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Structure–activity relationships of novel endomorphin-2 analogues with N–O turns induced by α-aminoxy acids

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Abstract—Endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2, EM-2) is a putative endogenous μ-opioid receptor ligand. To study the structure–activity relationship against its receptor, we introduced N–O turns into EM-2 and got the analogues with potent affinities for μ-opioid receptor. Our results indicated that N–O turn structures at the Pro^2-aminoxy-Phe^3 position of EM-2 analogues played important roles for their affinities. These novel analogues with N–O turns provided a new approach to develop potent analgesics related to EM-2.

Opioid peptides have been conjectured as secondary effect-free pharmacological tools to be used in place of morphine,[1–3] because of their roles as endogenous analgesics in mammals.[4–7] However, the inherent conformational flexibility of the opioid peptides has hampered numerous attempts to accurately assess the bioactive structures. Significant efforts have been devoted to the elucidation of the opioid receptor-bound structures through systematic studies of structurally constrained peptides[8] or peptidomimetics.[9] The goals of these efforts are to study the structure–activity relationships against their receptors and to develop potent analgesics without the well-known side effects of morphine. Several studies have suggested that turn structure is the potential biologically active structure of opioid peptides.[10–14]

Up to now, many unnatural building blocks have been designed to create novel foldamers whose oligomers or polymers are able to adopt well-defined structures.[15,16] Among them, an important novel turn mimic is N–O turn, which is induced by α-aminoxy acid (H_2N–O–CHR–CO_2H) (Fig. 1). In the peptide backbone, α-aminoxy acid induces a strong eight-membered-ring hydrogen bond formed between adjacent amino acid residues (the N–O turn). Furthermore, this turn structure is side-chain independent, and gives rigid and predictable secondary structure.[17] However, to the best of our knowledge, there are no reports of introducing N–O turns into opioid peptides. Most of the work has been limited to the studies of the structures of N–O turns. It is interesting to investigate whether the N–O turns are still favored when side chains are introduced to α-aminoxy acids in opioid peptides, and to probe whether these analogues have affinities for their opioid receptors. Also, the turn structures of opioid peptides can be simulated by this method.

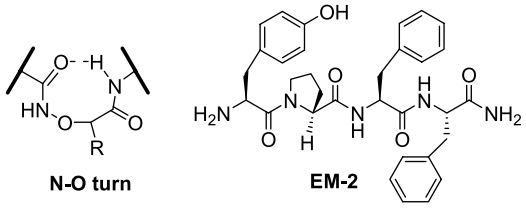

Figure 1. The primary structures of N–O turn and EM-2.

To demonstrate our concept, we chose endomorphin-2 (EM-2) as a target peptide (Fig. 1). Endomorphin-1,

Keywords: Endomorphin-2; Opioid peptide; N–O turn; α-Aminoxy acid; Simulation.
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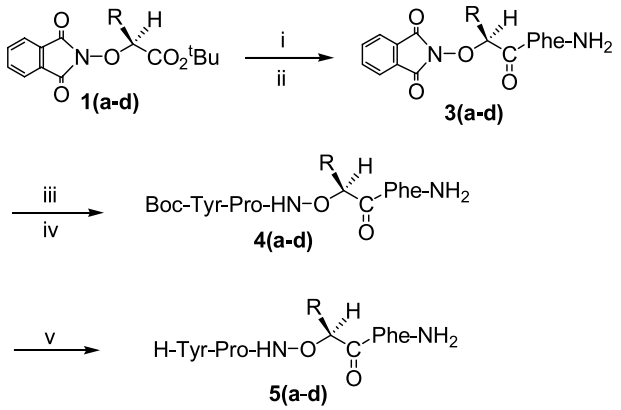
H-Tyr-Pro-Trp-Phe-NH_2, and endomorphin-2, H-Tyr-Pro-Phe-Phe-NH_2 were isolated from the bovine brain and human brain, which showed high affinities and selectivities for μ-opioid receptor (MOR).[18] Among opioid peptides, endomorphins (EMs) are regarded as the most effective endogenous analgesics that are released in response to pain stimuli. It is often convenient to study the corresponding peptidomimetics or peptide analogues by modifying the original structure of EMs.[19–22] The studies have indicated that a turn structure at the Pro^2–Phe^3 position is formed, which can be recognized by MOR, although EM-2 has a random conformation in water. The turn structure is EM-2's biologically active structure.[10] Thus, it is very important to simulate the turn structure and understand the structure–activity relationships between EM-2 and MOR. These studies can help us to find highly bioactive EM-2 analogues. Herein, we report a novel synthesis of a series of EM-2 analogues with different α-aminoxy acids by conventional solution phase method.

