Amino Acids and Peptides. X.[1] Leu-Enkephalin Analogues Containing a Fluorinated Aromatic Amino Acid

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Leu-enkephalin analogues containing a fluorinated derivative of Tyr[1] or Phe[4] were synthesized by the solid-phase method and their biological activities were studied. Fluorination of the *p*-position of Phe[4] resulted in a marked potentiation of the inhibitory effect on electrically induced contractions of guinea-pig ileum (GPI) and mouse *vas deferens* (MVD). On the other hand, substitution of fluorine for the hydroxyl group of Tyr[1] or introduction of a trifluoromethyl group at the *p*-position of Phe[4] markedly reduced the inhibitory effect in both preparations. Fluorination of other positions of Tyr[1] and Phe[4] did not cause substantial changes in the activities as compared with Leu-enkephalin.

Keywords Leu-enkephalin; fluorinated aromatic amino acid; fluorinated peptide; opioid peptide; analgesic peptide

Since fluorine has the smallest ionic radius and the highest electronegativity among halogens, fluorinated compounds are of interest in connection with the development of medicines. 5-Fluorouracil is well known as an antineoplastic agent and many fluorinated steroids are used as anti-inflammatory agents. Here we report the synthesis and biological activities of Leu-enkephalin analogues containing a fluorinated aromatic amino acid.

Gesellchen *et al.*[2a] reported that the analgesic activity of [Phe(*p*-F)[4], MeMet[5]]-enkephalin amide was stronger than that of [MeMet[5]]-enkephalin amide. They also reported that [Tyr(*m*-F)[1], D-Ala[2], MeMet[5]]-enkephalin amide[2b] showed weaker activity in the mouse *vas deferens* (MVD) test and stronger activity in the mouse hot plate-jump test compared with [D-Ala[2], MeMet[5]]-enkephalin amide. These results suggested the possible potentiation by fluorination of the opioid activity of enkephalin. Before starting the synthesis of fluorinated enkephalin, the stability of a fluorinated amino acid to various synthetic conditions was examined and the results are shown in Fig. 1. Tyr(*o*-F) was treated with trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA) in TFA, trimethylsilyltrifluoromethanesulfonate (TMSTf) in TFA, methanesulfonic acid (MSA), and HF, and subjected to hydrogenation over a Pd catalyst. One hundred-fold molar excess of the acids was used at room temperature for 2 h and hydrogenation was performed at room temperature for 1 h. The results were checked by high-performance liquid chromatography (HPLC) on an Asahipak GS column. As shown in Fig. 1, an extra peak corresponding to Tyr appeared after hydrogenation and minor peaks were detected after MSA, TFMSA, and TMSTf treatments. Since TFA and HF treatments did not give any extra peak, these treatments were employed in our synthesis.

α-Amino groups were protected with the *tert*-butoxycarbonyl(Boc) group which is removable by TFA treatment. The Boc group was introduced onto fluorinated Phe with 2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile (Boc–ON)[3] and onto fluorinated Tyr with *tert*-butyl *S*-4,6-dimethylpyrimidin-2-yl thiolcarbonate (Boc–SDP).[4] Boc–SDP gave only *N*-Boc–Tyr–OH, while Boc–ON gave a mixture of *N*,*O*-diBoc–Tyr–OH and *N*-Boc–Tyr–OH. Peptide synthesis was done by an automated peptide synthesizer and final deprotection was done by HF treatment. The deblocked materials were purified by reversed-phase HPLC. The following seven Leu-enkephalin analogues (KKF-1–KKF-7) were synthesized: KKF-1, [Tyr(*o*-F)[1]]; KKF-2, [Tyr(*m*-F)[1]]; KKF-3, [Phe(*p*-F)[1]]; KKF-4, [Phe(*o*-F)[4]];

