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Letters

Design, Synthesis, and Evaluation of Opioid Analogues with Non-Peptidic β-Turn Scaffold: Enkephalin and Endomorphin Mimetics

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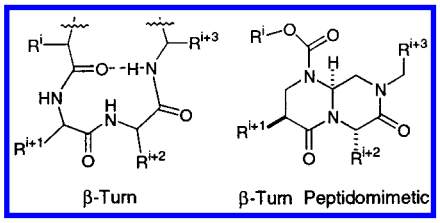
Abstract: We have identified a μ-selective opioid receptor agonist without a cationic amino group in the molecule from libraries of bicyclic β-turn peptidomimetics. The biologically active conformation of the lead is proposed to mimic an endomorphin type III 4 → 1 β-turn conformation.

The isolation and identification of the endogenous opioid peptides, methionine enkephalin and leucine enkephalin,[1] initiated an intensive effort to discern the biologically active conformation of enkephalin through comparison with the rigid morphine skeleton, which is believed to share a common binding motif.[2] It has been shown that the proper spatial orientation of a cationic amine, a phenol group, and an additional hydrophobic group is necessary to manifest biological activity through interaction with opioid receptors.[2a,c] However, the inherent conformational flexibility of the opioid peptides has hampered numerous attempts to accurately assess the bioactive conformation(s). Significant effort has been devoted to the elucidation of the receptor-bound conformation through systematic studies of conformationally constrained peptides[3] or peptidomimetics[4] or by conformational analyses of peptides in membrane-mimicking environments.[5] Several studies suggested reverse turn conformations as the potential biologically active conformation of the opioid peptides based upon computational models,[6] X-ray crystallography,[7] and spectroscopic analyses.[5b,8]

We have developed a β-turn peptidomimetic with four



sites of diversity readily accessible through solid-phase synthesis from commercially available diversity components. This privileged template was shown by X-ray crystal structural analysis[9a] and solution-phase 2D NMR spectroscopy[9] to mimic a type I β-turn conformation accurately on a rigid bicyclic structure. This highly constrained 6,6-bicyclic system incorporating functionality at the i to $i + 3$ positions affords us an opportunity to probe the biologically active conformation of peptides that potentially adopt a reverse turn conformation. Therefore, we applied this scaffold to the preparation of mimetics of Leu-enkephalin (YGGFL)[1] to identify potent and selective ligand(s) for opioid receptors (μ, δ, and κ)[10] and to elucidate their structure−activity relationship (SAR). In addition to the conformational stability induced by this template, its non-peptidic nature can potentially improve the undesirable therapeutic characteristics of peptides, which include poor bioavailability, short duration of action, and lack of oral activity.[11] During the course of our SAR studies, we found a striking similarity between the three-dimensional structure and the receptor selectivity profile of our lead compounds and those of the recently isolated opioid peptides, endomorphin-1 (YPWF) and endomorphin-2 (YPFF).[12] Herein, we report the design, synthesis, and evaluation of opioid peptide analogues utilizing our privileged bicyclic β-turn scaffold to discover potent

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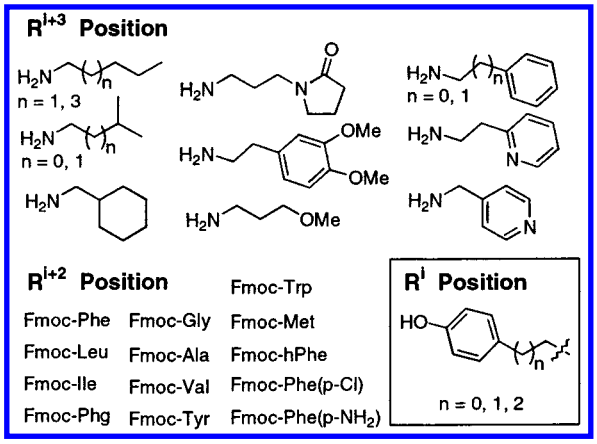
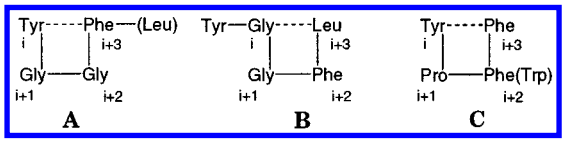


Figure 1. Diversity components for enkephalin and endomorphin mimetics.