All the α-aminoxy acids were synthesized using the method which had been recently developed.[23] The synthetic steps used to prepare EM-2 analogues **5a**–**5d** are shown in Scheme 1. Initially after *tert*-butyl deprotection of α-aminoxy acid **1**, the resulting acid was coupled to HCl·Phe-NH_2 by *N*-methylmorpholine (NMM) and isobutyl chloroformate (IBCF) to give dipeptide **3**. The phthaloyl (Pht) of **3** was removed by hydrazine hydrate; then the resulting amine was coupled to Boc-Tyr-Pro-OH under the same coupling conditions of preparing dipeptide **3** to give **4**. After removal of the Boc-group from **4**, we got final compound **5**. We synthesized EM-2 analogues **6a**–**d** (Fig. 2) with the similar methods of **5**. Furthermore, to illustrate the importance of N–O turns in these analogues, we synthesized EM-2 analogues **9a**–**b** with oxime links ($C_{\alpha1}$–CH=N–O–$C_{\alpha2}$). Analogues **9a**–**b** did not form N–O turn structures because of lacking the O atoms to function as acceptors in hydrogen bonding. The synthetic steps used to prepare **9a**–**b** are shown in Scheme 2. Dipeptide **3** was coupled to Boc-Pro-H aldehyde[24] in the presence of AcONa



Scheme 1. Total synthesis of EM-2 analogues **5a**–**d**. **a**–**d**: R = Bn(L), Bn(D), CH_3(D), H. Reagents and conditions: (i) 50% TFA, 0 °C, 2 h; (ii) NMM, IBCF, HCl·Phe-NH_2, overnight; (iii) NH_2–NH_2·H_2O, MeOH, rt, 2 h; (iv) NMM, IBCF, Boc-Tyr-Pro-OH, overnight; (v) 50% TFA, 0 °C, 2 h.

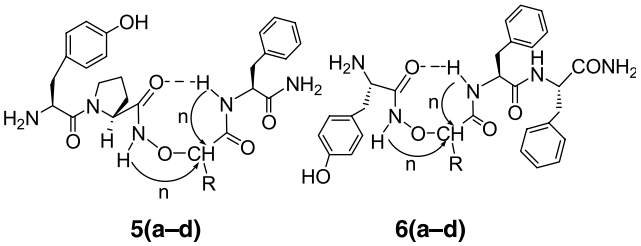
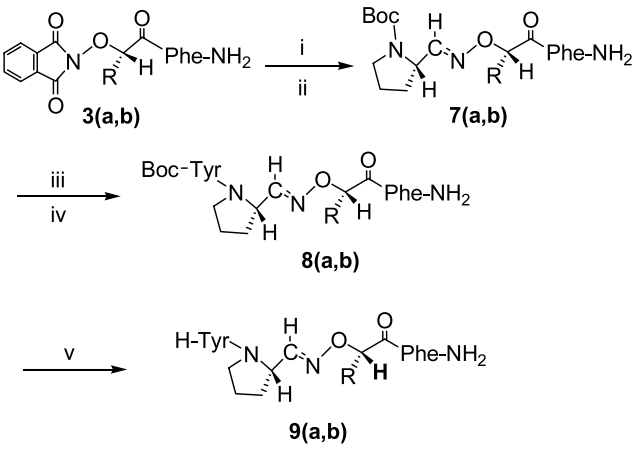


Figure 2. Summary of the NOEs observed (n, NOE) in the NOESY spectra of analogues **5a**–**d** and **6a**–**d** in DMSO at rt. **5a**, R = Bn(L); **5b**, R = Bn(D); **5c**, R = CH_3(D); **5d**, R = H; **6a**, R = Bn(L); **6b**, R = Bn(D); **6c**, R = CH_3(D); **6d**, R = H.



