Exclusive Affinity-Labeling of μ Opioid Receptors by Morphiceptin Analogs Containing *S*-(3-Nitro-2-pyridylthio)cysteine

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The *S*-(3-nitro-2-pyridylthio) (Npys) group only reacts with a free mercapto group to form a disulfide bond. A series of morphiceptin analogs containing SNpys–cysteine were designed and synthesized for specific affinity-labeling of μ opioid receptors. Affinity-labeling of opioid receptors was monitored and evaluated by radioligand receptor binding assays using rat brain membranes and specific tritium-labeled ligands of enkephalin analogs. It was found that analogs with Cys(Npys) at position 4 or 5 bind covalently to μ receptors, but not to δ receptors, resulting in a discriminative and exclusive affinity labeling of μ opioid receptor subtype.

The affinity-labeling of receptors is now an important method for identifying a ligand binding site.[1] Two different essential structural elements are required for affinity ligand to label the receptors: i.e., (i) an affinity core to bind to the specific binding site and (ii) a reactant for nucleophiles in the receptor protein. Affinity labeling usually results in irreversible cross-linking of a ligand to a receptor. The most utilized reactants are the electrophiles such as Michael acceptors, halomethyl ketones, and isocyanates, which react with nucleophiles in the receptor protein. Enkephalin analogs with C-terminal chloromethyl ketones were successfully utilized for affinity labeling of opioid receptors. Those include, for example, chloromethyl ketones of [Leu^5]-, [D-Ala^2, Leu^5]-, and [D-Ala^2, D-Leu^5]enkephalins[2,3] and H–Tyr–D-Ala–Gly–MePhe–chloromethyl ketone.[4] It should be noted, however, that the chloromethyl ketone moiety can react with the ε-amino group of lysine, the β-hydroxy group of serine, the β-mercapto group of cysteine, and the imidazolyl group of histidine.

Carbenes and nitrenes have been utilized as reactants for photoaffinity labeling. For instance, enkephalin analogs having azidophenylalanine at position 4 or a 2-nitro-4-azidophenyl group at the C-terminus were reported to label opioid receptors.[5—7] However, the azido groups react also with various amino acid residues, and photoirradiation often damages a protein, resulting in destruction of the receptor protein.

The best affinity ligand is a ligand that labels predominantly one of functional groups, which belongs to a certain amino acid residue. This is particularly important when the affinity technique is utilized for determining a ligand binding site. The 3-nitro-2-pyridylthio (Npys)[8] group attached to a mercapto group, namely the SNpys group, is highly electrophilic and only reacts with a free mercapto group via the thiol–disulfide exchange reaction.[9—11] If the SNpys group is loaded into the affinity core, this group only reacts with the β-mercapto group of cysteine, forming a disulfide covalent bond between the ligand and the receptor (Fig. 1). Several lines of evidence have indicated the existence of mercapto group(s) in opioid receptors.[12—14] At least two different types of mercapto groups sensitive to *N*-ethylmaleimide (NEM) were suggested: i.e., the cysteine β-mercapto group in GTP-binding proteins, and the one at or near the binding site of receptor protein.[13] Recently, we have demonstrated that SNpys-containing enkephalin

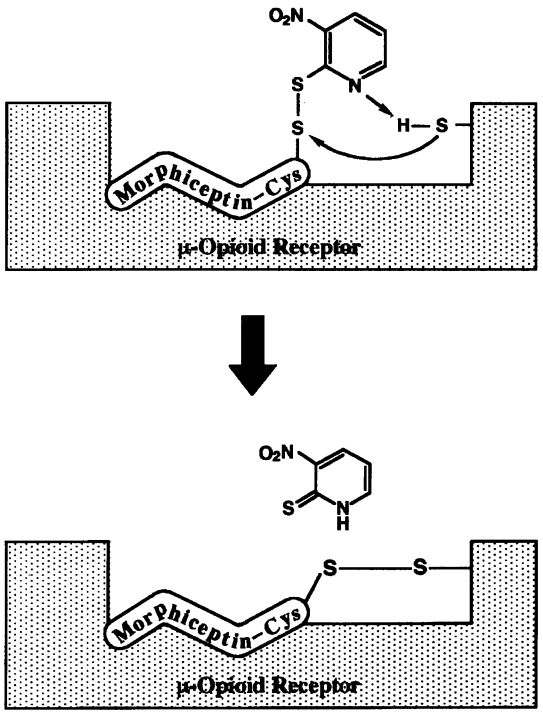

Fig. 1. Reaction mode of an Cys(Npys)-containing morphiceptin with opioid receptor.

