Studies on Analgesic Oligopeptides. VII.[1a,2] Solid Phase Synthesis and Biological Properties of Tyr-D-Arg-Phe-βAla-NH_2 and Its Fluorinated Aromatic Amino Acid Derivatives

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Tyr-D-Arg-Phe-βAla-NH_2 (I) and its six fluorinated analogs were synthesized. Their opioid receptor binding properties were examined *in vitro* and their analgesic activity *in vivo* using the mouse writhing test. It was found that I was one of the most selective and potent μ-receptor agonists reported to date. [Tyr(2F)^1](VI) and [Tyr(3F)^1](V) derivatives of I showed similar biological properties to those of I. Since these peptides resist enzymatic degradation, it is expected that they are excellent reagents for the studies of function of μ-receptor-mediated biological properties *in vivo* and *in vitro*.

Keywords solid phase synthesis; dermorphin analog; fluorinated aromatic amino acid; opioid receptor binding assay; mouse writhing test; high μ-affinity; high μ-selectivity; analgesics

Introduction

We have studied extensively the structure–activity relationships of [D-Arg^2]dermorphin analogs.[1] In a previous study on structure–opioid activity relationships of Tyr–D-Arg–Phe–NHR, we revealed that the introduction of an acid–amide group at the alkyl moiety (R) increases μ-opioid receptor selectivity and affinity compared to its acid derivative.[1a]

In this study, a potent analgesic tetrapeptide, Tyr–D-Arg–Phe–βAla, found in our laboratory,[1c] was selected as lead peptide and the tetrapeptide amide, Tyr–D-Arg–Phe–βAla–NH_2 (I) was synthesized by the solid phase method. I showed very high selectivity for μ-receptors, as expected (Table I), as well as analgesic activity (Table II).

On the other hand, in a previous study of δ-selective [D-Ala^2, D-Leu^5]enkephalin (DADLE) analogs containing a fluorinated aromatic amino acid, it was revealed that the fluorinated derivatives did not show a dramatic change in receptor selectivity in general.[3] In studies of fluorinated drug design,[4] it is also interesting that biologically active ^{19}F compounds are good reagents for the study of receptors,[5] and ^{18}F-containing compounds are good reagents for positron emission tomography (PET).[6] A direct fluorination method of Tyr–D-Ala–Phe–Gly–NH_2 to give Tyr(3F)–D-Ala–Phe–Gly–NH_2 in high yield has recently been developed.[7] The work suggests the usefulness of Tyr-containing peptides for PET.

In this study, six kinds of I analogs (II—VII) containing fluorinated aromatic amino acid(s) were synthesized using the solid phase method. Thus, [Phe(2F)^3](II), [Phe(3F)^3](III), [Phe(4F)^3](IV), [Tyr(3F)^1](V), [Tyr(2F)^1](VI) and [Tyr(3F)^1, Phe(4F)^3)](VII) derivatives of I were obtained and their biological properties examined. V and VI showed similar biological properties to I (Tables I and II).

Results and Discussion

Peptides were synthesized by the solid phase method starting with Boc–βAla–benzhydrylamine resin as described previously.[3] After cleavage of the peptide from resin and deprotection by treatment with an HF–anisole mixture, the peptide was purified by using a medium-pressured HPLC. Homogeneity of the peptides was checked by analytical HPLC, TLC and amino acid analysis after 6N HCl hydrolysis. Analytical data of peptides is shown in Table III.

Opioid receptor binding properties of the peptides were compared with those of DAGO,[8] DPDPE[9] and U-69593,[10] which are at present used routinely as one of the most selective ligands for μ, δ and κ-receptors, respectively, and the results are shown in Table I. Analogs synthesized in this study showed very weak κ-affinity, less than that of DAGO. The only exception is II which showed κ-affinity equi-potent to DAGO. The δ/μ selectivity ratio of 12952 of I makes compound (I) one of the most selective and potent μ-receptor agonists reported to date.[11] Among the fluorinated Phe^3 analogs of I, IV showed a marked decrease in both μ and δ-affinities, while II and III showed

