Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2027–2032

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESES OF POTENT LEU-ENKEPHALIN ANALOGS POSSESSING β-HYDROXY-α,α-DISUBSTITUTED-α-AMINO ACID AND THEIR CHARACTERIZATION TO OPIOID RECEPTORS

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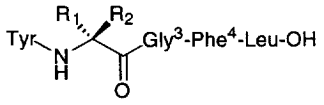
Received 28 May 1998; accepted 26 June 1998

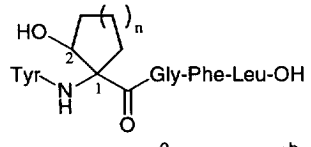
Abstract: Novel Leu-enkephalin (Leu-Enk) (**1**) analogs possessing various types of α-substituted serine instead of its glycine residue in the position 2 were synthesized via an efficient *O,N*-migration method. The binding characteristics of the synthetic analogs using Chinese hamster ovary (CHO) cells expressed cloned rat μ-, δ-, and κ-receptors revealed that [(1*R*,2*S*)-Ahh^2]Enk (**7**) was the most potent agonist of δ-opioid receptors among all the synthetic analogs tested, and was 10 times more potent than the native Leu-Enk.

It has been reported that an incorporation of 2-aminoisobutyric acid (Aib) to the Gly^2 residue of Leu-Enk (**1**) fixes its Tyr^1-Aib^2-Gly^3-Phe^4 moiety to a β-turn conformation in solution[1] which resembles a crystal structure of **1**.[2] However, [Aib^2]Enk (**2**) exhibits much weaker activity to opioid receptors than that of native **1**,[3] suggesting that the β-turn conformation is not a crucial factor for the activity. On the other hand, other substituted analogs at the Gly^2 residue such as [D-Ala^2]Enk or [D-Ser^2]Enk are known to be a potent agonist of opioid receptors.[3] We envisaged that an incorporation of serine analogs into the Gly^2 residue of **1**, in particular, α-substituted serine analogs which can be viewed as a conformational variant of serine,[4] would provide



Leu-Enk: R_1=R_2=H (**1**)
[Aib^2]Enk: R_1=R_2=Me (**2**)
[(*S*)-α-MeSer^2]Enk: R_1=Me, R_2=CH_2OH (**3**)[a]
[(*R*)-α-MeSer^2]Enk: R_1=CH_2OH, R_2=Me (**4**)[a]

[a]α-MeSer: α-methylserine
[b]Ahp: 1-amino-2-hydroxycyclopentanecarboxylic acid
[c]Ahh: 1-amino-2-hydroxycyclohexanecarboxylic acid



[(1*S*,2*R*)-Ahp^2]Enk: n=1 (**5**)[b]
[(1*R*,2*S*)-Ahp^2]Enk: n=1 (**6**)[b]
[(1*R*,2*S*)-Ahh^2]Enk: n=2 (**7**)[c]
[(1*R*,2*R*)-Ahh^2]Enk: n=2 (**8**)[c]

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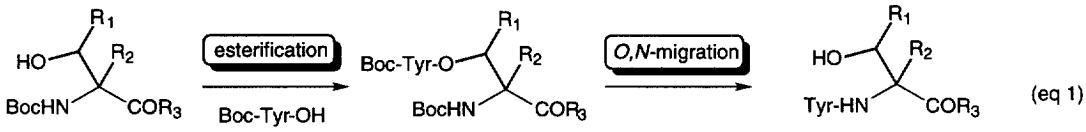
PII: S0960-894X(98)00349-7

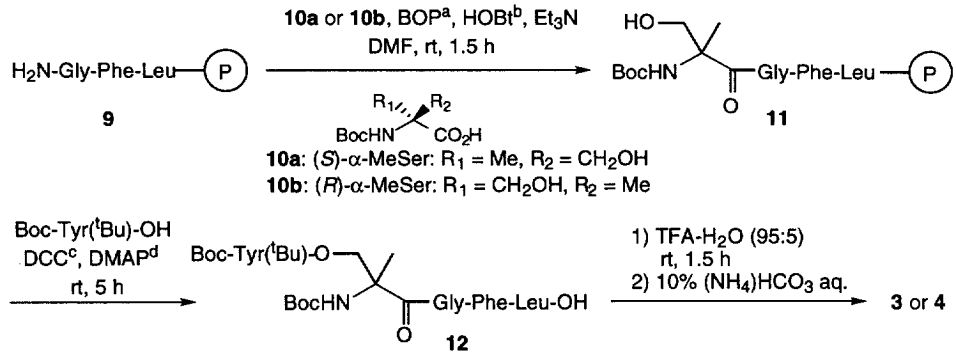
important insight into the structure-activity relationship of Leu-Enk. We wish to describe here the synthesis of highly potent enkephalin analogs **4**, **6** and **7**. The effect of α-substituted serine analogs on the conformation and binding affinity for opioid receptors are also disclosed.