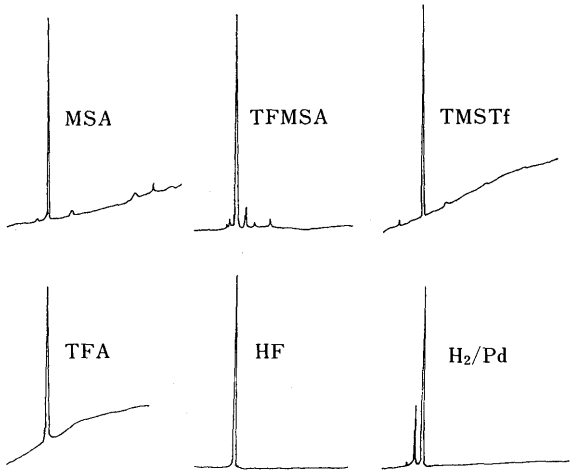

Fig. 1. HPLC of Acid-Treated and Hydrogenated H–Tyr(*o*-F)–OH on Asahipak GS-320 (7.6 × 500 mm)

Eluent, 0.1% TFA/H_2O. Flow rate, 2.0 ml/min. Absorption, 220 nm. Retention time for the major peak corresponding to H–Tyr(*o*-F)–OH was 12 min. Retention time for the minor peak corresponding to H–Tyr–OH in the hydrogenated sample was 9 min.

TABLE I. Amino Acid Ratios in Enzymatic Digests

	Tyr[a]	Gly	Phe[b]	Leu	Average recovery (%)
Leu-enkephalin[c]	1.00	2.11	0.92	0.91	73
KKF-1[c]	1.00	2.22	0.93	1.05	76
KKF-2[c]	1.00	2.25	0.92	1.00	82
KKF-3[c]	1.00	2.27	1.15	1.14	90
KKF-4[c]	1.00	2.08	0.71	0.89	76
KKF-5[c]	1.00	2.08	0.82	1.07	73
KKF-6[c]	1.00	2.00	0.87	1.00	79
KKF-7[c]	1.00	2.28	1.15	1.02	78
H–Phe–Leu–OH[d]			1.00	0.92	81
H–Phe(*p*-F)–Leu–OH[d]			1.00	1.06	79

a) Tyr or fluorinated Tyr. *b*) Phe or fluorinated Phe. *c*) AP-M digest, Tris buffer pH 7.5, 37 °C, 24 h. *d*) Chymotryptic digest, 0.1 M phosphate buffer pH 8.0, 24 h.

KKF-5, [Phe(*m*-F)[4]]; KKF-6, [Phe(*p*-F)[4]]; KKF-7, [Phe(*p*-CF_3)[4]]. Synthetic Leu-enkephalin analogues and H–Phe (*p*-F)–Leu–OH were exposed to enzymes, and the results are shown in Table I. Fluorinated Leu-enkaphalin analogues were hydrolyzed by aminopeptidase M (AP-M) and H–Phe (*p*-F)–Leu–OH was further hydrolyzed completely by chymotrypsin. These results showed that fluorination on the aromatic ring of the amino acid did not affect enzymatic hydrolysis of the peptide. The inhibitory effect of the synthetic fluorinated Leu-enkephalin analogues on electrically induced contractions of guinea pig ileum (GPI) and MVD[5] were examined and the results are summarized in Table II.

In contrast to the previous report[6] that modification of the Tyr^1 residue in enkephalin cause a decrease of opioid activities, KKF-1 showed almost equal inhibitory potency with Leu-enkephalin on the electrically induced contraction of GPI and MVD assays. However, as expected, KKF-2 was 4 times and KKF-3 was almost 100 times less effective than Leu-enkephalin in both tissue preparations. KKF-1 is the first enkephalin analogue that does not show decreased opioid activity of enkephalin as a consequence of modification of the phenol ring of Tyr.