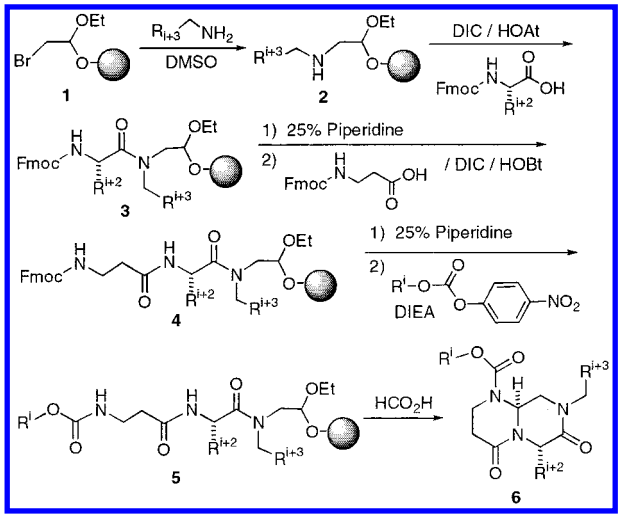
μ-receptor ligands that bear no cationic amine and we evaluate the relevance of the conformational and the functional requirements for biological activity.

Enkephalin can adopt any type of β-turn conformation in solution because of the presence of two glycine residues at the second and third positions. Indeed, various types of β-turn conformations (types I, I′, II′, and III)[5a−c,7,8a,b] of enkephalin have been observed in various media. By employing our β-turn scaffold, we can investigate the following hypothetical bioactive β-turn conformation models; enkephalin 4 → 1 β-turn model (A), enkephalin 5 → 2 β-turn model (B), and endomorphin 4 → 1 β-turn model (C). To examine these models,



we incorporate the designed diversity elements into the scaffold at the i to $i + 3$ positions. Twelve primary amines at the $i + 3$ position, 13 α-amino acids at the $i + 2$ position, one β-alanine at the $i + 1$ position, and a phenol component with three different lengths of spacer at the i position were selected as shown in Figure 1.

A focused library of opioid peptide mimetics with these components was prepared according to our previously published solid-phase synthetic protocol.[9] Nucleophilic displacement of the bromide, **1**, with a number of primary amines gave the corresponding secondary amine, **2**, which was then coupled with the appropriate Fmoc-α-amino acids with HOAt/DIC in NMP. Treatment of **3** with 25% piperidine in DMF followed by coupling with Fmoc-β-alanine afforded **4**. After deprotection of the Fmoc group of **4** with 25% piperidine, the resin was treated with the corresponding alkyl *p*-nitrophenyl carbonates in the presence of DIEA to produce **5**. Cleavage from the acetal resin followed by stereoselective tandem acyliminium cyclization was accomplished by treatment with formic acid at room temperature to give the 6,6-bicyclic β-turn mimetic, **6**. All products were purified by preparative TLC (silica gel) to afford the pure product in good overall yields (11−37%; see Supporting Information). The tryptophan derivatives at the $i + 2$ position and *p*-hydroxylbenzylcarbamate derivatives at the i position failed to afford the desired bicyclic scaffold.[13]



The inhibitory activity of each mimetic at 1 μM and/or 0.1 μM was evaluated through competition of specific binding of radioligand ([^{3}H]naloxone) for relatively nonselective opioid receptors in rat cerebral cortex.[14] The specific radioligand binding to the receptors is defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabeled naloxone (1 μM). Results are expressed as a percent inhibition of the specific binding obtained in the presence of the test compounds (see Supporting Information for the preliminary results). Among the 12 hydrophobic residues introduced at the $i + 3$ position, the benzyl and 2-pyridylmethyl groups showed the highest inhibitory activity and the other alkyl groups provided almost the same level of activity when the $i + 2$ position is benzyl. Diversification of the α-amino acids at the $i + 2$ position varies the inhibitory activity from 99% to 11% while holding the functionality at the $i + 3$ position as *n*-butyl. The phenethyl group at this position afforded the best result. The activities decrease in the order of phenethyl > *p*-aminobenzyl > benzyl > other alkyls, hydrogen, and phenyl groups > *p*-hydroxybenzyl group. Extension of the alkyl chain between the phenol group and the template at the i position (from ethyl to propyl) causes significant loss of inhibitory activity.

