ACS Medicinal Chemistry Letters

Letter

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Structural and Biological Exploration of Phe^3–Phe^4-Modified Endomorphin-2 Peptidomimetics

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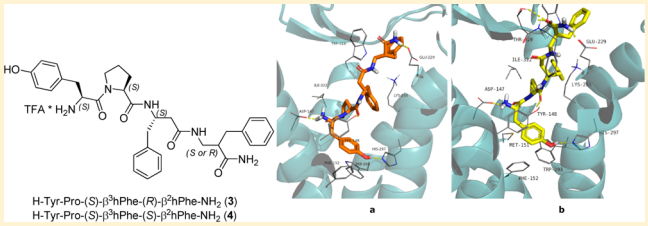
Supporting Information

ABSTRACT: This study reports on our ongoing investigation on hybrid EM-2 analogues, in which the great potential of β-amino acids was exploited to generate multiple conformational modifications at the key positions 3 and 4 of the parent peptide. The effect on the opioid binding affinity was evaluated, by means of ligand stimulated binding assays, which indicated a high nanomolar affinity toward the μ-receptor, with appreciable μ/δ selectivity, for some of the new compounds. The three-dimensional properties of the high affinity μ opioid receptor (MOR) ligands were investigated by proton nuclear magnetic resonance, molecular dynamics, and docking studies. In solution, the structures showed extended conformations, which are in agreement with the commonly accepted pharmacophore model for EM-2. From docking studies on an active form of the MOR model, different ligand–receptor interactions have been identified, thus confirming the ability of active compounds to assume a biologically active conformation.



KEYWORDS: *Peptidomimetics, opioid receptors, β-amino acids, conformational analysis, docking studies*

Most of the biological effects of endogenous opioid peptides are mediated through activation of three opioid receptors designated μ, δ, and κ in the peripheral or central nervous system.[1] One of the endogenous peptide ligands for the μ opioid receptor (MOR), endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2, Figure 1), exhibits high affinity and extraordinarily high selectivity relative to δ-opioid and κ-opioid receptor systems. This tetrapeptide has a strong antinociceptive effect on acute pain, similar to that of morphine, and it is also more effective than the majority of the opioid peptides against neuropathic pain even at low doses. However, as found in the case of most short linear natural peptides, native EM-2 lacks critical therapeutic characteristics such as bioavailability, duration of action, and oral activity.[2] Since MOR is proven to be the major target of analgesics[3] because of its particular physiological characteristics, development of synthetic EM-2 analogues is of great importance.

Research efforts are focused on the design of analogues and peptidomimetics in order to improve EM-2 properties as potential drug as well as to better understand the key structural and conformational features on which receptor recognition and binding are based.[4,5] It is well-known that the amino acid Pro represents a crucial factor in determining the structural and conformational properties of EM-2 analogues, acting as a stereochemical spacer, capable of favoring proper spatial orientation of the aromatic side chain groups.[6] In EM-2, aromatic amino acid side chains exhibit considerable conformational flexibility, especially the Phe^3 aromatic ring, which is free to adopt a bioactive conformation. Also the C-terminal address fragment Phe^4-NH_2 plays an important role in ligand recognition.[7]

Several chemical modifications performed on EM-2 have been described previously, and most of them were focused on single amino acid substitution.[8] However, in recent, multiple modifications in EM-2-based drug design are going to constitute the major interest.[9−13] Indeed, apart the changes in binding properties, most of these additional modifications tend to shift the pharmacokinetics and pharmacodynamics positively, facilitating a more feasible potential application of ligands for clinical purposes.

Aiming to help the definition of privileged structures exclusively associated with bioactivity, we recently investigated the biological activity and the conformational requirements of hybrid EM-2 analogues, in which the C-terminal Phe^3–Phe^4

