[Chem. Pharm. Bull. 36(3) 959—965 (1988)]

Synthesis and Analgesic Activity of Cholecystokinin-Heptapeptide Analogs with N-Terminal Substitution[1)]

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(Received June 29, 1987)

Analogs of cholecystokinin-heptapeptide (CCK-7), *i.e.*, two epimers of 3-(4-sulfooxyphenyl)-2-methylpropanoyl-Met–Gly–Trp–Met–Asp–Phe–NH_2, two epimers of 3-(4-sulfooxyphenyl)-2-methylpropanoyl-Nle–Gly–Trp–Met–Asp–Phe–NH_2 and [D-Tyr(SO_3H)^1]-CCK-7, were prepared by the solution method. The analgesic effects of these analogs were measured by means of the writhing test. These analogs produced analgesic effects after subcutaneous injection in mice. The replacement of the tyrosine(O-sulfate) residue at position 1 by a 3-(4-sulfooxyphenyl)-2-methylpropanoyl group enhanced the analgesic effect, and the configuration of these residues hardly influenced the effect. On the other hand, the replacement of the L-methionine residue at position 2 by an L-norleucine residue in addition to the exchange of the tyrosine(O-sulfate) residue at position 1 for a 3-(4-sulfooxyphenyl)-2-methylpropanoyl group reduced the activity.

Keywords——cholecystokinin; peptide synthesis; CCK-7 analog; writhing test; analgesic effect

Among various biological activities of cholecystokinin (CCK) and related peptides, analgesic activity has been reported for CCK-octapeptide (CCK-8) and caerulein.[2)] We have shown that CCK-heptapeptide (CCK-7) retained the analgesic effect on subcutaneous injection in mice and that the tyrosine(O-sulfate) residue at the N-terminal is essential for the activity of the native fragment.[3)] Further, Boc-CCK-7 appeared to show analgesic activity.[3)]

The above result on Boc-CCK-7 suggested that the α-amino function of the N-terminal might be exchangeable for other functions without loss of the activity, and a methyl function was considered to be suitable in place of the amino function because of the similarity of steric bulkiness. Thus, 3-(4-sulfooxyphenyl)-2-methylpropanoyl-Met–Gly–Trp–Met–Asp–Phe–NH_2 (hereafter abbreviated as [HMP(SO_3H)^1]-CCK-7) was synthesized to test the above hypothesis. Further, in order to examine the effect of the configuration of the N-terminal of CCK-7 on the analgesic effect, H–D-Tyr(SO_3H)–Met–Gly–Trp–Met–Asp–Phe–NH_2 (hereafter abbreviated as [D-Tyr(SO_3H)^1]-CCK-7) was prepared. Moreover, 3-(4-sulfooxyphenyl)-2-methylpropanoyl-Nle–Gly–Trp–Met–Asp–Phe–NH_2 (hereafter abbreviated as [HMP(SO_3H)^1, Nle^2]-CCK-7) was synthesized to examine the structural requirement at position 2 for the activity. This report describes these syntheses and the analgesic effect of the products.

The synthetic methods for [HMP(SO_3H)^1]-CCK-7 and [HMP(SO_3H)^1, Nle^2]-CCK-7 are summarized in Fig. 1.

In order to introduce a 3-(4-hydroxyphenyl)-2-methylpropanoyl residue into the N-terminal of the peptides, 3-(4-hydroxyphenyl)-2-methylpropanoic acid (I) was synthesized from methacrylonitrile. Thus, methacrylonitrile and phenol were reacted in the presence of anhydrous aluminum chloride and dry hydrogen chloride to give 3-(4-hydroxyphenyl)-2-methylpropiononitrile, which was hydrolyzed in the presence of hydrochloric acid to give I as