TABLE I. Opioid Receptor Binding Assay of Synthetic Dermorphin Analogs

Compound	[^{3}H]DAGO (μ) K_i (nM) (±S.E.)	[^{3}H] DPDPE (δ) K_i (nM) (±S.E.)	$K_i(\delta)/K_i(\mu)$	[^{3}H]U-69593 (κ) K_i (nM) (±S.E.)
DAGO	0.37 (0.03)	175 (16)	473	360 (80)
DPDPE	321 (54)	0.461 (0.067)	0.0014	—
U-69593	—	—	—	0.49 (0.34)
Tyr-D-Arg-Phe-βAla	0.020 (0.018)	42 (29)	2100	—[a]
Tyr-D-Arg-Phe-βAla-NH_2 (I)	0.021 (0.004)	272 (172)	12952	1365 (182)
[Phe(2F)^3] I (II)	0.144 (0.062)	656 (274)	4556	1292 (332)
[Phe(3F)^3] I (III)	0.082 (0.013)	353 (151)	4305	477 (167)
[Phe(4F)^3] I (IV)	0.87 (0.26)	2546 (1537)	2926	1079 (535)
[Tyr(3F)^1] I (V)	0.038 (0.008)	391 (214)	10289	1598 (162)
[Tyr(2F)^1] I (VI)	0.109 (0.037)	1060 (653)	9725	2375<
[Tyr(3F)^1, Phe(4F)^3] I (VII)	3.6 (1.4)	2571 (2183)	714	2375<

a) IC_{50} 10000< (see ref. 1a).

relatively little decrease at both receptors, indicating that the fluorination at position 4 of the Phe[3] ring is more effective for low affinity for μ and δ-receptors than that at position 2 or 3. On the other hand, the Tyr(3F)[1] analog, V, showed a high μ-affinity and selectivity nearly comparable to I.

The Tyr(2F)[1] analog, VI, also showed half the potency of V without a change in μ-selectivity. A multi-fluorinated analog, VII, resulted in a great decrease in μ-affinity, possibly due to Phe(4F)[3] residue as described above. In general, μ-selective I analogs containing fluorinated aromatic amino acids did not dramatically change receptor affinities as did the DADLE analogs described previously.[3] Incubation of all synthetic analogs with rat brain homogenate at 37 °C for 5 h produced no detectable degradation product on HPLC, showing good stability of these peptides against degradation enzymes in the brain. These results indicate that the data of the *in vitro* binding assay described above is reliable.

Analgesic activity in mice after s.c. injection of some highly μ-selective analogs in the PBQ writhing test is shown in Table II. Analogs I, V and VI showed low analgesic activity, similar to that of the lead peptide. Penetration to the brain across the blood brain barrier (BBB) of I, V and VI having two positive charges in the molecule after peripheral administration remains to be investigated in the future. Based on the assumption that highly polar, especially basic, peptide derivatives would not cross the BBB, analgesic compounds which act peripherally are being developed.[11,12] And it is advocated that one of these compounds, Tyr–D-Arg–Phe–Lys–NH_2, having three positive charges, is a peripherally active analgesic.[11] On the contrary, based on the assumption that there is a system of anionic receptor or binding sites in BBB-mediated transcytosis,[13] MeTyr–Gly–Gly–Phe–Leu–Arg–MeArg–D-Leu–NHC_2H_5, having three positive charges, has been shown to cross the BBB.[14] The apparent discrepancies need to be investigated further. For the solution of these problemes, I, V and VI may be good reagents.

In conclusion, the present study demonstrates that Tyr–D-Arg–Phe–βAla–NH_2 (I) is potent and one of the most selective μ-receptor agonists reported to date, and the fluorinated analogs of the Tyr[1] aromatic ring at position 2 or 3 retains potent μ-receptor affinity and high selectivity similar to I. Since I, V and VI resist enzymatic degradation, it is expected that these peptides are excellent reagents for studying the function of μ-receptor-mediated biological properties *in vitro* and *in vivo*.

TABLE II. PBQ-Induced Writhing Test of Highly μ-Selective Analogs

Peptide	ED_{50}[a] (μg/kg, s.c.)
Tyr–D-Arg–Phe–βAla	24.9 (18.3—33.9)
I	56.6 (46.2—69.3)
V	134 (107—168)
VI	140 (112—174)

a) The 95% confidence limits are given in parentheses.

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% trifluoroacetic acid (TFA) in H_2O and B, 80% acetonitrile in 0.06% TFA. A linear gradient from 10% B to 50% B over 40 min at a flow rate of 1.2 ml/min was used, and the eluate was monitored at 215 nm. 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. Fluorinated amino acids were a generous gift from Asahi Glass Co., Ltd.

