Opioid Activities of Morphiceptin Analogs Derived from Human β-Casein[1)]

Kazuyasu SAKAGUCHI, Hiroshi SAKAMOTO, Yoshiko TSUBAKI, Michinori WAKI,
Tommaso COSTA,† and Yasuyuki SHIMOHIGASHI*
Laboratory of Biochemistry, Department of Chemistry, Faculty of Science,
Kyushu University 33, Fukuoka 812
†National Institute of Child Health and Human Development, National Institutes of Health,
Bethesda, Maryland 20892, U.S.A.
(Received February 19, 1990)

Four tetrapeptide amides with the *N*-terminal Tyr-Pro sequence were synthesized as possible opioid agonists that can be produced by degradation of human β-casein. When these peptides were tested for their ability to bind to the μ and δ opioid receptors in rat brain, only H-Tyr-Pro-Phe-Val-NH_2 was active, showing the 60% increased affinity for the μ receptors as compared with morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2) derived from bovine β-casein. It was highly μ-selective, as well as morphiceptin, with the μ/δ-selectivity ratio of 285. Other three analogs, H-Tyr-Pro-Ser-Phe-NH_2, H-Tyr-Pro-Val-Arg-NH_2 and H-Tyr-Pro-Val-Pro-NH_2, were almost completely inactive. These results suggested that, for the morphiceptin-like tetrapeptide amides, the presence of Phe at position 3 is essential to elicit an activity to bind to the μ opioid receptors. Conformational considerations by measuring the CD spectra indicated that the sequence of tetrapeptide amide Tyr-Pro-Xxx-Pro(or Val)-NH_2 is an important structural requirement to interact with the μ opioid receptors.

Several peptides with high affinity for the opioid receptors have been isolated from the enzymatic hydrolysates of bovine casein. Those include β-casomorphin from β-casein[2)] and exorphins from α_{S1}-casein.[3)] When administered orally, β-casomorphin was shown to produce a moderate analgesia in rats, and its intracerebroventricular injection resulted in strong and naloxone-reversible analgesia.[4)] Morphiceptin, H-Tyr-Pro-Phe-Pro-NH_2, was originally reported as a synthetic tetrapeptide amide based on the sequence of bovine β-casein.[5,6)] However, Chang et al.[7)] found that morphiceptin per se can be detected biologically and immunochemically in the enzymatic hydrolysates of bovine β-casein. Morphiceptin is highly specific for the μ opioid receptors in rat brain membrane.[5,6)] It shows also the moderate affinities for opioid receptors in the peripheral tissues, when they are examined, for example, for the biological responses to inhibit the electrically stimulated contractions of smooth muscle preparations from the guinea pig ileum and mouse vas deferens.[5)] Since the existence of opioid receptors has been demonstrated in some intestinal systems,[8)] it is likely that these casein peptides possess some physiological roles, especially the nutritional functions by interacting with the receptors.

The amino acid sequence of human β-casein has been reported by Greenberg et al.[9)] Sequence comparison between the human β-casein and bovine β-casein revealed a 50% identity and a 10-residue shifted alignment relationship. Interestingly, there are four peptide portions with the Tyr-Pro sequence in this human β-casein. One of them is quite similar to the bovine casomorphin, having the sequence of Tyr-Pro-Phe. Thus, it was expected that the peptides having this Tyr-Pro-Phe sequence at the *N*-terminus may exhibit some opioid activities due to their possible interactions with the opioid receptors. Although a variety of potent morphiceptin analogs have been synthesized by many groups, most of them possess the D-Ala[2] and/or D-Pro[4] residues.[5,10,11)] In order to elucidate the possible nutritional functions, we intended to explore the more strict structural requirement of opioid receptors for peptides having the *N*-terminal Tyr-Pro sequence and thus to synthesize all the peptide fragments with the Tyr-Pro sequence in human β-casein.

In the present study, we describe the synthesis and the structure activity relationships of four morphiceptin-like tetrapeptide amides, namely H-Tyr-Pro-

Table 1. Binding Affinities of Morphiceptin Analogs Derived from Human β-casein

Compound	IC_{50} (nM)		μ-selectivity
	μ-affinity (^3H-DAGO)	δ-affinity (^3H-DADLE)	
Ia	5000	22000	—
Ib	35	10000	285
Ic	1400	16000	—
Id	14000	>100000	—
Morphiceptin	56	25000	446
DADLE	12	5	0.42
DAGO	1.5	400	267

H-Tyr-Pro-Phe-Pro-NH_2 morphiceptin
H-Tyr-Pro-Ser-Phe-NH_2 (Ia)
H-Tyr-Pro-Phe-Val-NH_2 (Ib)
H-Tyr-Pro-Val-Arg-NH_2 (Ic)
H-Tyr-Pro-Val-Pro-NH_2 (Id)

Fig. 1. Amino acid sequences of morphiceptin and morphiceptin-like peptides derived from human β-casein.