Scheme 2. Total synthesis of EM-2 analogues **9a** and **9b**. **a**: R = Bn(L), **b**: R = Bn(D). Reagents and conditions: (i) NH_2–NH_2·H_2O, MeOH, rt, 2 h; (ii) Boc-Pro-H aldehyde, AcONa, EtOH, anhydrous Na_2SO_4, rt, overnight; (iii) 50% TFA, 0 °C, 2 h; (iv) NMM, IBCF, Boc-Tyr-OH, overnight; (v) 50% TFA, 0 °C, 2 h.

and anhydrous Na_2SO_4 to give compound **7**. After Boc deprotection of **7**, the resulting amine was coupled to Boc-Tyr-OH by NMM and IBCF to give **8**. After removal of the Boc-group from **8**, we got final compound **9**. All the final products were TFA salts and were purified by preparative reversed-phase HPLC. The purity of all peptides was greater than 95%. The analytical data are listed in Table 1.

We initially investigated the structural features of novel EM-2 analogues using FT-IR. For the IR spectra of **5a**–**d** and **6a**–**d**, the ratio of hydrogen-bonded and non-hydrogen-bonded NH stretching bands reflected the position of the conformational equilibrium. The analogues of **5a**–**d** and **6a**–**d** displayed exclusively peaks at 3195–3302 cm^{-1} that corresponded to a hydrogen-bonded NH stretching band.[23,25] However, the analogues of **9a** and **9b** displayed peaks at 3397 and 3401 cm^{-1} that corresponded to no hydrogen-bonded NH stretching band (Table 1).

The clear evidence supporting analogues **5a**–**d** and **6a**–**d** adopting N–O turn structures was obtained from analysis of the 2D NOESY NMR spectra (Fig. 2). NOE between amide NH and $^{N-O}AaC_{\alpha}$-H, and NOE between oxyamide NH and $^{N-O}AaC_{\alpha}$-H showed the formation

Table 1. Analytical data of EM-2 analogues

Code	EM-2 analogues sequence	MS $[M+H]^+$		$[\alpha]_D^{20}$ (°)	IR (cm^{-1})[c]
		Calcd	Found		
5a	Tyr-Pro-(L-aminoxy Phe)-$PheNH_2$	588.2817	588.2823[a]	−42 (c = 0.5, MeOH)	3200
5b	Tyr-Pro-(D-aminoxy Phe)-$PheNH_2$	588.2817	588.2816[a]	+11 (c = 0.5, MeOH)	3207
5c	Tyr-Pro-(D-aminoxy Ala)-$PheNH_2$	512.2504	512.2469[a]	−24 (c = 0.125, MeOH)	3208
5d	Tyr-Pro-(aminoxy Gly)-$PheNH_2$	498.2347	498.2333[a]	−42 (c = 0.5, MeOH)	3206
6a	Tyr-(L-aminoxy Phe)-Phe-$PheNH_2$	638.2973	638.2969[a]	−15 (c = 0.5, MeOH)	3302, 3207
6b	Tyr-(D-aminoxy Phe)-Phe-$PheNH_2$	638.2973	638.2988[a]	+40 (c = 0.5, MeOH)	3195
6c	Tyr-(D-aminoxy Ala)-Phe-$PheNH_2$	562.2660	562.2663[a]	+40 (c = 0.5, MeOH)	3302, 3205
6d	Tyr-(aminoxy Gly)-Phe-$PheNH_2$	548.2504	548.2499[a]	+21 (c = 0.5, MeOH)	3193
9a	Tyr-Pro=(L-aminoxy Phe)-$PheNH_2$	572.28	572.2[b]	−32 (c = 0.5, MeOH)	3397
9b	Tyr-Pro=(D-aminoxy Phe)-$PheNH_2$	572.28	572.2[b]	+43 (c = 0.5, MeOH)	3401

[a] ESI-TOF-MS.
[b] FAB-MS.
[c] FT-IR data of EM-2 analogues between 3100 and 3500 cm^{-1} in MeOH at room temperature (1 mM solutions).

of N–O turn structures.[23,25] Combining with IR and NMR analysis, we concluded that the N–O turns were still favored in EM-2 analogues. However, in the structures of analogues **9a** and **9b** there were no N–O turn structures in the oxime links.

