Solid Phase Synthesis and Opioid Receptor Binding Activities of [D-Ala^2, D-Leu^5]Enkephalin Analogs Containing a Fluorinated Aromatic Amino Acid[1)]

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Five [D-Ala^2, D-Leu^5]enkephalin (DADLE) analogs containing fluorinated Tyr^1 or Phe^4 residue, that is, [Phe(2F)^4] (I), [Phe(3F)^4] (II), [Phe(4F)^4] (III), [Tyr(3F)^1] (IV) and [Try(2F)^1] (V), were synthesized by the solid phase method and their opioid receptor affinities were examined. Affinity profiles of five derivatives for the μ- and δ-receptor were similar to those of DADLE, and the affinity for κ-receptor was zero or negligible.

Keywords opioid peptide; fluorinated phenylalanine; fluorinated tyrosine; [D-Ala^2, D-Leu^5]enkephalin analog; receptor binding assay

Fluorine-19 containing compounds are of interest in connection with the development of medicine.[2)] Biologically active ^{19}F containing compounds are good reagents for the study of interaction with receptors using ^{19}F nuclear magnetic resonance.[3)] In addition, biologically active ^{18}F containing compounds are good reagents for positoron emission tomography (PET).[4)]

In this study, we synthesized [D-Ala^2, D-Leu^5]enkephalin (DADLE) analogs containing fluorinated phenylalanine or tyrosine and examined their affinities for opioid receptors, comparing them with the affinities of DADLE used as a reagent of a selective agonist for the δ-opioid receptor.[5)]

For the synthesis of fluorinated DADLE analogs, commercially available Phe(2F), Phe(3F), Phe(4F), Tyr(2F) and Tyr(3F) were used, and the peptides were synthesized by the solid phase method starting with Boc–D-Leu-Merrifield resin. After being cleaved from the resin, the peptides were purified by a medium pressure HPLC. Homogeneity of the peptides was checked by TLC, HPLC and amino acid analysis after 6 N HCl hydrolysis. The physicochemical data of synthetic analogs is shown in Table I.

Opioid receptor affinities of the peptides were determined by competition for binding with radioligands and synthetic peptides to rat brain (μ and δ) and guinea pig brain (κ) membrane fractions. [^{3}H][D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGO),[6)] [^{3}H]DADLE and [^{3}H]U-69593[7)] were respectively used as radioligands for μ-, δ- and κ-receptor bindings. The results are shown in Table II. I showed a 2- and 3-fold decrease in μ- and δ-receptor affinities, respectively, as compared with DADLE. II, IV and V showed equipotent μ-affinity and slightly lower δ-affinity compared to DADLE. III showed a 2-fold increased μ-affinity, but its δ- and κ-affinities were quite similar to those of DADLE. III was the only analog which exhibited weak κ-affinity comparable to that of DADLE.

TABLE I. Analytical Data of Synthetic Peptides

Analog No.	$[\alpha]_D$[a)] (°)	TLC[b)]		Amino acid ratios					HPLC[b)]	
		Rf (A)	*Rf* (B)	Tyr[c)]	D-Ala	Gly	Phe[d)]	D-Leu	t_R (min)[e)]	Purity (%)
I	+71.1	0.67	0.81	0.94	1.00	1.00	1.03	0.99	22.8	99.3
II	−23.0	0.65	0.81	0.98	1.00	1.00	0.94	1.00	24.5	99.5
III	+54.4[f)]	0.65	0.80	0.96	1.00	1.00	1.00	1.02	24.4	99.5
IV	+65.3	0.70	0.84	0.97	1.06	1.00	1.10	0.95	23.7	99.5
V	+70.7	0.70	0.82	0.93	1.00	0.95	1.12	1.10	23.8	95.6

a) Optical rotations were measured in 0.35 N AcOH (c=0.5) at 26 °C unless otherwise noted. *b*) See Experimental. *c*) Tyr or fluorinated Tyr (see Ref. 11). *d*) Phe or fluorinated Phe (see Ref. 11). *e*) DADLE=22.3 min. *f*) Measured in 30% ethanol/0.35 N AcOH.

TABLE II. Receptor Binding Assay of Fluorinated Analogs of DADLE

Peptides (DADLEs)	[^{3}H]DAGO (μ)		[^{3}H]DADLE (δ)		IC_{50} (δ) / IC_{50} (μ)	[^{3}H]U-69593 (κ)	
	IC_{50} (nM)	Relative potency[a)]	IC_{50} (nN)	Relative potency[b)]		IC_{50} (nN)	Relative potency[c)]
[Phe(2F)^4] (I)	78.0	3.6	32.0	32.8	0.41	10000 <	—
[Phe(3F)^4] (II)	34.0	8.2	16.0	65.6	0.47	10000 <	—
[Phe(4F)^4] (III)	21.0	13.3	10.5	100.0	0.50	7000	0.11
[Tyr(3F)^1] (IV)	44.0	6.4	13.3	79.0	0.30	10000 <	—
[Tyr(2F)^1] (V)	47.0	6.0	16.0	65.6	0.34	10000 <	—
DAGO	2.8	100	58.0	18.1	20.7	9400.0	0.09
DADLE	45.0	6.2	10.5	100	0.23	8200.0	0.08
U-69593	10000 <	—	9000.0	0.1	—	7.4	100

a) Relative potencies to DAGO (DAGO=100) on molar basis. *b*) Relative potencies to DADLE (DADLE=100) on molar basis. *c*) Relative potencies to U-69593 (U-69593=100) on molar basis.

