Evaluation of the Final Deprotection System for the Solid-Phase Synthesis of Tyr(SO_3H)-Containing Peptides with 9-Fluorenylmethyloxycarbonyl (Fmoc)-Strategy and Its Application to the Synthesis of Cholecystokinin (CCK)-12[1)]

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Acidolytic deprotection and cleavage conditions for an acid-labile Tyr(SO_3H)-containing peptide were systematically examined with respect to acid, temperature, and scavenger. The 90% aqueous trifluoroacetic acid (TFA)-based reagent systems (90% aqueous TFA/*m*-cresol and 90% aqueous TFA/*m*-cresol/2-methylindole) at 4 °C were found to minimize the deterioration of Tyr(SO_3Na) in the peptide. The latter deprotection/cleavage system was applied to the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase synthesis of cholecystokinin (CCK)-12 on an acid-labile PAL-linked support (PAL = peptide amide linker), with Fmoc–Tyr(SO_3Na)–OH as a building block.

Keywords tyrosine sulfate; acidolytic deprotection; Fmoc-based solid-phase synthesis; cholecystokinin-12; tyrosine sulfate sodium salt; acid-labile peptide-amide linker

Various proteins and peptides containing tyrosine sulfate [Tyr(SO_3H)] have been discovered and tyrosine sulfation is regarded as a widespread post-translational event in organisms.[2)] However, the roles of sulfate ester are poorly understood with the exception of cholecystokinin (CCK)[3)] and caerulein,[4)] the Tyr(SO_3H) moiety of which is crucial for the expression of their biological activities. To clarify the significance of tyrosine sulfation and the role of the sulfate ester moiety in the biological activity, synthetic Tyr(SO_3H)-containing peptides could serve as useful models. In this regard, much interest has been focused on establishment of a facile synthetic method for Tyr(SO_3H)-containing peptides, overcoming the acid-lability of Tyr(SO_3H).[5)]

In Fmoc-based solid-phase peptide synthesis[6)] [Fmoc = 9-fluorenylmethyloxycarbonyl], the amino-protecting group, Fmoc, is deprotected with base, and global deprotection of protecting groups and cleavage of peptides from the polymer support are conducted with trifluoroacetic acid (TFA) at the final stage of synthesis. In addition, some novel acid-labile peptide-resin linkers have been developed to give the peptide-amide upon TFA treatment.[7–9)] As tyrosine-*O*-sulfate derivatives, such as Tyr($SO_3Ba_{1/2}$)[10)] or Tyr(SO_3Na),[11)] are relatively stable in TFA, the approach in which preparation of Tyr(SO_3H)-containing peptides is accomplished through the Fmoc-strategy using Fmoc–Tyr(SO_3X)–OH (X = Na, $Ba_{1/2}$, *etc.*) as a building block looks attractive. Here, the use of Fmoc–Tyr(SO_3Na)–OH provides a practical approach because of its easy preparation. Previously, Penke and Rivier[11)] reported the synthesis of CCK-octapeptide (CCK-8) on a 2,4-dimethoxybenzhydrylamine support *via* a similar approach. They used TFA–thioanisole (4:1, 15 min at room temperature) as a deprotection/cleavage reagent, but found that the yield was low. Further, the desulfation rate at final acidolysis was not described. Therefore, we felt a systematic reexamination was necessary for the final deprotection and cleavage procedures of this approach. In this report, we describe acidolytic deprotection/cleavage conditions which minimize the deterioration of Tyr(SO_3H) in Tyr(SO_3H)-containing peptides and its application to the synthesis of CCK-dodecapeptide (CCK-12) on a recently developed acid-labile PAL-linked support[9)] using the Fmoc-based solid-phase method.

First, the effects of several deprotection procedures currently used in Fmoc-based peptide synthesis upon Tyr(SO_3Na) were examined by RP-HPLC using a model peptide substrate, Fmoc–Tyr(SO_3Na)–Ala–OMe. Tyr(SO_3Na) was chosen because of its easy preparation. This dipeptide derivative was obtained in a pure form after three reaction steps: (1) a mixed anhydride coupling[12)] of Fmoc–Tyr(^{t}Bu)–OH and H–Ala–OMe to give Fmoc–Tyr(^{t}Bu)–Ala–OMe, (2) subsequent deprotection of ^{t}Bu group with TFA, and (3) sulfation of the resulting Fmoc–Tyr–Ala–OMe with DMF–SO_3 complex[13)] followed by neutralization with Na_2CO_3. Direct coupling of Fmoc–Tyr(SO_3Na)–OH and H–Ala–OMe by the DCC–HOBt method[14)] resulted in a poor yield due to the lack of a suitable purification method.