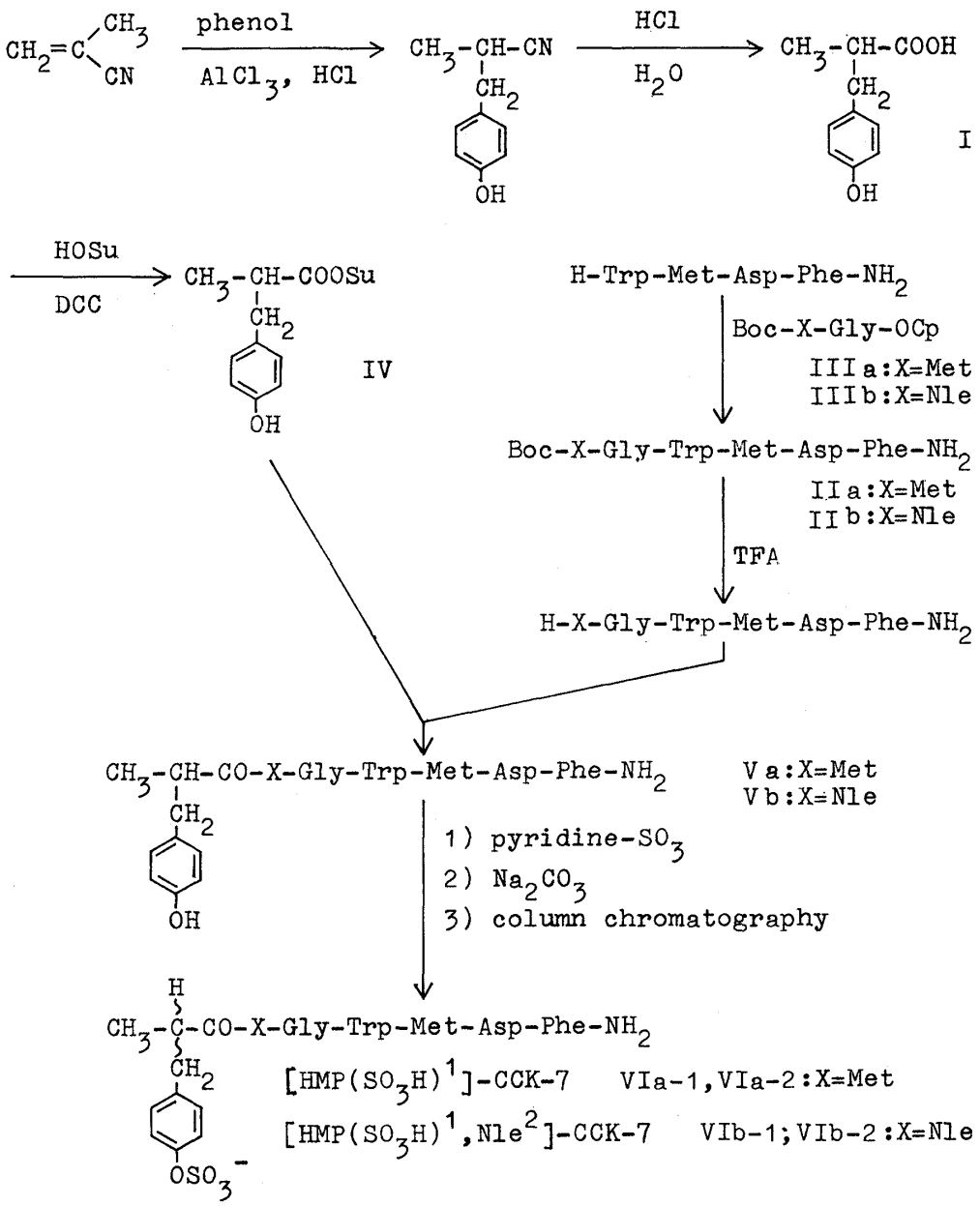

Fig. 1. Synthetic Methods for [HMP(SO_3H)^1]-CCK-7 and [HMP(SO_3H)^1,Nle^2]-CCK-7

a racemate. This compound (I) was used in the next reaction without optical resolution.

[HMP(SO_3H)^1]-CCK-7 was prepared as follows. Boc–Met–Gly–Trp–Met–Asp–Phe–NH_2 (IIa) was produced by the reaction of H-Trp–Met–Asp–Phe–NH_2 and Boc–Met–Gly–OCp (IIIa) as reported previously.[3] After deprotection of IIa with TFA, the resulting hexapeptide was coupled with the hydroxysuccinimide ester (IV) which was prepared from I and HOSu, to give the N-acylated peptide (Va). Sulfation of Va with pyridine–sulfur trioxide complex in anhydrous pyridine afforded a mixture of two products. The thin-layer chromatography (TLC) of this mixture on silica gel showed two spots (Rf^1 0.18, Rf^1 0.25) whose areas were nearly equal. After treatment of the reaction mixture with sodium carbonate, the two products were separated by column chromatography on silica gel. The infrared (IR) spectrum of the product with Rf^1 0.18 (compound VIa-1) was almost identical with that of the product with Rf^1 0.25 (compound VIa-2). Both VIa-1 and VIa-2 showed the characteristic band (1050 cm^{-1})[4] due to a phenolic sulfate ester. The secondary ion mass spectra of both

compounds showed the same protonated molecular ion peak. The results of amino acid analysis of acid hydrolysates of these compounds were in good agreement with the theoretically expected values for [HMP(SO_3H)^1]-CCK-7. On the other hand, the specific optical rotation of VIa-1 was different from that of VIa-2.

