dimer formation. Completion of the reaction was assessed by the TNBS test. S-Methylation of the thiourea was performed by three 1 h treatments of the resin with a 0.2 M MeI solution in DMF. Guanidinylation of the S-methyl-isothiurea bridge was performed by reaction with a 2 M solution of the amine compound in anhydrous DMSO for 16 h at 80 °C. In the case of amine hydrochlorides (for instance, methylamine), an equivalent amount of NMM was added. The resin was then washed with DMF and DCM.

Final cleavage of peptides from the resin and side chain deprotections were performed with TFA/TIS/H₂O (95:2.5:2.5, 10 mL/g resin) at room temperature for 2 h. After resin filtration, the filtrate was concentrated under vacuum and peptides were precipitated by diethyl ether addition and recovered after centrifugation. The pellet was washed twice with diethyl ether. All compounds were obtained with an average yield of 10%. They were purified by reverse-phase HPLC. All compounds were above 95% purity.

TNBS test: after each coupling and deprotection step and after isothiocyanate preparation and cyclization, an aliquot portion of resin beads was collected and treated with one drop of a 1 M TNBS solution in DMF and one drop of a 10% DIEA solution in DMF (v/v). Red beads after 1 min at room temperature indicated the presence of free amine groups.

Each intermediate step (peptide assembly, alloc removal, isothiocyanate preparation) was checked by cleaving 2–3 mg of resin with 500 μ L of the above-mentioned TFA mixture and the released products were analyzed by LC-MS.

Analysis and Purification

RP-HPLC analysis was performed using different sets of conditions: (A) Chromolith SpeedRod C18 column (0.46 cm $\times$ 5 cm), linear gradient (0–100%) of eluent B in A over 5 min (flow rate: 3 mL/min); (B) Merck Chromolith Performance C18 column (0.46 cm $\times$ 10 cm, 5 μ m), linear gradient (5–60%) of eluent B in A over 15 min (flow rate: 1 mL/min); (C) Waters Delta Pack column (0.39 cm $\times$ 15 cm, 5 μ m, 100 Å), linear gradient (10–40%) of eluent B in A over 20 min (flow rate: 1 mL/min). Detection was made at 214 nm.

Purification by preparative RP-HPLC was performed on a Waters Delta Pak C18 column (40 mm $\times$ 100 mm, 15 μ m, 100 Å), linear gradient of eluent B in A at a 1%/min rate (flow rate: 28 mL/min). Eluent A, 0.1% aqueous TFA; eluent B, acetonitrile/0.1% TFA.

Mass spectrometry: samples were prepared in acetonitrile/water (50/50 v/v) mixture. The LC-MS system consisted of a Waters Alliance 2690 HPLC, coupled to a Waters-Micromass ZQ spectrometer (electrospray ionization mode, ESI+). All analyses were carried out using a RP C18 monolithic Onyx Phenomenex column (25 mm $\times$ 4.6 mm), linear gradient (0–100%) of eluent B in A over 3 min (flow rate: 3 mL/min). Eluent A, 0.1% aqueous formic acid; eluent B, acetonitrile/0.1% formic acid. Positive ion electrospray mass spectra were acquired at a solvent flow rate of 100–500 μ L/min. Nitrogen was used as both the nebulizing and drying gas. The data were obtained in a scan mode in 0.1 s intervals; 10 scans were summed up to get the final spectrum.

The HPLC retention time in two condition sets, calculated monoisotopic mass, and measured high resolution mass of the compounds, and yields of purified products are reported in Table 1S, Supporting Information.