The effects of various acids, temperature, and scavengers on Tyr(SO_3Na) are shown in Figs. 1, 2, and 3, respectively. From these results, we concluded that: (i) TFA is not a

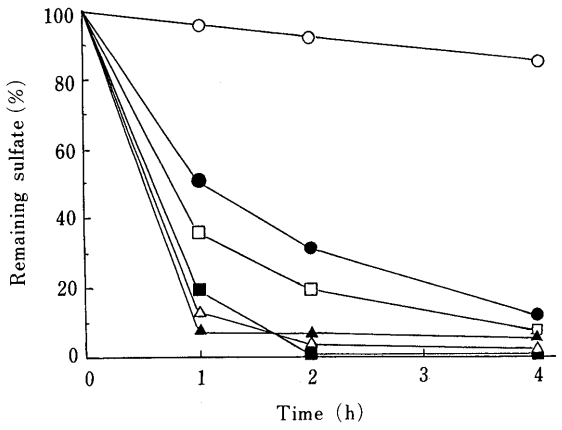

Fig. 1. Effect of Acid on the Stability of Tyr(SO_3Na) at 4 °C.

—○—, TFA (neat); —●—, 5 N HCl/DMF; —□—, 1 M TMSBr/TFA; —■—, AcOH (30 °C); —△—, 1 M MSA/TFA; —▲—, 1 M TFMSA/TFA.

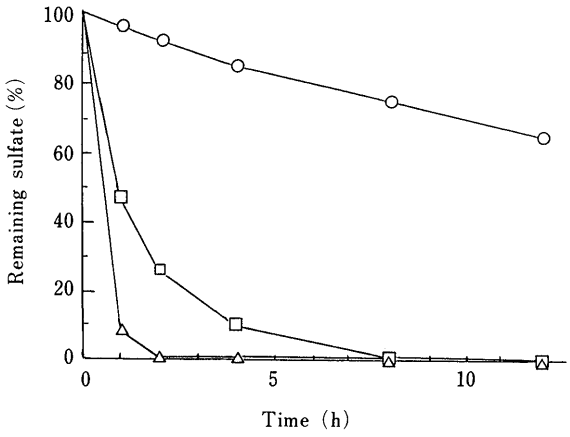

Fig. 2. Effect of Temperature on the Stability of Tyr(SO_3Na) in TFA (neat)

—○—, 4 °C; —□—, 18 °C; —△—, 30 °C.

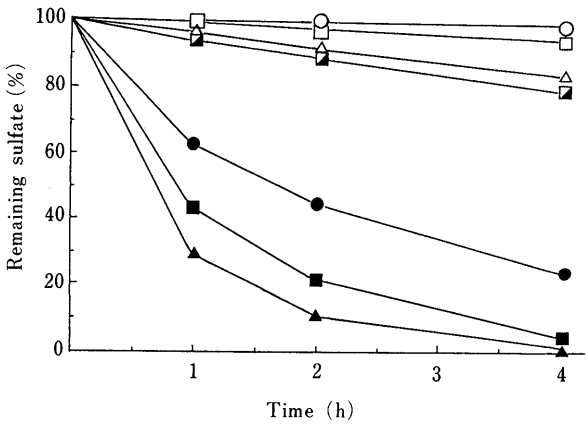

Fig. 3. Effect of Scavenger on the Stability of Tyr(SO_3Na) in TFA (4 °C)

—○—, 1 M 2-methylindole/TFA; —□—, 90% aqueous TFA; —△—, 1 M *m*-cresol/TFA; —◪—, 1 M anisole/TFA; —●—, 1 M dimethylsulfide/TFA; —■—, 1 M thioanisole/TFA; —▲—, 1 M ethanedithiol/TFA.

destructive acid for Tyr(SO_3Na), as already argued,[10] whereas strong acids such as trimethylsilyl bromide (TMSBr)[15] and trifluoromethanesulfonic acid (TFMSA)[16] can not be used for deprotection; (ii) temperature is a decisive determinant of the stability of Tyr(SO_3Na) in TFA; and (iii) addition of a sulfur compound as a scavenger in the TFA system promotes cleavage of the tyrosine-sulfate linkage, while scavengers such as H_2O, anisole, *m*-cresol, and 2-methylindole have little effect on the decomposition of Tyr(SO_3Na).