Because compound I was used without optical resolution, Va must be an epimeric mixture. Therefore, the sulfated product of Va is also an epimeric mixture. This consideration and the above analytical data of VIa-1 and VIa-2 indicate that VIa-1 and VIa-2 are epimers of [HMP(SO_3H)^1]-CCK-7.

[HMP(SO_3H)^1, Nle^2]-CCK-7 was synthesized as follows. Boc–Nle–Gly–OCp (IIIb) was prepared from HOCp and Boc–Nle–Gly–OH, which was obtained by the reaction of Boc–Nle–OSu[5] with Gly. Compound IIIb was reacted with H–Trp–Met–Asp–Phe–NH_2[6] to give Boc–Nle–Gly–Trp–Met–Asp–Phe–NH_2 (IIb). Compound IIb was deprotected and reacted with IV to afford the acylated peptide (Vb), then Vb was sulfated in the same manner as described for the synthesis of [HMP(SO_3H)^1]-CCK-7. This sulfation also produced two products, which were separated by column chromatography on silica gel, then purified by column chromatography on diethyl aminoethyl (DEAE) Sephadex A-25 using ammonium carbonate aqueous solution as the eluting solvent and lyophilized to give VIb-1 (Rf^1 0.20) and VIb-2 (Rf^1 0.29). The IR spectrum of VIb-1 was almost identical with that of VIb-2 and indicated the existence of a phenolic sulfate ester group (1050 cm^{-1}). In the secondary ion mass spectral examination of each compound, the molecular ion peak was difficult to detect, but the same peak due to the desulfated form was detected in each case. Amino acid analysis of an acid hydrolysate of each compound gave a molar ratio in good agreement with the theoretically expected value. Therefore, VIb-1 and VIb-2 were considered to be epimers of [HMP(SO_3H)^1, Nle^2]-CCK-7.

[D-Tyr(SO_3H)^1]-CCK-7 was prepared from H–Met–Gly–Trp–Met–Asp–Phe–NH_2[3] as follows. This hexapeptide amide was coupled with Boc–D-Tyr–OSu, which was prepared from Boc–D-Tyr–OH[7] and HOSu, to produce Boc–D-Tyr–Met–Gly–Trp–Met–Asp–Phe–NH_2. Then, this compound was sulfated with pyridine–sulfur trioxide complex, treated with sodium carbonate and deprotected with TFA. Finally, the resulting crude [D-Tyr(SO_3H)^1]-CCK-7 was purified successively by silica gel column chromatography and Sephadex G-10 column chromatography.

TABLE I. Analgesic Effects of CCK-7 Analogs

Peptides	Dose mg/kg, s.c.	Number of mice	Writhing (%) mean ± S.E.
[HMP(SO_3H)^1]-CCK-7 (VIa-1)	0.25	6	57.0 ± 5.4
	0.5	6	53.8 ± 15.1
	1.0	6	18.3 ± 6.5[a]
	2.5	6	6.8 ± 6.8[b]
[HMP(SO_3H)^1]-CCK-7 (VIa-2)	0.25	6	57.0 ± 14.0
	0.5	6	45.2 ± 8.6
	1.0	6	10.8 ± 5.4[a]
	2.5	6	6.8 ± 2.9[b]
[HMP(SO_3H)^1,Nle^2]-CCK-7 (VIb-1)	1.0	5	105.7 ± 40.6
	2.0	10	49.1 ± 12.3[a]
	4.0	5	37.7 ± 5.7[c]
	8.0	5	30.2 ± 6.6[c]
[D-Tyr(SO_3H)^1]-CCK-7	10.0	6	29.8 ± 10.6[b]

a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$ (*t*-test).

The analgesic effects of the synthetic peptides were examined by means of the writhing test according to Zetler.[2a] Writhing was elicited by intraperitoneal injection of 0.6% (v/v) acetic acid (10 ml/kg) into ddY male mice weighing 18—22 g. Solutions of the peptides in 0.05 M sodium carbonate were administered subcutaneously 10 min before the acetic acid injection. The number of writhings occurring between 10 and 20 min after injection of acetic acid was counted. Data were expressed as the ratio (%) with respect to the control value (Table I). [HMP(SO_3H)^1]-CCK-7 (VIa-1) and [HMP(SO_3H)^1]-CCK-7 (VIa-2) reduced the number of writhings dose-dependently, and the ED_{50} values were calculated from the regression analysis of the dose–response curves to be 0.39 mg/kg and 0.32 mg/kg, respectively. [HMP(SO_3H)^1, Nle^2]-CCK-7 (VIb-1) was also active and the ED_{50} value was calculated to be 3.0 mg/kg. The writhing test for [HMP(SO_3H)^1, Nle^2]-CCK-7 (VIb-2) was omitted, since the no influence of the configuration of the 3-(4-sulfooxyphenyl)-2-methylpropanoyl group on the activity was suggested from the result of the tests with VIa-1 and VIa-2. When [D-Tyr(SO_3H)^1]-CCK-7 was tested at a dose of 10 mg/kg, the writhing ratio with respect to the control value was 29.8%.