Receptor Binding Assay

Crude membrane fractions were prepared from Wistar rat brains according to the method of Pasternak with minor modifications.²⁵ All μ and δ receptor binding assays were performed as previously described.²⁶ Briefly, rat brain membranes were incubated at 35 °C for 45 min in 50 mM Tris-HCl (pH 7.4) in a final volume of 1 mL containing 1 mg of BSA and 0.2–0.4 mg/mL membrane protein, with the MOR agonist [³H]DAMGO (0.9–1.2 nM) or the DOR selective agonist [³H]DIDI (0.8–1.3 nM), in the presence of unlabeled test ligands (concentrations ranging from 10^{−5} to 10^{−11} M). Nonspecific binding was determined in the presence of 10 μ M naloxone. Bound radioactivity was determined in Packard Tricarb 2300TR liquid scintillation analyzer. Experiments were performed in duplicate and repeated at least three times. κ receptor binding affinities were determined by displacement of [³H]U69,593 (Amersham) from guinea pig brain membrane binding sites, as described.²⁷ Incubations were performed for 2 h at 0 °C with [³H]U69,593 at a concentration of 0.80 nM. IC₅₀ values were determined from log-dose displacement curves, and K_i values were calculated by means of the equation of Cheng and Prusoff,²⁸ using a value of 2.9 nM for the dissociation constant of [³H]U69,593.

Ligand-Stimulated [³⁵S]GTP γ S Functional Assay

Agonist-induced receptor-mediated G-protein stimulations were performed as previously described.²⁹ Briefly, rat brain membrane fractions (ref 24) (~10 μ g of protein/sample) were incubated at 30 °C for 60 min in Tris-EGTA buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl, pH 7.4) containing [³⁵S]GTP γ S (0.05 nM) and increasing concentrations (10^{−9} to 10^{−5} M) of the compounds tested in the presence of 30 μ M GDP. Agonist-induced receptor-mediated G-protein stimulation is given as a percentage over the specific [³⁵S]GTP γ S binding observed in the absence of receptor ligands (basal activity).

GPI/MVD Assays

The GPI and MVD functional assays were carried out as reported in detail elsewhere.^{30,31} A dose–response curve was determined with Leu-Enk as standard for each ileum and vas preparation, and IC₅₀ values of compounds being tested were normalized according to a published procedure.³²

Data Analysis

All radioligand receptor binding experiments were performed in duplicate, and the [³⁵S]GTP γ S binding assays were performed in triplicate. Experimental data were analyzed and graphically processed by GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com) research software package using standard office computers. Displacement curves were fitted by nonlinear regression using the one-site competition fitting option with no ligand depletion. The equilibrium inhibition constant (K_i value) was calculated from the IC₅₀ values according to the built-in Cheng–Prusoff equation module: $K_i = (IC_{50}) / (1 + ([L]) / (K_d))$ where $[L]$ is the concentration of free radioligand used in the assay and K_d is the dissociation constant of the radioligand for the receptor. G-Protein stimulation data were analyzed by the sigmoid dose–response curve fit option of Prism (version 4.0, San Diego, CA).

NMR Spectroscopy

The NMR samples contained ~1 mM of **7a**, **9**, **10a**, **12a**, **13a**, **13b**, and **13c** peptides dissolved in aqueous solutions (90% H₂O, 10% D₂O) at pH 4. All spectra were recorded on a Bruker Avance 600 spectrometer (Bruker Biospin) equipped with a 5 mm triple-resonance cryoprobe (¹H, ¹³C, ¹⁵N). Homonuclear 2-D spectra DQF-COSY, TOCSY (MLEV), ROESY, and NOESY were typically recorded in the phase-sensitive mode using the States-

TPPI method as data matrices of 256 real (t_1) $\times$ 2048 (t_2) complex data points; 32–48 scans per t_1 increment 6009.6 Hz in both dimensions were used. The mixing times were 100 ms for TOCSY and 450 ms for the ROESY/NOESY experiments. Spectra were processed with Topspin (Bruker Biospin) and visualized with Topspin or NMRView on a Linux station. Matrices were zero-filled to 1024 (t_1) $\times$ 2048 (t_2) points after apodization by shifted sine-square multiplication and linear prediction in the F1 domain. Chemical shifts were referenced to the solvent chemical shifts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