Next, we examined the degradation of synthetic Tyr(SO_3H)-containing peptides, leucosulfakinin (LSK)-II[17] and CCK-12,[18] which were prepared by the method newly developed by us. The details of the syntheses will be reported in a separate paper.[19] The degradation of both peptides in 90% aqueous TFA-based acidic media is shown in Fig. 4. In both cases, the degradation of Tyr(SO_3H)-peptides to the corresponding Tyr-peptides was minimized in these acidic media. In the case of LSK-II, prolonged treatment (16 h) in these media resulted in the loss of *ca.* 20% of the sulfate ester (data not shown).

Based on the results obtained here, we chose 90% aqueous TFA/*m*-cresol or 90% aqueous TFA/*m*-cresol/2-methylindole[20] (in the case of Trp-containing peptides) as a final

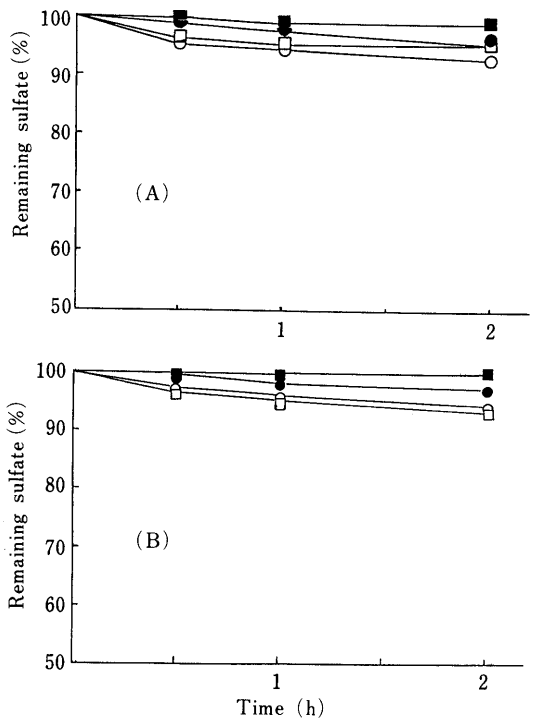

Fig. 4. Desulfation Rate of Leucosulfakinin II (A) and Cholecystokinin-12 (B) in Acidic Media at 4 °C

—○—, 90% aqueous TFA; —●—, 90% aqueous TFA/*m*-cresol; —□—, 90% aqueous TFA/2-methylindole; —■—, 90% aqueous TFA/*m*-cresol/2-methylindole. *m*-Cresol (50 eq) and 2-methylindole (25 eq) were added, with respect to the peptide. [Leucosulfakinin II: Pyr–Ser–Asp–Tyr(SO_3H)–Gly–His–Met–Arg–Phe–NH_2].

deprotection reagent for the synthesis of Tyr(SO_3H)-containing peptides.

We next performed the solid-phase synthesis of CCK-12, based on the following synthetic strategies: (a) the assembly of the resin-bound Tyr(SO_3Na)-containing peptide is carried out through Fmoc-based solid-phase synthesis, in which Tyr(SO_3Na) is introduced by the use of Fmoc–Tyr-(SO_3Na)–OH as a building block; (b) an acid-labile PAL-linked support [PAL = peptide amide linker, 5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenyl)valeric acid][9] is used to give a peptide-amide linkage after detachment of the peptide from the resin; and (c) the protected peptide-resin is simply deprotected and cleaved by 90% aqueous TFA/*m*-cresol/2-methylindole treatment. An outline of the synthesis is shown in Fig. 5. Prior to the synthesis, we had confirmed that the Pmc protecting group on Arg [Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl][21] could be deprotected completely with this deprotection system at 4 °C within 12 h.

The resin-bound Tyr(SO_3H)-containing peptide was assembled straightforwardly by the Fmoc-based solid-phase synthesis using a commercially available PAL-linked support. Repetitive Fmoc removal was conducted with 20% piperidine/DMF and peptide couplings were performed using 3 eq of the Fmoc-amino acid derivative and BOP reagent[22] (3 eq) [BOP = benzotriazoyloxytris(dimethylamino)phosphonium hexafluorophosphate] in DMF in the presence of NMM (9 eq) for 90 min. Complete incorporation of each amino acid was confirmed by a Kaiser test[23] of the peptide-resin after each coupling step. The incorporation of Fmoc–Tyr(SO_3Na)–OH was completed in 90 min, as in the case of other amino acid derivatives.