The ED_{50} value of CCK-7 in the writhing test has been reported as 1.8 mg/kg by us in the previous paper.[3] Our present results suggest that exchange of the tyrosine(O-sulfate) residue in CCK-7 for a 3-(4-sulfooxyphenyl)-2-methylpropanoyl group enhanced the analgesic activity and that the configuration of 3-(4-sulfooxyphenyl)-2-methylpropanoyl group was not significant for the activity. However, further exchange of the L-methionine residue at position 2 for an L-norleucine residue reduced the activity.

Exchange of the L-tyrosine(O-sulfate) residue of CCK-7 for a D-tyrosine(O-sulfate) residue could retain the analgesic effect.

The previous report showed that a tyrosine(O-sulfate) residue was essential for the native CCK fragment peptides to exhibit analgesic activity.[3] The present study has established a more precise structural requirement at the N-terminal of CCK-7 for the activity. Thus, it appears that the α-amino function of the tyrosine(O-sulfate) and its configuration are not essential for the analgesic activity.

It has been reported that the L-methionine residue at position 3 of CCK-8 was exchangeable for an L-norleucine residue with about 58% loss of pancreozymin-like activity.[9] In our study, the substitution of the L-methionine residue by an L-norleucine residue at position 2 of [HMP(SO_3H)^1]-CCK-7 resulted in a reduction of the analgesic activity to about 1/8 of that of [HMP(SO_3H)^1]-CCK-7. This reduction seems to be large in comparison with that of the pancreozymin-like activity. This phenomenon is not easy to explain, but we speculate that the structure of the analgesic receptor might not be the same as that of the pancreozymin-like receptor, and the substitution by an L-norleucine residue at position 2 in addition to the substitution at position 1 of CCK-7 might induce a change of the molecular conformation to a less suitable form for binding to the analgesic receptor.

Experimental

In order to prevent oxidation of the methionine residue, every reaction was performed under a nitrogen atmosphere, and peroxide-free ether stored over ferrous sulfate was used.[8]

The melting points are uncorrected. Optical rotations were measured with a DIP-181 polarimeter (Japan Spectroscopic Co.). Amino acid analyses of acid hydrolysates were performed with a JEOL JLC-6AH amino acid analyzer. IR spectra were measured with a Shimadzu IR-400 infrared spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi R-20 high-resolution NMR spectrometer; chemical shifts are given in δ (ppm) with tetramethylsilane as an internal standard (s, singlet; m, multiplet; br, broad). Secondary ion mass spectra (SIMS) were recorded on a Hitachi M-80B mass spectrometer. Elemental analyses were carried out with a Yanagimoto MT-3 CHN Corder. Ascending TLC was performed on silica gel TLC plates (Kieselgel 60 F_{254}, Merck) using the following solvent systems: Rf^1, AcOEt–pyridine–AcOH–H_2O (60 : 20 : 6 : 11); Rf^2, *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2); Rf^3, *n*-BuOH–AcOH–pyridine–H_2O (60 : 20 : 6 : 24).

3-(4-Hydroxyphenyl)-2-methylpropiononitrile——Anhydrous aluminum chloride (23.3 g) was added to a mixture of phenol (32.9 g) and methacrylonitrile (7 g) with stirring at 10 °C. While dry HCl gas was being passed into the mixture, methacrylonitrile (23.5 g) was added dropwise during 1 h at 60—70 °C. After being stirred for 1.5 h at the same temperature, the reaction mixture was poured over crushed ice (120 g) and extracted with ether (2 × 120 ml). The ether layer was washed with 10% KCl (3 × 100 ml), and dried over Na_2SO_4. The solvent was evaporated off and the residue was fractionated by distillation *in vacuo*. The fraction with boiling point 171—172 °C/3 mmHg was collected and recrystallized from benzene–hexane. Yield 11.2 g (19.9%), mp 72—73 °C. IR ν_{max}^{KBr} cm^{-1}: 3360 (OH), 2220 (CN). ^{1}H-NMR (in $CDCl_3$) δ: 1.15—1.45 (3H, m, methyl protons), 2.45—2.95 (3H, m, methylene and methine protons), 5.90 (1H, s, OH), 6.5—7.2 (4H, m, aromatic protons). *Anal.* Calcd for $C_{10}H_{11}NO$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.67; H, 6.95; N, 8.45.

