

Structural comparison of endomorphin-2 and its conformationally restricted analog Tyr-Pro-Phe-Phe-NH₂

Research Article

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Received 28 May 2011; Accepted 27 September 2011

Abstract: In the present study, the effect of a conformational constraint introduced into the endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂, EM-2) structure was studied using computational analysis and radioligand binding assay. EM-2 was modified by connecting nitrogen atoms of both phenylalanine residues by a methylene bridge. The obtained analog did not bind to the μ - or δ -opioid receptors in the *in vitro* studies. The computational analysis of this analog showed twisted, type IV turns and the absence of canonical β -turns typical for the EM-2 structure, which can be explained by the lack of hydrogen bonds involving Phe⁴. Our results show that the introduction of chemical constraint in the EM-2 structure has a significant effect on opioid receptor affinity and *in vitro* bioactivity.

Keywords: Conformational constraint • Endomorphin-2 • Hydrogen bond • μ -opioid receptor ligand • Structure-activity relationship study

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1. Introduction

Centrally acting opiates such as morphine are the most frequently used analgesics for the relief of severe pain, but their usefulness is limited by a number of well-known side effects, including tolerance, physical dependence, respiratory depression and diverse gastrointestinal effects. Two tetrapeptide amides, discovered by Zadina *et al.* in 1997, endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH₂, Fig. 1A) are the endogenous ligands that act at the μ -opioid receptor as morphine does [1]. Since the μ -opioid receptor mediates the most prominent pharmacological effects of morphine, EMs have aroused considerable interest as possible new therapeutics for pain management [2]. However, EMs like peptides in general, suffer from serious limitations, including short duration of action due to poor metabolic stability [3-5] and relative inability to cross the blood-brain barrier [6,7]. Therefore, the search

for EM analogs with improved pharmacological profile is still going on.

The structure of EMs consists of two biologically important fragments, the N-terminal message sequence (Tyr-Pro-Phe/Trp), related to signal transduction and receptor recognition, and the C-terminal address domain (Phe⁴-NH₂), affecting the receptor selectivity [8]. Elucidation of the role of side-chain residues located within the message and address domains and determination of their spatial arrangement are crucial for the structure-activity studies. It is well established that the phenolic OH function of Tyr¹ residue with a free cationic α -amino group, the aromatic side chains of Phe³/Trp³ and Phe⁴ together with the C-terminal amide are essential pharmacophoric elements required for μ -opioid receptor recognition and appropriate binding orientation [8-10]. A more subtle requirement is associated with the spatial disposition of each aromatic side chain [11,12]. However, in small peptides, such as EMs, the amino

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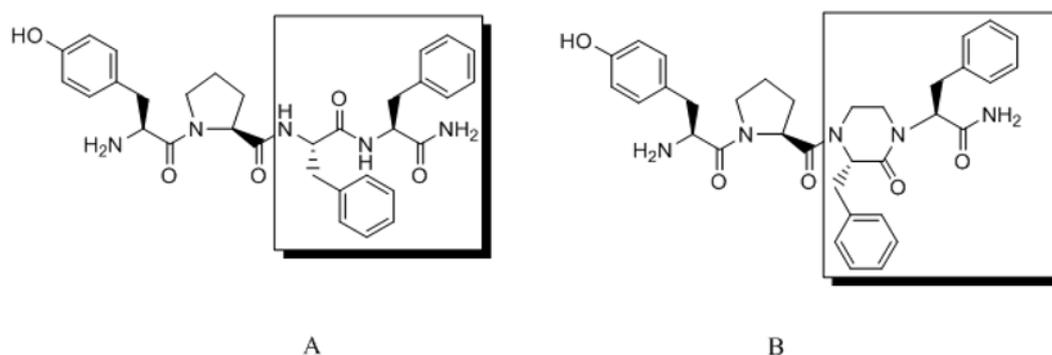


Figure 1. Structure of endomorphin-2 (A) and its conformationally restricted analog Tyr-Pro-Phe-Phe-NH₂ (B).