Ser-Phe-NH_2 (**Ia**), H-Tyr-Pro-Phe-Val-NH_2 (**Ib**), H-Tyr-Pro-Val-Arg-NH_2 (**Ic**) and H-Tyr-Pro-Val-Pro-NH_2 (**Id**) (Fig. 1). Their amino acid sequences correspond to the fragments of human β-casein 41—44, 51—54, 171—174, and 196—199, respectively.[9]

Results and Discussion

The synthetic scheme is shown in Fig. 2. The *C*-terminal Boc-dipeptide-NH_2 (**IVa—d**) were synthesized using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)[12] in the presence of 1-hydroxybenzotriazole (HOBt) (Table 2). These dipeptide amides were further elongated with Boc-Tyr-Pro-OH (**III**) by the same EDC-HOBt method. The resulting Boc-tetrapeptide amides were liberated with trifluoroacetic acid (TFA) to afford the desired morphiceptin-like peptide analogs (**Ia**, **b**, and **d**). In the case of **Ic**, the tetrapeptide was hydrogenated to remove the nitro group of Arg^3 after the TFA treatment. Purified tetrapeptide amides were verified by high-performance liquid chromatography (HPLC), elemental analyses and high-performance thin layer chromatography (HPTLC) (Table 3).

In rat brain, there are at least three distinct subtypes of opioid receptors designated as δ, μ, and κ.[13] Enkephalins and their analogs usually bind to the δ and/or μ opioid receptors, while dynorphins bind to the κ receptors. Morphiceptin binds predominantly to the μ receptors in rat brain, showing very high μ/δ-selectivity.[5,6] To examine the receptor preferences of ligands, it is useful to examine the ability to displace the radio-labeled analogs of such highly specific and selective ligands in rat brain. Since H-[^3H]-Tyr-D-Ala-Gly-Phe-D-Leu-OH (^3H-DADLE) is relatively δ-

Table 2. Physical Properties of Synthetic Boc-Dipeptide-Amides (**IVa—d**), Dipeptide Amides (**Va—d**) and Boc-Tetrapeptide Amides (**VIa—d**)

Compound	Yield %	Mp °C	$[\alpha]_D^{20}$/° (c 1.0, DMF)	HPTLC R_f
IVa	64	141—142	−6.8	0.81
IVb	84	187—189	+2.1	0.93
IVc	78	102—104	−25.7	0.79
IVd	87	(Oil)	—	0.74
Va	95	204—205	+17.2	0.60
Vb	92	213—216	+27.2	0.69
Vc	83	153—155	−15.1	0.48
Vd	87	204—206	(Insoluble)	0.45
VIa	64	138—140	−22.8	0.61
VIb	71	221—223	(Insoluble)	0.71
VIc	64	149—150	−32.3	0.49
VId	80	126—128	−59.7	0.46

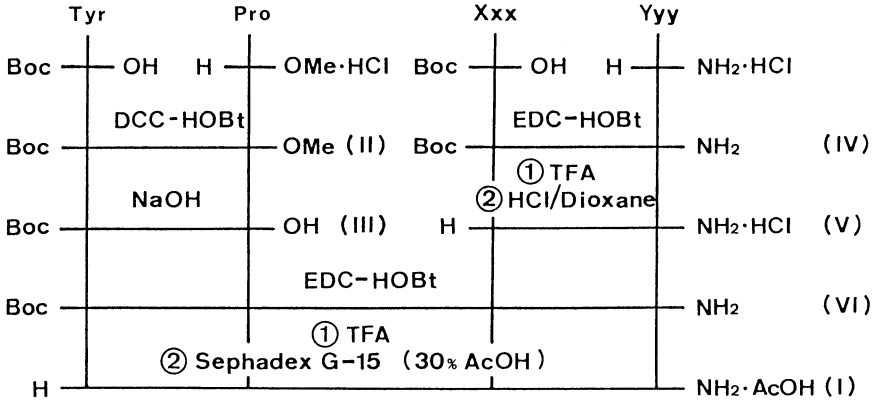

Fig. 2. Synthetic scheme of morphiceptin-like peptides. Xxx and Yyy denote the amino acid residues in position 3 and 4 of the tetrapeptides, respectively: (Xxx, Yyy)=(Ser, Phe) for **a**; (Phe, Val) for **b**; (Val, Arg (NO_2)) for **IVc**, **Vc**, and **VIc**, and (Val, Arg) for **Ic**; and (Val, Pro) for **d**. For preparation of **Ic** from **VIc**, amino-liberated tetrapeptide by TFA was hydrogenated in the presence of Pd-black.