3-(4-Hydroxyphenyl)-2-methylpropanoic Acid (I)——3-(4-Hydroxyphenyl)-2-methylpropiononitrile (15.1 g) was added to concentrated hydrochloric acid (187 ml) and the mixture was refluxed for 3 h. Hydrochloric acid was evaporated off *in vacuo*. The residue was dissolved in hot benzene and treated with activated charcoal (1 g). The solution was cooled to precipitate white needles. Yield 13.3 g (79.2%), mp 99—100 °C. IR ν_{max}^{KBr} cm^{-1}: 1700 (C=O). ^{1}H-NMR (in $CDCl_3$) δ: 0.95—1.25 (3H, m, methyl protons), 2.2—3.0 (3H, m, methylene and methine protons), 6.5—7.1 (4H, m, aromatic protons), 9.0 (2H, br, phenolic proton and proton of carboxylic acid). *Anal.* Calcd for $C_{10}H_{12}O_3$: C, 66.65; H, 6.71. Found: C, 66.68; H, 6.70.

Succinimidyl 3-(4-Hydroxyphenyl)-2-methylpropanoate (IV)——DCC (1.06 g) was added to a mixture of I (900 mg) and N-hydroxysuccinimide (575 mg) in AcOEt (40 ml) under cooling with ice, then the whole was stirred at room temperature overnight. After removal of dicyclohexylurea by filtration, the filtrate was washed successively with 3% sodium bicarbonate (2 × 30 ml) and brine (2 × 10 ml), then dried over Na_2SO_4. Evaporation of the solvent *in vacuo* gave an oil. Yield 940 mg (68%). ^{1}H-NMR (in $CDCl_3$) δ: 1.1—1.4 (3H, m, methyl protons), 2.76 (4H, s, methylene protons), 2.6—3.3 (3H, m, methylene and methine protons), 6.6—7.2 (4H, m, aromatic protons).

3-(4-Hydroxyphenyl)-2-methylpropanoyl-Met–Gly–Trp–Met–Asp–Phe–NH_2 (Va)——Et_3N (46 mg) was added to a solution of H–Met–Gly–Trp–Met–Asp–Phe–NH_2·TFA[3] (405 mg) in DMF (3 ml). The mixture was stirred for 30 min under cooling with ice, a solution of IV (125 mg) in DMF (1 ml) was added and the whole was stirred at 7—8 °C for 3 d. The solvent was evaporated off *in vacuo* and the residue was triturated with ice-water (30 ml) containing AcOH (0.2 ml). The resulting powder was washed successively with H_2O (2 × 10 ml), EtOH (5 ml) and AcOEt (5 ml), then dried over P_2O_5 *in vacuo*. Yield 282 mg (66%), mp 215—217 °C (dec.). This material was used for the next reaction (sulfation) without further purification. A sample for analysis was purified by silica gel column chromatography (column size, 2.2 × 35 cm) with AcOEt–pyridine–AcOH–H_2O (60:20:6:11) as the eluent, and recrystallized from EtOH–H_2O. mp 215—216 °C (dec.), $[\alpha]_D^{20}$ −21° (c=1, DMF). Rf^1 0.40, Rf^2 0.65, Rf^3 0.68. Amino acid ratio in an acid hydrolysate: Asp, 0.99; Gly, 1.02; Met, 1.96; Phe, 1.02; NH_3, 0.96 (average recovery, 89%). *Anal.* Calcd for $C_{46}H_{58}N_8O_{10}S_2$: C, 58.33; H, 6.17; N, 11.83. Found: C, 58.07; H, 6.18; N, 11.57.