Alloc	allyloxycarbonyl
Boc	<i>tert</i> -butyloxycarbonyl
BSA	bovine serum albumin
COSY	correlation spectroscopy
DAMGO	[D-Ala ² , N-MePhe ⁴ , Gly-ol ⁵]-enkephalin
Dab	diaminobutyl
Dap	diaminopropionyl
DCM	dichloromethane
DIDI	[Ile ^{5,6}]-deltorphin-II
DIEA	diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DOR	δ -opioid receptor
DPT	di-2-pyridylthionocarbonate
DQF-COSY	double quantum filter COSY
eGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
ESI-MS	electron spray ionization mass spectrometry
FAB	fast atom bombardment
Fmoc	9-fluorenylmethyloxycarbonyl
GPI	guinea pig ileum
GTPγS	guanosine-5'-O-(3-thiotriphosphate)

HBTU	<i>N</i> -[(1 <i>H</i> -benzotriazol-1-yloxy) (dimethylamino)-methylene]- <i>N</i> -methylmethanaminium hexafluorophosphate
HEK	human embryonic kidney
HPLC	high performance liquid chromatography
HRMS	high resolution mass spec-trometry
LC-MS	liquid chromatography coupled to mass spectrometry
Leu⁵-Enk	Leu ⁵ -enkephalin
Met-Enk	Met ⁵ -enkephalin
MLEV	Malcom Levitt's decoupling cycle
MOR	μ -opioid receptor
Mtt	methyltrityl
MVD	mouse vas deferens
NMM	<i>N</i> -methyl-morpholine
NMP	<i>N</i> -methyl pyrrolidone
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
Pip	piperidine
PS	polystyrene
Pyr	pyrrolidine
RGD	Arg-Gly-Asp tripeptide
ROESY	rotating frame Overhauser spectroscopy
RP-HPLC	reverse phase-high performance liquid chromatography
rt	room temperature
R_t	retention time
<i>t</i>Bu	<i>tert</i> -butyl
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	trifluoroethanol
THF	tetrahydrofuran
TIS	triisopropylsilane
TNBS	2,4,6-trinitro-benzene-1-sulfonic acid
TOCSY	total correlation spectroscopy
TPPI	time-proportional phase incrementation
Tris	tris(hydroxymethyl)aminomethane

