Amino Acids and Peptides. XXIII.[1] Leu-Enkephalin Analogs Containing a Fluorinated Amino Acid at Position 2, 4 or 5

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Received February 25, 1994; accepted April 12, 1994

Fluorinated analogs of Leu-enkephalin were synthesized by the solution method and the solid-phase method. The synthetic peptides were examined for opioid activities on mouse *vas deferens* and guinea pig ileum. Among the synthetic peptides,[D-Ala^2,Leu(F_3)(2*R*,4*S*)^5]enkephalin and [D-Ala^2,Leu(F_3)(2*S*,4*R*)^5]enkephalin exhibited potent opioid activity, and [Leu(F_3)(2*S*,4*R*)^5]enkephalin exhibited high δ-receptor selectivity.

Keywords opioid peptide; fluorinated amino acid; enkephalin; opioid receptor; mouse *vas deferens*; guinea pig ileum

Fluorination of organic compounds has been used to modify pharmacological and biological activities. Previously we synthesized Leu-enkephalin(Leu-Enk, H–Tyr–Gly–Gly–Phe–Leu–OH) analogs containing fluorine in the aromatic ring of Tyr[1] or Phe[4], and examined their opioid activity.[2] The *p*-fluorophenylalanine[4] analog (H–Tyr–Gly–Gly–Phe(*p*-F)–Leu–OH) exerted more potent activity than Leu-Enk on mouse *vas deferens* (MVD) and on guinea pig ileum (GPI). Sasaki *et al.* reported that [D-Ala^2, Phe(*p*-F)^4, D-Leu^5]Enk had similar affinity to H–Tyr–D-Ala–Gly–Phe–D-Leu–OH (DADLE) in opioid receptor binding assay.[3] Coy *et al.* found that [D-Ala^2, pentafluorophenylalanine^4]Met-enkephalinamide exhibited more potent analgesic activity than the nonfluorinated parent compound.[4] Stimulated by these studies, we have synthesized several new Leu-Enk analogs with a fluorinated amino acid at position 2, 4 or 5.

H–Tyr–Val(F_6)–Gly–Phe–Leu–OH [I] and H–Tyr–D-Val(F_6)–Gly–Phe–Leu–OH [II], each containing the fluorinated aliphatic amino acid, 4,4,4,4,4,4-hexafluorovaline (Val(F_6)) at position 2, were synthesized by an automatic solid-phase method. H–Tyr–Gly–Gly–Phg(*o*-F)–Leu–OH [III] and H–Tyr–Gly–Gly–D-Phg(*o*-F)–Leu–OH [IV], each containing *o*-fluorophenylglycine (Phg(*o*-F)) at position 4, were also similarly synthesized. The starting materials were Boc–L-amino acids and alternatives, *i.e.* Boc–DL-Val(F_6)–OH and Boc–DL-Phg(*o*-F)–OH, which were derived from DL-Val(F_6) and DL-Phg(*o*-F), respectively. Solid phase peptide synthesis started with Boc–Leu–resin, to which Boc-amino acids or alternatives were sequentially coupled in the usual manner. Each protected peptide-resin was treated with HF,[5] and purified by HPLC. Crude H–Tyr–Gly–Gly–DL-Phg(*o*-F)–Leu–OH showed two main peaks (Fig. 1). The first and second peaks gave almost identical amino acid ratios after 6 N HCl hydrolysis. But after aminopeptidase-M (AP-M) digestion, the amino acid ratio of the first peak was Tyr : Gly + Phg(*o*-F) : Leu = 1.00 : 2.72 : 0.95, while that of the second peak was Tyr : Gly + Phg(*o*-F) : Leu = 1.00 : 1.15 : (not detected). This implies that the first peak was due to H–Tyr–Gly–Gly–Phg(*o*-F)–Leu–OH [III], and the second peak to H–Tyr–Gly–Gly–D-Phg(*o*-F)–Leu–OH [IV]. H–Tyr–Val(F_6)–Gly–Phe–Leu–OH [I] and H–Tyr–D-Val(F_6)–Gly–Phe–Leu–OH [II] were each purified on a preparative HPLC column in the same way as described for III and IV.