[HMP(SO_3H)^1]-CCK-7 (VIa-1 and VIa-2)——Pyridine–sulfur trioxide complex (454 mg) was added to a solution of Va (270 mg) in anhydrous pyridine (15 ml) under cooling with ice and the mixture was stirred at 7—8 °C overnight. TLC of the reaction mixture showed that this reaction produced two products (Rf^1 0.18, Rf^1 0.25). Pyridine was evaporated off *in vacuo* and a solution of sodium carbonate (406 mg) in ice-water (25 ml) was added to the residue. After being stirred for 30 min, the mixture was extracted with aqueous *n*-BuOH (3 × 30 ml), and the extract was evaporated *in vacuo*. The residue was chromatographed on a silica gel column (4 × 80 cm) with AcOEt–pyridine–AcOH–H_2O (60:20:6:11) as the eluent, and each fraction (11 ml) was monitored by TLC. Fractions No. 156—180 (Rf^1 0.18) and fractions No. 118—140 (Rf^1 0.25) were collected separately, and the solvent was evaporated off *in vacuo*. The residue with Rf^1 0.18 was dissolved in H_2O–MeOH (1:1, 20 ml), and 0.1 M ammonium carbonate was added for neutralization. The solution was concentrated *in vacuo* to about 7 ml. The resulting gelatinous precipitate was collected by filtration and washed with water to give VIa-1 as a white powder. Yield 66 mg, mp 188—200 °C (dec.), $[\alpha]_D^{20}$ −78° (c=0.1, 1 N NH_4OH). Rf^1 0.18, Rf^2 0.55, Rf^3 0.60. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). SIMS m/z: 1067 (MH^+). Amino acid ratio in an acid hydrolysate: Asp, 0.97; Gly, 1.05; Met, 1.97; Phe, 1.01; NH_3, 2.29 (average recovery, 92%). *Anal.* Calcd for $C_{46}H_{57}N_8NaO_{13}S_3 \cdot NH_3 \cdot 2H_2O$: C, 50.10; H, 5.85; N, 11.43. Found: C, 50.20; H, 6.07; N, 11.29.

On the other hand, the residue with Rf^1 0.25 was treated in the same manner to give VIa-2 as a white powder. Yield 69 mg, mp 180—182 °C, $[\alpha]_D^{20}$ −8° (c=0.15, 1 N NH_4OH). Rf^1 0.25, Rf^2 0.59, Rf^3 0.62. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). SIMS m/z: 1067 (MH^+). Amino acid ratio in an acid hydrolysate: Asp, 0.97; Gly, 1.05; Met, 1.95; Phe, 0.98; NH_3, 2.31 (average recovery, 93%). *Anal.* Calcd for $C_{46}H_{57}N_8NaO_{13}S_3 \cdot NH_3 \cdot 3H_2O$: C, 49.32; H, 5.94; N, 11.25. Found: C, 49.06; H, 5.95; N, 11.20.

Boc–Nle–Gly–OCp (IIIb)——A solution of Boc–Nle–OSu[5] (1.31 g) in THF (12 ml) was added to a mixture of Et_3N (404 mg) and Gly (300 mg) in water (6 ml) under cooling with ice, and the whole was stirred at room temperature overnight. After concentration of the reaction mixture to about 6 ml *in vacuo*, water (40 ml) was added. The aqueous layer was washed with AcOEt (2 × 40 ml), acidified with citric acid and extracted with AcOEt (3 × 50 ml). The extract was washed with water (3 × 60 ml) and dried over Na_2SO_4. The solvent was evaporated off *in vacuo* to give Boc–Nle–Gly–OH (600 mg) as an oil. This compound (570 mg) was dissolved in dichloromethane (6 ml), then HOCp

(391 mg) and DCC (433 mg) were added under cooling with ice. The mixture was stirred at 7—8 °C overnight. After removal of the resulting dicyclohexylurea by filtration, the solvent of the filtrate was evaporated off *in vacuo*. The residue was recrystallized from AcOEt to give IIIb as white needles. Yield 570 mg, mp 101—103 °C, $[\alpha]_D^{23}$ −21° ($c=1$, MeOH). *Anal.* Calcd for $C_{19}H_{25}Cl_3N_2O_5$: C, 48.79; H, 5.39; N, 5.99. Found: C, 48.99; H, 5.47; N, 5.99.

Boc–Nle–Gly–Trp–Met–Asp–Phe–NH_2 (IIb)——Et_3N (131 mg) was added to a solution of H–Trp–Met–Asp–Phe–NH_2·TFA[6] (923 mg) in DMF (8 ml) under cooling with ice, and the mixture was stirred for 20 min. Compound IIIb (608 mg) and HOBt (176 mg) were added to the mixture, then the whole was stirred at 7—8 °C for 2 d. The reaction mixture was poured into 1% AcOH (100 ml) under cooling with ice to give a precipitate, which was collected by filtration, washed with water, then recrystallized from EtOH to give a white powder. Yield 687 mg (60%), mp 203—204 °C (dec.), $[\alpha]_D^{20}$ −27° ($c=1$, DMF). Rf^1 0.53, Rf^2 0.67, Rf^3 0.72. Amino acid ratio in an acid hydrolysate: Asp, 1.01; Gly, 1.01; Met, 0.99; Nle, 0.99; Phe, 1.00; NH_3, 1.13 (average recovery, 81%). *Anal.* Calcd for $C_{42}H_{58}N_8O_{10}S\cdot 0.5H_2O$: C, 57.58; H, 6.79; N, 12.79. Found: C, 57.61; H, 6.87; N, 12.79.