Received: May 17, 2013
Accepted: July 11, 2013
Published: July 11, 2013

   dx.doi.org/10.1021/ml400189r | ACS Med. Chem. Lett. 2013, 4, 795−799

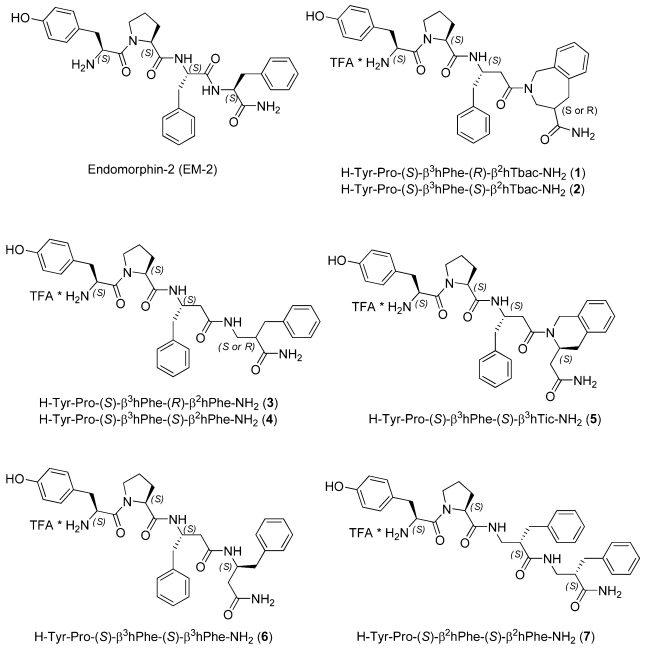


Figure 1. Molecular structure of EM-2 and analogues **1**−**7**.

dipeptide is simultaneously substituted by various β-peptidomimetic elongated structures. In some cases, we could observe the maintenance of opioid affinity and we also could highlight common binding modes for active compounds, confirming the necessity for specific binding interactions, for instance, between Asp 147 and Tyr[1], as evidenced by docking studies.[14] As recently outlined,[15] peculiar advantages are connected with the employment of heterogeneous (mixed α and β) backbones in peptidomimetic chemistry, such as a high stability toward hydrolytic enzymes, more possibility of chemical diversity and, in most cases, more predictable secondary structures, each of which offers a potentially distinctive way to project sets of side chains in space and to generate preferential conformations.[16,17]

Being aware that the potential of such strategic substitutions were not fully explored in the key Phe[3]−Phe[4] fragment of EM-2, we went on in our research project, and we report here on the EM-2 analogues given in Figure 1.

They are obtained by replacing the Phe amino acids in positions 3 and 4 with various different β-homologues residues, characterized as β^2- or β^3-aa's, according to Seebach's nomenclature,[18] and in some cases endowed with skeletal constraints in the fourth residue. They were thought able to deeply alter the tetrapeptide backbone conformation and thus modulate in new ways the bioactivity of the reference EM-2 peptide. Their synthesis, opioid receptor binding affinities and selectivities, NMR and molecular dynamics conformational behavior, and binding modes from docking are here reported.

Orthogonally protected intermediates **8**−**14** (Figure 2) were synthesized from the key precursors β^3-hPhe and β^2-hPhe amino acids and their constrained Tbac (2,3,4,5-tetrahydro-1*H*-benzo[c]azepine-4-carboxylic acid) and Tia (2-(1,2,3,4-tetrahydroisoquinolin-3-yl)acetic acid) derivatives. Preparation of *N*-Boc-β^3-hPhe and β^3-hPhe methyl ester was accomplished following the protocol of stereospecific classical homologation[19] of the corresponding L-αPhe, by means of the Kolbe reaction. *N*-Boc-β^2-hPhe and β^2-hPhe methyl ester could be obtained in both the enantiomeric forms following an efficient protocol developed by Gellman.[20] The constrained Tbac- and

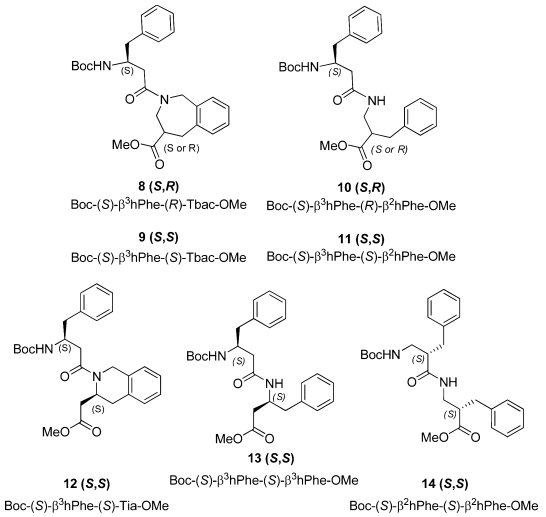


Figure 2. Molecular structure of the β-dipeptidomimetics **8**−**14** used to replace the native Phe[3]−Phe[4]-based residue in EM-2.