H–Tyr–Gly–Gly–Phe–Leu(F_3)–OH, [Leu(F_3)^5]Enk, contains 5,5,5-trifluoro-leucine(Leu(F_3)) instead of the *C*-terminal leucine. Since Leu(F_3) has two chiral carbons in the molecule, [Leu(F_3)^5]Enk comprises four isomers (2*R*,4*R*), (2*R*,4*S*), (2*S*,4*S*) and (2*S*,4*R*), which correspond to H–Tyr–Gly–Gly–Phe–Leu(F_3)(2*R*,4*R*)–OH [V], H–Tyr–Gly–Gly–Phe–Leu(F_3)(2*R*,4*S*)–OH [VI], H–Tyr–Gly–Gly–Phe–Leu(F_3)(2*S*,4*S*)–OH [VII] and H–Tyr–Gly–Gly–Phe–Leu(F_3)(2*S*,4*R*)–OH [VIII], respectively. The 2*S*-isomer is a substitute for L-Leucine, and the 2*R*-isomer for D-Leucine. [D-Ala^2,Leu(F_3)^5]Enk also com-

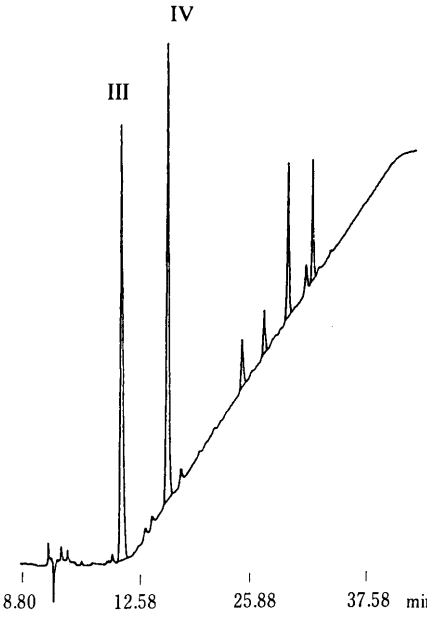

Fig. 1. HPLC Profile of Crude H–Tyr–Gly–Gly–Phe(*o*-F)–Leu–OH

III: H–Tyr–Gly–Gly–Phg(*o*-F)–Leu–OH, IV: H–Tyr–Gly–Gly–D-Phg(*o*-F)–Leu–OH, Column: YMC-ODS (AM-303, 4.6 × 250 mm). Solvent A, 0.1% TFA/H_2O; B, 0.1% TFA/CH_3CN. A linear gradient: 25% B/A (0 min)→45% B/A (30 min). Flow rate, 1.0 ml/min. UV, 220 nm.

prises four isomers: H–Tyr–D-Ala–Gly–Phe–Leu(F_3)-(2*R*,4*R*)–OH [IX], H–Tyr–D-Ala–Gly–Phe–Leu(F_3)-(2*R*,4*S*)–OH [X], H–Tyr–D-Ala–Gly–Phe–Leu(F_3)-(2*S*,4*S*)–OH [XI] and H–Tyr–D-Ala–Gly–Phe–Leu(F_3)-(2*S*,4*R*)–OH [XII]. These eight compounds (V, VI, VII, VIII, IX, X, XI, XII) were synthesized as shown in Fig. 2. Z–Tyr(Bzl)–Gly–Gly–Phe–N_2H_3 and Z–Tyr(Bzl)–D-Ala–Gly–Phe–N_2H_3, each prepared *via* stepwise elongation by the solution method, were each coupled with the corresponding Leu(F_3)(2*R*,4*R*; 2*R*,4*S*; 2*S*,4*S*; 2*S*,4*R*) by the usual azide method.[7] The crude protected peptides were hydrogenated over Pd catalyst and purified. We also synthesized [Leu(F_3)^2]Leu-Enk analogs, H–Tyr–Leu(F_3)(2*R*,4*R*)–Gly–Phe–Leu–OH [XIII], H–Tyr–Leu(F_3)(2*R*,4*S*)–Gly–Phe–Leu–OH [XIV], H–Tyr–Leu(F_3)(2*S*,4*S*)–Gly–Phe–Leu-OH [XV], H–Tyr–Leu(F_3)(2*S*,4*R*)–Gly–Phe–Leu–OH [XVI] by a manual solid-phase method using benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)[8] as the coupling agent. Four isomers of Boc–Leu(F_3)–OH were used for the synthesis. The final deprotection was done by HF treatment and products were purified by HPLC. Each isomeric peptide had a distinct retention time on HPLC.

In a previous study, substitution with *o*-fluorotyrosine (Tyr(*o*-F)) or *m*-fluorotyrosine (Tyr(*m*-F)) for Tyr at position 1, and substitution with *p*-fluorophenylalanine (Phe(*p*-F)) for Phe at position 4, tended to increase opioid