analogs, [D-Ala^2, Leu(CH$_2$SNpys)^5]enkephalin and [D-Ala^2, Leu^5]enkephalyl-Cys(Npys)^6, affinity label selectively the μ and δ opioid receptors in peripheral nervous[15,16] and central nervous systems.[17] In the binding assay using rat brain membrane preparations, [D-Ala^2, Leu(CH$_2$SNpys)^5]-enkephalin labeled μ receptors much more preferentially (ca. 100-fold) than δ receptors, while [D-Ala^2, Leu^5]enkephalyl-Cys(Npys)^6 labeled δ receptors much more effectively (150-fold) than μ receptors.[17]

For exclusive affinity labeling of one of the receptor subtypes, it is an absolute requisite to modify a highly selective ligand. In the present study, a series of morphiceptin analogs containing Cys(Npys) were designed and synthesized to obtain more effective affinity ligand for μ opioid receptors (Fig. 2). Morphiceptin, H–Tyr–Pro–Phe–Pro–NH$_2$, is a highly selective ligand for μ receptors.[18] Since the N-terminal Tyr–Pro sequence is essential for receptor activation, Cys(Npys) was incorporated into the positions 3 and 4 of morphiceptin, and also into the position 5 corresponding to the C-terminal extension. We here report the receptor binding characteristics of these Cys(Npys)-containing morphiceptin analogs.

Results and Discussion

Figure 3 depicts a representative schematic profile showing the synthesis of Cys(Npys)-containing morphiceptin analog, Cys(Npys)^4-morphiceptin. Cysteine was first introduced as *S*-(*p*-methoxybenzyl) (MBzl) derivative. The C-terminal tri- or dipeptide amide containing Cys(MBzl) was coupled with Boc–Tyr–Pro–OH using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of 1-hydroxybenzotriazole (HOBt). At near the final step, the MBzl protecting group was converted into the Npys group with 3-nitro-2-pyridinesulfenyl chloride (Npys-Cl), and the resulting Cys(Npys)-containing penta- or tetrapeptides were treated with trifluoroacetic acid (TFA) to afford the desired morphiceptin analogs.

Table 1 summarizes the binding affinity of Cys(Npys)-containing morphiceptin analogs in the μ and δ binding assays.

(A) Tyr Pro Phe Pro NH_2

(B) Tyr Pro Cys(Npys) Pro NH_2

(C) Tyr Pro Phe Cys(Npys) NH_2

(D) Tyr Pro Phe Pro Cys(Npys) NH_2

(E) Tyr Pro Phe Asp NH_2

(F) Tyr Pro Phe Lys NH_2

Fig. 2. Structures of Cys(Npys)-containing morphiceptins. (A) morphiceptin, (B) Cys(Npys)^3-morphiceptin, (C) Cys-(Npys)^4-morphiceptin, (D) morphiceptinoyl-Cys(Npys)^5, (E) Asp^4-morphiceptin, and (F) Lys^4-morphiceptin.

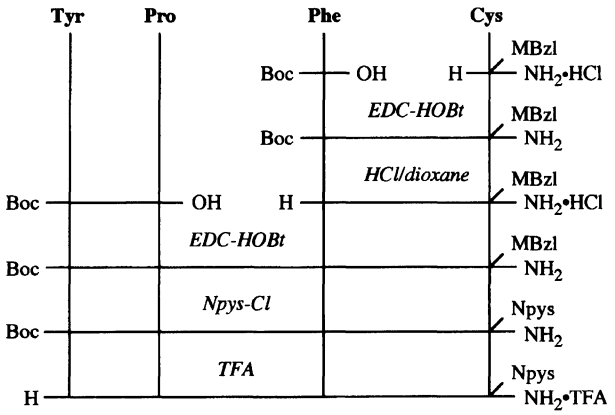

Fig. 3. Synthetic scheme of Cys(Npys)^4-morphiceptin.