To validate this preliminary SAR result, we resynthesized four of the most active compounds. β-Turn mimetics with a benzyl group or phenethyl group at the $i + 2$ position and a benzyl group or *n*-butyl group at the $i + 3$ position were prepared, and the IC_{50} values of these four compounds versus the relatively nonselective opioid receptors in rat cerebral cortex were determined as shown in Table 1. These compounds represent an enkephalin 5 → 2 β-turn model with one methylene length shorter at the i position and an endomorphin type III 4 → 1 β-turn model with one methylene length longer at the i position.[15] The IC_{50} value of **6a** was measured as 149 nM. The replacement of the *n*-butyl group of **6a** at the $i + 3$ position with a benzyl group, **6b**, improved the inhibitory activity almost 2-fold to 80 nM, and the replacement of the benzyl group of **6a** at the $i + 2$ with a phenethyl group, **6c**, improved the inhibitory activity almost 5-fold to 27 nM. The inhibitory activity of **6c** was further enhanced 3-fold to 9 nM by replacing the *n*-butyl group at the $i + 3$ position with a benzyl group, **6d**. All four compounds are lipophilic and bear no cationic

Table 1. Effects of the Mimetics on the Specific Radioligand ([^{3}H]Naloxone) Binding to the Relatively Nonselective Opioid Receptor

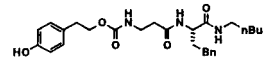
	Compounds			Inhibition (%)		
	R^i	R^{i+2}	R^{i+3}	0.1 μM	1 μM	IC_{50} (nM)
6a	*p*-OH-Phenethyl	Bn	nBu		84	149 ± 14
6b	*p*-OH-Phenethyl	Bn	Bn		96	80
6c	*p*-OH-Phenethyl	Phenethyl	nBu	79	99	27
6d	*p*-OH-Phenethyl	Phenethyl	Bn	91		9
7					<10	>1000

Table 2. Effects of the **6c** and **6d** on the Specific Radioligand Binding to the Human Opioid Receptors and IC_{50} Values for the Reference Compounds[a]

	6c	**6d**	reference compounds	
receptors	0.1 μM	0.1 μM		IC_{50} (nM)
δ (h)	<10%	<10%	DPDPE	3.5
κ (h)	<10%	<10%	U 50488	0.69
μ (h)	91%	101%	DAMGO	1.6

[a] Radioligands [^{3}H]DPDPE, [^{3}H]U 69593, and [^{3}H]DAMGO were used for assays for human δ-, κ-, and μ-opioid receptors, respectively. IC_{50} values are within accepted limits of historic averages obtained ± 0.5 log unit.

amino group in the molecule.[16] Compounds **6c** and **6d** were further studied using cloned human μ-, δ-, and κ-receptors to examine the receptor selectivity as shown in Table 2. Both of the compounds, **6c** and **6d**, showed excellent μ-selectivity for the human opioid receptors.

For comparison, a linear peptide **7** corresponding to **6c** was prepared and tested for its affinity for the rat cerebral cortex relatively nonselective opioid receptors. Although the same functional groups, such as *p*-hydroxyphenethyl, (*S*)-homophenylalanyl, and *n*-butyl groups, are displayed on the surface of the linear amide backbone structure, no binding activity was observed at the 1 μM level. This result suggests that the correct spatial orientations and conformational restrictions of these functional groups by the rigid template are critical for high receptor affinity.

We carried out an in vivo evaluation of the analgesic effect of **6c**. Three groups of 10 male CD-1 mice were administered 0.1, 1.0, or 10 mg/kg of **6c** intravenously (iv) at 5.0 mL/kg. One group of 10 male CD-1 mice was administered vehicle (propylene glycol and saline, 1:1) iv at 5.0 mL/kg. Another group of 10 male CD-1 mice received the positive control, morphine sulfate at 10 mg/kg, iv, at 5.0 mL/kg. The mice were sequentially placed on a hot plate analgesia meter (set at 55 °C) and observed for a reaction to the heat stimulus. The reaction times were obtained at 5, 15, 30, 60, and 120 min following iv administration. The administration of morphine sulfate at 10 mg/kg, iv, produced statistically significant ($p \leq 0.05$) increases in mean reaction times of 279%, 234%, 164%, 113%, and 22% analgesia respectively at 5, 15, 30, 60, and 120 min postadministration. The administration of **6c** at 1.0 mg/kg, iv, produced a statistically significant ($p \leq 0.05$) increase in the mean reaction time of analgesia 104% at 5 min postdose. The administration of **6c** at 10 mg/kg, iv, showed statistically significant ($p \leq 0.05$) increases in the mean reaction times of 337%, 189%, and 75% analgesia respectively at 5, 15, and 30 min postadministration. The results of the intravenous mouse hot plate analgesia assay are