3-(4-Hydroxyphenyl)-2-methylpropanoyl-Nle–Gly–Trp–Met–Asp–Phe–NH_2 (Vb)——Compound IIb (750 mg) was added to TFA (3.5 ml) containing thioanisole (0.2 ml), and the mixture was stirred under cooling with ice for 1 h. The reaction mixture was poured into ether (100 ml) to cause a precipitate, which was washed with ether and dried over KOH *in vacuo* to give H–Nle–Gly–Trp–Met–Asp–NH_2·TFA (706 mg). From this compound (554 mg), Et_3N (64 mg) and IV (175 mg), Vb was prepared and chromatographed according to the procedure described for Va, then the product was triturated with water. Yield 279 mg (43%), mp 211—213 °C, $[\alpha]_D^{20}$ −20° ($c=1$, DMF). Rf^1 0.45, Rf^2 0.66, Rf^3 0.70. Amino acid ratio in an acid hydrolysate: Asp, 1.04; Gly, 1.01; Met, 1.00; Nle, 0.98; Phe, 0.98: NH_3, 1.19 (average recovery, 89%). *Anal.* Calcd for $C_{47}H_{60}N_8O_{10}S\cdot H_2O$: C, 59.60; H, 6.60; N, 11.83. Found: C, 59.46; H, 6.54; N, 11.62.

[$HMP(SO_3H)^1$, Nle^2]-CCK-7 (VIb-1 and VIb-2)——According to the procedure described for VIa-1 and VIa-2, Vb (220 mg) was sulfated with pyridine–sulfur trioxide complex (377 mg). TLC showed that two products (Rf^1 0.20, Rf^2 0.29) were formed in about equal amounts. The product was treated with sodium carbonate (251 mg), then the reaction mixture was worked up and chromatographed on a silica gel column in the same manner as described for VIa-1 and VIa-2. Fractions of 13 ml of the eluate were collected and monitored by TLC. Fractions No. 130—180 contained the compound with Rf^1 0.29, fractions No. 181—199 contained two compounds (Rf^1 0.29 and Rf^1 0.20) and fractions No. 200—255 contained the compound with Rf^1 0.20.

Fractions No. 200—255 (Rf^1 0.20) were combined and the solvent was evaporated off *in vacuo*. The residue was further purified on a DEAE Sephadex A-25 column (1.8 × 25 cm). The column was eluted with a linear gradient of ammonium carbonate (from 0.1 to 1.5 M) and the eluate was monitored by measuring the ultraviolet (UV) absorption at 280 nm. Fractions (No. 110—200, 10 ml each) were collected and lyophilized to give VIb-1 as a powder. Yield 29 mg, mp 198—208 °C, $[\alpha]_D^{20}$ −58° ($c=0.1$, 1 N NH_4OH). Rf^1 0.20, Rf^2 0.60, Rf^3 0.61. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). SIMS *m/z*: 930 ($M-SO_4$). Amino acid ratio in an acid hydrolysate: Asp, 1.00; Gly, 1.01; Met, 1.03; Nle, 0.96; Phe, 1.00; NH_3, 1.89 (average recovery, 88%).

On the other hand, fractions No. 130—180 (Rf^1 0.29) mentioned before were collected, the solvent was evaporated off *in vacuo* and the residue was further purified by column chromatography on a DEAE-Sephadex A-25 column in the same manner as described for VIb-1 to give VIb-2 as a powder. Yield 24 mg, mp 183—188 °C, $[\alpha]_D^{20}$ +3° ($c=0.06$, 1 N NH_4OH). Rf^1 0.29, Rf^2 0.64, Rf^3 0.63. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). SIMS *m/z*: 930 ($M-SO_4$). Amino acid ratio in an acid hydrolysate: Asp, 1.05; Gly, 1.05; Met, 0.99; Nle, 0.99; Phe, 1.00; NH_3, 2.20 (average recovery, 75%).

Boc–D-Tyr–OSu——DCC (11.3 g) was added to a solution of Boc–D-Tyr–OH[7] (18.6 g) and HOSu (6.3 g) in dioxane (300 ml) under cooling with ice, and the mixture was stirred at 7—8 °C overnight. The resulting dicyclohexylurea was removed by filtration and washed with THF (300 ml). The filtrate and washings were combined and the solvent was evaporated off *in vacuo*. The residue was washed with ether (30 ml) and recrystallized from MeOH–iso-PrOH. Yield 12.7 g (61%), mp 187—189 °C (dec.), $[\alpha]_D^{20}$ +46° ($c=1$, DMF).

