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Synthesis and evaluation of new endomorphin analogues modified at the Pro² residue

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Abstract

Six new endomorphin analogues, incorporating constrained amino acids in place of native proline have been synthesized. Residues of (S)-azetidine-2-carboxylic acid (Aze), 3,4-didehydro-(S)-proline (Δ^3 Pro), azetidine-3-carboxylic acid (3Aze) and didehydro-alanine (Δ Ala) have been used to prepare [Δ^3 Pro²]EM-2 (**1**), [Aze²]EM-1 (**2**), [Aze²]EM-2 (**3**), [3Aze²]EM-1 (**4**), [3Aze²]EM-2 (**5**) and [Δ Ala²]EM-2 (**6**). Binding assays and functional bioactivities for μ - and δ -receptors are reported. The highest affinity, bioactivity and selectivity is shown by peptides **2** and **3** containing the Aze residue.

Keywords

azetidine carboxylic acids; 3,4-didehydro-(S)-proline; didehydro-alanine; endomorphins; μ -receptors

The endogenous neuropeptides endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂; EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂; EM-2), originally isolated from bovine brain¹ and subsequently from the human brain cortex,² continue to attract considerable attention in the field of opioid peptides. In comparison with the other endogenous peptide ligands of opioid receptors, endomorphins (EMs) combine potency and efficacy with high affinity and selectivity towards the μ -opioid receptor,^{1,3,4} the receptor most involved with acute analgesic effects in the central nervous system.⁵ Furthermore, the EMs proteolytic stability, although low on a general scale, is the highest among the known endogenous opioid peptides^{6,7} and their activity is accompanied by reduced cardiorespiratory side effects, as compared with the reference alkaloid ligand morphine.^{8,9} All of these properties, render EMs the object of continuous investigations and a promising target for the development of new opioid analgesics. However,

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as found in the case of most short linear natural peptides, native EMs lack critical therapeutic characteristics such as bioavailability, duration of action and oral activity.¹⁰ Research efforts are then focused on the design of analogues and peptidomimetics in order to improve EMs properties as potential drugs as well as to better understand the key structural and conformational features on which receptor recognition and binding are based.¹¹

Several chemical modifications performed on EMs have been described previously and many of them were focused on the Pro at position 2.^{12–20} The presence of Pro, the sole proteinogenic amino acid possessing a cyclic structure and a secondary amino group, represents a crucial factor in determining the structural and conformational properties of proteins and peptides. In the specific context of studies on EMs analogues, the Pro² is considered a stereochemical spacer, capable of favouring proper spatial orientation of the aromatic side chain groups, a key factor for ligand recognition and interaction with the receptor.^{21,22}

This paper reports on the EM analogues given in Fig. 1, which are obtained by replacing the Pro in position 2 with one of the four amino acid residues depicted in Fig. 2. These amino acids, characterized by different constraints and structural features, can deeply alter the tetrapeptide backbone conformation and thus modulate in new ways the bioactivity of the peptide.

A first modification, which leads to the EM-2 analogue **1**, is based on the introduction of the 3,4-didehydro-(S)-proline (Δ^3 Pro) residue. This amino acid, as compared with Pro, has an almost planar and less flexible cyclic side chain ring, which does not allow rapid interconversion between ring-puckered forms typical of Pro residues and enhances, at the same time, the population of *cis* conformers around the CO-N tertiary amide bond.^{23,24} In addition to this, the presence of the double bond may significantly improve, through π - π interactions, its binding to opioid receptors.^{25,26} A related Δ^3 Pro² containing EM-2 analogue, namely [Dmt¹, Δ^3 Pro²]EM-2, has been previously reported by Toth and Szemenyei²⁷ and utilized for the synthesis of the corresponding tritium labelled [³H₂]Pro² analogue. Two of the other modified ligands here reported analogues contain a cyclic lower homologue of Pro. In particular, in analogues [Aze²]EM-1 (**2**) and [Aze²]EM-2 (**3**) the (S)-azetidine-2-carboxylic acid (Aze) has been incorporated. Literature data show that the four-membered ring system of *N*-acylated (S)-azetidine-2-carboxylic acid (Aze) is either planar or only slightly puckered^{28–30} and presents, as compared with proline, reduced side chain steric effects, with increased backbone flexibility.³¹ This latter property generates a larger number of low energy conformers with a population of the *cis* rotamers around the CO-N amide bond which has been suggested to be higher than that observed in Pro-containing peptides.³² Furthermore, the different ring size may significantly influence the type of folding with β -turns and γ -turns preferentially induced by Pro and Aze, respectively.³¹