Table 3. Physical Properties of Tetrapeptide Amides (**Ia—d**)

Compound	Yield /%	Mp /°C	$[\alpha]_D^{20}$/° (c 0.5, AcOH)	HPTLC R_f	HPLC* (% MeOH)
Ia	92	123—126	−14.2	0.60	47.2
Ib	69	99—100	−28.7	0.73	51.6
Ic	65	94—98	−45.6	0.45	26.9
Id	78	108—110	−89.2	0.66	37.1

*) Conditions for HPLC: column, Hitachi 3063-C_{18}; flow rate, 1.0 ml min^{-1}; and solvent, a linear gradient of 10—90% MeOH in 0.1% TFA for 40 min followed by the isocratic elution with 90% MeOH in 0.1% TFA for 10 min.

selective and H-[^3H]-Tyr-D-Ala-Gly-MePhe-Gly-ol (^3H-DAGO) is μ-selective,[13] using these radio-labeled ligands we have evaluated the receptor binding affinities of morphiceptin-like peptides for the opioid receptors in rat brain. The potencies expressed by IC_{50}, the dose which produces a 50% displacement of the tritiated ligands, were estimated from the logarithmic dose-response curves constructed with 6—8 doses. Table 1 shows the binding affinities of morphiceptin and its analogs derived from human β-casein. It was found that, among the peptides synthesized, only H-Tyr-Pro-Phe-Val-NH_2 (**Ib**) was active and selective for the μ receptors. The IC_{50} values of **Ib** were 35 nM in the ^3H-DAGO assay and 10,000 nM in the ^3H-DADLE assay. Based on these IC_{50} values, the μ-selectivity ratio of peptide **Ib** was calculated to be 285. Morphiceptin showed the binding characteristics similar to that of peptide **Ib**: IC_{50}=56 nM against ^3H-DAGO; 25,000 against ^3H-DADLE; and μ-selectivity =446 (Table 1). It should be noted that **Ib** and morphiceptin are very active for the μ receptors, but almost inactive for the δ receptors.

Peptide **Ia**, H-Tyr-Pro-Ser-Phe-NH_2, was almost completely inactive for both δ and μ receptors. Peptides **Ic** and **Id** were also inactive. Since the structural difference between peptides **Ib** and **Id** is the amino acid residues at position 3, namely Phe^3 in peptide **Ib** and Val^3 in peptide **Id** (Fig. 1), and since the amino acid residue at position 3 in morphiceptin is Phe, the Tyr-Pro-Phe sequence appears to be the most important structural element to elicit the binding ability to the μ opioid receptors. The Phe residue at position 3 is likely to be the essential requisite of morphiceptin-like peptides for recognition of the μ-opioid receptors. In contrast, the position 4 is not so restricted to replace the amino acids. It is clear that the substitution of Pro^4 in morphiceptin with Val increases (about 60%) the receptor binding activity. There must be a specific structural requirement of the μ receptors at the site corresponding to this amino acid residue^4 in the morphiceptin analogs. It is interesting to elucidate such a structural element for obtaining more specific ligands.

CD spectra of morphiceptin-like peptides in the two different solvents (H_2O and 2,2,2-trifluoroethanol (TFE)) are shown in Fig. 3. In H_2O (Fig. 3A), peptide **Ib** and morphiceptin exhibited very similar CD spectra with the negative Cotton effect at 217 nm and positive one at 230 nm. Also, they showed similar CD patterns in TFE (Fig. 3B). Interestingly, inactive peptide **Id** exhibited similar CD spectra to those of morphiceptin in both H_2O and TFE, suggesting that **Id** is in a conformation similar to that of morphiceptin. Since the peptide **Id** lacks the binding affinity for both δ and μ receptors, this inactiveness is certainly due to the substitution of morphiceptin Phe^3 with Val^3. It is thus likely that the bioactive conformation of morphiceptin peptides is sustained by the Tyr-Pro-Xxx-Pro(or Val)-NH_2 sequence, while the Phe^3 residue in morphiceptin plays an essential role in receptor recognition and affinity.