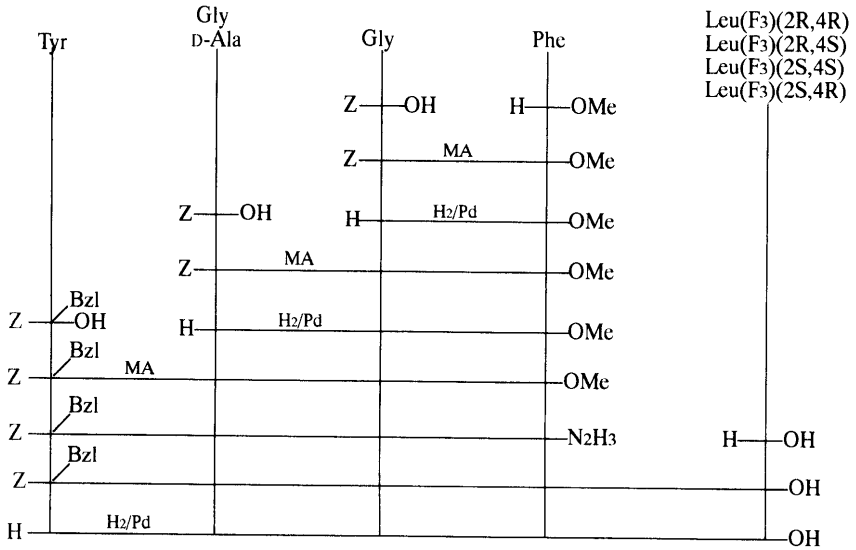

Fig. 2. Synthetic Scheme for V—XII

MA: mixed anhydride method.

TABLE I. Inhibitory Effects of the Synthetic Peptides on the Electrically Induced Contractions of Isolated Smooth Muscle Preparations

	Compound	GPI		MVD		GPI (IC_{50}) / MVD (IC_{50})
		IC_{50} (nM)	Relative activity	IC_{50} (nM)	Relative activity	
	Leu-Enk	209	100	10	100	21
	[D-Leu^5]Enk	1000	21	21	48	48
	DALDA	155	135	830	1	0.2
	DTLET	20	1045	0.07	14300	286
I	[Val(F_6)^2]Leu-Enk	Inactive	—	26800	0.04	—
II	[D-Val(F_6)^2]Leu-Enk	1300	16	775	1	2
III	[Phg(*o*-F)^4]leu-Enk	Inactive	—	>100 μM	<0.01	—
IV	[D-Phg(*o*-F)^4]Leu-Enk	>100 μM	<0.2	8400	0.1	—
V	[Leu(F_3)(2*R*,4*R*)^5]Enk	2650	8	113	9	23
VI	[Leu(F_3)(2*R*,4*S*)^5]Enk	4310	5	74	14	58
VII	[Leu(F_3)(2*S*,4*S*)^5]Enk	398	53	25	40	16
VIII	[Leu(F_3)(2*S*,4*R*)^5]Enk	1010	21	7	143	144
IX	[D-Ala^2,Leu(F_3)(2*R*,4*R*)^5]Enk	43	486	9	127	5
X	[D-Ala^2,Leu(F_3)(2*R*,4*S*)^5]Enk	35	597	2	500	18
XI	[D-Ala^2,Leu(F_3)(2*S*,4*S*)^5]Enk	43	486	5	200	9
XII	[D-Ala^2,Leu(F_3)(2*S*,4*R*)^5]Enk	33	633	2	500	17
XIII	[Leu(F_3)(2*R*,4*R*)^2]Leu-Enk	75	279	117	9	0.6
XIV	[Leu(F_3)(2*R*,4*R*)^2]Leu-Enk	303	69	558	2	0.5
XV	[Leu(F_3)(2*S*,4*S*)^2]Leu-Enk	22557	1	12708	0.1	2
XVI	[Leu(F_3)(2*S*,4*R*)^2]Leu-Enk	3467	6	5041	0.2	0.7
XVII	[Tyr(*o*-F)^1,Phe(*p*-F)^4]Leu-Enk	360	58	19	53	19
XVIII	[Tyr(*m*-F)^1,Phe(*p*-F)^4]Leu-Enk	3000	7	398	3	8
XIX	[Phe(*p*-F)^3]DALDA	2100	10	106	9	20
XX	[Phe(*p*-F)^4]DTLET	24	871	0.4	2500	60

activity. This encouraged us to synthesize the analogs, H–Tyr(*o*-F)–Gly–Gly–Phe(*p*-F)–Leu–OH [XVII], H–Tyr(*m*-F)–Gly–Gly–Phe(*p*-F)–Leu–OH [XVIII], H–Tyr–D-Arg–Phe(*p*-F)–Lys–NH_2 [XIX], whose parent peptide H–Tyr–D-Arg–Phe–Lys–NH_2(DALDA)[9] has high μ-selectivity, and H–Tyr–D-Thr–Gly–Phe(*p*-F)–Leu–Thr–OH [XX], whose parent peptide H–Tyr–D-Thr–Gly–Phe–Leu–Thr–OH (DTLET)[10] has high δ-selectivity. These syntheses were also carried out by the solid-phase method as mentioned above.

The IC_{50} values, the relative activity and the IC_{50} (GPI)/IC_{50} (MVD) ratio are summarized in Table I. All compounds, except I and III in the GPI test, dose-dependently inhibited electrically induced contraction of both GPI and MVD preparations.