Maeda *et al.*[8] reported that [Phe(4F)^4]Leu-enkephalin showed much stronger activity in the guinea-pig ileum and mouse *vas deferens* assays as compared with Leu-enkephalin. The different degree of enhancement between the opioid activity and the present binding affinity may be due to differences of the lead compound and the biological assay method.

In conclusion, the result of opioid receptor binding activities of DADLE analogs containing fluorinated Phe^4 or Tyr^1, together with the biological properties of fluorinated Leu-enkephalin analogs reported by the others,[8] shows that the replacement of a carbon–hydrogen bond at any position of the aromatic ring in Phe^4 or Tyr^1 residue by the highly polarized carbon–fluorine bond does not drastically change their biological activities, although there are a few exceptions of highly active enkephalin analogs containing Phe(4F)^4.[8,9] This fact seems to give an expectation for the development of ^{18}F containing opioid peptides for PET, if the fluorination procedure of Phe or Tyr residue at the final step of peptide synthesis is developed, since the half life of ^{18}F is very short (110 min). In addition, it is of interest that the enhanced phenol acidity of Tyr(2F)^1-DADLE (V) changes few biological properties, although the phenolic hydroxyl group in the Tyr^1 residue of enkephalins is considered to be essential to the biological activity. Literature pK_a values for related compounds are: phenol 9.98 and *o*-fluorophenol 8.81.[10]

Experimental

Optical rotations were measured with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel plates (Merck, Kieselgel 60F$_{254}$, 5 × 10 cm) with the following solvent systems: *Rf*(A), 1-BuOH–AcOH–H_2O (4:1:5, upper phase); *Rf*(B), 1-BuOH–AcOH–pyridine–H_2O (15:3:10:12). Analytical HPLC was performed on a YMC-Pack AM-303 ODS column (4.6 × 250 mm) by gradient elution with the following solvent system: A, 0.06% TFA in H_2O and B, 0.06% TFA in 80% acetonitrile. A linear gradient from 25% B to 55% B over 40 min at a flow rate of 1.2 ml/min was used, and the eluate was monitored at 215 nm. Purity of the peptide was assessed by HPLC with a Chromatocorder 12 recording integrator. Amino acid analysis was carried out on a Hitachi 835 analyzer using a high separation column after 6 N HCl hydrolysis of the peptide at 110 °C for 20 h.[11] Fluorinated amino acids were a generous gift from Asahi Glass Co., Ltd.

Solid Phase Peptide Synthesis Peptides were constructed on Boc–D-Leu-Merrifield resin (0.50 mmol/g) by the usual DIPCDI method using a Biosearch SAM II peptide synthesizer. Boc-amino acid with the side chain protecting groups 2-bromobenzyloxycarbonyl for Tyr was used. The following schedule was used to introduce each amino acid: 1) CH_2Cl_2 (× 2); 2) 45% TFA and 5% anisole/CH_2Cl_2 (× 2, 1 and 20 min); 3) CH_2Cl_2 (× 2); 4) DMF (× 2); 5) CH_2Cl_2 (× 2); 6) 10% DIPEA/CH_2Cl_2 (× 3, 1 min each); 7) CH_2Cl_2 (× 4); 8) CH_2Cl_2–DMF (1:1, × 1); 9) Boc-amino acid (4 eq)/DMF and DIPCDI (4 eq)/CH_2Cl_2 (× 1, 120 min); 10) CH_2Cl_2–DMF (1:1, × 2); 11) CH_2Cl_2 (× 2); 12) DMF (× 2); 13) 0.4 M acetylimidazole/DMF (× 1, 30 min); 14) DMF (× 2). The protected peptide resin was treated with anhydrous HF containing 10% anisole at 0 °C for 60 min. After evaporation of excess HF under vacuum, the crude peptide was extracted with 10% AcOH and the extract was washed with ether and then evaporated to dryness under vacuum. The crude peptides were purified on a column (2.8 × 25 cm) of Develosil (Nomura Kagaku, ODS-silica), which was eluted with a linear gradient of 20—60% acetonitrile in 0.1% TFA over 150 min at a flow rate of 3 ml/min. Eluate was monitored by the ninhydrin reaction on a filter paper. The main fraction was checked by analytical HPLC and pure parts were collected and freeze-dried. The synthetic peptide was converted to its AcOH salt by treatment with Dowex 1 × 2 (AcOH form) resin.

Opiate Receptor Binding Assay Binding assays were performed by the same method described in detail elsewhere.[12] The IC_{50} values were determined from log dose-displacement curves.

References and Notes

1) Amino acids, peptides and their derivatives in this study are of L-configuration unlesss otherwise stated. Abbreviations used are: Boc = *tert*-butoxycarbonyl, TFA = trifluoroacetic acid, DIPCDI = diisopropylcarbodiimide, DIPEA = diisopropylethylamine, DMF = dimethylformamide, TLC = thin-layer chromatography, HPLC = high performance liquid chromatography, Phe(2F) = 2-fluorophenylalanine, Phe(3F) = 3-fluorophenylalanine, Phe(4F) = 4-fluorophenylalanine, Tyr(3F) = 3-fluorotyrosine, Tyr(2F) = 2-fluorotyrosine.
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