Inactive peptides **Ia** and **Ic** showed completely different CD spectra in H_2O, while in TFE they show CD profiles relatively similar to those of active analogs, suggesting these peptides are flexible. Chang and Cuatrecasas[14] postulated the model for binding sites

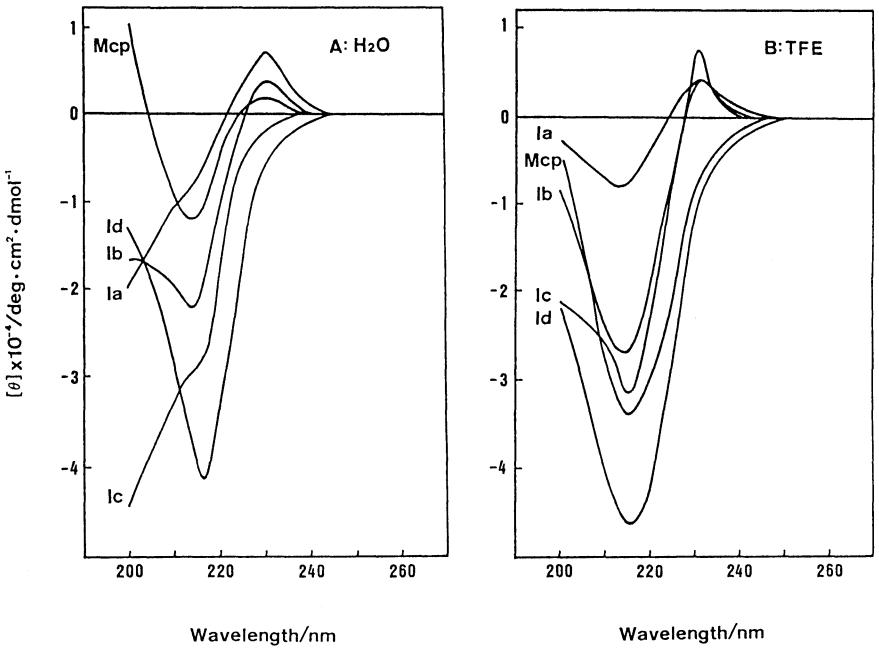

Fig. 3. CD spectra of morphiceptin and morphiceptin-like peptides. Solvents: A, H_2O; and B, TFE. Peptide concentrations: 1.0×10^{-4} M (1 M=1 mol dm^{-3}). Temperature: 25 °C.

in the μ receptors and explained the activity and selectivity of morphiceptin and enkephalins. Loew et al.[15] analyzed the conformation of morphiceptin to exhibit the μ-selectivity by the energy calculation. These studies showed the importance of sterical orientation and distance between two aromatic rings of Tyr[1] and Phe[3]. The conformation depicted by CD measurements may represent such a conformation. Recently, Doi et al.[16] reported the conformational analysis of peptide **Ib** by ^{1}H NMR, noting our preliminary report[1] to select **Ib** as μ-selective peptide ligand. According to this report, **Ib** appears to be in a folded conformation stabilized with 5 (amide)-1 (Tyr-C=O) hydrogen bond.

Yoshikawa et al.[17] reported the binding affinities of morphiceptin and their synthetic peptide corresponding to **Ib** using rat brain and ^3H-naloxone, an antagonist specific for the μ opioid receptors. Their potencies, however, seem to be weak (IC_{50}=2,000—3,000 nM) as compared with the results using agonists such as ^3H-DAGO (Table 1) and other radio-ligands; for morphiceptin, IC_{50}=45 nM using ^3H-dihydromorphine;[5] and 19 nM using ^{125}I-FK33-824 enkephalin analog.[6] On the other hand, Koch et al.[18] have shown that the synthetic fragment of human β-casein 41—44, which correspond to the free acid of peptide **Ib**, was extremely weakly active (IC_{50}=27,600 nM) in the binding assay using rat brain and ^3H-DAGO. The activity of this tetrapeptide acid appears to be almost three-order magnitude weaker than that of the corresponding tetrapeptide amide synthesized in the present study. Brantl[19] also reported the biological activity of this fragment in the assay utilizing the guinea pig ileum. It was also very weak (IC_{50}=56,200 nM) in inhibiting the electrically stimulated contractions. These results indicate that the *C*-terminal amidation confers a conformation preferable to fit the μ opioid receptors upon the resulting tetrapeptide amide. Unfortunately, this tetrapeptide amide would not be in an enzymatic digest of human β-casein, since no glycine residue that can be a substrate of α-amidating enzyme exists at the position 55 following the tetrapeptide (51—54).[9] Gly is present at position 60, and thus Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr-NH_2 may appear in the digested mixture to exhibit some physiological roles in the intestinal opioid system.