In line with the earlier reports on DALDA, and DTLET, we found that DALDA possesses typical μ-opioid receptor agonistic activity and DTLET a typical δ-receptor agonist characteristic; the value of IC_{50} (GPI)/IC_{50} (MVD) ratio of DALDA and DTLET were 0.2 and 286, respectively. The inhibitory effects of compounds I—IV were lower than that of Leu-Enk in GPI and MVD preparations. The IC_{50} values of GPI and MVD indicate that the [Leu(F_3)^5]Enk compounds V—VIII, as compared with the parent compound Leu-Enk, showed decreased potencies in inhibiting the contractions of the tissue preparations with the exception of a slight increase in the potency of VIII in MVD preparations. Consequently, the δ-receptor selectivity of compound VIII was 7 times that of Leu-Enk.[11] Meanwhile, [D-Ala^2,Leu(F_3)^5]Enk analogs IX—XII, obtained by introducing D-Ala^2 into [Leu(F_3)^5]Enk analogs V—VIII possessing weak activity, produced higher inhibitory activity than Leu-Enk in both preparations, and more stable potentiation of the activity was found in the GPI preparation than in the MVD. Thus, the potentiation by the D-Ala^2 substitution may be attributed to the increase of resistance to enzymatic hydrolysis of the Tyr^1–Gly^2 bond. Fluorination of a drug molecule may modify the hydrophobic character and the electron density of the drug molecule[12] and may increase the activity of the drug. Coy *et al.*[4] and Walker *et al.*[13] reported enhancement of the pharmacological effect by the fluorination of [D-Ala^2]Met-enkephalinamide and dynorphin 1—13, respectively. The substitution of fluorinated Leu for Gly^2, as in compounds XIII—XVI, led to a substantial decrease in ability to inhibit the contraction of both preparations, except that compound XIII showed a somewhat increased activity in GPI preparation. The selectivity of a series of [Leu(F_3)^2]Enk analogs XIII—XVI, for μ-receptor was at least 10 times that of [Leu(F_3)^5]Enk and Leu-Enk. Fluorination at Tyr^1 and Phe^4 of Leu-Enk, analogs XVII and XVIII, and that at Phe^3 of DALDA, compound XIX, resulted in decreased inhibitory activity in both preparations. Similarly, monofluorinated DTLET at Phe^4, compound XX, showed a weaker inhibitory effect on the contractions of MVD preparation than the parent nonfluorinated DTLET.

The results, taken together, suggest that the structural changes of the peptide including the alteration of molecular size, conformation, electron density and hydrophobic character of the peptide, due to the introduction of fluorine tends to decrease the affinity for opioid receptors, and in some cases, changes the selectivity for the receptor types *in vitro*; however, from the differences among Leu-Enk and [Leu(F_3)^5]Enk analogs V—VIII in inhibiting the contractions of the preparations, the configuration of [Leu(F_3)(2*S*,4*R*)^5]Enk seems to be more capable of binding to δ-opioid receptors of MVD than that of Leu-Enk or other [Leu(F_3)^5]Enk. Additionally, the slight differences in the binding to GPI preparation among V—VIII as well as IX—XII indicate that the μ-opioid receptors in GPI preparation seem to be more flexible than the δ-receptors in MVD in accommodating the structural changes caused by fluorination of the ligands.

Experimental

Melting points are uncorrected. Solvent systems for ascending TLC on Silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = *n*-BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^3 = $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf^5 = $CHCl_3$–AcOH–MeOH (90:2:8). Optical rotations were measured with a JASCO DIP-360 polarimeter. RP-HPLC was conducted with a Waters 600 on YMC Pack AQ-ODS-5 using a mixture of 0.1% TFA-containing CH_3CN/H_2O as an eluent. Synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h. The amino acid compositions of acid and enzymatic hydrolysates were determined with a Hitachi 835 amino acid analyzer. Positive ion mass spectra were measured with a Hitachi model M-1000 quadrupole mass spectrometer. Negative ion mass spectra were measured with a Hitachi model M-2000 double-focusing mass spectrometer. 4,4,4,4,4,4-Hexafluoro-DL-valine and *o*-fluoro-DL-phenylglycine were purchased from PCR Incorporated. AP-M was purchased from Boehringer Mannheim GmbH. AP-M digestion was done by the method reported in our previous paper.[14]

Solid-Phase Peptide Synthesis Peptides were prepared by use of the Boc strategy. Resins were purchased from Peptide Institute, Inc. *p*-Methylbenzhydrylamine-resin (NH_2 content, 0.63 meq/g) was used for preparation of XIX. Chloromethylated polystyrene (divinylbenzene 1%, Cl content, 0.77 meq/g) was used for preparation of C-terminal carboxyl-free peptides. Boc-amino acid was introduced on chloromethylated resin by the cesium salt method.[15] For side chain protection, the Bzl group was used for Tyr and Thr, and the tosyl or nitro group was used for Arg. Final deprotection was performed by HF treatment. Synthetic peptide-resin was treated with 10% anisole/HF at 0 °C for 90 min. After removal of HF, the residue was extracted with 5% AcOH and the mixture was filtered. The filtrate was washed with ether and lyophilized. Crude peptides were purified by RP-HPLC. Peptides I, II, III, IV, XVII and XVIII were prepared by the automatic peptide synthesizer. Other peptides were prepared by the manual method using the protocol shown below. Peptides XVIII, XIV, XV and XVI were prepared using BOP[7] as a coupling agent. Final deprotection was performed by the HF method as described.