acid side chains exhibit considerable conformational flexibility. Hence, the three-dimensional arrangement of pharmacophoric elements vital for μ -opioid activity is not yet determined, despite the large number of data reported on the conformational analysis of EMs. A promising approach in identifying the spatial arrangement of side chain groups is the design of conformationally restricted analogs, since such analogs adopt conformations that are better defined. To restrict the freedom of side chains, two approaches have been used; synthesizing cyclic amino acids [13] or constraining torsion angles by suitable substitutions [14–18]. Use of unnatural amino acids with constraint side chain groups allows finding low-energy conformations for the peptides to which they are introduced, which are likely to be the bioactive conformations. Such approach was used by Tömböly *et al.* [19], who incorporated β -methylated amino acids to restrict conformational mobility of aromatic residues in position 3 and 4 of EMs. The NMR measurements of a very potent EM-2 analog, [(2S,3S)- β MePhe⁴]EM-2, showed that the preferred low-energy conformation of β MePhe⁴ side chain was *gauche(-)* which implied the presence of the $\chi^1=60^\circ$ rotamer of Phe⁴ in the binding conformer of EMs. These results indicated that the proper spatial disposition of the side chain of Phe⁴ is important for the binding to the receptor. Yu *et al.* [20,21] came to a similar conclusion, performing structure-activity studies of a series of EM analogs, in which Phe⁴ was substituted by non-aromatic residues of L- or D-configuration and the C-terminal end was terminated by NH-benzyl moiety. The obtained results showed that the appropriate orientation of the Phe⁴ aromatic ring is substantial for the pharmacological activities of EMs and for their interaction with the μ -opioid receptor, as well as for the determination of the positions of other aromatic side chains. Finally, the C-terminal amide group of EMs was found to be an important contributor to the formation of the bioactive conformation and to the proper receptor-ligand binding [22].

Two general spatial models of opioid ligands active at the μ -opioid receptor were proposed previously on the basis of structural analysis of morphiceptin and its stereoisomeric analogs and of cyclic somatostatin analogs. The first model is defined by fixed distances between the pharmacophores; the free cationic α -amino and phenolic OH groups of the Tyr¹ residue and the aromatic side chain of Trp³/Phe³ [8]. In the second model the optimal spatial arrangement of the pharmacophore groups is furnished by a bent backbone structure, in which the first aromatic side chain was shown to prefer the *gauche(+)* conformation, while a predominance of the *gauche(-)* conformation of the second, and increased flexibility of the third aromatic side chain were observed [23]. This latter model was confirmed partially by our recent structure-activity study based on molecular dynamics (MD) simulations of the selected μ -opioid peptides [24].

Here we report the synthesis of a new EM-2 analog, in which the Phe³-Phe⁴ fragment was replaced by a conformationally restricted dipeptide, -Phe-Phe, containing an ethylene bridge between nitrogen atoms of the α -amino groups (Fig. 1B). Furthermore, we characterized the effect of the chemical constraint on receptor-ligand interactions using computational analysis and radioligand binding studies on rat brain membrane preparations.

2. Experimental procedure

2.1. General

All reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Poznan, Poland). Protected amino acids were obtained from Bachem (Bubendorf, Switzerland). (2S,3'S)-2-(4'-Boc-3'-benzyl-2'-oxopiperazin-1-yl)-3-phenyl-propionic acid (-Phe-Phe), which was first synthesized by Tong *et al.* [25], was obtained from PolyPeptide Group (Hilleroed, Denmark).

EM-2 was synthesized as described previously [26]. The conformationally restricted analog Tyr-Pro-Phe-Phe-NH₂ was prepared by the manual solid-phase technique using benzhydrylamine (BHA) resin (100–200 mesh, 0.6 mM g⁻¹, Advanced ChemTech, Louisville, KY, USA). The peptide was assembled using N- α -*t*-butoxycarbonyl (*t*-Boc)-protected amino acids and 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) coupling reagents [27]. The coupling reaction times for N-Boc-Phe-Phe and N-Boc-Pro were 24 h and repeated couplings were done. Simultaneous deprotection and cleavage from the resin was accomplished by treatment with 90% anhydrous hydrofluoric acid (HF) and 10% anisole scavenger (10 mL of HF and 1 mL of anisole per g of resin) at 0°C for 1 h. After the evaporation of HF the resin was washed with diethyl ether, the peptide was extracted with 20% acetic acid and then lyophilized.