[^{3}H]–[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin ([^{3}H]DAGO)[19] and [^{3}H]–[D-Ser^2, Leu^5]enkephalyl–Thr^6 ([^{3}H]DSLET)[20] were utilized as radiolabeled ligands specific for μ and δ receptors, respectively. It is clear that only Cys(Npys)^4-morphiceptin binds to μ receptors as strongly as morphiceptin does. Cys(Npys)^4-morphiceptin binds to μ receptors highly selectively, and the ratio in receptor selection of μ versus δ is about 140-fold (Table 1). Although this selectivity is smaller than that of morphiceptin, it appears to be sufficient to label the receptors predominantly. Cys(Npys)^5-morphiceptin was also fairly potent for μ receptors, while Cys(Npys)^3-morphiceptin was almost 80-fold less active than morphiceptin (Table 1). Their μ/δ-selectivity was not as high as that of Cys(Npys)^4-morphiceptin.

Asp^4- and Lys^4-morphiceptin[19] were assayed as references. These analogs were expected not to label the receptors via the disulfide bonding because of the lack of the Npys group, although it is possible for them to interact electrostatically with the charged groups of amino acids in the receptor. Their binding affinity to μ receptors was not strong, and they were only slightly μ-selective (Table 1).

When Cys(Npys)-containing morphiceptin was incubated with rat brain membranes, they would bind to the ligand binding sites of receptors in the first instance. Meanwhile, if there is a receptor mercapto group near the SNpys group, SNpys would react with this free mercapto group, resulting in the formation of a disulfide bond. Such affinity labeling of receptors would reduce the number of receptors available for ligands added afterwards. Thus, after preincubation of membranes with Cys(Npys)-containing morphiceptins, receptor binding assays would reveal the loss of μ and δ receptors, and consequently the amounts of the receptors labeled.

In the present study, rat brain membranes were first incubated with Cys(Npys)^4-morphiceptin for 30 min at 25 °C. After four consecutive washings of membranes by centrifugation, they were further incubated with DAGO and then with its tritium-labeled derivative [^{3}H]DAGO to determine the amount of μ receptors remaining unlabeled. When membranes were incubated with 1.0 μM of Cys(Npys)^4-morphiceptin (1 M = 1 mol dm^{-3}), about 30% μ receptors were reduced, indicating that 30% μ receptors were occupied by

Table 1. Binding Affinity of Cys(Npys)-Containing Morphiceptin Analogs for Opioid μ and δ Receptors.

Peptides	IC_{50} (μM)		Selectivity
	[^{3}H]DAGO	[^{3}H]DSLET	μ/δ
Tyr–Pro–Phe–Pro–NH_2(morphiceptin)	0.030	53	1800
Tyr–Pro–Cys(Npys)–Pro–NH_2	2.4	32	13
Tyr–Pro–Phe–Cys(Npys)–NH_2	0.051	7.3	140
Tyr–Pro–Phe–Pro–Cys(Npys)–NH_2	0.27	12	44
Tyr–Pro–Phe–Asp–NH_2	3.3	14	4.2
Tyr–Pro–Phe–Lys–NH_2	0.98	17	17

a) DAGO;[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin. DSLET; [D-Ser^2, Leu^5]enkephalyl-Thr^6.

Cys(Npys)^4-morphiceptin (Fig. 4). With increasing concentration of Cys(Npys)^4-morphiceptin, the receptors available for DAGO decreased sharply (Fig. 4). At the 100 μM concentration, Cys(Npys)^4-morphiceptin appeared to occupy almost all the μ receptors. Scatchard analyses gave monophasic straight lines in all cases (data not shown), and the affinity constants of DAGO were estimated to be about 6.8×10^{10} M^{-1} in all DAGO/[^{3}H]DAGO binding assays. These results imply that the receptors occupied by Cys(Npys)^4-morphiceptin are homogenous and highly specific for DAGO-enkephalin, indicating that they are μ opioid receptors.

When the amount of receptors labeled (% labeling) was plotted against the concentrations of Cys(Npys)^4-morphiceptin for preincubation, a sigmoid curve was obtained, as shown in Fig. 5. From this curve, the effective concentration (EC_{50}) which is enough to label the half-maximal amount of total receptors was estimated to be 4.2 μM (Table 2). This EC_{50} value of Cys(Npys)^4-morphiceptin is approximately 80 times larger than its IC_{50} value (0.051 μM; Table 1) in the ordinary binding assay for μ receptors. The SNpys group of Cys(Npys)^4-morphiceptin does not necessarily direct to the cysteine β-mercapto group of μ receptors, and thus their interaction becomes somewhat disadvantageous stereochemically to make a disulfide bond.