REFERENCES

1. Touati-Jallabe Y, Chiche L, Hamzée A, Aumelas A, Lisowski V, Berthomieu D, Martinez J, Hernandez J-F. Cyclic peptides with a diversely substituted guanidine bridge: solid-phase synthesis and structural analysis. *Chemistry*. 2011; 17:2566–2570. [PubMed: 21294201]
2. Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature*. 1975; 258:577–580. [PubMed: 1207728]
3. Mosberg HI, Fowler CB. Development and validation of opioid ligand–receptor interaction models: the structural basis of mu vs delta selectivity. *J. Pept. Res.* 2002; 60:329–335. [PubMed: 12464111]
4. Hruby VJ, Agnes RS. Conformation–activity relationships of opioid peptides with selective activities at opioid receptors. *Biopolymers*. 1999; 51:391–410. [PubMed: 10797229]
5. Malicka J, Czaplewski C, Groth M, Wicz W, Oldziej S, Lankiewicz L, Ciarkowski J, Liwo A. Use of NMR and fluorescence spectroscopy as well as theoretical conformational analysis in conformation–activity studies of cyclic enkephalin analogues. *Curr. Top. Med. Chem.* 2004; 4:123–133. [PubMed: 14754380]
6. DiMaio J, Schiller PW. A cyclic enkephalin analog with high in vitro opiate activity. *Proc. Natl. Acad. Sci. U. S. A.* 1980; 77:7162–7166. [PubMed: 6261242]
7. Shreder K, Zhang L, Dang T, Yaksh TL, Umeno H, DeHaven R, Daubert J, Goodman M. Synthesis and biological activity of a novel methylamine-bridged enkephalin analogue (MABE): a new route to cyclic peptides and peptidomimetics. *J. Med. Chem.* 1998; 41:2631–2635. [PubMed: 9651168]
8. Sarantakis, D. Analgesic polypeptides.. 1979. U.S. Patent 4148786
9. Schiller PW, Eggimann B, DiMaio J, Lemieux C, Nguyen TM. Cyclic enkephalin analogs containing a cystine bridge. *Biochem. Biophys. Res. Commun.* 1981; 101:337–343. [PubMed: 6272756]
10. Mosberg HI, Hurst R, Hruby VJ, Gee K, Yamamura HI, Galligan JJ, Burks TF. Bis-penicillamine enkephalins possess highly improved specificity toward delta opioid receptors. *Proc. Natl. Acad. Sci. U. S. A.* 1983; 80:5871–5874. [PubMed: 6310598]
11. Mosberg HI, Haaseth RC, Ramalingam K, Mansour A, Akil H, Woodard RW. Role of steric interactions in the delta opioid receptor selectivity of [D-Pen2, D-Pen5]enkephalin. *Int. J. Pept. Protein Res.* 1988; 32:1–8. [PubMed: 2851561]
12. Berezowska I, Chung NN, Lemieux C, Wilkes BC, Schiller PW. Dicarba analogues of the cyclic enkephalin peptides HTyr-c[d-Cys-Gly-Phe-d(or l)-Cys]NH(2) retain high opioid activity. *J. Med. Chem.* 2007; 50:1414–1417. [PubMed: 17315860]
13. Mollica A, Guardiani G, Davis P, Ma S-W, Porreca F, Lai J, Mannina L, Sobolev AP, Hruby VJ. Synthesis of stable and potent delta/mu opioid peptides: analogues of H-Tyr-c[d-Cys-Gly-Phe-d-Cys]-OH by ring-closing metathesis. *J. Med. Chem.* 2007; 50:3138–3142. [PubMed: 17539621]
14. Pawlak D, Oleszczuk M, Wójcik J, Pachulska M, Chung NN, Schiller PW, Izdebski J. Highly potent side-chain to side-chain cyclized enkephalin analogues containing a carbonyl bridge: synthesis, biology and conformation. *J. Pept. Sci.* 2001; 7:128–140. [PubMed: 11297348]
15. Mosberg HI, Omnaas JR, Medzihradsky F, Smith CB. Cyclic, disulfide- and dithioether-containing opioid tetrapeptides: development of a ligand with high delta opioid receptor selectivity and affinity. *Life Sci.* 1988; 43:1013–1020. [PubMed: 2845204]
16. Hansen DW Jr. Stapelfeld A, Savage MA, Reichman M, Hammond DL, Haaseth RC, Mosberg HI. Systemic analgesic activity and delta-opioid selectivity in [2,6-dimethyl-Tyr1,D-Pen2,D-Pen5]enkephalin. *J. Med. Chem.* 1992; 35:684–687. [PubMed: 1311764]
17. Bryant SD, Jinsmaa Y, Salvadori S, Okada Y, Lazarus LH. Dmt and opioid peptides: a potent alliance. *Biopolymers*. 2003; 71:86–102. [PubMed: 12767112]
18. Weltrowska G, Berezowska I, Lemieux C, Chung NN, Wilkes BC, Schiller PW. N-Methylated cyclic enkephalin analogues retain high opioid receptor binding affinity. *Chem. Biol. Drug Des.* 2010; 75:182–188. [PubMed: 20028398]
19. Przydzial MJ, Pogozheva ID, Ho JC, Bosse KE, Sawyer E, Traynor JR, Mosberg HI. Design of high affinity cyclic pentapeptide ligands for kappa-opioid receptors. *J. Pept. Res.* 2005; 66:255–262. [PubMed: 16218993]