step	reagents	reaction time (min)	
1	5% NMM/DCM	10	×2
2	DCM	3	×3
3	Boc-amino acid (2 eq) in DMF (or DCM) 1 M DCC/DCM, 1 M HOBt/DMF [or 1 M BOP/DMF (2 eq) and 1.5% DIEA (4 eq)]	120	
4	DMF	3	×2
5	50% MeOH/DCM	5	×3
6	DCM	2	1
7	50% TFA/DCM	2	1
		45	1
8	DCM	3	×3

Boc-DL-Val(F_6)-OH Prepared from 4,4,4,4,4,4-hexafluoro-DL-valine (0.8 g, 3.6 mmol) and 2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile[16] (Boc-ON, 1.1 g, 4.3 mmol) in the usual manner. Precipitated from AcOEt/petroleum ether. Yield 1.07 g (93.1%), mp 116 °C, Rf^5 0.92. MS *m/z*: 324 $(M-H)^-$.

Boc-DL-Phg(*o*-F)-OH Prepared from H-DL-Phg(*o*-F)-OH (0.5 g,

3 mmol) and Boc-ON (0.9 g, 3.6 mmol) in the usual manner.[16] Precipitated from AcOEt/petroleum ether. Yield 0.8 g (99%), mp 121—124 °C, Rf^3 0.66. MS *m/z*: 268 $(M-H)^-$.

H-Tyr-Val(F_6)-Gly-Phe-Leu-OH [I] Prepared by the synthesizer and obtained in a pure form from the crude mixture of I and II by preparative HPLC. Yield 55 mg (7.4%), Rf^1 0.75, $[\alpha]_D^{27}$ +15.4° (*c* = 1.0, H_2O). MS *m/z*: 705 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.67, Val(F_6) 0.68, Gly 1.00, Phe 1.00, Leu 1.08 (average recovery 80%). Amino acid ratios in AP-M digest: Tyr 1.00, Val(F_6) 0.22, Gly 0.24, Phe 0.26, Leu 0.32 (recovery of Tyr 70%).

H-Tyr-D-Val(F_6)-Gly-Phe-Leu-OH [II] Prepared as described above. Yield 58 mg (15.6%), Rf^1 0.78, $[\alpha]_D^{27}$ +22.9° (*c* = 1.0, H_2O). MS *m/z*: 705 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.63, Val(F_6) 0.84, Gly 0.95, Phe 1.00, Leu 0.98 (average recovery 68%). No amino acid was detected in an AP-M digest.

H-Tyr-Gly-Gly-Phg(*o*-F)-Leu-OH [III] Prepared by the synthesizer and obtained from a mixture of crude III and IV by HPLC. Yield 45 mg (7.6%), Rf^1 0.76, $[\alpha]_D^{27}$ +8.4° (*c* = 1.0, H_2O). MS *m/z*: 559 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.98, Gly + Phg(*o*-F) 3.38, Leu 1.08 (average recovery 83%). Amino acid ratios in an AP-M digest: Tyr 1.00, Gly + Phg(*o*-F) 2.72, Leu 0.95 (recovery of Tyr 77%).

H-Tyr-Gly-Gly-D-Phg(*o*-F)-Leu-OH [IV] Prepared as described above. Yield 51 mg (8.6%), Rf^1 0.76, $[\alpha]_D^{27}$ +16.8° (*c* = 1.0, H_2O). MS *m/z*: 559 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.74, Gly + Phg(*o*-F) 3.21, Leu 1.00 (average recovery 81.4%). Amino acid ratios in AP-M digest: Tyr 1.00, Gly + Phg(*o*-F) 1.15, Leu not detected (recovery of Tyr 92%).

Z-Gly-Phe-OMe Z-Gly-OH (2.1 g, 10 mmol) and H-Phe-OMe·HCl (2.2 g, 10 mmol) were coupled in *N*,*N*-dimethyl formamide (DMF) by the mixed anhydride method in the usual manner.[17] Yield 3.63 g (98%), sirupy material, Rf^3 0.90, $[\alpha]_D^{27}$ −19.4° (*c* = 1.0, MeOH). MS *m/z*: 371 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Gly 1.02, Phe 1.00 (average recovery 87%).