When the binding assay was carried out using [^{3}H]DSLET, a highly specific and selective ligand for δ receptors, no labeling was observed even after incubation with 100 μM Cys(Npys)^4-morphiceptin (Fig. 5). This means that Cys(Npys)^4-morphiceptin completely lacks an ability to cross-link δ receptors and can label only μ receptors. Recently, we found that [D-Ala^2, Leu(CH_2SNpys)^5]enkephalin labels μ receptors highly selectively. However, it still labeled δ receptors rather strongly (EC_{50} = 5.0 μM).[17] Since rat brain contains almost no κ receptors, Cys(Npys)^4-morphiceptin is the first affinity ligand which labels only one of multiple opioid receptors in rat brain.

Figure 6 also shows the dose-response curves of other Cys(Npys)-containing morphiceptin analogs. Table 2 summarized the EC_{50} values calculated from these curves. Morphiceptinoyl-Cys(Npys)^5 was moderately active (Table 2) as compared to Cys(Npys)^4-morphiceptin. In contrast, Cys(Npys)^3-morphiceptin was very weak, showing only 30%

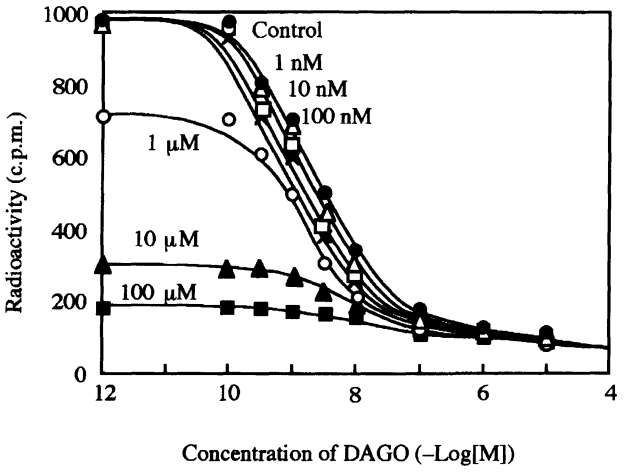

Fig. 4. Loss of receptor binding sites on preincubation of rat brain membranes with Cys(Npys)^4-morphiceptin. Dose-response curves of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGO) displacing [^{3}H]DAGO after preincubation with Cys(Npys)^4-morphiceptin at different concentrations are shown. The concentrations are the concentrations of Cys(Npys)^4-morphiceptin used for preincubation.

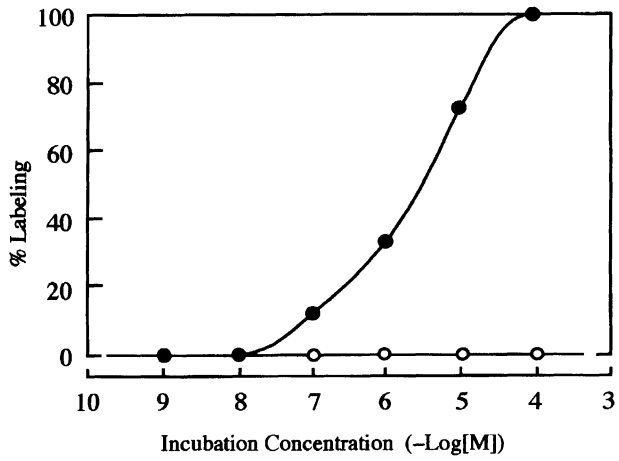

Fig. 5. Affinity labeling of opioid receptors by Cys(Npys)^4-morphiceptin. The percentage labeling was calculated by subtracting the total binding of DAGO or DSLET from that without preincubation with Cys(Npys)^4-morphiceptin. The affinity labeling of μ receptors was determined by evaluating the binding of DAGO (●), and that of δ receptors by evaluating the binding of DSLET (○).

Table 2. Affinity-Labeling of μ and δ Opioid Rceptpors by Cys(Npys)-Containing Morphiceptin Analogs.

Compounds	EC_{50} (μM)	
	^3H – DAGO	^3H – DSLET
Tyr–Pro–Phe–Pro–NH_2(morphiceptin)	N.I.[a]	N.I.
Tyr–Pro–Cys(Npys)–Pro–NH_2	> 100	N.d.[b]
Tyr–Pro–Phe–Cys(Npys)–NH_2	4.2	N.d.
Tyr–Pro–Phe–Pro–Cys(Npys)–NH_2	13	N.d.
Tyr–Pro–Phe–Asp–NH_2	N.I.	N.I.
Tyr–Pro–Phe–Lys–NH_2	> 100	N.I.

a) No labeling was observed. b) Extremely weakly labeled, and unable to estimate the EC_{50} values.