The crude peptide was purified by preparative reversed-phase HPLC on a Vydac C₁₈ column (10 μ m, 22×250 mm), equipped with a Vydac guard cartridge. A solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% B over 15 min was used. Peptide purity was verified by analytical HPLC employing a Vydac C18 column (5 μ m, 4.6×250 mm) and a solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B). A linear gradient of 0–100% solvent B over 25 min at a flow rate of 1 mL min⁻¹ was used for the analysis. Final purity of both peptides was >98%. Calculated values for protonated molecular ions were in agreement with those determined by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS).

The physicochemical parameters for EM-2 analog: t_R 19.4 min, HR-ESI-MS m/z [M+H]⁺ 598.25 (calcd for C₃₄H₃₉N₅O₅ 597.30).

The μ - and δ -opioid receptor binding studies were performed according to the modified method described elsewhere [28]. Briefly, crude membrane preparations, isolated from Wistar rat brains, were incubated at 25°C for 120 min with the appropriate concentration of a tested peptide in the presence of 0.5 nM [³H]DAMGO (as a μ -selective radioligand) or 0.5 nM [³H][Ile^{5,6}]deltorphin-2 (as a δ -selective radioligand), in a total volume of 0.5 mL of 50 mM Tris/HCl (pH 7.4), containing MgCl₂ (5 mM), EDTA (1 mM), NaCl (100 mM), and bacitracin (20 mg L⁻¹). Non-specific binding was determined in the presence of 1 μ M naloxone. Incubations were terminated by rapid filtration through Whatman GF/B (Brentford, UK) glass fiber strips, which had been pre-soaked for 2 h in 0.5% polyethylamine, using Millipore Sampling

Manifold (Billerica, USA). The filters were washed three times with 4 mL of ice-cold Tris buffer solution. The bound radioactivity was measured in Packard Tri-Carb 2100 TR liquid scintillation counter (Ramsey, MN, USA) after overnight extraction of the filters in 4 mL of Perkin Elmer Ultima Gold scintillation fluid (Wellesley, MA, USA). Three independent experiments for each assay were carried out in duplicate.

2.2. Computational details

All simulations were performed using the AMBER 9 program package and the AMBER ff99 force field parameter set [29]. Parameters for unnatural amino acid residues were supplemented from the generalized Amber force field (gAFF) [30] and partial charges were determined at the HF/6-31G(d) level using the RESP method. At first, a 1000 member low-energy structural ensemble was generated by repeated simulated annealing, using a previously described protocol [31]. For annealing runs, the GB/SA implicit solvent model [32] was used and the long-range non-bonded interactions were calculated with a 10 Å cut-off. Then, for each resultant structure 1000 steps of steepest descent followed by 1500 steps conjugate gradient minimization was done where the convergence criteria for the energy gradient was 10⁻⁴ kcal mol⁻¹ Å. The minimized structures were then clustered to eliminate duplicate structures and 5 markedly different structures were chosen for MD simulations. For MD simulations the starting structures were immersed in a truncated octahedral box of pre-equilibrated TIP3P water [33] where box sides were at least 8.0 Å from the closest atom of the solute. Solvent molecules were removed from the box when the distance between any atom of the solute molecule and any atom of the solvent molecule was less than the sum of the Van der Waals radii of both atoms. Charged N-termini were neutralized by Cl⁻ ions at the positions of the first solvent molecule with the most favorable electrostatic potential. All systems were subjected to 500 steps of steepest descent followed by 500 steps of conjugate gradient energy minimization having all atoms of the solute molecule fixed with a 500 kcal mol⁻¹ Å² force constant. Then the position restraint was removed and 1000 steps steepest descent, followed by 1500 steps conjugate gradient energy minimization was performed on the whole system. 100 ps NVT molecular dynamics was then performed with slow heating from 0 to 300 K and by anchoring the peptide in the center of the box with a force constant of 10 kcal mol⁻¹ Å² on each atom to allow solvent density to equilibrate around the solute molecule. This was followed by 40.1 ns NPT molecular dynamics simulations of each system at constant temperature (300 K) and pressure (1 bar), with the

following parameters: the time step was set to 2 fs; the SHAKE algorithm was used to constrain all bonds at their correct length; temperature was regulated with the weak coupling algorithm with a relaxation constant of 1.0 ps; constant pressure was maintained using isotropic scaling with a relaxation constant of 2.0 ps and $4.46 \times 10^{-5} \text{ bar}^{-1}$ isothermal compressibility. Non-bonded interactions were calculated using the PME method with all cutoff values set at 10 Å. The coordinates of the system were stored after every 2 ps and the first 100 ps, regarded as the equilibration period, was excluded resulting in 40 ns trajectories of 20000 sampled conformations for each starting structure. Trajectories were then combined and analyzed at once.