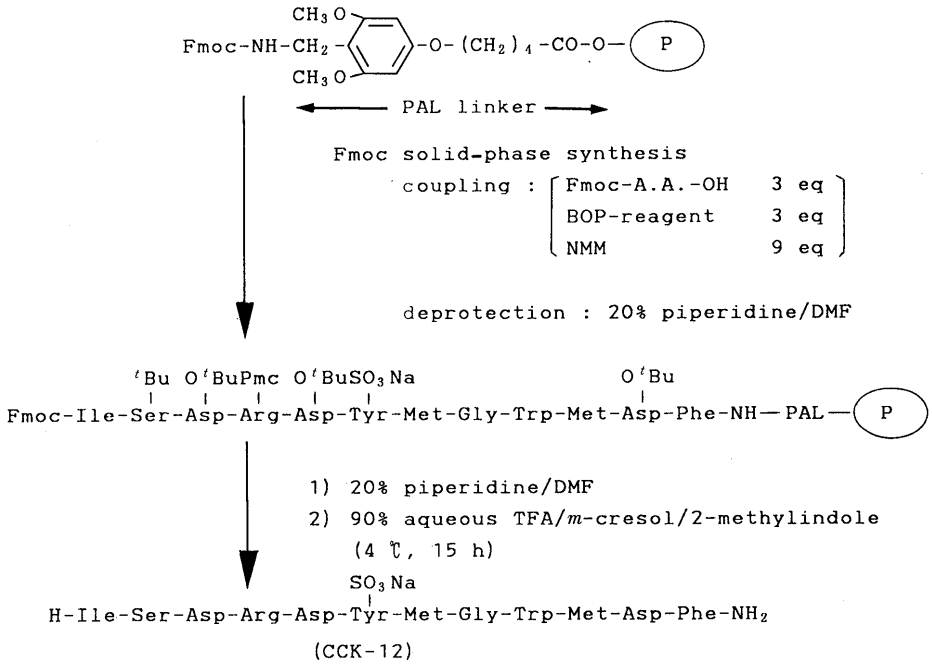

Fig. 5. Fmoc-Based Solid-Phase Synthesis of Cholecystokinin-12 (CCK-12)
(P), polymer support; A.A., amino acid.

After removal of the Fmoc group on Ile, deprotection of the protecting groups and peptide-resin cleavage were concurrently effected with 90% aqueous TFA/*m*-cresol/2-methylindole[24] (4 °C, 15 h) and the crude CCK-12 was obtained in 37% yield after gel-filtration on Sephadex G-10. The crude peptide was shown to contain about 15% desulfated peptide (CCK-12 non-sulfate form) by RP-HPLC analysis (Fig. 6a). Subsequent purification on RP-HPLC readily gave homogeneous CCK-12 (sulfate form) in 9.3% yield from the first incorporation of Phe onto the resin. The purity of the obtained peptide and the existence of the sulfate ester on Tyr were ascertained by analytical HPLC (Fig. 6b), FAB-MS, FT-IR, and amino acid analysis after leucine aminopeptidase (LAP) digestion.[25]

Thus, CCK-12 was easily obtained with a satisfactory purity, though the cleavage efficiency of the peptide-resin bond remained at only about 35% based on amino acid analysis of the residual resin after deprotection. We have conducted the present synthesis focusing on the final deprotection conditions to minimize the loss of the sulfate ester on Tyr. Total yield would be improved by using a more sophisticated linker system such as PAL-Nle linker (Nle = norleucine), as pointed out by Albericio *et al.*[9]

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were determined with a Union PM-201 polarimeter. HPLC was conducted with a Hitachi L-6200 system. FAB mass spectra were recorded on a JEOL JMS-D 300 spectrometer. FT-IR spectra were obtained on a Perkin Elmer 1720 spectrophotometer. Amino acid compositions in acid hydrolysates [12 N HCl/propionic acid (1 : 1, v/v), 110 °C, 20 h] and in LAP (Sigma No. L-6007) digests [enzyme (*ca.* 1 U) was used for digestion of peptide (*ca.* 100 μg) at 37 °C for 24 h] were determined with a Hitachi 8500 model amino acid analyzer.

Rf values were determined on precoated Silica gel plates 60 F_{254} [1 × 8 cm, 0.25 mm thickness (Merck)] with the following solvent systems (v/v): Rf_1, $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower layer); Rf_2, $CHCl_3$–MeOH–AcOH (9 : 3 : 0.5); Rf_3, *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1). For detection, ninhydrin, UV light, and I_2 vapor were used.

DMF–SO_3 complex was purchased from Fluka, and Fmoc–Arg(Pmc)–OH·IPE was purchased from Novabiochem. Other Fmoc amino acid derivatives and PAL-linked support were purchased from MilliGen.