To determine the affinities toward the μ- and δ-opioid receptors, the opioid receptor binding assays were performed in 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 0.5 mL containing 250–400 μg of protein (Synaptosomal brain membrane P2 was prepared from Wistar rats). In competition experiments, the following conditions were used for incubations: [^{3}H]DAMGO (0.5 nM, 25 °C, 1 h), [^{3}H]DPDPE (1 nM, 25 °C, 3 h). Nonspecific binding was determined in the presence of 10 μM naxolone. K_d values of [^{3}H]DAMGO and [^{3}H]DPDPE were 0.533 and 2.75 nM, respectively. K_i values were calculated according to the equation of Cheng and Prusoff.[26] The K_i values of EM-2 in [^{3}H]DAMGO and [^{3}H]DPDPE assays agreed with Toth and co-workers[27] and Okada et al.,[21b] respectively. The data are listed in Table 2. The data shown in Table 2 are the means of at least three experiments. Functional bioactivities of these analogues were evaluated in guinea pig ileum (GPI) and mouse vas deferens (MVD) assays, respectively. Stand compounds (EM-2 for the GPI and deltorphin I for the MVD) were assayed in each preparation to permit estimation of relative potencies. The analogues acted as μ-opioid receptor agonists and weak δ-opioid receptor agonists (data are not shown here). The results are the means of at least three experiments.

From the opioid receptor binding data shown in Table 2, we got the following results. The μ-opioid receptor (MOR) binding affinities of **5a** and **5b**, which formed N–O turns at the Pro^2-aminoxy-Phe^3 position, were about 10-fold better than that of **9a** and **9b**. Compounds **9a** and **9b** did not form N–O turn structures at the Pro^2-aminoxy-Phe^3 position. Compounds **5a** and **5b** both remained good selectivities for MOR over δ-opioid receptor (δ/μ). But **6a**–**d**, which formed N–O turns at the Tyr^1-aminoxy-Aa^2 position, decreased the MOR affinities of **5a**–**b** by more than 20-fold. At the same time, **6a**–**d** decreased the MOR affinities of **9a**–**b** by more than 2-fold. Hence, we got the structure–activity relationships between these analogues and affinities for MOR. First, the N–O turns at the Tyr^1-aminoxy-Aa^2 position in analogues **6a**–**d** were the unfavorable structures for MOR affinities. Second, the N–O turns at the Pro^2-aminoxy-Phe^3 position in analogues **5a**–**b** were the most favorable structures for MOR affinities in the three types of these analogues, although **5a**–**b** decreased the MOR affinities of EM-2. Based on these results, we thought that when EM-2 bind to MOR, it is likely to form a turn structure at the Pro^2-Phe^3 position, and hardly forms a turn structure at the Tyr^1-Pro^2 position.

Table 2. Binding affinity in competition with [^{3}H]DAMGO and [^{3}H]DPDPE in rat brain membranes

EM-2 analogs	[^{3}H]DAMGO (μ) K_i ± SE (μM)	[^{3}H]DPDPE (δ) K_i ± SE (μM)	Selectivity (δ/μ)
EM-2	$(8.23 \pm 0.5) \times 10^{-3}$	8.36 ± 1.3	1016
5a	0.434 ± 0.07	>10 (15%[a])	>23
5b	0.6 ± 0.07	>10 (30%[a])	>17
5c	>10 (38%[a])	>10 (30%[a])	—
5d	>10 (35%[a])	>10 (21%[a])	—
6a	>10 (22%[a])	>10 (18%[a])	—
6b	na	>10 (30%[a])	—
6c	>10 (40%[a])	>10 (35%[a])	—
6d	>10 (34%[a])	>10 (13%[a])	—
9a	4.8 ± 0.05	19.8 ± 2.3	4.1
9b	4.04 ± 0.03	37.1 ± 3.5	9

na: no activity.
[a] Percent decrease of maximum binding at 10 μM peptide.

These results might help us to increase the binding affinities of EM-2 by introducing better turn structures into the Pro^2-Phe^3 position of EM-2, and to develop the structure–activity relationships between EM-2 and MOR.

In conclusion, it was the first time α-aminoxy acids were introduced into opioid peptides. A series of novel EM-2 analogues containing N–O turns were designed and synthesized. Their N–O turn structures were proved by NMR and FT-IR. Some of these analogues had potent affinities for MOR. The affinities of the analogues that formed N–O turns at the Pro^2-aminoxy-Phe^3 position were much higher than that of the analogues that formed N–O turns at the Tyr^1-aminoxy-Aa^2 position. We developed the structure–activity relationships between the MOR affinities and the positions of N–O turns. The relationships suggest that when EM-2 binds to MOR, it is likely to form turn structure at the Pro^2-Phe^3 position. According to these relationships, we can develop more potent analgesics with less side effects related to EM-2. Further studies are currently in progress in our laboratory to investigate the relationships between the structures of the different analogues and receptor affinities, in particular the turn structures and their positions.

Acknowledgments

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