All analysis was done using programs of the GROMACS 3.3 suite [34] and Perl scripts written in-house. Structures along the trajectory were probed for the presence of intramolecular hydrogen bonds by the `g_hbond` utility of GROMACS 3.3. The cutoff distance between a donor and an acceptor atom was set to 3.5 Å and 60° was used as the cutoff for the donor-hydrogen-acceptor angle. Distances between the putative pharmacophore groups were measured for each structure along the trajectory using `g_dist`. Backbone structure was examined by the measurement of the $C(1)-C_\alpha(2)-C_\alpha(3)-N(4)$ virtual dihedral angle and the distance between the terminal C_α -atoms. Bent structure was assigned when this dihedral angle was between -80° and 80° and the distance was less than 7 Å [35]. Secondary structure was assigned by using the STRIDE algorithm as well [36]. The evolutions of backbone and side chain χ^1 dihedral angles along the trajectories were examined utilizing the `g_chi` program of the GROMACS package. Clustering of structures along the trajectory, comparing main chain and C_β atoms was performed using the `g_cluster` utility and the `gromos` method [37], having the RMSD cutoff set to 1.0 Å.

3. Results and discussion

Based on the “message-address” concept, the C-terminal fragment in EM-2 structure ($\text{Phe}^4\text{-NH}_2$) affects its conformational stability and selectivity at μ -opioid receptors [38]. The aim of present study was to investigate the effect of a chemical constraint, introduced by the $-\text{CH}_2\text{-CH}_2-$ bridge between the amide nitrogens of Phe^3 and Phe^4 on EM-2 binding affinity and receptor-ligand interaction. Therefore, computational structural analysis was performed on this novel EM-2 analog. The data for EM-2 were taken from our results reported earlier [24] having the values obtained for *cis* and *trans* EM-2 weighted by their ratio of occurrence

(1:2, respectively) and combined. The analysis of specific intramolecular hydrogen bonds showed that γ -turns involving the C-terminal amide can be formed in EM-2, and a hydrogen bond formed between the C-terminal amide N and the carbonyl O of the Tyr¹ or Pro² residues can contribute to the stabilization of bent backbone structures.

We have observed that the chemical constraint introduced in the EM-2 structure does not allow the Φ and Ψ backbone dihedral angles of Phe^3 to rotate freely. Furthermore, this modification blocks the formation of hydrogen bonds involving the aforementioned amide nitrogens. During the MD simulation, the Φ angle of Phe^3 was locked around -150° and the Ψ angle varied between 0° and -60° , mainly because of the puckering of the ring introduced by the chemical constraint (Fig. 2). These values are not compatible with the majority of the canonical β -turn types, and no stabilizing $1 \leftarrow 4$ hydrogen bond formation is possible due to the absence of the amide hydrogen in Phe^4 . Chain topography was analyzed via the measurement of the carbonyl $C(\text{Tyr}^1)-C_\alpha(\text{Pro}^2)-C_\alpha(\text{Phe}^3)-N(\text{Phe}^4)$ virtual dihedral angle and the distance between the $C_\alpha(\text{Tyr}^1)$ and $C_\alpha(\text{Phe}^4)$ atoms, according to the guidelines of Ball *et al.* [35]. Results indicated that despite the lack of $1 \leftarrow 4$ hydrogen bonds, chain reversal occurs frequently in the compound, even slightly more frequently than in EM-2 (Table 1).

The occurrence of secondary structural elements as determined by STRIDE is shown in Fig. 3 and Table 2, and contains the populations of different secondary structural elements. Compared to EM-2, fewer types of secondary structures were formed, mainly due to the lack of possibility for the formation of H-bonds. EM-2 forms type I β -turns mainly, while in the studied derivative mostly non-canonical bends, or twisted type IV turns were identified. As opposed to the case of EM-2, bent structures of the studied analog are not stabilized by specific H-bonds between the corresponding amides but most likely induced by the rigid kink in the chain, furnished by the chemical constraint. The representative structures of the dominant conformational families are shown in Fig. 4.