In spite of the isosteric character of –F and $–O^-$, fluorine substitution for the –OH group (KKF-3) caused a marked loss of activity. This result with KKF-3 supports Ling's report that substitution of phenolic –OH for –I, –Cl, $–NO_2$, and $–NH_2$ in Met-enkephalin caused a reduction in opioid activity.[7]

Similarly, in a series of Leu-enkephalin analogues fluorinated at the Phe^4 residue, fluorination at the *m*-position (KKF-5) did not affect the potency of the parent compound, but fluorination at the *p*-position resulted a marked increase of the inhibitory effect on both GPI and MVD preparations. Introduction of a CF_3 group into the *p*-position of Phe^4 (KKF-7) reduced the inhibitory effect on both isolated preparations.

The inhibition of the contractions by Leu-enkephalin and all synthetic peptides in GPI and MVD preparation was prevented by naloxone. The pA_2 values[8] of fluorinated analogues with naloxone were 8.33—8.54 and 7.33—7.79 for GPI and MVD, respectively.

TABLE II. Opioid Activity of the Synthetic Peptides on GPI and MVD

	GPI IC_{50} (nM)	MVD IC_{50} (nM)
Morphine	68.0±5.0	290±50
Leu-enkephalin	216±36	18.9±2.1
KKF-1	194±40	19.3±2.3
KKF-2	750±90	93.0±21.0
KKF-3	21000±1500	2000±300
KKF-4	280±52	15.2±2.1
KKF-5	218±32	11.8±3.5
KKF-6	28.0±7.5	1.2±0.2
KKF-7	2400±600	275±15

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography (TLC) on Silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^2 = BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf^3 = $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf^4 = AcOEt–benzene (1:1), Rf^5 = $CHCl_3$–AcOH–MeOH (90:2:8). Amino acid compositions of acid and enzymatic hydrolysates were determined with a Kyowa K-101 AS amino acid analyzer and a Hitachi 835 amino acid analyzer. Fluorinated amino acids were purchased from Asahi Glass Inc. Stabilities of these fluorinated amino acids to the conditions of acid hydrolysis (6 N HCl, 20 h) were not studied in detail, but no extra peak was detected in acid hydrolysates of fluorinated amino acid-containing peptides by the amino acid analyzer. Furthermore, no extra peak was detected by the amino acid analyzer when H–Phe (*p*-F)–OH and H–Phe (*p*-CF_3)–OH were exposed separately to the above conditions.

Stability of H–Tyr(*o*-F)–OH to Various Deprotection Procedures H–Tyr(*o*-F)–OH (1 mg, 5 μM) was treated with the reagents listed below at room temperature for 2 h. Chilled ether was added to the mixture and the resulting precipitate was collected by centrifugation, washed with ether and dried. The dried sample was checked by HPLC using Asahipak GS-320 (7.6 × 500 mm) as shown in Fig. 1.

1, MSA (33 μl, 0.5 mM); 2, TFMSA (44 μl, 0.5 mM) + TFA (39 μl, 0.5 mM); 3, TMSTf (98 μl, 0.5 mM) + TFA (39 μl, 0.5 mM).

HF Treatment was done under the following conditions.

H–Tyr(*o*-F)–OH (5 mg, 25 μM) in HF (5 ml), 0 °C, 90 min.

H–Tyr(*o*-F)–OH (5 mg, 25 μM) was hydrogenated over Pd catalyst in 10% AcOH (3 ml) for 30 min.

Samples treated with HF and H_2/Pd were checked by HPLC as described above.

