Receptor Interactions of Synthetic Morphiceptin Analogs Containing Phenylalanine Homologs in Position 4

Kazuyasu SAKAGUCHI, Tommaso COSTA,† Hiroshi SAKAMOTO, and Yasuyuki SHIMOHIGASHI*
Laboratory of Biochemistry, Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812
†National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, U.S.A.
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A series of phenylalanine homologs with elongated phenylalkyl side chains (R=$-(CH_2)_n-C_6H_5$, n=1—4) have been incorporated into opioid peptide morphiceptin at position 4 in order to elucidate a role of the amino acid residue in the molecular mechanism of receptor activation. The receptor specificity and selectivity of peptides synthesized were examined in the radio-ligand receptor binding assays using tritiated DAGO- and DADLE-enkephalins. [Phe^4]Morphiceptin was most potent for the μ receptors and showed the most pronounced μ/δ-receptor selectivity with a specific ratio of 410. Its μ-affinity was about three times stronger than morphiceptin. All analogs showed very similar CD spectra, suggesting that these peptides have a similar backbone conformation. The results strongly suggest that, besides the backbone conformation, the side-chain aromatic rings at positions 1 (Tyr), 3 (Phe), and 4 (Phe) array in a specific stereoorientation and this array is important to activate μ-receptors. Analogs with longer phenylalkyl side chains appeared not to retain such an arrangement due to the steric hindrance caused by the presence of more than two methylene groups.

Morphiceptin (Mcp) is a tetrapeptide amide found in the enzymatic hydrolysate of bovine β-casein.[1] Mcp binds strongly to the μ subtype of opioid receptors in the central brain membranes, while its biological activity for the peripheral μ receptors is not so strong as expected from its receptor affinity.[2,3] This suggests that the Mcp molecule itself is missing important structural requirements to activate μ receptors despite its high binding affinity. In our recent studies on morphiceptin-like peptides,[4,5] it was suggested that the μ receptor possesses the two sites to which morphiceptin binds specifically. They appear to be the sites responsible for receptor recognition and activation, respectively.

Mcp analogs having a hydrophobic amino acid residue in position 4 exhibit much more enhanced receptor affinity and biological activity than Mcp itself.[4,5] In particular, when the aromatic group is present at this position, μ receptors are strongly activated as demonstrated for [Thr(Bzl)^4]morphiceptin.[5] It is thus likely that there is an acitivity-eliciting site in the μ receptors to interact preferentially with the aromatic amino acid residue at position 4 of Mcp.

In the present study, in order to confirm the presence of the activity-eliciting site, a series of phenylalanine homologs (Fig. 1) were incorporated into Mcp. Receptor specificity and affinity of Mcp analogs synthesized

Mcp	H-Tyr-Pro-Phe-Pro-NH_2
[Thr(Bzl)^4]Mcp	H-Tyr-Pro-Phe-Thr(Bzl)-NH_2
[Phe^4]Mcp	H-Tyr-Pro-Phe-Phe-NH_2
[Apb^4]Mcp	H-Tyr-Pro-Phe-Apb-NH_2
[App^4]Mcp	H-Tyr-Pro-Phe-App-NH_2
[Aph^4]Mcp	H-Tyr-Pro-Phe-Aph-NH_2
[Apb^3,Phe^4]Mcp	H-Tyr-Pro-Apb-Phe-NH_2

Fig. 2. Amino acid sequences of morphiceptin and its analogs containing Thr(Bzl) or phenylalanine homologs in position 4.

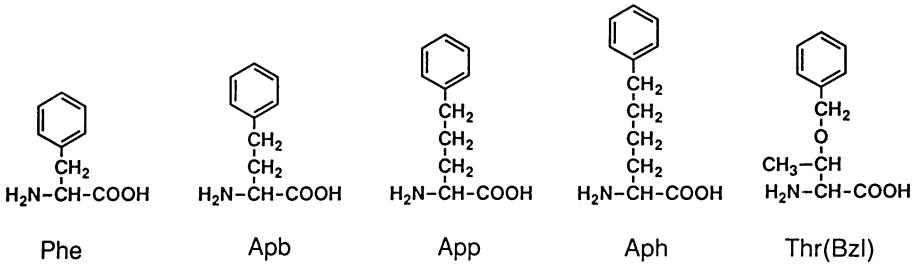

Fig. 1. Structure of phenylalanine, its homologs, and *O*-benzylthreonine. Abbreviations for amino acids are as follows: Apb, 2-*a*mino-4-*p*henyl*b*utanoic acid; App, 2-*a*mino-5-*p*henyl*p*entanoic acid; and Aph, 2-*a*mino-6-*p*henyl*h*exanoic acid.