It can be seen that the chemical constraint facilitates the formation of the bent backbone structure, which was found previously to be important for the μ -opioid activity [24,39,40]. On the other hand, the fraction of structures fulfilling the previously established distance criteria between pharmacophore groups [8] is low. However, it was found recently that those criteria may not apply generally to all μ -opioid peptides [24]. Another set of previously proposed parameters of the μ -opioid activity [24] are apparently fulfilled by the studied EM-2 derivative, as the rotamer populations of its aromatic

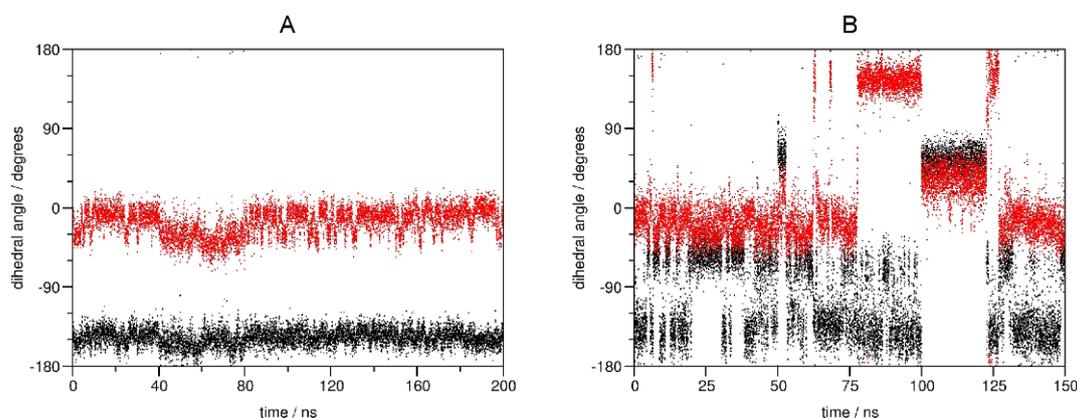


Figure 2. Fluctuation of Φ (black) and Ψ (red) dihedral angles of Phe³ in (A) [Phe³-Phe⁴]EM-2 and (B) EM-2.

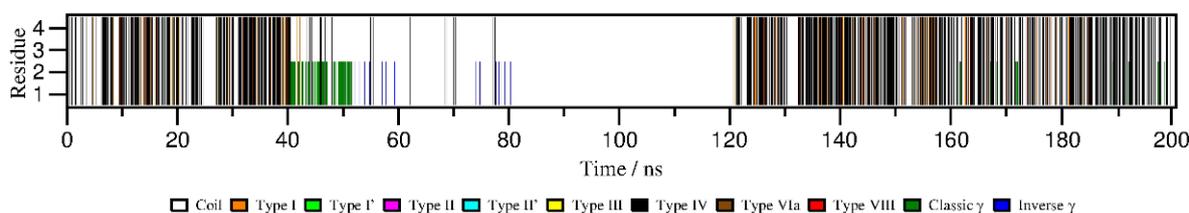


Figure 3. Occurrence of secondary structural elements determined by STRIDE.

Table 1. Occurrence of specific H-bonds, bent structure and the fraction of structures fulfilling the previously proposed distance criteria between pharmacophores.

	[Phe ³ -Phe ⁴]EM-2	EM-2	
Occurrence of specific intramolecular hydrogen bonds / %	CO(Tyr ¹)---HN(C-term.)	0.6	15.7
	CO(Pro ²)---HN(C-term.)	0.0	15.9
	CO(Phe ³)---HN(Tyr ¹)	0.0	0.1
	CO(Phe ⁴)---HN(Tyr ¹)	0.6	4.0
Occurrence of bent structure / %	47.2	33.2	
Pharmacophore distances / %	0.2	8.4	

side chains are almost identical to those of EM-2 (Table 3). However, the Phe⁴ side chain appears to be less flexible compared to Phe⁴ in EM-2, which may interfere during the formation of the receptor-ligand complex.