TABLE III. Yields, Rotations, and Analysis of Synthetic Peptides

	Yields (%)[a]	$[\alpha]_D^{23}$ (°)	Elemental analysis	Calcd (Found) C	H	N	Amino acid ratios in an acid hydrolysate Tyr[b]	Gly	Phe[c]	Leu (Average recovery, %)
KKF-1	41	+13.6 (c=0.5, H_2O)	$C_{28}H_{36}FN_5O_7 \cdot HCl \cdot 8H_2O$	44.6 (44.4)	7.1 6.8	9.3 9.4)	0.91	2.19	1.00 (84)	1.00
KKF-2	32	+11.9 (c=0.5, H_2O)	$C_{28}H_{36}FN_5O_7 \cdot HCl \cdot 3/4\,H_2O$	52.4 (52.1)	6.4 6.1	10.9 11.3)	0.91	2.06	0.91 (80)	1.00
KKF-3	42	+16.6 (c=0.5, H_2O)	$C_{28}H_{35}FN_5O_6 \cdot HCl \cdot 2H_2O$	53.4 (53.4)	6.6 6.3	11.1 11.1)	0.79	2.18	0.92 (86)	1.00
KKF-4	72	+13.2 (c=1.0, H_2O)	$C_{28}H_{36}FN_5O_7 \cdot HCl \cdot 3/2\,H_2O$	52.8 (52.8)	6.3 6.1	11.0 11.0)	1.05	2.06	1.15 (87)	1.00
KKF-5	78	+18.8 (c=1.0, H_2O)	$C_{28}H_{36}FN_5O_7 \cdot HCl \cdot 3/2\,H_2O$	52.8 (52.6)	6.3 6.2	11.0 11.0)	1.00	2.01	1.07 (88)	1.00
KKF-6	83	+16.7 (c=1.0, H_2O)	$C_{28}H_{36}FN_5O_7 \cdot HCl \cdot 4H_2O$	49.3 (49.2)	6.7 6.7	10.3 10.2)	0.97	2.00	1.07 (84)	1.00
KKF-7	80	+12.0 (c=1.0, 0.2 N HCl)	$C_{29}H_{36}F_3N_5O_7 \cdot HCl \cdot 3/2\,H_2O \cdot 1/2\,C_4H_8O_2$[d]	50.2 (50.5)	6.1 5.9	9.6 9.2)	0.97	2.00	1.13 (78)	1.00

a) Yields were calculated from starting resins. *b*) Tyr or fluorinated Tyr. *c*) Phe or fluorinated Phe. *d*) $C_4H_8O_2$, dioxane. Peptides except KKF-7 were lyophilized from H_2O in the presence of 1 N HCl. KKF-7 was lyophilized from a mixture of H_2O, dioxane, and 1 N HCl.

Boc–Tyr(*o*-F)–OH Prepared from H–Tyr (*o*-F)–OH(1 g, 5 mM) and Boc–SDP(1.33 g, 5.5 mM) according to the usual method. Precipitated from AcOEt–petroleum ether. Yield 1.26 g (84%), mp 71 °C, Rf^5 0.42, $[\alpha]_D^{23}$ +4.0° (c=1.0, MeOH). *Anal.* Calcd for $C_{14}H_{18}FNO_5$: C, 56.2; H, 6.1; N, 4.7. Found: C, 55.9; H, 6.3; N, 4.5.

Boc–Tyr(*m*-F)–OH Prepared from H–Tyr(*m*-F)–OH(1 g, 5 mM) and Boc–SDP(1.33 g, 5.5 mM) in the usual manner. Precipitated from AcOEt–petroleum ether. Yield 1.48 g (98%), mp 68°C, Rf^5 0.31, $[\alpha]_D^{23}$ +9.1° (c=1.0, MeOH). *Anal.* Calcd for $C_{14}H_{18}FNO_5$: C, 56.2; H, 6.1; N, 4.7. Found: C, 55.9; H, 6.3; N, 4.7.

Boc–Phe(*o*-F)–OH Prepared from H–Phe(*o*-F)–OH(1 g, 5.5 mM) and Boc–ON (1.48 g, 6 mM) in the usual manner. Precipitated from AcOEt/petroleum ether. Yield 1.46 g (93%), mp 82 °C, Rf^5 0.54, $[\alpha]_D^{23}$ −2.4° (c=1.0, MeOH). *Anal.* Calcd for $C_{14}H_{18}FNO_4$: C, 59.3; H, 6.4; N, 5.0. Found: C, 59.5; H, 6.4; N, 5.4.