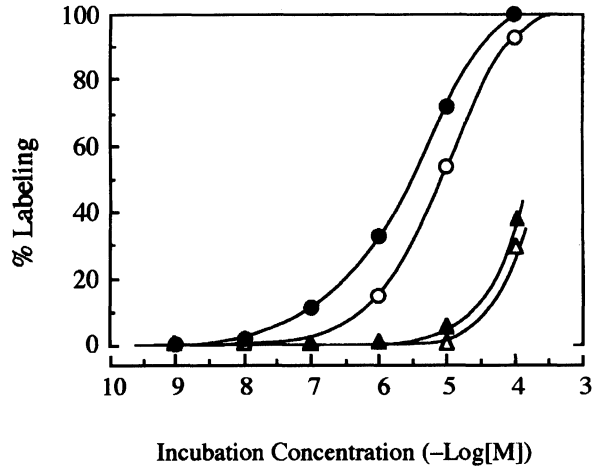

Fig. 6. Affinity labeling of opioid receptors by Cys(Npys)-containing morphiceptins and Lys[4]-morphiceptin. The percentage labeling was calculated by subtracting the total binding of DAGO from that without preincubation with morphiceptin analogs. Cys(Npys)[4]-morphiceptin (●), morphiceptinoyl-Cys(Npys)[5] (○), Cys(Npys)[3]-morphiceptin (△), and Lys[4]-morphiceptin (▲). The affinity labeling of μ receptors was determined by evaluating the binding of DAGO.

labeling of μ receptors at its 100 μM concentration. It is notable that all these morphiceptin analogs were completely inactive in labeling the δ receptors.

Morphiceptin and its analog with Asp[4] were completely devoid of ability to label the opioid receptors. Clearly, the SNpys group is a requisite for covalent attachment of Cys-(Npys)-containing morphiceptin analogs to the μ receptors. However, at the 100 μM concentration, Lys[4]-morphiceptin appeared to label (about 40%) μ receptors (Fig. 6). If this was a kind of affinity labeling, the labeling might be due to the electrostatic interaction between Lys[4] and presumably the acidic amino acid residue such as Asp and Glu in the receptor. Nonetheless, this was an extremely weak effect, unlike affinity labeling observed for Cys(Npys)[4]-morphiceptin and morphiceptinoyl-Cys(Npys)[5].

It should be noted that an exclusive occupation of μ receptors without affecting δ receptors is now feasible with Cys(Npys)-containing morphiceptins. Morphiceptin analogs containing Cys(Npys) at position 4 or 5 can label predominantly the μ opioid receptors. The one point to be improved is the affinity of morphiceptin. For a complete labeling of μ receptors, high concentrations are required. By reinforcing the affinity of morphiceptin, it will be possible to obtain highly specific affinity ligands.

Experimental

Peptide Synthesis. Melting points were uncorrected. High-performance thin-layer chromatography was carried out on silica gel G (Merck, Frankfurt) with the following solvent systems (v/v): R_f^1, $CHCl_3$–MeOH (9 : 1); R_f^2, $CHCl_3$–MeOH–aq NH_3 (50 : 10 : 2); R_f^3, $CHCl_3$–MeOH–AcOH (95 : 5 : 1); R_f^4, *n*-BuOH–AcOH–water (4 : 1 : 5, organic layer). Optical rotations were measured with a Union High Sensitivity Polarimeter PM-71 (Unikon Giken, Osaka).

Boc–Cys(MBzl)–NH_2 (1): To a solution of Boc–Cys-(MBzl)–OH (3.14 g, 10 mmol) in tetrahydrofuran (20 ml) and triethylamine (Et_3N) (1.41 ml, 10 mmol) was added isobutylchloroformate (1.32 ml, 10 mmol) at −15 °C. After vigorous stirring for 10 min, 28% aqueous ammnonia solution (2.0 ml) was added and the reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature, and then evaporated in vacuo. The residue dissolved in EtOAc was washed successively with 4% $NaHCO_3$, 5% $KHSO_4$, and water, and the residue from the organic solution was recrystallized from EtOAc–ether–pet. ether: Yield 2.18 g (83%); mp 149—150 °C; $[\alpha]_D^{20}$ −33.9° (*c* 1.0, DMF); R_f^1 0.65. Found: C, 56.24; H, 7.26; N, 8.18%. Calcd for $C_{16}H_{24}O_4N_2S$: C, 56.44; H, 7.12; N, 8.23%.