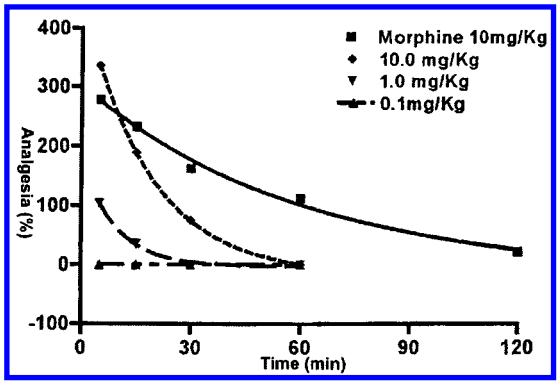


Figure 2. Intravenous mouse hot plate analgesia assay of **6c**.

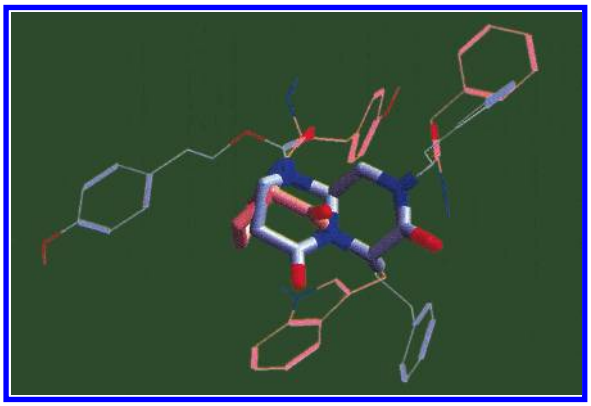


Figure 3. Overlay of the dominant low-energy conformer of **6d** (azure) and type III β-turn conformation of endomorphin (rose, ref 18).

summarized in Figure 2. It appears that the initial level of analgesic activity of **6c** is the same as the initial activity level of morphine, although the in vivo half-life of **6c** was almost 2-fold less than that of morphine.

Our 2D NMR experiments in $CDCl_3$ at −20 °C showed that the bicyclic scaffold of **6c** and **6d** adopts the same ring conformation as observed by X-ray crystallography for the corresponding sulfonamide derivative[9a] and that **6c** and **6d** exist as a mixture of two rotamers about the urethane linkage. A Monte Carlo conformational search for **6d** using the MMFF force field in vacuo as implemented in MacroModel 7.1[17] generated the bicyclic ring structure of the lowest energy conformer that matched the core ring structure deduced from the 2D NMR experiments. The overlaid structure of **6d** with a trans-endomorphin-1 β-turn type III like structure[18] revealed the structural similarity of these molecules with an rmsd value of 0.39 Å at six atom positions[19] as shown in Figure 3. Judging from the similarity of the three-dimensional structure and the μ-selectivity profile of **6d**, it would be reasonable to deduce that the opioid analogue **6d** mimics the endomorphin type III 4 → 1 β-turn model.

In conclusion, we have identified a nanomolar affinity μ-opioid receptor agonist lacking a positive charge in the molecule by employing a privileged β-turn scaffold. The stereochemistries of **6c** and **6d** in solution were analyzed by 2D NMR in combination with molecular mechanics, which suggest that the biologically active conformation of endomorphin is a type III 4 → 1 β-turn conformation and that the μ-active conformation of enkephalin is a 5 → 2 β-turn conformation. Finally, we have demonstrated the application of our β-turn pepti-

domimetic library for the discovery of potent and selective ligands for a member of the G-protein-coupled receptor superfamily.

Supporting Information Available: Experimental details and a list of abbreviations. This material is available free of charge via the Internet at http://pubs.acs.org.

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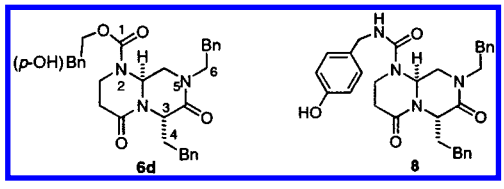
(19) C(1), N(2), C(3), C(4), N(5), and C(6) atoms of **6d**, and the corresponding C(Tyr^1), N(Pro^2), Cα(Trp^3), Cβ(Trp^3), N(Phe^4), and Cα(Phe^4) atoms of endomorphin-2 were compared.



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