Z-Gly-Gly-Phe-OMe Prepared from Z-Gly-OH (480 mg, 2.2 mmol) and H-Gly-Phe-OMe·HCl (600 mg, 2.2 mmol) by the mixed anhydride method. Yield 654 mg (70%), sirupy material, Rf^3 0.79, $[\alpha]_D^{27}$ −45.6° (*c* = 1.0, MeOH). MS *m/z*: 428 $(M+H)^-$. Amino acid ratios in an acid hydrolysate: Gly 2.02, Phe 1.00 (average recovery 84%).

Z-D-Ala-Gly-Phe-OMe Prepared from Z-D-Ala-OH (760 mg, 3.4 mmol) and H-Gly-Phe-OMe·HCl (930 mg, 3.4 mmol) by the mixed anhydride method. Precipitated from AcOEt/petroleum ether. Yield 1.18 g (78%), mp 102 °C, Rf^5 0.64, $[\alpha]_D^{27}$ −56.9° (*c* = 1.0, MeOH). MS *m/z*: 442 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Ala 1.00, Gly 1.04, Phe 1.00 (average recovery 93%).

Z-Tyr(Bzl)-Gly-Gly-Phe-OMe Prepared from Z-Tyr(Bzl)-OH (1.05 g, 2.6 mmol) and H-Gly-Gly-Phe-OMe (prepared from 1.11 g of Z-Gly-Gly-Phe-OMe by hydrogenation) by the mixed anhydride method. Precipitated from AcOEt/petroleum ether. Yield 1.28 g (72%), mp 140—142 °C, Rf^3 0.64, $[\alpha]_D^{27}$ −23.1° (*c* = 1.0, MeOH). MS *m/z*: 681 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.84, Gly 2.06, Phe 1.00 (average recovery 81%).

Z-Tyr(Bzl)-D-Ala-Gly-Phe-OMe Prepared from Z-Tyr(Bzl)-OH (1.05 mg, 2.6 mmol) and H-D-Ala-Gly-Phe-OMe (prepared from 840 mg of Z-D-Ala-Gly-Phe-OMe by hydrogenation) by the mixed anhydride method. Yield 0.98 g (54%), mp 140—145 °C, Rf^3 0.72, $[\alpha]_D^{27}$ −46.0° (*c* = 1.0, MeOH). MS *m/z*: 695 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.82, Ala 0.99, Gly 1.10, Phe 1.00 (average recovery 85%).

Z-Tyr(Bzl)-Gly-Gly-Phe-N_2H_3 Prepared from Z-Tyr(Bzl)-Gly-Gly-Phe-OMe (680 mg, 1 mmol) and $NH_2NH_2 \cdot H_2O$ (0.15 ml, 3 mmol) in MeOH (15 ml) in the usual manner. Yield 470 mg (69%), mp 194 °C, Rf^3 0.59, $[\alpha]_D^{27}$ −23.9° (*c* = 1.0, MeOH). MS *m/z*: 681 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.78, Gly 2.06, Phe 1.00 (average recovery 80%).

Z-Tyr(Bzl)-D-Ala-Gly-Phe-N_2H_3 Prepared from Z-Tyr(Bzl)-D-Ala-Gly-Phe-OMe (695 mg, 1 mmol) and $NH_2NH_2 \cdot H_2O$ (0.15 ml, 3 mmole) in MeOH (15 ml) in the usual manner. Yield 540 mg (78%), mp 204 °C, Rf^3 0.46, $[\alpha]_D^{27}$ −50.4° (*c* = 1.0, MeOH). MS *m/z*: 695 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.77, Ala 0.98, Gly 1.00, Phe 1.00 (average recovery 75%).

H-Tyr-Gly-Gly-Phe-Leu(F_3)(2*R*,4*R*)-OH [V] Isoamyl nitrite (60 μl, 0.6 mmol) was added to a DMF solution (5 ml) of Z-Tyr(Bzl)-Gly-Gly-Phe-N_2H_3 (410 mg, 0.6 mmol) and 6 N HCl/dioxane (0.5 ml) at −10 °C and the mixture was stirred for 10 min. H-Leu(F_3)(2*R*,4*R*)-OH (92.5 mg, 0.5 mmol) dissolved in a mixture of Et_3N (91 μl, 0.66 mmol), H_2O (5 ml) was added to the mixture and the whole was stirred for 20 h in a cold room. The solvent was removed *in vacuo* and the residue was washed with H_2O. The protected peptide was then hydrogenated on Pd catalyst in MeOH to give H-Tyr-Gly-Gly-Phe-Leu(F_3)(2*R*,4*R*)-OH, followed by HPLC. Yield 232 mg (59%), Rf^1 0.35, $[\alpha]_D^{27}$ +46.1° (*c* = 1.0, H_2O). MS *m/z*: 610 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.79, Gly 2.00, Phe 1.00, Leu(F_3) 0.82 (average recovery 75%).

The following compounds were prepared in the same way.