Tia-methyl esters were obtained starting from *N*-Cbz-β^2-hPhe methyl ester and from *N*-Cbz-β^3-hPhe methyl ester, respectively, by means of TFA-catalyzed Pictet−Spengler condensation, followed by Cbz removal. Peptide coupling was better performed by means of BOP-Cl and TEA in CH_2Cl_2 in the case of constrained amino acids derivatives (compounds **8**, **9**, and **12**) and by means of EDC, HOBt, and DIPEA in CH_2Cl_2 in all other cases (compounds **10**, **11**, **13**, and **14**).

With the dipeptidomimetic scaffolds **8**−**14** in our hands, EM-2 analogues **1**−**7** were obtained by conversion of the terminal methyl ester to the corresponding primary amide, followed by standard peptide coupling chemistry and isolation of the target compounds as trifluoroacetate salts. All new products were characterized by ^{1}H NMR and HR-MS data.

Amides **1**−**7** have been evaluated for their affinity and selectivity for μ and δ opioid receptors in radioligand binding assays under standard conditions, using cloned receptors stably expressed on Chinese hamster ovary (CHO) cells, and [^{3}H]DPDPE (cyclo[D-Pen2,DPen5]enkephalin) and [^{3}H]DAMGO ([D-Ala2,NMePhe4,glyol5-enkephalin), respectively, as the radioligands. The data are summarized in Table 1.

All compounds **1**−**7** exhibit a very good nanomolar affinity toward the μ-opioid receptor, with appreciable μ/δ selectivity in most cases. The highest affinity, coupled with notable selectivity, is shown by peptidomimetics **3** and **4**, reaching the low nanomolar scale as the parent EM-2. It should be noted that such a finding is indeed remarkable for EM-2 analogues

Table 1. Opioid Receptor Binding Affinities and Selectivities of EM-2 Analogues 1−7

	selectivity		affinity K_i (nm)	
compd	K_i^μ/K_i^δ	K_i^δ/K_i^μ	[^{3}H]DAMGO (μ)	[^{3}H]DPDPE (δ)
EM-2	0.002	639	5.3 ± 0.4 (4)	3385 ± 309 (3)
1	0.03	33	21 ± 1.4 (3)	695 ± 21.4 (3)
2	0.075	13	14 ± 1.2 (3)	187 ± 14.5 (4)
3	0.004	245	5.5 ± 0.18 (4)	1347 ± 112 (3)
4	0.007	140	3.3 ± 0.15 (5)	463 ± 38.5 (3)
5	0.012	83	20 ± 1.9 (3)	1650 ± 14 (3)
6	0.007	147	19 ± 1.4 (3)	2798 ± (3)
7	0.011	9	101 ± 8.4 (3)	922 ± 10.0 (4)

dx.doi.org/10.1021/ml400189r | ACS Med. Chem. Lett. 2013, 4, 795−799

with multiple structural modifications in the Phe−Phe portion, comprising amino acid homologation and variation upon the configuration of the incorporated β^2hPhe-NH_2 at the fourth position.

The conformationally constrained mimics **1**, **2**, and **5** are not as active as **3** and **4**, disclosing how deep modifications in the key address fragment (C-terminal Phe^4-NH_2), leading to an appreciable reduction of the conformational freedom, would result in a decrease in affinity. Finally, the low activity of peptidomimetic **7** clearly highlights the detrimental effect of β^2-homologation at Phe^3, which alters significantly the pharmacophore distances[21] of the aromatic ring in the message fragment (N-terminal tripeptide unit). This date confirms once again that the proper aromatic ring distance and spatial orientation of the third residue is highly influencing the binding process, discriminating a potent lead from a much less active derivative.

Structural studies, in particular 1D and 2D proton NMR, molecular dynamics, and docking analysis, have been performed in order to fully investigate the three-dimensional properties of synthesized analogues (see Supporting Information). At present, a large number of data have been reported on the conformational analysis of endomorphins, but conclusions are rather contradictory as both extended and folded structures have been suggested as the bioactive conformation. For instance, the importance of the Tyr^1−Pro^2 amide bond conformation of EM-2 in its bioactive form is a controversial and maybe an overstressed problem. In fact, if the opioid receptor protein selects its ligand by conformational selection in a dynamic environment, the conformation of a certain amide bond alone cannot be a strict determinant for a stable ligand−receptor interaction. However, relying on total energy measurements, it was recently proposed that the trans isomer of EM-2 and analogues could be the mainly bioactive form and that the cis isomer is mainly an artifact under the solution conditions.[22]