H–Cys(MBzl)–NH_2·HCl (2·HCl): Boc–Cys(MBzl)–NH_2 (10.2 g, 30 mmol) was dissolved in 4.5 M HCl/dioxane (130 ml). The reaction mixture was allowed to stand for 1 h at 0 °C, and evaporated in vacuo. The residue was recrystallized from MeOH–ether: Yield 7.70 g (93%); mp 211—212 °C (decomp); $[\alpha]_D^{20}$ −37.9° (*c* 1.0, DMF); R_f^2 0.68. Found: C, 47.85; H, 5.79; N, 10.10%. Calcd for $C_{11}H_{16}O_2N_2S$·HCl: C, 47.73; H, 5.84; N, 10.12%.

Boc–Phe–Pho–OH (4): To a solution of Boc–Phe–OH (2.17 g, 10 mmol), H–Pro–OMe·HCl (1.65 g, 10 mmol) in DMF (20 ml) and Et_3N (1.4 ml, 10 mmol) were added HOBt (1.62 g, 12 mmol) and EDC·HCl (2.30 g, 12 mmol) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature, and evaporated in vacuo. The residue was treated as described for compound **1** to afford an oily product of Boc–Phe–Pro–OMe (**3**): Yield 3.27 g (86%). The obtained Boc-dipeptide methyl ester was dissolved in MeOH (30 ml), and 1 M NaOH (9 ml) was added. The reaction mixture was allowed to stand for 2 h at room temperature and then was evaporated. The residue was dissolved in water to

acidify with citric acid, and the resulting oil was extracted with EtOAc. The organic solution was washed with water, dried over Na_2SO_4, and then evaporated in vacuo. The residue was crystallized from ether: Yield 2.88 g (98%); mp 180—181 °C; $[\alpha]_D^{20}$ −53.9° (*c* 1.0, MeOH); R_f[3] 0.50. Reported values: mp 179—180 °C; $[\alpha]_D^{20}$ −55° (ethanol).[22]

Boc–Phe–Pro–Cys(MBzl)–NH$_2$ (5): This was prepared from Boc–Phe–Pro–OH (**4**) (1.45 g, 4 mmol), H–Cys(MBzl)–NH$_2$·HCl (**2·HCl**) (1.11 g, 4 mmol) as described for compound **3**. Purification was carried out using a silica-gel column (2.2 × 45 cm) eluted with $CHCl_3$–MeOH (20 : 1): Yield 1.93 g (82%); mp 71—72 °C; $[\alpha]_D^{20}$ −61.0° (*c* 1.0, MeOH); R_f[1] 0.62. Found: C, 60.74; H, 6.93; N, 9.27%. Calcd for $C_{30}H_{40}O_6N_4S\cdot 1/2H_2O$: C, 60.68; H, 6.80; N, 9.44%.

H–Phe–Pro–Cys(MBzl)–NH$_2$·HCl (6·HCl): This was prepared from compound **5** (1.75 g, 3 mmol) and 4.5 M HCl/dioxane (13.7 ml) as described for **2·HCl**: Yield 1.50 g (96%); mp 65—66 °C; $[\alpha]_D^{20}$ −41.5° (*c* 1.0, MeOH); R_f[2] 0.67. Found: C, 57.82; H, 6.38; N, 10.69%. Calcd for $C_{25}H_{32}O_4N_4S\cdot HCl$: C, 57.62; H, 6.40; N, 10.75%.

Boc–Phe–Cys(MBzl)–NH$_2$ (7): This was prepared from Boc–Phe–OH (0.87 g, 4 mmol) and H–Cys(MBzl)–NH$_2$·HCl (**2·HCl**; 1.11 g, 4 mmol) by the EDC–HOBt method as describe for compound **3**: Yield 1.74 g (88%); mp 171—173 °C; $[\alpha]_D^{20}$ −32.9° (*c* 1.0, DMF); R_f[1] 0.66. Found: C, 61.54; H, 6.79; N, 8.54%. Calcd for $C_{25}H_{33}O_5N_3S$: C, 61.58; H, 6.84; N, 8.62%.