The computational analysis of a novel EM-2 analog was followed by radioligand binding assays, in which the affinity and selectivity were evaluated in the rat brain membranes, using [³H]DAMGO and [³H][Ile^{5,6}]deltorphin-2 as the μ - and δ -opioid receptor radioligands, respectively. The conformationally restricted analog displayed no significant affinity either at the μ - ($IC_{50} > 1000$ nM vs. 0.74 ± 0.04 nM for EM-2) or δ -opioid receptor ($IC_{50} > 1000$ nM for both peptides). The results of the binding studies are thus in good agreement with the predicted features of the EM-2 derivative and

clearly show biological and structural implications of the introduced chemical constraint. The obtained results indicate that the amide NH groups of the Phe³ and Phe⁴ play a significant role in ligand-receptor interactions, since their absence clearly diminishes μ -opioid receptor affinity even when the backbone conformation and the spatial orientation of the side chains are in agreement with previously established three-dimensional structural criteria of μ -opioid activity. This observation can be further confirmed by our previous data on EM-2 analogs containing *N*-Me-Phe in position 3 or 4. *N*-methylation in position 3 did not influence the μ -opioid receptor affinity of EM-2⁴¹, DAMGO [42] or morphiceptin [43], while [N-Me-Phe⁴]EM-2 showed greatly decreased affinity [41]. Our results clearly indicate that the possibility of intra- or intermolecular (ligand-receptor) hydrogen

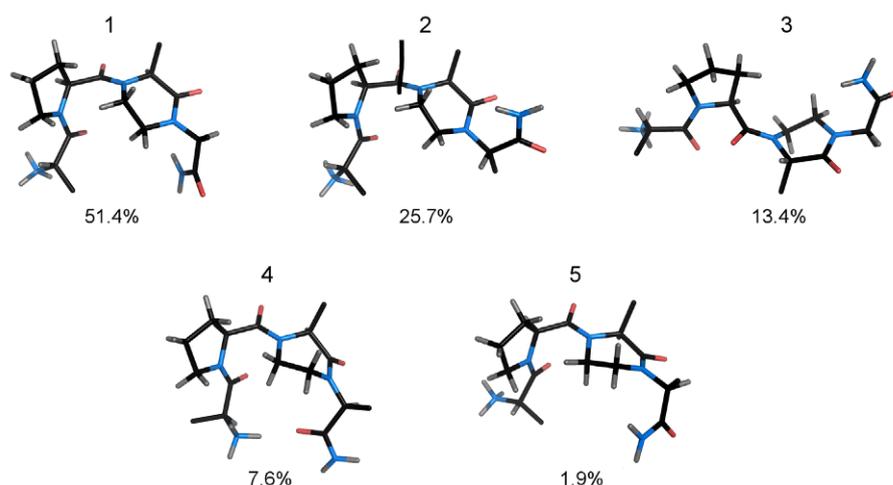


Figure 4. Representative structures and relative populations of the dominant conformational families. Populations are expressed in percentages as fractions of the total structural ensemble. Aromatic side chains are omitted for clarity.

Table 2. Occurrence of canonical secondary structural elements as identified by STRIDE analysis.

	γ -turn location and type						β -turn type						
	1 - 3		2 - 4		3 - 5		I	I'	II	II'	III	IV	VIII
	c	i	c	i	c	i							
[Phe³-Phe⁴]EM-2	-	-	-	-	3.9	1.0	3.9	-	-	-	-	31.6	-
EM-2	-	1.5	< 0.5	< 0.5	< 0.5	< 0.5	17.3	-	< 0.5	-	6.0	7.4	< 0.5

c, classic; i, inverse

Table 3. Populations of the three rotameric states of the aromatic side chains expressed as percentages of the total conformational ensemble.

		[Phe³-Phe⁴]EM-2	EM-2
Tyr¹	g ⁺	8.7	4.1
	g ⁻	19.8	11.1
	t	71.5	84.8
Phe³	g ⁺	19.5	18.4
	g ⁻	28.7	27.9
	t	51.8	53.7
Phe⁴	g ⁺	2.0	35.7
	g ⁻	77.4	38.3
	t	20.6	26.8

bonding, involving the amide hydrogen in Phe⁴ is crucial for the high affinity of EMs at the μ -opioid receptor.