(Fig. 2) have been evaluated in terms of side chain length of phenylalanine homologs and molecular conformation.

Results and Discussion

Phenylalanine homologs, 2-amino-5-phenylpentanoic acid (App)[6] and 2-amino-6-phenylhexanoic acid (Aph), were synthesized by the method of Hashimoto et al.[7] A synthetic scheme of Mcp analogs is illustrated in Fig. 3. Boc-Tyr-OH was coupled with H-Pro-OBzl to give Boc-Tyr-Pro-OBzl by the water-soluble carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of 1-hydroxybenzotriazole (HOBt). This dipeptide ester was hydrogenated to afford the corresponding free acid. *C*-Terminal dipeptide amides Boc-Phe-Xxx-NH_2, where Xxx represents a series of phenylalanine homologs such as Phe (n=1), 2-amino-4-phenylbutanoic acid (Apb) (n=2), App (n=3), and Aph (n=4) (Fig. 1), were prepared from Boc-Phe-OH and H-Xxx-NH_2 by the EDC-HOBt method. The amides of phenylalanine homologs were prepared by methyl-esterification followed by amidation in ammonia/MeOH. After removal of the Boc group by treatment with trifluoroacetic acid (TFA), liberated dipeptide amides were coupled with Boc-Tyr-Pro-OH to afford the tetrapeptide amides. These amides were treated with TFA to remove the Boc group, and amino-free peptides were purified by gel filtration on a Sephadex G-15 column eluted with 30% acetic acid.

The receptor affinities of peptides were evaluated in the radio-ligand receptor binding assays using rat brain membrane preparations.[8] [^{3}H]-[D-Ala^2, D-Leu^5]-enkephalin (^{3}H-DADLE) and [^{3}H]-[D-Ala^2,MePhe^4, Gly-ol^5]enkephalin (^{3}H-DAGO) were utilized as tracers of δ- and μ-receptors, respectively. Table 1 shows the binding affinities of Mcp analogs for both μ and δ

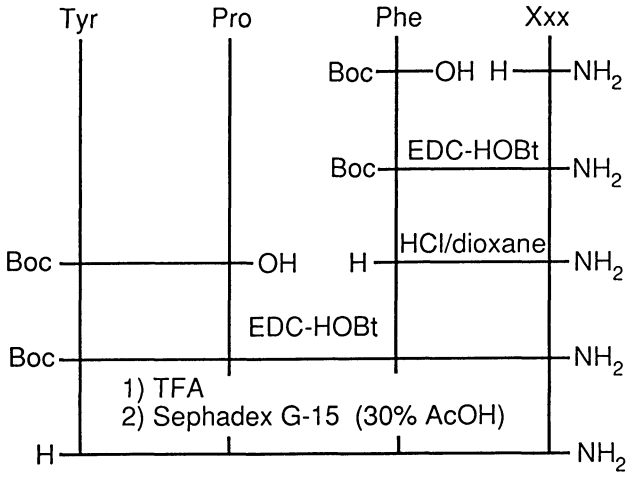

Fig. 3. General synthetic scheme of morphiceptin analogs containing phenylalanine homologs in position 4.

Table 1. Binding Affinities of Morphiceptin Analogs in Rat Brain Membrane

Peptide	IC_{50} (nM)		μ-Selectivity
	^{3}H-DAGO	^{3}H-DADLE	
Mcp	18.7	5800	310
[Thr(Bzl)^4]Mcp	2.9	710	245
[Phe^4]Mcp	6.6	2700	409
[Apb^4]Mcp	175.3	7700	44
[App^4]Mcp	86.8	10400	120
[Aph^4]Mcp	81.5	4800	59
[Apb^3,Phe^4]Mcp	79.5	10500	132
DADLE	10.3	3.3	0.32
DAGO	1.05	55.0	52.4

receptors together with their μ-receptor selectivity ratio. Among the Mcp analogs containing phenylalanine homologs, [Phe^4]Mcp was the most potent for μ receptors. It was about 3-fold more potent than Mcp. Other analogs with longer side chains at position 4 exhibited much weaker (12—26 fold) affinity than [Phe^4]Mcp. Obviously, the binding affinity of a series of Mcp analogs for μ receptors is maximized with phenylalanine (n=1).