Boc–Phe(*m*-F)–OH Prepared from H–Phe(*m*-F)–OH(1 g, 5.5 mM) and Boc–ON (1.48 g, 6 mM) in the usual manner. Precipitated from AcOEt–petroleum ether. Oily material. Yield 0.92 g (59%), Rf^5 0.64, $[\alpha]_D^{23}$ +8.2° (c=1.0, MeOH). The oily material (100 mg, 0.35 mM) was converted to its cyclohexylamine salt in ether and recrystallized from ether. Yield 46 mg (35%), mp 106 °C, $[\alpha]_D^{25}$ +32.6° (c=1.0, MeOH). *Anal.* Calcd for $C_{14}H_{18}FNO_4 \cdot C_6H_{11}N \cdot H_2O$: C, 62.8; H, 8.2; N, 7.3. Found: C, 62.6; H, 8.4; N, 7.4.

Boc–Phe(*p*-F)–OH Prepared from H–Phe(*p*-F)–OH(1 g, 5.5 mM) and Boc–ON(1.48 g, 6 mM) in the usual manner. Precipitated from AcOEt–petroleum ether. Yield 1.24 g (79%), mp 68 °C, Rf^5 0.57, $[\alpha]_D^{23}$ +7.8° (c=1.0, MeOH). *Anal.* Calcd for $C_{14}H_{18}FNO_4$: C, 59.3; H, 6.4; N, 4.9. Found: C, 59.4; H, 6.1; N, 4.5.

Boc–Phe(*p*-CF_3)–OH Prepared from H–Phe(*p*-CF_3)–OH (0.5 g, 2.1 mM) and Boc–ON (0.58 g, 2.4 mM) in the usual manner. Precipitated from AcOEt–petroleum ether. Yield 0.62 g (89%), mp 86 °C, $Rf^5$0.71, $[\alpha]_D^{23}$ +3.9° (c=1.0, MeOH). *Anal.* Calcd for $C_{15}H_{18}F_3NO_4$: C, 54.0; H, 5.5; N, 4.2. Found: C, 54.2; H, 5.0; N, 4.4.

Peptide Synthesis A peptide synthesizer, model 430A (Applied Biosystems Inc.) was used. Boc–Leu–PAM resin (0.5 mM/cartridge) was purchased from Applied Biosystems Inc. The α-amino group was protected with a Boc group and the phenolic group of Tyr was protected with a Z(2-Br) group. The phenolic group of fluorinated Tyr was not protected. These amino acids were reacted in the presence of HOBt. Final deprotection was done by HF treatment in the presence of anisole at 0 °C for 90 min and the product was purified by HPLC using a YMC D-ODS-5 column (20 × 250 mm) and 0.1% TFA-containing 30% CH_3CN–H_2O as the eluent. The analytical data of the products are summarized in Table III.

Opioid Activities of the Synthetic Peptides Inhibitory effects of the synthetic peptides on the electrically induced contraction of GPI and MVD were determined according to the methods reported in the previous paper.[5] The results are given in Table II.

References and Notes

1) Standard abbreviations for amino acids, protecting groups, and peptides are used [*Eur. J. Biochem.*, **138**, 9 (1984)]. Other abbreviations include: DMF = dimethylformamide, Tyr(*o*-F) = L-*o*-fluorotyrosine, Tyr(*m*-F) = L-*m*-fluorotyrosine, Phe(*p*-F) = L-*p*-fluorophenylalanine, Phe(*o*-F) = L-*o*-fluorophenylalanine, Phe(*m*-F) = L-*m*-fluorophenylalanine, Phe(*p*-CF_3) = L-*p*-trifluoromethylphenylalanine.
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