4. Conclusion

In conclusion, the results of the present investigation demonstrated the detrimental effect of the chemical constraint, introduced into the C-terminal part of EM-2, on the pharmacological activity of the analog. The

conformational restriction, in the form of an ethylene bridge connecting the nitrogen atoms of the α -amino groups of Phe³-Phe⁴ enforced the conformation of twisted, type IV turns and the absence of canonical β -turns typical for the EM-2 structure. The conformational change, caused by the absence of the NH group of Phe⁴, which can form hydrogen bonds with the residues of the message sequence, resulted in the lack of affinity of this ligand at the μ -opioid receptor. These results should be taken into consideration in the design of new EM-based therapeutics.

Acknowledgements

This work was supported by a grant POLONIUM, a grant from the Polish Ministry of Science No. 730/N-POLONIUM/2010/0 and a grant from the Medical University of Lodz No. 503/1-156-02/503-01. Research of A. Borics is funded by a Hungarian OTKA-73081 grant. The authors wish to thank Jozef Cieslak for his excellent technical assistance.

References

- [1] J.E. Zadina, L. Hackler, L.J. Ge, A.J. Kastin, *Nature* 386, 499 (1997)
- [2] G. Horváth, *Pharmacol. Ther.* 88, 437 (2000)
- [3] A. Janecka, R. Staniszewska, K. Gach, J. Fichna, *Peptides* 29, 2066 (2008)
- [4] C. Tomboly, A. Peter, G. Toth, *Peptides* 23, 1573 (2002)
- [5] C. Sakurada, S. Sakurada, T. Hayashi, S. Katsuyama, K. Tan-No, T. Sakurada, *Biochem. Pharmacol.* 66, 653 (2003)
- [6] A.J. Kastin, M.B. Fasold, R.R. Smith, K.A. Horner, J.E. Zadina, *Exp. Brain Res.* 139, 70 (2001)
- [7] Y. Koda, K. Shiotani, I. Toth, Y. Tsuda, Y. Okada, J.T. Blanchfield, *Bioorg. Med. Chem. Lett.* 17, 2043 (2007)
- [8] T. Yamazaki, S. Ro, M. Goodman, N.N. Chung, P.W. Schiller, *J. Med. Chem.* 36, 708 (1993)
- [9] M.G. Paterlini, F. Avitabile, B.G. Ostrowski, D.M. Ferguson, P.S. Portoghese, *Biophys. J.* 78, 590 (2000)
- [10] I. Lengyel, G. Orosz, D. Biyashev, L. Kocsis, M. Al-Khrasani, A. Ronai, C. Tomboly, Z. Furst, G. Toth, A. Borsodi, *Biochem. Biophys. Res. Commun.* 290, 153 (2002)
- [11] V.J. Hruby, R.S. Agnes, *Biopolymers* 51, 391 (1999)
- [12] Y. Okada, A. Fukumizu, M. Takahashi, Y. Shimizu, Y. Tsuda, T. Yokoi, S. D. Bryant, L. H. Lazarus, *Biochem. Biophys. Res. Commun.* 276, 7 (2000)
- [13] P.W. Schiller, T.M. Nguyen, G. Weltrowska, B.C. Wilkes, B. J. Marsden, C. Lemieux, N.N. Chung, *Proc. Natl. Acad. Sci. U.S.A* 89, 11871 (1992)
- [14] R. Dharanipragada, K. Van Hulle, A. Bannister, S. Bear, L. Kennedy, V.J. Hruby, *Tetrahedron* 48, 4733 (1992)
- [15] E. Nicolas, K.C. Russell, J. Knollenberg, V.J. Hruby, *J. Org. Chem.* 58, 7565 (1993)
- [16] L.W. Boteju, K. Wegner, X. Qian, V.J. Hruby, *Tetrahedron* 50, 2391 (1994)
- [17] V.J. Hruby, G. Toth, C.A. Gehrig, L.F. Kao, R. Knapp, G.K. Lui, H. Yamamura, T.H. Kramer, P. Davis, T.F. Burks, *J. Med. Chem.* 34, 1823 (1991)