R_1 = H, R_2 = Me, R_3 = -NH-Gly-Phe-Leu
R_1 = R_2 = -$(CH_2)_n$-, R_3 = -NH-Gly-Phe-Leu

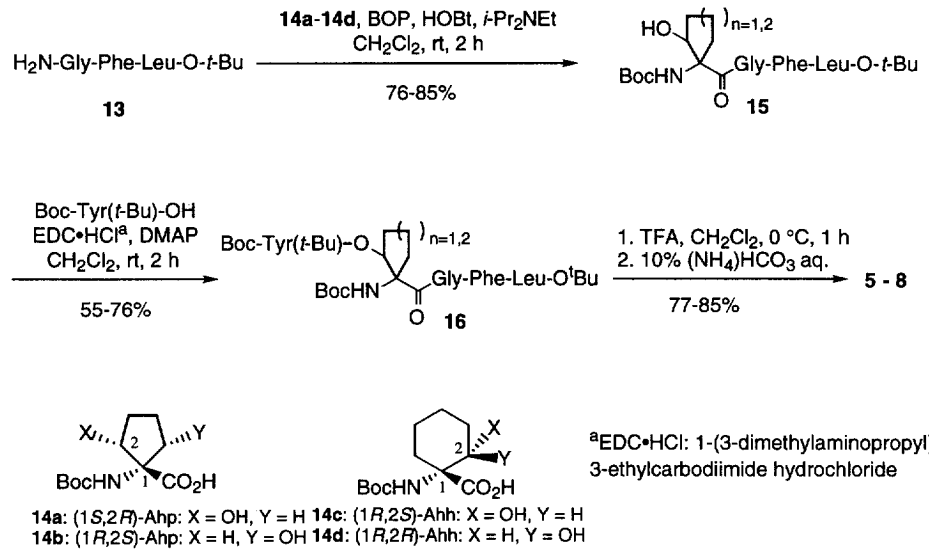
Syntheses of Leu-Enk analogs 3-8. H-Gly-Phe-Leu residues (**9** and **13**), common to all synthetic analogs of this study, were prepared either on a solid support or in a liquid phase using a standard coupling method. Condensation of **9** or **13** with the *C*-terminal of the *N*-Boc-α-substituted serines (**10** or **14**) was effected by BOP, HOBt, and triethylamine or *N,N*-diisopropylethylamine to give the desired *N*-Boc-tetrapeptides (**11** or **15**) in excellent yields. Since the coupling of the *N*-terminal of the α-substituted serine residue with Boc-Tyr(*t*-Bu)-OH encountered difficulty due, probably, to the presence of the sterically bulky substituents at the α position, we used the following sequence of reactions which have been recently developed by us: (i) ester formation of the *C*-terminal of the tyrosine residue with the hydroxy group of the *N*-Boc-α-substituted serine residue, (ii) removal of the Boc group, and (iii) internal migration of the ester group to the amino group (*O,N*-migration),[5] as shown in eq 1. An example of the above transformation was represented by the synthesis of [(1*R*,2*S*)-Ahh[2]]Enk (**7**). Esterification was effected by the use of EDC-HCl and DMAP to give tyrosyl ester **16**. Initial removal of the protecting groups with TFA and subsequent treatment with a 10% aqueous solution

Scheme 1. Syntheses of [α-MeSer[2]]Enk (**3**, **4**).



[a]BOP: benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate,
[b]HOBt: 1-hydroxybenzotriazole hydrate, [c]DCC: 1,3-dicyclohexylcarbodiimide,
[d]DMAP: 4-dimethylamino-pyridine

Scheme 2. Syntheses of [Ahp^2 or Ahh^2]Enk (**5-8**).



of ammonium bicarbonate effected the *O,N*-migration to give the desired **7** in 77% yield (Scheme 2).[6] Other analogs, **3-6**, and **8**, were prepared efficiently in a similar manner to the synthesis of **7** (Scheme 1 and 2).[6] Thus, a series of the α-substituted serines was efficiently introduced to the Gly^2 residue of Leu-Enk using the *O,N*-migration method.