H-Tyr-Gly-Gly-Phe-Leu(F_3)(2*R*,4*S*)-OH [VI] Yield 187 mg (52%), Rf^1 0.35, $[\alpha]_D^{27}$ +31.0° (*c* = 1.0, H_2O). MS *m/z*: 610 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.80, Gly 1.99, Phe 1.00, Leu(F_3) 0.80 (average recovery 68%).

H-Tyr-Gly-Gly-Phe-Leu(F_3)(2*S*,4*S*)-OH [VII] Yield 192 mg (59%), Rf^1 0.35, $[\alpha]_D^{27}$ +8.8° (*c* = 1.0, H_2O). MS *m/z*: 610 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.78, Gly 1.99, Phe 1.00, Leu(F_3) 0.81 (average recovery 70%).

H-Tyr-Gly-Gly-Phe-Leu(F_3)(2*S*,4*R*)-OH [VIII] Yield 181 mg (50%), Rf^1 0.35, $[\alpha]_D^{27}$ +15.9° (*c* = 1.0, H_2O). MS *m/z*: 610 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.72, Gly 2.02, Phe 1.00, Leu(F_3) 0.79 (average recovery 75%).

H-Tyr-D-Ala-Gly-Phe-Leu(F^3)(2*R*,4*R*)-OH [IX] Prepared from Z-Tyr(Bzl)-D-Ala-Gly-Phe-N_2H_3 (410 mg, 0.6 mmol) and the corresponding H-Leu(F_3)-OH (92.5 mg, 0.5 mmol) in the same way as described for V. Yield 99 mg (64%), Rf^1 0.31, $[\alpha]_D^{27}$ +69.1° (*c* = 1.0, H_2O). MS *m/z*: 624 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.97, Ala 0.94, Gly 1.12, Phe 1.00, Leu(F_3) 1.03 (average recovery 96%).

The following compounds were prepared in the same way.

H-Tyr-D-Ala-Gly-Phe-Leu(F_3)(2*R*,4*S*)-OH [X] Yield 88 mg (56%), Rf^1 0.34, $[\alpha]_D^{27}$ +58.4° (*c* = 1.0, H_2O). MS *m/z*: 624 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ala 0.96, Gly 1.10, Phe 1.00, Leu(F_3) 1.00 (average recovery 96%).

H-Tyr-D-Ala-Gly-Phe-Leu(F_3)(2*S*,4*S*)-OH [XI] Yield 97 mg (62%), Rf^1 0.34, $[\alpha]_D^{27}$ +32.9° (*c* = 1.0, H_2O). MS *m/z*: 624 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.86, Ala 0.99, Gly 0.95, Phe 1.00, Leu(F_3) 1.04 (average recovery 86%).

H-Tyr-D-Ala-Gly-Phe-Leu(F_3)(2*S*,4*R*)-OH [XII] Yield 114 mg (73%), Rf^1 0.34, $[\alpha]_D^{27}$ +40.2° (*c* = 1.0, H_2O). MS *m/z*: 624 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.87, Ala 0.97, Gly 0.99, Phe 1.00, Leu(F_3) 1.05 (average recovery 90%).

Boc-Leu(F_3)(2*R*,4*R*)-OH Prepared from H-Leu(F_3)(2*R*,4*R*)-OH (52 mg, 0.28 mmol) and $(Boc)_2O$ (180 mg, 0.84 mmol) in the usual manner.[18] Precipitated from AcOEt/petroleum ether. Yield 80 mg (100%), amorphous material, Rf^3 0.69, $[\alpha]_D^{24}$ +31.4° (*c* = 1.0, MeOH). MS *m/z*: 284 $(M-H)^-$.

Boc-Leu(F_3)(2*R*,4*S*)-OH Prepared from H-Leu(F_3)(2*R*,4*S*)-OH (101 mg, 0.55 mmol) and $(Boc)_2O$ (360 mg, 1.65 mmol) in the usual manner. Precipitated from AcOEt/petroleum ether. Yield 157 mg (100%), amorphous material, Rf^3 0.80, $[\alpha]_D^{24}$ +1.2° (*c* = 1.0, MeOH). MS *m/z*: 284 $(M-H)^-$.

Boc-Leu(F_3)(2*S*,4*S*)-OH Prepared from H-Leu(F_3)(2*S*,4*S*)-OH (39 mg, 0.21 mmol) and $(Boc)_2O$ (138 mg, 0.63 mmol) in the usual manner. Precipitated from AcOEt/petroleum ether. Yield 61 mg (100%), amorphous material, Rf^3 0.76, $[\alpha]_D^{24}$ −35.6° (*c* = 1.0, MeOH). MS *m/z*: 284 $(M-H)^-$.