H–Phe–Cys(MBzl)–NH$_2$·HCl (8·HCl): This was prepared from compound **7** (980 mg, 2 mmol) as described for compound **2·HCl**: Yield 860 mg (93%); mp 171—172 °C; $[\alpha]_D^{20}$ +7.1° (*c* 1.0, MeOH); R_f[2] 0.71. Found: C, 54.21; H, 6.32; N, 9.48%. Calcd for $C_{20}H_{25}O_3N_3S\cdot HCl\cdot H_2O$: C, 54.35; H, 6.40; N, 9.51%.

Boc–Cys(MBzl)–Pro–NH$_2$ (9): This was prepared from Boc–Cys(MBzl)–OH (1.13 g, 7.5 mmol) and H–Pro–NH$_2$·HCl (2.56 g, 7.5 mmol) by the EDC–HOBt method as described for compound **3**: Yield 3.20 g (97%); mp 49—50 °C; $[\alpha]_D^{20}$ +48.5° (*c* 1.0, MeOH); R_f[1] 0.58. Found: C, 57.93; H, 7.33; N, 9.49%. Calcd for $C_{21}H_{31}O_5N_3S$: C, 57.64; H, 7.16; N, 9.61%.

H–Cys(MBzl)–Pro–NH$_2$·HCl (10·HCl): This was prepared from compound **9** (2.63 g, 6 mmol) as described for **2·HCl**: Yield 2.18 g (95%); mp 67—68 °C; $[\alpha]_D^{20}$ −42.5° (*c* 1.0, MeOH); R_f[2] 0.68. Found: C, 51.23; H, 6.53; N, 11.54%. Calcd for $C_{16}H_{23}O_3N_3S\cdot HCl$: C, 51.39; H, 6.48; N, 11.24%.

Cys(MBzl)-Containing Morphiceptine Analogs: Boc–Tyr–Pro–OH was coupled with H–Phe–Pro–Cys(MBzl)–NH$_2$·HCl (**6·HCl**), H–Phe–Cys(MBzl)–NH$_2$·HCl (**8·HCl**), and H–Cys(MBzl)–Pro–NH$_2$·HCl (**10·HCl**) by the EDC/HOBt method. Protected tetra or pentapeptides were purified on a silica-gel column (2.0×45 cm) eluted with $CHCl_3$–MeOH (20 : 1). The fractions containing pure product were collected and evaporated, and the residue was crystallized from ether–petroleum ether.

Boc–Tyr–Pro–Phe–Pro–Cys(MBzl)–NH$_2$: Yield 81%; mp 124—125 °C; $[\alpha]_D^{20}$ −93.7° (*c* 1.0, MeOH); R_f[1] 0.52. Found: C, 62.60; H, 6.77; N, 9.73. Calcd for $C_{44}H_{56}O_9N_6S$: C, 62.54; H, 6.69; N, 9.95.

Boc–Tyr–Pro–Phe–Cys(MBzl)–NH$_2$: Yield 93%; mp 106—107 °C; $[\alpha]_D^{20}$ −61.7° (*c* 1.0, MeOH); R_f[1] 0.52. Found: C, 62.35; H, 6.71; N, 9.11%. Calcd for $C_{39}H_{49}O_8N_5S$: C, 62.63; H, 6.62; N, 9.37%.

Boc–Tyr–Pro–Cys(MBzl)–Pro–NH$_2$: Yield 87%; mp 112—113 °C; $[\alpha]_D^{20}$ −87.5° (*c* 1.0, MeOH); R_f[1] 0.43. Found: C, 60.17; H, 6.95; N, 9.77%. Calcd for $C_{35}H_{47}O_8N_5S$: C, 60.24; H, 6.80; N, 10.04%.

Cys(Npys)–Containing Mophiceptine Analogs: Boc-tetra- or pentapeptide amides (0.5 mmol) containing Cys(MBzl) were dissolved in CH_2Cl_2 (5 ml) at 0 °C. After addition of Npys-Cl (190 mg, 1.0 mmol), the reaction mixture was stirred for 1—3 h and evaporated in vacuo. The residue was purified on a Sephadex LH-20 column (2.0×140 cm) eluted with DMF, and then on a silica-gel column (2.2×45 cm) eluted with $CHCl_3$–MeOH (20 : 1). Crystallization was carried out using a solvent system of ether–petroleum ether.