Experimental

Synthesis. HPTLC was carried out on Silica Gel G (Merck) with the following solvent system (v/v): R_f, *n*-BuOH-AcOH-EtOAc-H_2O (1 : 1 : 1 : 1). Optical rotations were measured with a Union high sensitivity polarimeter PM-71. Amino acid analyses were performed on a Hitachi KLA-5. HPLC was performed on a Hitachi 655A-11 liquid chromatograph.

Boc-Tyr-Pro-OMe (II): To a solution of H-Pro-OMe·HCl (2.15 g, 13 mmol) and Et_3N (1.8 ml, 13 mmol) in DMF (30 ml) were added Boc-Tyr-OH (3.66 g, 13 mmol), dicyclohexylcarbodiimide (3.22 g, 16 mmol) and HOBt (3.51 g, 26 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and at room temperature overnight. After evaporation in vacuo, the residue was suspended in EtOAc and dicyclohexylurea was removed by filtration. The filtrate was washed successively with 5% $KHSO_4$, 5% $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated to dryness. The solid obtained (4.69 g) was purified on a silica-gel column (4×50 cm) eluted with $CHCl_3$-EtOAc (2—1 : 1). The fractions containing a pure product were collected and evaporated. The residual oil was crystallized from ether-pet.ether: Yield, 3.85 g (75%); mp 61—63 °C; $[\alpha]_D^{20}$—25.3° (*c* 1.0 DMF); R_f 0.85. Found: C, 61.01; H, 7.29; N, 6,90%. Calcd for $C_{20}H_{28}O_6N_2$: C, 61.21; H, 7.19; N, 7.14%.

Boc-Tyr-Pro-OH (III): To a solution of Boc-Tyr-Pro-OMe (3.14g, 8 mmol) in MeOH was added 1 M NaOH (16 ml) (1 M=1 mol dm^{-3}). The reaction mixture was incubated for 9 h at room temperature and evaporated. The residue was triturated with 10% citric acid and the separated oil was extracted twice with EtOAc. The solution combined was washed with water and dried. After filtration and evaporation, the residue was crystallized from ether-pet.ether: Yield, 2.67 g (96%); mp 115—117 °C; $[\alpha]_D^{20}$—19.2° (*c* 1.0, DMF); R_f 0.77. Found: C, 57.72; H, 7.16; N, 7.01%. Calcd for $C_{19}H_{26}O_6N_2 \cdot H_2O$: C, 57.56; H, 7.12; N, 7.07%.

Boc-dipeptide-NH_2 (IV): Boc-Ser-Phe-NH_2 (**IVa**), Boc-Phe-Val-NH_2 (**IVb**), Boc-Val-Arg(NO_2)-NH_2 (**IVc**), and Boc-Val-Pro-NH_2 (**IVd**) were prepared from Boc-amino acid (5 mmol) and amino acid amide hydrochloride (5 mmol) by essentially the same method as described for compound **II** by the EDC-HOBt method. Physical properties of each compound purified are shown in Table 2.

H-dipeptide-NH_2·HCl (Va—d): Compound IVa—d (2 mmol) were treated with TFA (2 ml) for 1 h at 0 °C. After evaporation, the residue was dissolved in MeOH to add 5.6 M HCl in dioxane (2 ml). After incubation followed by evaporation, the residue was solidified with the aid of ether to afford the hydrochloride. Physical properties of each compound are shown in Table 2.

Boc-Tyr-Pro-dipeptide-NH_2 (VIa—d): Boc-Tyr-Pro-OH (**III**) (0.52g, 1.5 mmol) was coupled with H-dipeptide-NH_2 (**Va—d**) (1.5 mmol) by the EDC-HOBt method as described for **II**. Physical properties of Boc-tetrapeptide-NH_2 (**VIa—d**) are shown in Table 2.