20. Hutchinson EG, Thornton JM. A revised set of potentials for beta-turn formation in proteins. *Protein Sci.* 1994; 3:2207–2216. [PubMed: 7756980]
21. Möhle K, Gusmann M, Hofmann H-J. Structural and energetic relations between g turns. *J. Comput. Chem.* 1997; 18:1415–1430.
22. Robey FA. Selective and facile cyclization of N-chloroacetylated peptides from the C4 domain of HIV Gp120 in LiCl/DMF solvent systems. *J. Pept. Res.* 2000; 56:115–120. [PubMed: 11007268]
23. Ye Y, Liu M, Tang Y, Jiang X. Promotion of cyclization of linear pentapeptides and heptapeptide by different univalent metal ions. *Chem. Commun. (Cambridge, U. K.).* 2002:532–533.
24. Roberts KD, Ede NJ. Examination of methodology for the synthesis of cyclic thioether peptide libraries derived from linear tripeptides. *J. Pept. Sci.* 2007; 13:811–821. [PubMed: 17880033]
25. Simon J, Benyhe S, Abutidze K, Borsodi A, Szücs M, Tóth G, Wollemann M. Kinetics and physical parameters of rat brain opioid receptors solubilized by digitonin and CHAPS. *J. Neurochem.* 1986; 46:695–701. [PubMed: 3005492]
26. Tóth G, Ioja E, Tömböly C, Ballet S, Tóourwe D, Péter A, Martinek T, Chung NN, Schiller PW, Benyhe S, Borsodi A. beta-Methyl substitution of cyclohexylalanine in Dmt-Tic-Cha-Phe peptides results in highly potent delta opioid antagonists. *J. Med. Chem.* 2007; 50:328–333. [PubMed: 17228874]
27. Lu Y, Nguyen TM, Weltrowska G, Berezowska I, Lemieux C, Chung NN, Schiller PW. [2',6'-Dimethyltyrosine]-dynorphin A(1–11)-NH₂ analogues lacking an N-terminal amino group: potent and selective kappa opioid antagonists. *J. Med. Chem.* 2001; 44:3048–3053. [PubMed: 11543672]
28. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50% inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
29. Spetea M, Monory K, Tömböly C, Tóth G, Tzavara E, Benyhe S, Hanoune J, Borsodi A. In vitro binding and signaling profile of the novel mu opioid receptor agonist endomorphin 2 in rat brain membranes. *Biochem. Biophys. Res. Commun.* 1998; 250:720–725. [PubMed: 9784412]
30. Schiller PW, Lipton A, Horrobin DF, Bodanszky M. Unsulfated C-terminal 7-peptide of cholecystokinin: a new ligand of the opiate receptor. *Biochem. Biophys. Res. Commun.* 1978; 85:1332–1338. [PubMed: 217386]
31. DiMaio J, Nguyen TM, Lemieux C, Schiller PW. Synthesis and pharmacological characterization in vitro of cyclic enkephalin analogues: effect of conformational constraints on opiate receptor selectivity. *J. Med. Chem.* 1982; 25:1432–1438. [PubMed: 6296388]
32. Waterfield AA, Leslie FM, Lord JA, Ling N, Kosterlitz HW. Opioid activities of fragments of beta-endorphin and of its leucine65-analogue. Comparison of the binding properties of methionine- and leucine-enkephalin. *Eur. J. Pharmacol.* 1979; 58:11–18. [PubMed: 499333]

Figure 1.
Structure of the two series of cyclic enkephalins.

^aReagents and conditions. (a) 0.2 equiv Pd(PPh₃)₄, 24 equiv phenylsilane, dry DCM, 2 × 4 h; (b) 5 equiv DPT, dry DCM; (c) DCM/TFE/AcOH (7/2/1), 6 × 20 min; (d) 4 equiv TEA, dry THF; (e) 0.2 M MeI in DMF, 3 × 1 h; (f) 2 M HN(R¹)R² (NH₄⁺, AcO⁻ in the case of R¹=R²=H), dry DMSO, 80 °C; (g) TFA/TIS/H₂O (95/2.5/2.5).

Scheme 1.

Synthesis of the Small Cycle Series (Compounds 7–14)^a

^aReagents and conditions. (a) 0.2 equiv Pd(PPh₃)₄, 24 equiv phenylsilane, dry DCM, 2 × 4 h; (b) 5 equiv DPT, dry DCM; (c) DCM/TFE/AcOH (7/2/1), 6 × 20 min; (d) 4 equiv TEA, dry THF; (e) 0.2 M MeI in DMF, 3 × 1 h; (f) 2 M HN(R¹)R² (NH₄⁺, AcO⁻ in the case of R¹=R²=H), dry DMSO, 80 °C; (g) TFA/TIS/H₂O (95/2.5/2.5).

Figure 2.
 ^1H NMR spectra of **7a**, **9a**, **10a**, **12a**, and **13a** in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1) at 298 K, pH 4.