Characterization of Leu-enkephalin analogs to opioid receptors. We investigated the binding affinities of the synthetic enkephalin analogs and their inhibitory effects on the cAMP production using chinese hamster ovary (CHO) cells expressing cloned rat μ-, δ- or κ-opioid receptors.[7] Binding affinities of the synthetic enkephalin analogs for μ-, δ- and κ-opioid receptors were determined by a competitive inhibition of the radioligands, [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69593, respectively, and were shown as IC_{50} values in Table 1. The binding characteristics of the synthetic enkephalin analogs clearly indicated the following points,: (i) All the synthetic enkephalin analogs had higher affinities for δ-opioid receptors than for μ-opioid receptors, and exhibited very weak binding affinities for κ-opioid receptors. (ii) The (*R*)-isomers **4**, **6**, **7** and **8** potently activated the δ-opioid receptors. In particular, [(1*R*,2*S*)-Ahh^2]Enk (**7**) had the highest affinity for δ-opioid receptors among the assayed analogs **2-8**, which was 10 times more potent than native Leu-Enk. (iii) The (*S*)-isomers showed much lower activity than those of the corresponding (*R*)-isomers. (iv) (*R*)-Isomers **4**, **6** and **7** also activated μ-opioid receptors to almost the same degree with that of Leu-Enk. To confirm whether the synthetic enkephalin analogs are agonists or not, we investigated their effects on the inhibition of forskolin-stimulated cAMP production using CHO cells expressing cloned rat δ-opioid receptors.[7] IC_{50} values of the synthetic analogs in the cAMP assay were well compatible with those obtained by the radioligand binding studies (Table 2). Thus, all the synthetic enkephalin analogs were found to be agonists of δ-opioid receptors.

These binding studies revealed that the α-substituted serine residue with the 1*R* configuration is a crucial factor for the high affinity binding to δ-opioid receptors. Among the 1*R* isomers, the carbocyclic analogs with a 1*R*,2*S* configuration, **6** and **7**, enhanced the binding activity of acyclic [(*R*)-α-MeSer^2]Enk (**4**), almost ten-

and forty-fold, respectively. It is of particular interest to note that [(1*R*,2*S*)-Ahh[2]]Enk (**7**), the most potent isomer among the tested analogs, fixed its hydroxy group to an equatorial orientation on the cyclohexane ring (chair conformation). This was ascertained by the *J* values (δ 3.7, dd, *J* = 4.4, 11.2 Hz) of its ^{1}H NMR (400 MHz in D_2O), indicating the importance of the local conformation of the hydroxy group at the α-substituted serine residue (Figure 1).[8] Therefore, we examined the conformation of the synthetic analogs by the ^{1}H NMR experiments.

Table 1. Receptor binding affinities of Leu-Enk analogs.

Ligands	IC_{50} (nM)[d]		
	[^{3}H]DAMGO (μ-activity)	[^{3}H]DPDPE (δ-activity)	[^{3}H]U69593 (κ-activity)
1	1.8	0.6	400
2	158	50	>10000
3	>10000	2500	>10000
4	6.3	0.4	>10000
5	320	10	>10000
6	2.5	0.04	>10000
7	0.25	0.01	6310
8	50	1.0	>10000
DAMGO[a]	3.0	—	—
DPDPE[b]	—	0.63	—
U69593[c]	—	—	0.63

[a]DAMGO: [D-Ala[2], *N*-Me-Phe[4], Gly-ol[5]]-Enkephalin; [b]DPDPE:[D-Penicillamine[2,5]]-Enk; [c]U69593: (5a,7a,8b)-(+)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide; [d]IC_{50} values were determined by the competitive inhibition of specific binding of 1 nM [^{3}H]DAMGO, 1 nM [^{3}H]DPDPE, or 2 nM [^{3}H]-U69593 in membrane prepared from CHO cells expressing μ-, δ-, or κ-receptors, respectively.

Table 2. Inhibitory effects of Leu-Enk analogs on forskolin-stimulated cAMP production in CHO cells expressing δ-opioid receptors.

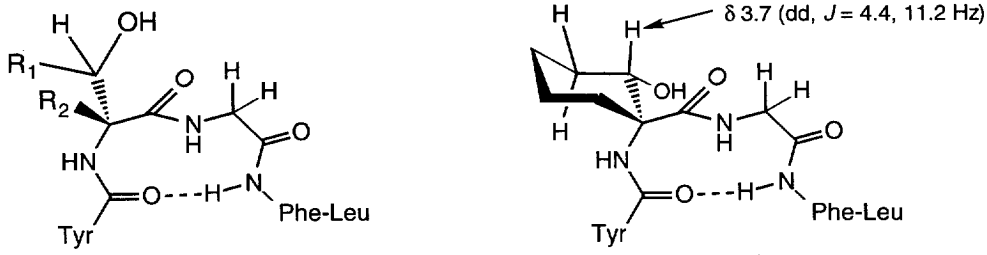
Ligands	IC_{50} (nM) δ-receptor	Ligands	IC_{50} (nM) δ-receptor
2	140	**6**	0.010
3	5000	**7**	0.005
4	0.071	**8**	2.0
5	10	DPDPE	4.0