- [18] M. Keller, C. Boissard, L. Patiny, N. N. Chung, C. Lemieux, M. Mutter, P. W. Schiller, *J. Med. Chem.* 44, 3896, (2001)
- [19] C. Tomboly, K.E. Kover, A. Peter, D. Tourwe, D. Biyashev, S. Benyhe, A. Borsodi, M. Al-Khrasani, A.Z. Ronai, G. Toth, *J. Med. Chem.* 47, 735 (2004)
- [20] Y. Yu, X. Shao, Y. Cui, H.M. Liu, C.L. Wang, Y.Z. Fan, J. Liu, S.L. Dong, Y.X. Cui, R. Wang, *Chem. Med. Chem.* 2, 309 (2007)
- [21] Y. Yu, X. Shao, C.L. Wang, H.M. Liu, Y. Cui, Y.Z. Fan, J. Liu, R. Wang, *Peptides* 28, 859 (2007)
- [22] Y. In, K. Minoura, K. Tomoo, Y. Sasaki, L.H. Lazarus, Y. Okada, T. Ishida, *FEBS J.* 272, 5079 (2005)
- [23] W.M. Kazmierski, R.D. Ferguson, A.W. Lipkowski, V.J. Hruby, *Int. J. Pept. Protein Res.* 46, 265 (1995)
- [24] A. Borics, G. Toth, *J. Mol. Graph. Model.* 28, 495 (2010)
- [25] Y. Tong, Y. M. Fobian, M. Wu, N.D. Boyd, K.D. Moeller, *J. Org. Chem.* 65, 2484 (2000)
- [26] J. Fichna, J.C. do-Rego, N.N. Chung, C. Lemieux, P.W. Schiller, J. Poels, J.V. Broeck, J. Costentin, A. Janecka, *J. Med. Chem.* 50, 512 (2007)
- [27] B. Lammek, M. Czaja, I. Derdowska, E. Lempicka, P. Sikora, W. Szkrobka, H.J. Trzeciak, *J. Pept. Res.* 51, 149 (1998)
- [28] J. Fichna, J.C. do-Rego, P. Kosson, J. Costentin, A. Janecka, *Biochem. Pharmacol.* 69, 179 (2005)
- [29] J. Wang, P. Cieplak, P.A. Kollman, *J. Comp. Chem.* 21, 1049 (2000)
- [30] J. Wang, R.M. Wolf, J.W. Caldwell, P.A. Kollman, D.A. Case, *J. Comput. Chem.* 25, 1157 (2004)
- [31] S. Lovas, R.F. Murphy, In: Irvine, Williams (Eds.), *Methods of Molecular Biology Neuropeptide Protocols* (Humana Press, Totowa, NJ, 1997) Vol. 73, 209
- [32] W.C. Still, A. Tempczyk, R.C. Hawley, T. Hendrickson, *J. Am. Chem. Soc.* 112, 6127 (1990)
- [33] W.L. Jorgensen, J. Chandrasekhar, J.D. Madura, R.W. Impey, M.L. Klein, *J. Chem. Phys.* 79, 926 (1983)
- [34] E. Lindahl, B. Hess, D. van der Spoel, *J. Mol. Mod.* 7, 306 (2001)
- [35] J.B. Ball, R.A. Hughes, P.F. Alewood, P.R. Andrews, *Tetrahedron* 49, 3467 (1993)
- [36] D. Frishman, P. Argos, *Proteins* 23, 566 (1995)
- [37] X. Daura, K. Gademann, B. Jaun, D. Seebach, W.F. van Gunsteren, A.E. Mark, *Angew. Chem. Int. Ed.* 38, 236 (1999)
- [38] T.G. Metzger, M.G. Paterlini, P.S. Portoghese, D.M. Ferguson, *Neurochem. Res.* 21, 1287 (1996)
- [39] M. Eguchi, R.Y. Shen, J.P. Shea, M. Lee, M. Kahn, *J. Med. Chem.* 45, 1395 (2002)
- [40] C. Tomboly, S. Ballet, D. Feytens, K.E. Kover, A. Borics, S. Lovas, M. Al-Khrasani, Z. Furst, G. Toth, S. Benyhe, D. Tourwe, *J. Med. Chem.* 51, 173 (2008)
- [41] R. Kruszynski, J. Fichna, J.C. do-Rego, T. Janecki, P. Kosson, W. Pakulska, J. Costentin, A. Janecka,

Bioorg. Med. Chem. 13, 6713 (2005)

[42] H.W. Kosterlitz, J.A. Lord, S.J. Paterson,
A.A. Waterfield, Br. J. Pharmacol. 68, 333 (1980)

[43] K.J. Chang, E.T. Wei, A. Killian, J.K. Chang,
J. Pharmacol. Exp. Ther. 227, 403 (1983)