Figure 3.
¹H NMR spectra of **13a–c** in H₂O/D₂O (9:1) at 298K, pH 4.

Figure 4.
Schematic representation of the **13a–c** long-range ROE correlations (gray arrays).

Table 1

Cyclic Enkephalin Analogues

large cycle		small cycle	
compd	X	compd	X
1	=S	7	=S
2	-S-Me	8	-S-Me
3	-NH ₂	9	-NH ₂
4	-NH-Me	10	-NH-Me
		11	-NH-Et
5	-N(Me) ₂	12	-N(Me) ₂
6	pyrrolidine	13	pyrrolidine
		14	piperidine

Table 2

LC-MS Retention Time (min) of the Isomers of Compounds 7-14

compound	isomer a	isomer b	isomer c	ratio a/b/c ^a
7	0.89	0.80		85/15
8	0.70			NA
9	0.69	0.75		70/30
10	0.70	0.74		75/25
11	0.72	0.78		83/17
12	0.70	0.76		80/20
13	0.75	0.87	0.91	40/40/20
14	0.79	0.92	0.97	45/35/20

^aEstimated from RP-HPLC and LC-MS analyses. NA: not applicable.

Table 3

Equilibrium Competition Binding Affinities toward μ and δ Opioid Receptors and G-Protein Activation, Compounds 1-6 (Large Cycle)

ligands (X)	K_i^a , nM (equilibrium affinity, $\log IC_{50} \pm SEM$)			$[^{35}S]GTP\gamma S$ binding	
	MOR assay, $\mu[^3H]DAMGO$	DOR assay, $\delta[^3H]DIDI$	selectivity δ/μ	EC_{50} , nM ($\log EC_{50} \pm SEM$)	E_{max} (%) ^b
DAMGO	1.7 (8.62 ± 0.04)				
DIDI		2.34 (8.51 ± 0.06)			
Met-Enk				304 (6.51 ± 0.08)	205.3 $\pm$ 3.8
1 (=S)	9.1 (7.91 ± 0.06)	75.7 (7.01 ± 0.07)	8.3	589 (6.22 ± 0.20)	139.3 $\pm$ 5.6
2 (-S-Me)	21 (7.54 ± 0.07)	191 (6.60 ± 0.08)	9.1	652 (6.18 ± 0.16)	156.4 $\pm$ 4.9
3 (-NH ₂)	18.4 (7.60 ± 0.08)	108 (6.84 ± 0.08)	5.9	3012 (5.52 ± 0.16)	159.3 $\pm$ 8.4
4 (-NH-Me)	17.9 (7.62 ± 0.06)	185 (6.61 ± 0.07)	10.3	1231 (5.91 ± 0.42)	140.1 $\pm$ 8.2
5 (-N(Me) ₂)	14.3 (7.71 ± 0.05)	146 (6.71 ± 0.05)	10.2	1516 (5.81 ± 0.13)	179.7 $\pm$ 7.6
6 (-Pyr)	175 (6.63 ± 0.07)	628 (6.08 ± 0.04)	3.6	8005 (5.09 ± 0.51)	150.9 $\pm$ 7.2

^a K_i was calculated by the Cheng-Prusoff equation.

^b Stimulation over basal activity (%).

Table 4

Equilibrium Competition Binding Affinities toward μ and δ Opioid Receptors and G-Protein Activation, Compounds 7-14 (Small Cycle)