H-Tyr-Pro-dipeptide-NH_2·AcOH (Ia—d): Compound **VIa**, **b**, and **d** (0.2 mmol) were treated with TFA (1 ml) for 30 min at 0 °C. After evaporation of TFA, the residue was dissolved in a small amount of 30% AcOH and the solution was put on a column (1.8×140 cm) of Sephadex G-15 eluted with 30% AcOH. The fractions containing a pure product were collected and twice lyophillized from water. In the case of compound **VIc**, which contains Arg(NO_2), the amino-liberated derivative by treatment of TFA was hydrogenated for 9 h in MeOH-water-AcOH (5 : 3 : 2, 10 ml) in the presence of Pd-black. The catalyst-free filtrate was evaporated and the residue was reconstituted in 30% AcOH to put a Sephadex G-15 column for purification as described above. The chromatographic purity was verified by HPTLC and HPLC. Physical properties of tetrapeptide amides are shown in Table 3.

CD Measurements. CD was measured at room temperature with a JASCO J-40A spectropolarimeter equipped with a data processor. TFE of spectroscopic grade and twice

distilled water were used as solvents for the peptides. The CD spectra (Fig. 3) were obtained by plotting the molar ellipticity (deg $cm^2 dmol^{-1}$) against the wave length (nm).

Receptor Binding Assays. Receptor binding assays using rat brain membrane preparations were carried out essentially as reported previously.[1] ^3H-DADLE (1.5 TBq/mmol, Amersham) and ^3H-DAGO (1.5 TBq/mmol, Amersham) were used at a final concentration of 0.25 nM. Incubations were carried out for 1 h at 25 °C in 50 mM Tris·HCl buffer (pH 7.4) containing bacitracin (100 $\mu mg\,ml^{-1}$) as an enzyme inhibitor.

References

1) A part of the results were preliminary reported in the Proceedings of the 23rd Symposium on Peptide Chemistry; Peptide Chemistry 1985, 197 (1986).

2) V. Brantl, H. Teschemacher, A. Henschen, and F. Lottspeich, *Hoppe-Seyler's Z. Physiol. Chem.*, **306**, 1211 (1979).

3) S. Loukas, D. Varoucha, C. Zioudrou, K. A. Streaty, and W. A. Klee, *Biochemistry*, **22**, 4567 (1983).

4) V. Brantl, H. Teschemacher, J. Blasig, A. Henschen, and F. Lottspeich, *Life Sci.*, **28**, 1903 (1981).

5) V. Brantl, A. Pfeiffer, A. Herz, A. Henschen, and F. Lottspeich, *Peptides*, **3**, 793 (1982).

6) K. -J. Chang, A. Killian, E. Hazum, P. Cuatrecasas, and J. -K. Chang, *Science*, **212**, 75 (1981).

7) K. -J. Chang, Y. Fu Su, D. A. Brent, and J. -K. Chang, *J. Biol. Chem.*, **260**, 9706 (1985).

8) J. A. H. Lord, A. A. Waterfield, J. Hughes, and H. W. Kosterlitz, *Nature*, **261**, 495 (1977).

9) R. Greenberg, M. L. Groves, and H. J. Dower, *J. Biol. Chem.*, **259**, 5132 (1984).

10) K. -J. Chang, E. T. Wei, A. Killian, and J. -K. Chang, *J. Pharmacol. Exp. Ther.*, **227**, 403 (1983).

11) H. Matthies, H. Stark, B. Harerodt, H. -L. Ruethrich, H. -T. Spieler, A. Barth, and K. Neubert, *Peptides*, **5**, 463 (1984).

12) Abbreviations: DADLE, [D-Ala^2, D-Leu^5]enkephalin; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; DMF, *N,N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

13) Y. Shimohigashi, C. H. Stammer, and T. Costa, in "Advances in Biotechnological Process, Synthetic Peptides in Biotechnology," ed by A. Mizrahi and A. L. van Wezl, Alan R. Liss Inc., New York (1988), Vol. 10, p. 203.

14) K. -J. Chang and P. Cuatrecasas, *Federation Proc.*, **40**, 2729 (1981).

15) G. Loew, C. Keys, B. Luke, W. Polgar, and L. Toll, *Mol. Pharmacol.*, **29**, 546 (1983).

16) M. Doi, M. Tanaka, K. Ikuma, M. Nabae, K. Kitamura, M. Inoue, and T. Ishida, *Biochem. J.*, **251**, 581 (1988).

17) M. Yoshikawa, T. Yoshimura, and H. Chiba, *Agric. Biol. Chem.*, **48**, 3185 (1984).

18) G. Koch, K. Wiedemann, and H. Teschemacher, *Arch. Pharmacol.*, **331**, 351 (1985).

19) V. Brantl, *Eur. J. Pharmacol.*, **106**, 213 (1985).