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# Design and Synthesis of Highly Specific and Selective Enkephalin Analog Containing S-Npys-Cysteine for δ Opioid Receptors

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Enkephalin analogs containing S-(3-nitro-2-pyridinesulfenyl)cysteine at positions of 1, 5, or 6 were synthesized for searching possible thiol groups in the opioid receptors. In the radio-ligand receptor assay and biological assays, [D-Ala$^2$, Leu$^5$]enkephalyl-Cys(Npys)$^6$ exhibited a very high affinity and selectivity for δ over μ receptors, and its covalent attachment to δ receptors through the disulfide bonding was evidenced.

S-(3-Nitro-2-pyridinesulfenyl)cysteine, Cys(Npys), has been introduced as an amino acid derivative which selectively reacts with the free thiol group by a disulfide bond formation.[1] Its incorporation into enzyme substrates of thiol proteases was found to change them to specific inhibitors due to their covalent attachment to the enzyme thiol group.[1,2] Thus, Cys(Npys) can be utilized as an amino acid constituent of peptides when the peptide probe is required for searching a possible thiol group in its accepting substance. Since the Npys group is stable as a protecting group of cysteine, Cys(Npys) *per se* can be used directly for synthesis.

Larsen et al.[3] have suggested that in the opioid receptors there are at least two different thiol groups sensitive to *N*-ethylmaleimide (NEM). One of such thiols was found to be the cysteine β-thiol contained in the α-subunit of GTP-binding protein $G_i$. The other was suggested to be present in the receptor protein. Simon et al.[4] also suggested the existence an essential thiol group in the rat brain opioid receptors. Recently, using enkephalin analogs containing *S*-activated leucinthiol Leu($CH_2$SNpys) we demonstrated the presence of a thiol group in the binding site of μ receptors in the guinea pig ileum (GPI).[5] We have shown that *S*-activated [D-Ala$^2$,Leu($CH_2$SNpys)$^5$]enkephalin bound to μ receptors through a covalent disulfide linkage by the thiol-disulfide exchange reaction, but not to δ receptors. In the present study, in order to examine the thiol group in δ receptors, we have designed and synthesized several enkephalin analogs containing Cys(Npys) (Fig. 1).

| 1 | 2 | 3 | 4 | 5 | 6 | |
|---|---|---|---|---|---|---|
| H-Cys(Npys)- | Gly- | Gly- | Phe- | Met-OH | | (1) |
| H-Tyr- | Gly- | Gly- | Phe- | Cys(Npys)-OH | | (2) |
| H-Tyr- | D-Ala- | Gly- | Phe- | Cys(Npys)-OH | | (3) |
| H-Tyr- | D-Ala- | Gly- | Phe- | Leu- | Cys(Npys)-OH | (4) |
| H-Tyr- | D-Ala- | Gly- | Phe- | D-Leu- | Cys(Npys)-$NH_2$ | (5) |

Fig. 1. Amino acid sequences of enkephalin analogs containing the Cys(Npys) residue.

All the peptides were prepared using a general procedure for the solid-phase peptide synthesis described by Stewart and Young.[6] Amino groups were protected with the Boc group and the following side chain protecting groups were used: 2-bromobenzyloxycarbonyl (Br-Z) for tyrosine and Npys for cysteine. Hydroxymethyl resin for synthesis of carboxy-free peptides was purchased from Bachem Feinchemikalien AG (Bubendrof, Switzerland), and benzhydrylamine resin for synthesis of peptide amide from Peptide Institute Inc. (Osaka). As a typical example, the synthesis of [D-$Ala^2$,$Leu^5$]enkephalyl-$Cys(Npys)^6$ (**4**) (Fig. 1) is described in detail.

Hydroxymethyl resin (2 g, 0.96 mmol) was treated with Boc-Cys(Npys)-OH (1.5 equiv.), *N*,*N*-dicyclohexylcarbodiimide (DCC) (1.5 equiv.) and 4-dimethylaminopyridine (1.5 equiv.) in *N*,*N*-dimethylformamide (DMF) for 8 hrs at room temperature. Remaining free hydroxyl groups were blocked by acetylation using acetic anhydride and triethylamine (3 equiv. each). The subsequent residues were coupled using the Boc derivatives of Leu, Phe, Gly, D-Ala, and Tyr(Br-Z) in that order. The couplings were carried out in DMF with DCC (3 equiv.) and 1-hydroxybenzotriazole (6 equiv.) for 2—5 h. The Boc group was removed with 50% trifluoroacetic acid (TFA) in $CH_2Cl_2$. Coupling efficiency was monitored by the Kaiser test.[7] The completed resin was dried and then treated with HF (20 ml) containing anisole (2 ml) for 30 min at 0 °C. After removal of HF, the mixture of peptide and resin was washed with several portions of ethyl ether, and the peptide was then extracted twice with TFA (30 ml). The extract was concentrated and the residue was triturated with ether. The crude precipitate was purified on a Sephadex LH-20 column (MeOH) followed by a reversed-phase HPLC C-18 column (a linear gradient of 0.1% TFA-$CH_3CN$ (0—50%) over 60 min at a flow rate of 7 ml/min). Fractions showing the yellow color due to the Npys group and showing a single spot on TLC were pooled and evaporated. The pure product of H-Tyr-D-Ala-Gly-Phe-Leu-Cys(Npys)-OH was obtained as a TFA salt by recrystallization from MeOH-ether-petroleum ether; 52% yield from the initiated Boc-Cys(Npys)-resin. The amino acid ratio was as follows: $CySO_3H$ 0.83, Tyr 0.87, Ala 1.00, Gly 1.05, Phe 0.96, and Leu 1.05. The physicochemical properties are shown in Table 1 together with those of other peptides synthesized.