We performed NMR studies in DMSO-d_6 solution, as this solvent is considered a good physical approximation of transport fluids environments[23] and is claimed to be a better approximation for the mechanical and electrostatic environment of binding to the MOR than D_2O is.[24] Moreover, being a good hydrogen bond acceptor, it may reveal intrinsic conformational preferences, mimicking at the same time the physical circumstances of receptor−ligand interactions. The spectra show the presence of two conformers (cis and trans with respect to the Tyr−Pro peptide bond) for all compounds. A higher prevalence of the trans conformers (trans/cis ratio ranging from 1 for most compounds to 4 for **7**) has been observed according to the intensities of OH peaks in ^{1}H NMR spectra.

From relevant inter-residue ROE interactions, some common conformational behaviors can be highlighted within all compounds. In particular, the Tyr^1 aromatic ring seems to be always folded over the Pro^2 segment (ROE between Pro H-δ and various Tyr protons), and in most cases, the NH of the β-$hPhe^3$ interacts with the Pro's H-α. Constrained compounds **1** and **2** present additional ROE contacts, involving the β^2-$hTbac^4$ residue from one side, and the benzylic or H-α protons of β^3-$hPhe^3$, on the other side, and clearly underlining a folding between the third and fourth residue. No significant inter-residue ROE interaction could be observed for linear derivatives, revealing preferentially extended conformations. The only exception regards compound **6** where the presence of multiple ROE contacts would suggest a prominent folding of the overall structure. Back to biological results, high activity of **3** and **4** seems to go hand in hand with an essentially extended conformation, such as that adopted as a result of β^3-hPhe-β^2-hPhe insertion in place of the third and fourth EM-2 residues.

To evaluate the pharmacophoric ability of our analogues **3** and **4**, we decided to explore their conformational behavior by means of computational tools, focusing our attention on the intramolecular distances outlined by the pharmacophore model, assembled for bioactivity and MOR selectivity. It suggests as ideal the key distance of 10−13 Å (*C* distance) between the aromatic rings of residues 1 and 3. In addition, two other distances concerning the message domain have been considered conclusive for activity, namely, the Tyr^1 N−Tyr^1 O distance (about 8 Å) and the Tyr^1 N−Phe^3 ring distance (about 7 Å).[25,26]

First a conformational analysis was performed, then the lowest energy conformers were submitted to a 10 ns molecular dynamics at 300 K to test their conformational stability. Calculations were executed on the Tyr^1 protonated form of **3** and **4** in water and in DMSO. The conformations were also clustered according to folded or extended conformations by measuring the interatomic Cα distance between the first and the fourth residue. Results showed a preference for folded conformations in water for the two ligands, both after Monte Carlo conformational analysis (MC) and molecular dynamics (MD) simulations. In DMSO, a substantial equilibrium between folded and extended conformations is established, with a slight preference for the latter. The pharmacophore *A*, *B*, and *C* distances were measured and averaged on all found conformations for both compounds. A good agreement of all values with the proposed model could be observed for water environment, while lower C distances have been measured in DMSO. In both environments, comparison between compounds **3** and **4** highlights a greater value of the *C* distance for compound **4**, accordingly with its slightly higher activity.

In order to better evaluate the binding orientations and to study the key ligand−receptor interactions involved in the molecular recognition process with the μ opioid receptor (MOR), most active compounds **3** and **4** were submitted to docking studies. Recently the crystal structure of the mouse MOR with the irreversible morphinan antagonist β-funaltrexamine (β-FNA) has been reported.[27] Since it can be supposed that the reported structure was referred to an inactive form of the receptor, we rather decided to use an active form of the MOR model as described by Mosberg.[28] The ligands were docked in to the MOR model flexibly with the Molegro Virtual Docker software (Figure 3).