Boc–D-Tyr–Met–Gly–Trp–Met–Asp–Phe–NH_2——Et_3N (0.1 ml) was added to a solution of H–Met–Gly–Trp–Met–Asp–Phe–NH_2·TFA[3] (550 mg) in DMF (15 ml) under cooling with ice, and the mixture was stirred for 30 min. After addition of Boc–D-Tyr–OSu (230 mg), the mixture was stirred at 7—8 °C for 3 d. The solvent was evaporated off *in vacuo*. The residue was dissolved in *n*-BuOH (100 ml), and washed successively with 3% $NaHCO_3$ (30 ml), 5% citric acid (30 ml) and water. After evaporation of the solvent *in vacuo*, the residue was triturated with H_2O (30 ml) to give a powder (440 mg). This compound (300 mg) was chromatographed on a silica gel column (2.2 × 40 cm) in the same way as described for Va and triturated with water. Yield 240 mg. This material was used for the next sulfation. A sample for analysis was further purified by Sephadex LH-20 column chromatography (column size, 2.2 × 40 cm) with MeOH as the eluent, then triturated with water. mp 183—187 °C, $[\alpha]_D^{20}$ −18° ($c=0.75$, DMF). Rf^1 0.50, Rf^2 0.66, Rf^3 0.66. Amino acid ratio in an acid hydrolysate: Asp, 1.01; Gly, 0.98; Met, 1.99; D-Tyr, 1.01; Phe, 1.01; NH_3, 1.15 (average recovery, 85%). *Anal.* Calcd for $C_{50}H_{65}N_9O_{12}S_2\cdot 3H_2O$: C, 54.48; H, 6.49; N, 11.44. Found: C, 54.74; H, 6.29; N, 11.14.

[D-Tyr(SO_3H)^1]-CCK-7——Pyridine–sulfur trioxide complex (280 mg) was added to a solution of Boc–D-Tyr–Met–Gly–Trp–Met–Asp–Phe–NH_2 (180 mg) in anhydrous pyridine (7 ml) under cooling with ice, and the mixture

was stirred at 7—8 °C for 2 d. After evaporation of the solvent *in vacuo*, a cold solution of sodium carbonate (250 mg) in H_2O (20 ml) was added to the residue, and the mixture was stirred for 30 min. The solvent was evaporated off *in vacuo*, and the residue was treated with TFA (3 ml) in a presence of thioanisole (0.1 ml) under cooling with ice for 1 h. After evaporation of TFA *in vacuo*, the residue was triturated with ether, washed with ether and dried over KOH *in vacuo* to give crude [D-Tyr(SO_3H)[1]]-CCK-7. This material was chromatographed on a silica gel column (2.8 × 50 cm) with AcOEt–pyridine–AcOH–H_2O (60 : 20 : 6 : 11) as the eluent. The eluate was monitored by TLC. The desired fractions were collected, the solvent was evaporated off *in vacuo* and the residue was further purified by Sephadex G-10 column chromatography (column size, 3 × 37 cm) with 3% NH_4OH as the eluent. The eluate was monitored by measuring the UV absorption at 254 nm. The The desired fractions were combined and lyophilized repeatedly to give a hygroscopic powder. Yield 54 mg, mp 200—205 °C, $[\alpha]_D^{20}$ −49° (c=0.7, 0.3 N NH_4OH). Rf^1 0.10, Rf^2 0.47, Rf^3 0.56. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Asp, 1.02; Gly, 0.96; Met, 2.04; D-Tyr, 1.01; Phe, 1.02 (average recovery, 90%). *Anal.* Calcd for $C_{45}H_{56}N_9NaO_{13}S_3 \cdot NH_3 \cdot 4H_2O$: C, 47.44; H, 5.93; N, 12.29. Found: C, 47.36; H, 5.57; N, 12.08.

Acknowledgement We are grateful to Dr. T. Nose, general manager of the Pharmaceuticals Center, Kanebo Ltd., for his encouragement during this investigation, Mr. K. Noumi, Pharmaceuticals Research Center, Kanebo Ltd., for the secondary ion mass spectral examination, and Mr. H. Yoshidome, Development Laboratories, Kanebo Ltd., for elemental analyses.

References and Notes

1) The customary L indication for amino acid residues is omitted. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967); *11*, 1726 (1972)]. Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; OCp, 2,4,5-trichlorophenyl ester; OSu, *N*-hydroxysuccinimide ester; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; TFA, trifluoroacetic acid; AcOEt, ethyl acetate; AcOH, acetic acid; iso-PrOH, 2-propanol; *n*-BuOH, 1-butanol; HOBt, 1-hydroxybenzotriazole.
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