The azetidine ring system also has been incorporated into the backbone of the analogues [3Aze²]EM-1 (**4**) and [3Aze²]EM-2 (**5**). Azetidine-3-carboxylic acid (3Aze) is an achiral conformationally restricted analogue of β -alanine which, although not used in opioid chemistry, has been employed for the synthesis of other pharmaceutical compounds.^{33–35} As compared with Aze and Pro residues, 3Aze maintains the cyclic structure and the secondary amino group in the ring but is devoid of both chirality and restricted rotation around the N-C α bond. Among 5-membered chiral β -residues related to 3Aze, both β -proline (pyrrolidine-3-carboxylic acid) and β -homoproline (2-pyrrolidineacetic acid) have already been used to replace Pro² in EM analogues.^{14,15} Furthermore, EM-2 analogues containing cyclic 6-membered higher homologues of β -proline and 3Aze [*viz* nipecotic acid (piperidine-3-carboxylic acid) and isonipecotic acid (piperidine-4-carboxylic acid), respectively]] have been recently reported.²⁰

The last analogue under study here is [Δ Ala²]EM-2 (**6**) which contains the rigid skeleton of dehydro-alanine (Δ Ala). The local conformational restriction imposed on the backbone by the planar structure of Δ Ala moiety, as well as its electronic distribution, significantly influences the properties of this compound. Although a variety of molecular modifications performed on native EMs have been reported, analogues containing α,β -unsaturated amino acid residues are very scarce.³⁶ Thus, the reported data on compound **6** represent, to the best of our knowledge, the first information available in the literature on receptor binding affinity and selectivity of this type of EM analogues.

The solution phase synthesis of peptides **1–5** were performed following standard literature methods³⁷ which are given in Scheme 1. The synthesis of the dehydroalanine-containing analogue **6**, was accomplished by exploiting the reactivity of the methylcysteine sulfoxide³⁸ as illustrated in Scheme 2. All new compounds were characterized by ¹H-NMR data and elemental analysis.³⁹

Receptor binding affinities to the δ - and μ -opioid receptors were performed using cell membrane preparations from transfected cells that stably express the respective receptors. The radiolabeled ligands used were [³H]DPDPE and [³H]DAMGO for δ - and μ -receptors, respectively (for more details about the procedure see ref. ⁴⁰). The *in vitro* tissue bioassays (MVD and GPI/LMMP) were performed as described previously.⁴¹ IC₅₀ values represent means of no less than four experiments. IC₅₀ values, relative potency estimates, and their associated standard errors were determined by fitting the data to the Hill equation by a computerized non-linear least-squares method.

As shown in Table 1 all analogues, except [3Aze²]EM-1 (**4**), which can be considered a μ/δ mixed agonist, show high selectivity for μ -receptors and functional bioactivities in good agreement with the binding values. The highest affinity and selectivity is shown by the Aze-containing peptides **2** and **3**. This finding, together with the good activity of the [Δ^3 Pro²]EM-2 analogue **1**, confirms the role of the residue at position 2 as a stereochemical spacer.

The two analogues containing a β -residue, namely [3Aze²]EM-1 (**4**) and [3Aze²]EM-2 (**5**) exhibit highly different activity profiles (Table 1). The much lower activity shown by **5**, as compared to the Trp³ containing analogue [3Aze²]EM-1 (**4**), can be interpreted on the basis of the well recognized role of the aromatic residues on ligand-receptor interactions. Several studies on EM-1 and EM-2 clearly show that aromatic side chains with different size, distinct noncovalent interactions and H-bonding capability, can highly influence conformations⁴³ and binding.^{21,44,45} Results of ligands **4** and **5** indicate that the beneficial effect on the activity, constantly observed in EM analogues containing the indole ring system at position 3, gives results that are particularly efficient as compared to the Phe³ residue in models containing the achiral 3Aze residue.