Conformational analysis of [α-substituted Ser[2]]Enk. It is well accepted that the temperature coefficient

values (-dδ/dT, ppm/K) provide useful information on the inter- or intramolecular hydrogen bonding of the NH group: low -dδ/dT values (<0.003 ppm/K) at variable temperature are characteristic of solvent-shielded and/or intramolecularly hydrogen-bonded NH groups.[1,9] Thus, we measured the temperature dependence of NH chemical shifts of the synthetic analogs in $(CD_3)_2SO$. The resulting -dδ/dT values of each amide hydrogen were calculated (Table 3). The NH groups of the residue-2, Gly^3, and Leu^5 in all peptides tested were exposed to the solvent because their -dδ/dT values were >0.0045 ppm/K. On the other hand, the NH group at the Phe residue of **3-8** gave small -dδ/dT values (less than 0.003 ppm/K), respectively, suggesting the presence of an intramolecular hydrogen bonding at this position (Figure 1). Since these data were quite similar to those of the Aib^2 analog **2**,[9,10] all the peptides with α-substituted serine would have β-turn conformation. Therefore, the α-substituted serine residue employed in this study would play a role in fixing the conformation of the peptides to the β-turn conformation. These results are inconsistent with the fact that the Aib^2 analog **2** is a weak agonist of δ-opioid receptors.[3] The high affinity binding to the receptor would require both the β-turn conformation and the presence of a β-hydroxy group with an appropriate configuration at the Gly^2 residue.

Table 3. Temperature coefficients (-dδ/dT, ppm/K) of NH resonances of Leu-Enk analogs in DMSO-d_6.

	Residue^2-NH	Gly^3-NH	Phe^4-NH	Leu^5-NH
Leu-Enk (**1**)	0.0055	0.0046	0.0054	0.0067
[Aib^2]Enk (**2**)	0.0055	0.0049	**0.0021**	0.0067
3	0.0075	0.0049	**0.0023**	0.0079
4	0.0074	0.0039	**0.0027**	0.0059
5	0.0070	0.0049	**0.0021**	0.0065
6	0.0075	0.0040	**0.0025**	0.0067
7	0.0073	0.0042	**0.0023**	0.0072
8	0.0070	0.0039	**0.0021**	0.0069



[(1*R*)-α-substituted Ser^2]Enk (**4**, **6**, **8**) [(1*R*, 2*S*)-Ahh^2]Enk (**7**)

Figure 1. Proposed conformation of [(1*R*)-α-substituted Ser^2]Enk analogs.

In conclusion, novel Leu-Enk analogs **3-8** were synthesized in an efficient manner. Among the synthetic

analogs, the *R*-isomers **4**, **6** and **7** exhibited potent binding affinity to δ-opioid receptors. The binding characteristics of the synthetic analogs led us to propose that the high affinity binding for δ-opioid receptor requires the β-turn conformation at the Tyr[1]-[(1*R*)- or (1*R*,2*S*)-α-substituted Ser[2]]-Gly[3]-Phe[4] moiety, although the role of the hydroxy group can not be determined at this stage, that is, whether this group functions as a binding group or simply as a stabilizing factor of the β-turn conformation.

Acknowledgments: We are grateful to Professor Masamichi Satoh and Dr. Masabumi Minami, Graduate School of Pharmaceutical Sciences, Kyoto University, for the generous gift of cloned opioid receptors. This work was financially supported in part by New Energy and Industrial Technology Development Organization (NEDO), Japan.

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6. **3**: HRMS (FAB) calcd for $C_{30}H_{42}O_8N_5$ $(M+H)^+$ 600.3033, Found 600.3038; **4**: HRMS (FAB) calcd for $C_{30}H_{42}O_8N_5$ $(M+H)^+$ 600.3033, Found 600.3041; **5**: HRMS (FAB) calcd for $C_{32}H_{44}O_8N_5$ $(M+H)^+$ 626.3189, Found 626.3211; **6**: HRMS (FAB) calcd for $C_{32}H_{44}O_8N_5$ $(M+H)^+$ 626.3189, Found 626.3172; **7**: HRMS (FAB) calcd for $C_{33}H_{46}O_8N_5$ $(M+H)^+$ 640.3346, Found 640.3334; **8**: HRMS (FAB) calcd for $C_{33}H_{46}O_8N_5$ $(M+H)^+$ 640.3346, Found 640.3330.
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