As shown in Table 1, all enkephalin analogs synthesized exhibited very weak activity for μ receptors in GPI. In contrast, D-$Ala^2$-enkephalin analogs containing Cys(Npys) at

Table 1. Physical and biological properties of synthetic enkephalin analogs

| Enkephalins | Mp | $[\alpha]_D^{22}$/° | TLC[a] | | Relative potency[b] | | Receptor selectivity[c] |
|---|---|---|---|---|---|---|---|
| | °C | (*c* 0.2, MeOH) | $R_f^1$ | $R_f^2$ | μ-receptor | δ-receptor | |
| **1** | 172-176 | +125.8 | 0.24 | 0.74 | 0.0013 | 0.029 | 1.5 |
| **2** | 211-214 | -4.4 | 0.32 | 0.47 | 0.0017 | 0.38 | 15 |
| **3** | 215-219 | +9.5 | 0.27 | 0.79 | 0.078 | 32 | 27 |
| **4** | 216-221 | -36.3 | 0.53 | 0.82 | 0.056 | 120 | 140 |
| **5** | 199-202 | +22.7 | 0.62 | 0.84 | 0.019 | 0.74 | 2.6 |

a) TLC was carried out on a silica gel (Kieselgel G, Merck) in the following solvent systems (by volume): $R_f^1$, n-BuOH-AcOH-$H_2O$ (4:1:1); and $R_f^2$, n-BuOH-AcOH-$H_2O$-pyridine (15:3:12:10). b) The muscle assays were carried out using the strips of GPI and MVD field-stimulated (60V, 0.5 msec, 0.1 Hz) in Krebs-Ringer solution. Tests were repeated at least three times for each compund to calculate the concentration required to produce a half-maximal inhibitory effect ($IC_{50}$). Relative potency was estimated using the $IC_{50}$ values[5] of each enkephalin analog by calculating their ratio against the $IC_{50}$ of [D-Ala$^2$,MePhe$^4$,Gly-ol$^5$]enkephalin as standard for both δ and μ receptors. c) Receptor selectivity was assessed by calculating the ratio of $IC_{50}$ values for δ versus μ receptors.

positions 5 (**3**) or 6 (**4**) were very active for δ receptors in mouse vas deferens (MVD). Analog **4** was particularly potent in MVD and showed a very high δ/μ affinity ratio (140-fold) in receptor selection. When 1 μM **4** was incubated with MVD tissue for 30 min, it was found that about 70% of receptors responding to enkephalins were occupied to elicit continuous opioid activity even after more than 30 washings. This sustained activity was completely reversed with 1 μM naloxone and disappeared by treatment with 1 mM dithiothreitol, a disulfide-reducing reagent. These results indicate that **4** was crosslinked to δ receptors via disulfide bonding (Fig. 2). Since **4** did not show such an affinity labelling in GPI, **4** appears to be very specific and selective in selection of δ receptors. Similar results were also obtained by the radio-ligand receptor binding assays using rat brain membrane. Detailed biochemical results will be reported elsewhere.

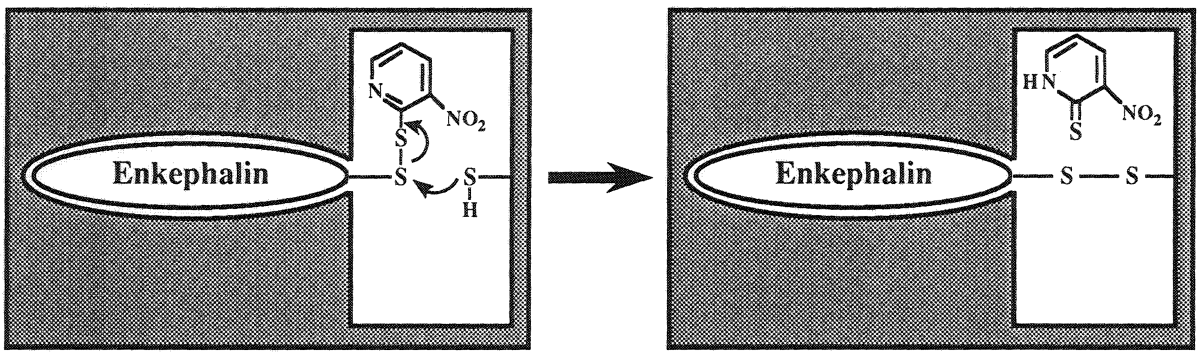


Fig. 2. Schematic model of affinity labelling of δ opioid receptor by Npys-enkephalin.

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