Boc-Leu(F_3)(2*S*,4*R*)-OH Prepared from H-Leu(F_3)(2*S*,4*R*)-OH (48 mg, 0.26 mmol) and $(Boc)_2O$ (170 mg, 0.77 mmol) in the usual manner. Precipitated from AcOEt/petroleum ether. Yield 74 mg (100%), amorphous material, Rf^3 0.69, $[\alpha]_D^{24}$ −8.4° (*c* = 1.0, MeOH). MS *m/z*: 284 $(M-H)^-$.

H-Tyr-Leu(F_3)(2*R*,4*R*)-Gly-Phe-Leu-OH [XIII] Prepared by the manual solid-phase method using BOP as the coupling agent. Yield 38 mg (21%), Rf^1 0.46, $[\alpha]_D^{27}$ +13.8° (*c* = 1.0, H_2O). MS *m/z*: 666 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.73, Leu(F_3) 1.09, Gly 1.08, Phe 1.00, Leu 1.01 (average recovery 95.0%).

The following compounds were prepared in the same way.

H-Tyr-Leu(F_3)(2*R*,4*S*)-Gly-Phe-Leu-OH [XIV] Yield 53 mg (29%), Rf^1 0.46, $[\alpha]_D^{27}$ +38.5° (*c* = 1.0, H_2O). MS *m/z* 666 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.73, Leu(F_3) 0.89, Gly 1.08, Phe 1.00, Leu 1.00 (average recovery 78%).

H-Tyr-Leu(F_3)(2*S*,4*S*)-Gly-Phe-Leu-OH [XV] Yield 25 mg (48%), Rf^1 0.46, $[\alpha]_D^{27}$ −53.2° (*c* = 1.0, H_2O). MS *m/z*: 666 $(M+H)^+$. Amino

acid ratios in an acid hydrolysate: Tyr 0.73, Leu(F_3) 1.03, Gly 0.92, Phe 1.00, Leu 1.01 (average recovery 68%).

H-Tyr-Leu(F_3)(2*S*,4*R*)-Gly-Phe-Leu-OH [XVI] Yield 34 mg (60%), Rf^1 0.46, $[\alpha]_D^{27}$ −6.0° (c=1.0, H_2O). MS m/z: 666 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.72, Leu(F_3) 1.03, Gly 0.92, Phe 1.00, Leu 1.05 (average recovery 80%).

H-Tyr(*o*-F)-Gly-Gly-Phe(*p*-F)-Leu-OH [XVII] Prepared by the synthesizer. Yield 72 mg (12%), Rf^1 0.52, $[\alpha]_D^{27}$ +18.2° (c=1.0, H_2O). MS m/z: 591 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr(*o*-F) 0.72, Gly 1.86, Phe(*p*-F) 0.73, Leu 1.00 (average recovery 77%).

The following compounds were prepared in the same way.

H-Tyr(*m*-F)-Gly-Gly-Phe(*p*-F)-Leu-OH [XVIII] Yield 150 mg (24%), Rf^1 0.58, $[\alpha]_D^{27}$ +16.3°(c=1.0, H_2O). MS m/z: 591 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr(*m*-F) 0.94, Gly 1.88, Phe(*p*-F) 1.04, Leu 1.00 (average recovery 70%).

H-Tyr-D-Arg-Phe(*p*-F)-Lys-NH_2 [XIX] For side chain protection, a nitro group was used for Arg and a 2-chlorobenzyloxycarbonyl group was used for Lys. Yield 242 mg (32%), Rf^1 0.64, $[\alpha]_D^{27}$ +3.8° (c=1.0, H_2O). MS m/z 765 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.97, Lys 1.01, Phe(*p*-F) 0.96, Arg 1.00 (average recovery 89%).

H-Tyr-D-Thr-Gly-Phe(*p*-F)-Leu-Thr-OH [XX] Yield 420 mg (58%), Rf^1 0.34, $[\alpha]_D^{27}$ +20.5° (c=1.0, H_2O). MS m/z: 720 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.87, Thr 1.67, Gly+Phe(*p*-F) 1.97, Leu 1.03 (average recovery 91%).

Bioassay Male guinea pigs weighing 300 to 350 g (Otsubo Exp. Animal) were killed by a blow to the head and segments (about 4 cm) of the ileum 10 to 15 cm from the ileo-cecal valve were isolated. The longitudinal muscle with myenteric plexus of GPI was prepared as described by Rang.[19] MVD was isolated from male ddY mice weighing 30—35 g (Otsubo Exp. Animal). Both preparations were mounted in a 10 ml organ bath filled with Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM KH_2PO_4, 2.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose) for GPI or Mg^{2+}-free Krebs–Henseleit solution for MVD, and kept at 37°C. Inhibitory effects of the test compounds on the electrically induced contractions of both preparations were estimated from concentration–response curves and expressed as IC_{50}. Details of the procedures were reported in our previous paper.[2b]

This work was supported in part by a grant from The Science Research Promotion Fund of the Japan Private School Promotion Foundation.

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 = *N*,*N*-dimethylformamide, Bzl = benzyl, DCM = dichloromethane.
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