ligands (X)	K_i^a nM (equilibrium affinity, logIC ₅₀ $\pm$ SEM)		selectivity δ/μ	[³⁵ S]GTP γ S binding	
	MOR assay, μ [³ H]DAMGO	DOR assay, δ [³ H]DIDI		EC ₅₀ , nM (logEC ₅₀ $\pm$ SEM)	$E_{\max}$ (%) ^b
DAMGO	1.9 (8.62 $\pm$ 0.04)				
DIDI		2.5 (8.64 $\pm$ 0.05)			
Met-Enk				304 (6.51 $\pm$ 0.08)	205.3 $\pm$ 3.8
7a (=S)	0.4 (9.31 $\pm$ 0.04)	5.4 (8.13 $\pm$ 0.07)	13.5	250 (6.60 $\pm$ 0.18)	187.5 $\pm$ 6.4
7b (=S)	29 (7.44 $\pm$ 0.05)	304 (6.38 $\pm$ 0.08)	10.5	ND	ND
8 (-S-Me)	4.2 (8.28 $\pm$ 0.05)	11 (7.83 $\pm$ 0.07)	2.6	2235 (5.65 $\pm$ 0.19)	174.5 $\pm$ 11.2
9a (-NH ₂)	19 (7.62 $\pm$ 0.08)	739 (6.01 $\pm$ 0.08)	38.9	2274 (5.64 $\pm$ 0.26)	143.0 $\pm$ 7.6
10a (-NH-Me)	1.5 (8.72 $\pm$ 0.04)	62 (7.08 $\pm$ 0.06)	41.3	278 (6.55 $\pm$ 0.09)	163.9 $\pm$ 2.5
10b (-NH-Me)	19 (7.63 $\pm$ 0.03)	245 (6.47 $\pm$ 0.06)	12.9	1592 (5.79 $\pm$ 0.14)	153.5 $\pm$ 4.6
11a (-NH-Et)	21 (7.59 $\pm$ 0.03)	263 (6.44 $\pm$ 0.06)	12.5	1233 (5.91 $\pm$ 0.11)	147.8 $\pm$ 3.6
12a (-N(Me) ₂)	57 (7.15 $\pm$ 0.04)	738 (6.03 $\pm$ 0.07)	12.9	1833 (5.73 $\pm$ 0.11)	144.4 $\pm$ 2.9
13a (-Pyr)	39(7.32 $\pm$ 0.04)	580 (6.11 $\pm$ 0.05)	14.9	1384 (5.97 $\pm$ 0.12)	145.9 $\pm$ 7.3
13b (-Pyr)	288 (6.45 $\pm$ 0.08)	1646 (5.65 $\pm$ 0.09)	5.7	2283 (5.55 $\pm$ 0.41)	115.5 $\pm$ 4.8
13c (-Pyr)	351 (6.37 $\pm$ 0.04)	2884 (5.41 $\pm$ 0.09)	8.2	2457 (5.32 $\pm$ 0.39)	110.5 $\pm$ 4.3
14a (-Pip)	107 (6.88 $\pm$ 0.07)	1097 (5.82 $\pm$ 0.07)	10.2	1945 (5.84 $\pm$ 0.23)	151.7 $\pm$ 6.8
14b (-Pip)	341 (6.38 $\pm$ 0.05)	3591 (5.31 $\pm$ 0.12)	10.5	2071 (5.38 $\pm$ 0.22)	120.0 $\pm$ 6.5
14c (-Pip)	637 (6.11 $\pm$ 0.05)	3504 (5.32 $\pm$ 0.11)	5.5	2692 (5.12 $\pm$ 0.17)	110.8 $\pm$ 4.9

^a K_i was calculated by the Cheng-Prusoff equation.

^b Stimulation over basal activity (%). ND, not determined.

Table 5

Biological Activity of Compounds 7a-10a and 14a in Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays

ligands (X)	GPI		MVD	
	IC ₅₀ (nM) ^a	relative potency	IC ₅₀ (nM) ^a	relative potency
Leu-Enk	246 ± 38	1	11.4 ± 1.1	1
7a (=S)	1.76 ± 0.05	140 ± 4	2.37 ± 0.24	4.81 ± 0.45
8 (-S-Me)	17.8 ± 3.2	13.8 ± 2.5	20.7 ± 2.6	0.550 ± 0.068
9a (-NH ₂)	244 ± 27	1.01 ± 0.11	347 ± 33	0.0329 ± 0.0031
10a (-NH-Me)	41.8 ± 1.1	5.89 ± 0.16	76.5 ± 16.4	0.149 ± 0.032
14a (-Pip)	398 ± 64	0.618 ± 0.099	306 ± 27	0.0373 ± 0.0033

^aMean of 3-5 determinations ± SEM.