The low activity shown by [3Aze²]EM-2 (**5**) appears, however, is quite unexpected when compared to the high activity shown by EM analogues containing other cyclic β -residues in place of the native Pro². In particular, among the recently examined EM-2 analogues containing positional isomers of the 6-membered piperidine-carboxylic acid, replacement of Pro² with the chiral nipecotic acid (*viz* piperidine-3-carboxylic acid) led, in accordance with the results concerning other related cyclic β -residues,^{14,15} to a highly active analogue.²⁰ On the contrary, Pro² replacement with the achiral piperidine-4-carboxylic acid (*viz* isonipecotic acid) resulted, as in the case of the here reported analogue **5**, to a practically inactive ligand.²⁰ It can be argued that the high conformational restriction and the reduced flexibility of the 4-membered achiral 3Aze are detrimental features which prevail over the advantages which, in larger cyclic systems, derive from the presence of a β -positioned carboxylic group.

Peptide (6), containing the rigid skeleton of Δ Ala residue, has only modest affinity and bioactivity at the μ -opioid receptor, and this suggests that an efficient constraint at position 2 involves an amino acid residue with a cyclic structure.

Further studies on the synthesis and activity of EM analogues obtained by replacing native amino acid residues with synthetic mimetics are under way in our laboratories and will be reported in due course.

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Figure 1.
Molecular structure of the EM analogues **1–6**

Figure 2.

Molecular structure of the amino acids used in the present paper to replace the native Pro² residue in endomorphins: (S)-Azetidine-2-carboxylic (Aze); 3,4-Didehydro-(S)-proline (Δ³Pro); Azetidine-3-carboxylic acid (3Aze); Didehydro-alanine (ΔAla).

Scheme 1.

Synthesis of EM analogues **1–5**. Xaa = Δ^3 Pro; Yaa = Phe (**1**); Xaa = Aze; Yaa = Trp (**2**); Xaa = Aze; Yaa = Phe (**3**); Xaa = 3Aze; Yaa = Trp (**4**); Xaa = 3Aze; Yaa = Phe (**5**).

Scheme 2.

Reagents and conditions: a) SOCl₂, MeOH, 15 min at 0° C then 3h at rt; b) Boc-Tyr-OH, EDC, HOBT, DIPEA, CH₂Cl₂ 30 min at 0° C then 12h at rt; c) 1N NaOH, MeOH 5h at rt; d) HCl-Phe-OMe, EDC, HOBT, DIPEA, CH₂Cl₂ 30 min at 0° C then 12h at rt; e) NaIO₄, H₂O, Dioxane 1h at 0° C; f) DBU, MeOH, 1.5h at rt; g) 1N NaOH, MeOH 5h at rt; h) HCl-Phe-NH₂, EDC, HOBT, DIPEA, CH₂Cl₂ 30 min at 0° C then 12h at rt; i) TFA, CH₂Cl₂ 1.5h at rt under N₂.

Table 1

Binding affinity and in vitro activity for compounds 1–6.

Compound	Receptor affinity ^{a,b} (nM)		Selectivity	Functional Bioactivity	
	K_i^{δ}	K_i^{μ}		MVD ^b (IC ₅₀)	GPI ^b (IC ₅₀)
1 [Δ^3 Pro ²]EM-2	n.c. ^c	6.5 ± 2.3	--	70 ± 12	14 ± 2.1
2 [Aze ²]EM-1	3500 ± 360	2.3 ± 0.23	1500	140 ± 21	7.9 ± 0.83
3 [Aze ²]EM-2	5100 ± 600	5.6 ± 1.2	920	150 ± 60	6.4 ± 1.2
4 [3Aze ²]EM-1	40 ± 8.5	34 ± 5.1	1.2	170 ± 32	140 ± 32
5 [3Aze ²]EM-2	6900 ± 1200	210 ± 51	32	13 % at 1 μ M	1400 ± 280
6 [Δ Ala ²]EM-2	710 ± 130	34 ± 6.3	21	490 ± 150	170 ± 23
EM-1 ^d	--	10.70 ± 1.5	--	1710 ± 110	28 ± 3
EM-2 ^d	--	9.56 ± 0.98	--	510 ± 35	15 ± 2

^a Displacement of [³H]DAMGO (μ -selective) and [³H]DPDPE (δ -selective) from rat brain membrane binding sites.

^b ± S.E.M.

^c n.c. = data not collected.

^d Data from ref. 42.