There is a tendency of increase in affinity between analogs with longer side chains, where the order of affinity is [Apb^4]Mcp (n=2)<[App^4]Mcp (n=3)< [Aph^4]Mcp (n=4) (Table 1). However, the exact significance of this affinity tendency is not clear, since these analogs are apparently less potent than Mcp having Pro at position 4 (Fig. 1). The presence of benzene ring on the head of methylene chains longer than Phe seems to destabilize the interaction of Mcp with μ receptors.

The most active [Phe^4]Mcp was about twice less potent than [Thr(Bzl)^4]Mcp (Table 1). On the other hand, receptor selectivity of [Phe^4]Mcp was higher than that of [Thr(Bzl)^4]Mcp. Such a discrepancy in activity and selectivity is due presumably to subtle difference in conformation of side chain at position 4. When CD spectra of Mcp analogs were compared, all analogs containing phenyl group in the side chain at position 4 showed very similar profiles in both MeOH (Fig. 4) and water. This implies that these Mcp peptides have very similar backbone conformations. From the result of energy calculations, Loew et al.[9] have recently postulated a possible backbone conformation of Mcp peptides. Mcp was suggested to have a type II-β-like turn structure. CPK model building study based on this conformation suggested that three aromatic rings of [Thr(Bzl)^4]Mcp (Tyr^1, Phe^3, and Thr(Bzl)^4) can array in a specific stereoorientation and that the corresponding aromatic ring of [Phe^4]Mcp can retain a similar stereochemical arrangement. On the other hand, Apb^4, App^4, and Aph^4 in Mcp appear not to hold such an arrangement because of steric hindrance of methylene groups in the side chains. Thr(Bzl)^4 has three atoms (C_β-O-CH_2) between α-carbon and phenyl group. The presence of the oxygen atom and β-methyl group