Boc–Tyr–Pro–Phe–Pro–Cys(Npys)–NH$_2$: Yield 52%; mp 140—141 °C; $[\alpha]_D^{20}$ −34.5° (*c* 1.0, MeOH); R_f[1] 0.73. Found: C, 56.25; H, 5.87; N, 12.55%. Calcd for $C_{41}H_{50}O_{10}N_8S_2$: C, 56.02; H, 5.75; N, 12.75%.

Boc–Tyr–Pro–Phe–Cys(Npys)–NH$_2$: Yield 94%; mp 138—139 °C; $[\alpha]_D^{20}$ −84.4° (*c* 1.0, MeOH); R_f[1] 0.44. Found: C, 54.25; H, 5.70; N, 12.08%. Calcd for $C_{36}H_{43}O_9N_7S_2\cdot H_2O$: C, 54.05; H, 5.43; N, 12.26%.

Boc–Tyr–Pro–Cys(Npys)–Pro–NH$_2$: Yield 75%; mp 134—135 °C; $[\alpha]_D^{20}$ −100.0° (*c* 1.0, MeOH); R_f[1] 0.30. Found: C, 52.64; H, 5.76; N, 13.23%. Calcd for $C_{32}H_{41}O_9N_7S_2$: C, 52.51; H, 5.65; N, 13.40%.

Boc-tetra- or pentapeptide amides containing Cys(Npys) (0.1 mmol) were dissolved in trifluoroacetic acid (TFA) (1 ml) and the reaction mixture was allowed to stand for 1 h at 0 °C. After evaporation, the residue was crystallized from MeOH–ether.

H–Tyr–Pro–Phe–Pro–Cys(Npys)–NH$_2$·TFA: Yield 96%; mp 145—146 °C; $[\alpha]_D^{20}$ −118° (*c* 1.0, MeOH); R_f[4] 0.66.

H–Tyr–Pro–Phe–Cys(Npys)–NH$_2$·TFA: Yield 86%; mp 141—142 °C; $[\alpha]_D^{20}$ −98.5° (*c* 1.0, MeOH); R_f[4] 0.60.

H–Tyr–Pro–Cys(Npys)–Pro–NH$_2$·TFA: Yield 82%; mp 149—150 °C; $[\alpha]_D^{20}$ −110° (*c* 1.0, MeOH); R_f[4] 0.34.

Receptor Binding Assays. Radio-ligand receptor binding assays involving rat brain preparations were carried out essentially as described.[23] [^{3}H]DAGO (1.80 TBq/mmol; New England Nuclear, Boston, Mass. USA) and [^{3}H]DSLET (1.51 TBq/mmol; New England Nuclear) were used as tracers selective for μ and δ opioid receptors, respectively, at the final concentration of 0.25 nM. Incubations were carried out at 25 °C for 60 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% bovine serum albumin. Bacitracin (100 μg/ml) was added to buffer as an enzyme inhibitor. Morphiceptin and its analogs were tested for receptor binding assays. Dose response curves were constructed utilizing seven to ten doses. The results were analyzed with the computer program, ALLFIT,[24] and the data were used to construct least-square estimate of the logistic curves relating the binding of labeled ligands [^{3}H]DAGO and [^{3}H]DSLET to the concentrations of the nonlabeled ligands.

Affinity-Labeling of Opioid Receptors. Assays for affinity-labeling were carried out essentially as described previously.[17] Briefly, rat brain membranes in 50 mM Tris-buffer (pH 7.5) were incubated with Npys-peptides (1 nM—100 μM) or without ligands (controls) in the presence of bacitracin (100 μg/ml) at 25 °C for 30 min. The membranes were then centrifuged (18000 rpm, 15 min) and resuspended in the same buffer for homogenization with Polyton homogenizer for a few minutes. This washing process was repeated successively four times, and the washed membranes were finally assayed for binding of DAGO using [^{3}H]DAGO or of DSLET using [^{3}H]DSLET as described above. The total amount of free μ or δ receptors was estimated from the amount of DAGO or DSLET that displaces radio-labeled ligands.

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8) The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9—37 (1984), are used through out. Unless otherwise specified, the amino acids are L-stereoisomers. Additional abbreviations are as follows: Boc, *t*-butoxycarbonyl; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; DSLET, [D-Ala^2, Leu^5]enkephalyl-Thr^6; DMF, *N*,*N*-dimethylformamide; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Et_3N, triethylamine; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; Npys, 3-nitro-2-pyridinesulfenyl or 3-nitro-2-pyridylthio; TFA, trifluoroacetic acid.

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