Both compound **3** and **4** displayed high negative MolDock score (−260 and −339, respectively), indicating a strong favorable interaction of the ligands with the receptor, with a lower energy for **4**, thus in accordance with biological activity results. Most of the interactions found to be relevant for the EM-2 activity are observed in the binding mode of **3** and **4**. The Tyr^1 residue is deeply placed in the binding site providing strong interactions with the receptor. An H-bond/electrostatic interaction between the protonated amino group and the carboxylate moiety of the Asp147 residue is present, and the Tyr-OH is involved in an H-bond with the nitrogen of His297. A π−π interaction with Trp293 is established. Further H-bonds are formed with Tyr148 and Glu229. Also a stabilizing interaction with Lys 233 is established. Other important interactions are found between lipophilic regions of the ligand and Met151, Phe152, Ile296, Val300, and Ile322. The binding mode of **4** showed additional π−π interactions between Tyr^1

dx.doi.org/10.1021/ml400189r | ACS Med. Chem. Lett. 2013, 4, 795−799

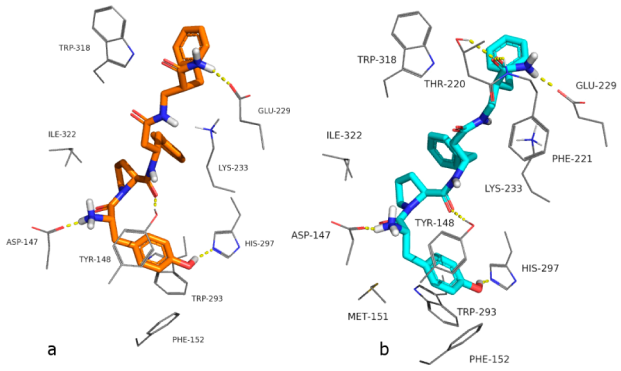


Figure 3. Binding mode of peptides **3** (a) and **4** (b) as obtained by docking simulations. Hydrogen bonds are represented as yellow dashed lines.

and Trp293 and Phe152 and Tyr148. The terminal amide moiety is involved with a range of H-bonds: the NH_2 group is bonded with Glu229, and the carbonyl oxygen interacts with both the backbone nitrogen of Phe221 and the OH of Thr220. These strong H-bond interactions in the recognized C-terminal address domain, could be related to the higher affinity showed by peptide **4**. It is worth to notice that these results are in agreement with site-directed mutagenesis studies, which already highlighted the importance of some residues as Asp147, Tyr148, Glu229, and His297.[4]

As a further consideration, some relevant analogies with the binding pocket of the recently reported crystal structure of the (β-FNA)-MOR complex can also be highlighted. Some of the residues most interacting with β-FNA (Asp147, Tyr148, Met151, Ile296, Glu229, Lys233, Trp293, His 297, Val300, and Ile322) were also found to be in close contact with **3** and **4**, thus confirming the validity of our model. Comparison of binding modes of less active compounds confirmed that the sequence β^3hPhe^3–β^2hPhe^4–NH_2 is critical for the effectiveness of the ligands. In fact, when a β^2hPhe^3 is present (compound **7**) the binding is less effective due to the lack of the H-bond with Tyr148; in the case of β^3hPhe^4–NH_2 substitution (compound **6**), a conformational change in the terminal residue make not possible the important H-bond interaction with Glu229. The same considerations can be done for compounds **1**, **2**, and **5**, thus confirming the hypothesis that constraining the phenyl ring of the C-terminal residue is detrimental to the activity of the peptides.

In conclusion, a small collection of EM-2 analogues were synthesized, by incorporating various β-dipeptidomimetics to replace the native Phe^3–Phe^4-based residue in the reference compound. In two cases, the multiple structural modifications resulted in highly active and selective analogues, thanks to the retention of the structural properties required for MOR binding.

On the basis of NMR analysis and molecular dynamics studies, the solution structures of the selected high affinity MOR ligands **3** and **4** were found to show prevalent extended conformations and to be in full agreement with the commonly accepted pharmacophore model of EM-2. Since conformational studies on isolated molecules in the absence of their receptor do not necessarily provide unambiguous information on the bioactive structure, these data were complemented with docking studies. Different intramolecular interactions have been identified, which confirm the ability of active compounds **3** and **4** to assume a biologically active correct conformation. Thanks to these outcomes, peptidomimetics **3** and **4** may validate further study for the development of a peptide analgesic based on the EM-2 sequence.

Even if caution must be still used in providing key structural parameters for bioactivity versus opioid receptors, which exist as dynamic entities that can occupy multiple conformations, we believe that the recent report of the crystal structure of the mouse MOR and the joint investigation of the receptor and its ligands will soon provide conclusive evidence with regard to the structural features of the biologically active form of EM-2 and analogues.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed procedure for the synthesis of all compounds and their characterization; copy of NMR spectra for all compounds; radioligand binding assay; and computational details. 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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