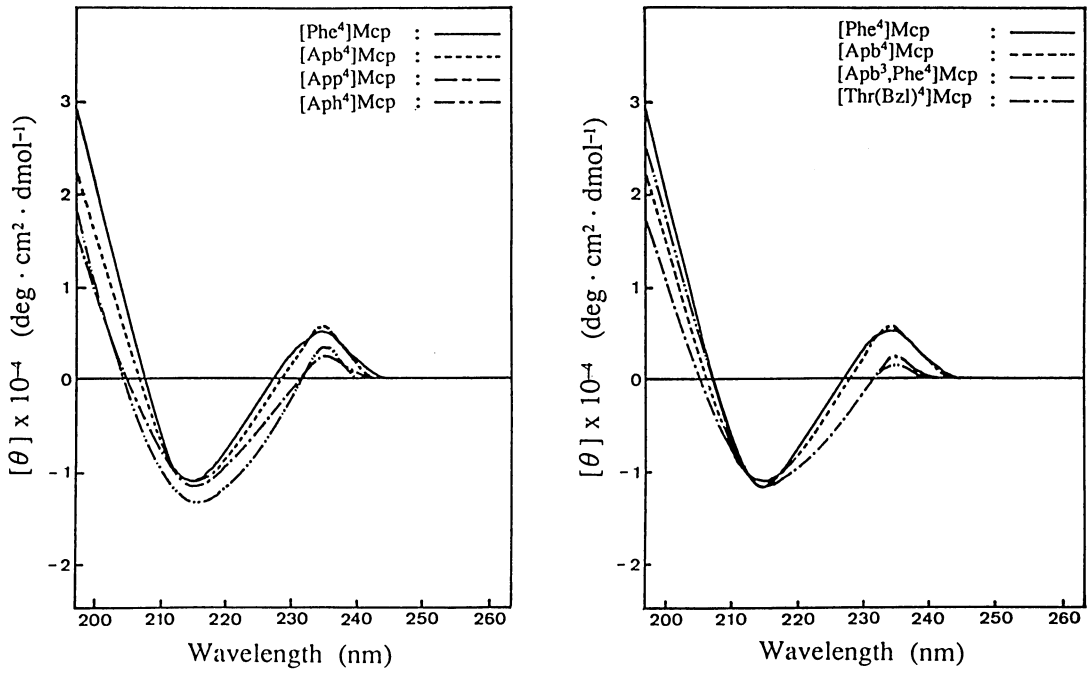

Fig. 4. CD spectra of morphiceptin and its analogs containing phenyalanine homologs in position 4. Solvent: MeOH. Peptide concentration: 1.0×10^{-4} M. Temperature: 25 °C.

seems to restrict severely the rotation of the phenyl group. On the contrary, the phenyl group of Phe⁴ of [Phe⁴]Mcp is more flexible than Thr(Bzl). This may explain the activity difference between [Thr(Bzl)⁴]Mcp and [Phe⁴]Mcp.

The Phe³ residue of Mcp is very important for recognition of receptors. No substitution was allowed at this position.[4] We incorporated there Apb. Apb has two methylene groups in the side chain, that is only one methylene longer than Phe. However, as shown in Table 1, [Apb³, Phe⁴]Mcp diminished sharply the affinity (12-fold) especially for μ receptors as compared with [Phe⁴]Mcp. This result further demonstrates a very restricted structural requirement at position 3 of Mcp.

The present study indicates the importance of phenyl group in the side chain of positions 3 and 4 for opioid receptor recognition and activation. Since [Phe⁴]Mcp possesses high specificity and selectivity for μ receptors (Table 1), it can be utilized as a novel standard for studies of Mcp peptides together with [Thr(Bzl)⁴]Mcp.

Experimental

Synthesis. High-performance (HP)-TLC was carried out on Silica Gel G (Merck, Frankfurt) with the following systems (v/v): R_f^1, $CHCl_3$–MeOH (9 : 1); R_f^2, *n*-BuOH–AcOH–EtOAc–H_2O (1 : 1 : 1 : 1). Optical rotations were measured with a Union high sensitivity polarimeter PM-71. All melting points were uncorrected.

H-Xxx-NH_2·HCl: Phenylalanine homologs App and Aph were prepared by the method of asymmetric hydrogenation of cyclic dehydrodipeptides.[7,10] Apb was purchased from Bachem (Bubendorf, Switzerland). Amino acids (1.5 mmol) were esterificated in thionyl chloride/MeOH and the methyl esters obtained were directly amidated in ammonia/MeOH. Physical properties are shown in Table 2.

Boc-Phe-Xxx-NH_2(I): The title compound with Xxx(=Phe, Apb, App, and Aph) was prepared from Boc-Phe-OH and respective amino acid amide by the EDC-HOBt method. To a chilled solution of amino acid amide hydrochloride (2 mmol) and Et_3N (0.28 ml, 2 mmol), EDC·HCl (422 mg, 2.2 mmol) and HOBt (324 mg, 2.4 mmol) were added 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 dissolved in EtOAc and the solution was washed successively with 4% $NaHCO_3$, 5% $KHSO_4$, and H_2O, and dried over Na_2SO_4. After filtration and evaporation, the residue was crystallized from EtOAc–ether–petroleum ether. Boc-Apb-Phe-NH_2 was also prepared from Boc-Apb-OH and H-Phe-NH_2·HCl as described above. Physical properties of purified compounds are shown in Table 2.

H-Phe-Xxx-NH_2·HCl (II): Compound I (1.5 mmol) was treated with 4 M HCl in dioxane (7.5 ml) at 0 °C for 1 h. After evaporation, the residue was solidified with the aid of ether to afford the hydrochoride. H-Apb-Phe-NH_2·HCl was prepared in the same way. Physical properties of each compound are shown in Table 2.

Boc-Tyr-Pro-Phe-Xxx-NH_2 (III): Boc-Tyr-Pro-OH (245 mg, 1 mmol)[4] was coupled with compound II (1 mmol) by the EDC-HOBt method as described for compound I. Physical properties of purified compounds including Boc-Tyr-Pro-Apb-Phe-NH_2 are shown in Table 2.