Solid Phase Peptide Synthesis The peptide was constructed on Boc–βAla–benzhydrylamine resin (0.85 meq/g, 1% cross-linked, 100—200 mesh) according to the coupling schedule described previously.[3] Boc amino acids with side chain protecting groups, 2-bromobenzyloxycarbonyl for Tyr and tosyl for D-Arg, were used. The hydroxyl group of fluorinated Tyr was left unprotected. 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 resulting residue was extracted with 5% AcOH. The extract was washed with ether and evaporated to dryness under vacuum. The crude peptide was purified on a Develosil LOP ODS 24S (Nomura Kagaku) column (24 × 360 cm) which was eluted with a linear gradient of 12—32% acetonitrile in 0.1% TFA over 150 min at a flow rate of 3 ml/min. The eluate was monitored at 280 nm. Fractions around the main peak were checked by analytical HPLC and the 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. Analytical data of peptides synthesized are shown in Table III.

Opioid Receptor Binding Assay The binding assay was performed by the method previously described in detail.[1a] [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]U-69593 were used as radioligands for μ, δ and κ-receptors, respectively. Inhibition constants (K_i) were calculated from IC_{50} values using the relation of $K_i = IC_{50}/(1 + L/K_d)$,[15] where L is a concentration of a radioligand and K_d is its equilibrium dissociation constant. The IC_{50} values were determined from log dose-displacement curves. The K_d values of [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]U-69593 used are 0.46, 3.43 and 0.62, respectively.

TABLE III. Analytical Data of Synthetic Peptides

Compound	$[\alpha]_D$[a] (°)	TLC[b]		Amino acid analysis				HPLC[b]
		Rf(A)	*Rf*(B)	Tyr[c]	D-Arg	Phe[c]	βAla[d]	t_R (min)
I	+29.8	0.33	0.68	0.97	1.14	1.00	0.95	19.0
II	+30.7	0.37	0.75	0.68	1.00	0.55[c]	1.00	20.0
III	+35.1	0.36	0.74	0.88	1.00	+[e]	+[e]	20.9
IV	+28.3	0.36	0.76	0.92	1.00	0.89[c]	1.12	20.9
V	+29.3	0.38	0.74	+[f]	1.00	0.93	+[f]	20.0
VI	+29.1	0.35	0.74	0.92[c]	1.00	0.98	1.00	19.9
VII	+33.9	0.41	0.74	+[f]	1.00	0.87	+[f]	22.0

a) Optical rotation was measured in 1% AcOH (c = 0.5) at 20 °C. *b*) See Experimental. *c*) See ref. 3 for retention times of fluorinated Tyr and Phe on amino acid analyzer. *d*) βAla was eluted just before Phe. *e*) Phe(3F) + βAla = 1.19 as Phe. *f*) Tyr(3F) + βAla = 2.20 as Phe.

Enzymatic Stability of Peptides in a Rat Brain Homogenate A peptide (100 μg) was incubated with 400 μl of crude rat synaptosomal membrane fractions[1a] (0.73 mg protein/ml) in 50 mM Tris–HCl buffer at pH 7.40 at 36 °C for 5 h. The reaction was stopped by addition of 0.2 M HCl (50 μl). After centrifugation at 10000 r.p.m. for 10 min, an aliquot of the supernatant was applied to analytical HPLC. No degradation products were detected on HPLC in any sample of peptides synthesized in this study. Under the incubation conditions, Met-enkephalin was degraded completely within 40 min.

PBQ Writhing Test Groups of six male mice of the ICR Strain weighing 15—18 g were used. Peptides dissolved in saline solution or saline alone were administered subcutaneously in a dose volume of 10 ml/kg, and 15 min later the mice were injected with PBQ (2.5 mg/kg) intraperitoneally. Five minutes after the PBQ injection, abdominal torsion and contraction was counted for 10 min. ED_{50} values were defined as the dose of peptides capable of reducing writhing in saline-injected mice by 50%. ED_{50} and 95% confidence limits were determined by the method of Litchfield and Wilcoxon.[16]

References and Notes

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2) Amino acids and peptides are of L-configuration unless otherwise noted. Abbreviations used are: Phe(2F) = 2-fluorophenylalanine, Phe(3F) = 3-fluorophenylalanine, Phe(4F) = 4-fluorophenylalanine, Tyr(2F) = 2-fluorotyrosine, Tyr(3F) = 3-fluorotyrosine, Boc = *tert*-butoxycarbonyl, DAGO = [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, DPDPE = [D-Pen^2, D-Pen^5]enkephalin (Pen = penicillamine), U-69593 = (5α, 7α, 8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide, PBQ = phenyl-1,4-benzoquinone, TLC = thin layer chromatography, HPLC = high-performance liquid chromatography.

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