H-Tyr-Pro-Phe-Xxx-NH_2 (Morphiceptin Analogs): Boc-protected tetrapeptide amide III (0.5 mmol) was treated with trifluoroacetic acid (TFA) (2.5 ml) at 0 °C for 1 h. After evaporation of TFA, the residue was dissolved in a small amount of 30% AcOH and the solution was put on a column

Table 2. Physical Properties of Synthetic Peptides

Peptide	Yield	Mp	$[\alpha]_D^{20}$/°	(HP)-TLC
	%	°C	(*c* 1.0, DMF)	R_f
H-Xxx-NH_2·HCl				
Xxx=Apb	97	248—250	+14.3	R_f^2=0.67
App	85	208—210	+19.8	0.71
Aph	96	238—243	+19.4	0.71
Boc-Phe-Xxx-NH_2 (I)				
Xxx=Phe	81	194—195	−20.7	R_f^1=0.88
Apb	94	179—181	−22.2	0.88
App	69	164—165	−12.3	0.87
Aph	79	142—145	−11.5	0.86
Boc-Apb-Phe-NH_2	48	143—145	−10.0	0.88
H-Phe-Xxx-NH_2·HCl (II)				
Xxx=Phe	100	278—280	+12.5	R_f^2=0.80
Apb	99	250—251	+20.0	0.82
App	100	172—175	+17.3	0.83
Aph	99	180—182	+14.9	0.83
H-Apb-Phe-NH_2·HCl	95	179—180	+13.3	0.83
Boc-Tyr-Pro-Phe-Xxx-NH_2 (III)				
Xxx=Phe	68	126—128	−41.8	R_f^1=0.43
Apb	67	112—113	−36.3	0.43
App	69	110—114	−32.9	0.44
Aph	77	111—113	−32.9	0.44
Boc-Tyr-Pro-Apb-Phe-NH_2	75	113—116	−27.8	0.40

Table 3. Physical Properties of Morphiceptin Analogs

Peptide	Yield	Mp	$[\alpha]_D^{20}$/°	(HP)-TLC	HPLC[a)]
	%	°C	(*c* 0.5, AcOH)	R_f^2	%MeOH
H-Tyr-Pro-Phe-Xxx-NH_2					
Xxx=Phe	45	70—73	−10.9	0.70	44.2
Apb	92	96—98	−13.3	0.75	47.7
App	90	79—82	−12.9	0.74	51.5
Aph	90	89—92	−11.6	0.75	56.3
H-Tyr-Pro-Apb-Phe-NH_2	58	88—92	−4.0	0.75	47.5

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

(1.8×105 cm) of Sephadex G-l5 eluted with 30% AcOH. The fractions containing a pure product were pooled and lyophilized from water. The chromatographic purity was verified by (HP)-TLC and HPLC. Physical properties of tetrapeptide amide are shown in Table 3.

CD Measurements. CD was measured at room temperature with a JASCO J-40A spectrometer equipped with a data processor. Spectroscopic grade MeOH and twice-distilled water were used as solvents for the peptides. CD spectra (Fig. 4) were obtained by plotting the molar ellipticity (deg cm^2 $dmol^{-1}$) versus the wavelength (nm).

Receptor Binding Assays. Receptor binding assays by using rat brain membranes were carried out essentially as described previously.[8] [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-Enkephalin (^{3}H-DAGO, 1.4 TBq/mmol, New England Nuclear) and [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin (^{3}H-DADLE, 1.5 TBq/mmol, New England Nuclear) were utilized as tracers specific 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 bacitracin (100 μg/ml) as an enzyme inhibitor. Dose-response curves were constructed using 7—10 dose levels in duplicate. The data were utilized to construct the least-square estimates of the logistic curves. Since DADLE binds to both δ and μ receptors and Mcp analogs gave dose-responce curves with two phases, lower affinity was evaluated as affinities for δ receptors. Higher affinity were almost equal to that obtained from DAGO assays, indicating that these represent the affinities for μ receptors. The results are shown in Table 1.

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6) Abbreviations: Apb, 2-amino-4-phenylbutanoic acid; Aph, 2-amino-6-phenylhexanoic acid; App, 2-amino-5-phenylpentanoic acid; DADLE, [D-Ala^2, D-Leu^5]enkephalin; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; Mcp, morphiceptin; and TFA, trifluoroacetic acid.

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