Fmoc–Tyr(ᵗBu)–Ala–OMe Fmoc–Tyr(ᵗBu)–OH (1.00 g, 2.18 mmol) was dissolved in DMF (10 ml) containing Et_3N (0.31 ml, 2.18 mmol), and isobutyl chloroformate (0.29 ml, 2.18 mmol) was added at −30 °C. The reaction mixture was stirred for 15 min under cooling, then a solution of H–Ala–OMe [prepared from 0.34 g (2.40 mmol) of its hydrochloride and Et_3N (0.34 ml, 2.40 mmol) in DMF (20 ml)] was added. The reaction mixture was stirred for 2 h with ice-cooling. The solvent was removed by evaporation *in vacuo* and the residue was partitioned between AcOEt (50 ml) and H_2O (20 ml). The organic phase was washed with 5% Na_2CO_3 (× 2), 5% citric acid (× 3), and H_2O (× 3), then dried over Na_2SO_4. The AcOEt was evaporated off under reduced pressure and the residual solid was collected by filtration. Recrystallization from AcOEt with ether afforded crystals; yield 1.14 g (96%), mp 176—178 °C, $[\alpha]_D^{20}$ −6.6° (c = 0.6, DMF), Rf_2 0.42. *Anal.* Calcd for $C_{32}H_{36}N_2O_6 \cdot 1/4H_2O$: C, 69.99; H, 6.70; N, 5.10. Found: C, 70.04; H, 6.82; N, 4.95.

Fmoc–Tyr–Ala–OMe Fmoc–Tyr(ᵗBu)–Ala–OMe (1.00 g, 1.84 mmol) was treated with TFA (2.0 ml) in the presence of anisole (0.5 ml) for 1.5 h at room temperature, then TFA was evaporated off *in vacuo*. The residue was triturated with ether to afford a powder, which was dissolved in AcOEt (50 ml). The organic phase was washed with brine (× 3) and H_2O (× 3), then dried over Na_2SO_4. The AcOEt was removed by evaporation *in vacuo* to afford crystals, which were collected by filtration after standing overnight; yield 0.87 g (97%), mp 151—154 °C, $[\alpha]_D^{15}$ +1.5° (c = 0.6, DMF), Rf_1 0.80. *Anal.* Calcd for $C_{28}H_{28}N_2O_6 \cdot 1/2H_2O$: C, 67.59; H, 5.88; N, 5.63. Found: C, 67.93; H, 5.90; N, 5.30.

Fmoc–Tyr(SO_3Na)–Ala–OMe Fmoc–Tyr–Ala–OMe (0.41 g, 0.84 mmol) was dissolved in a mixture of DMF–pyridine–dioxane (8 ml–2 ml–1 ml) and, to this solution, DMF–SO_3 complex (0.39 g, 2.52 mmol) was added in one portion. The reaction mixture was stirred overnight at room temperature, then the solvent was removed by evaporation *in vacuo*. The residue was dissolved in 50% aqueous MeOH (50 ml) and the pH of the solution was adjusted to 7 with 5% Na_2CO_3. After evaporation of the solvent, the gelatinous residue was triturated with ether, then dissolved in DMF. The insoluble material was filtered off and the filtrate was concentrated *in vacuo*. The residue was triturated with ether to afford a solid, which was further purified by silica gel column chromatography. The sample was dissolved in a mixture of $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase, 5 ml) and applied to a column (3.3 × 13 cm), which was eluted

with the same solvent system. The faster-moving material (Rf_1 0.85) was eluted and the fractions containing the desired material (Rf_1 0.34) were pooled. The solvent was evaporated off *in vacuo*, and the crystalline residue was triturated with ether, then collected by filtration; yield 0.24 g (49%), mp 152—157 °C, $[\alpha]_D^{18}$ −7.7° ($c=1$, MeOH), Rf_1 0.34, Rf_2 0.52. *Anal.* Calcd for $C_{28}H_{27}N_2NaO_9S \cdot 3/2H_2O$: C, 54.45; H, 4.90; N, 4.54. Found: C, 54.75; H, 4.64; N, 4.90. FAB-MS *m/z*: 591 $(M+H)^+$, 613 $(M+Na)^+$. FT-IR (KBr): 1053, 1240 cm^{-1}.

Examination of Stability of Tyr(SO_3Na) Using Fmoc–Tyr(SO_3Na)–Ala–OMe (i) Effect of Acid: Fmoc–Tyr(SO_3Na)–Ala–OMe (10 mg) was treated with the following reagents (1 ml) at 4 °C; (1) TFA (neat), (2) 1 M TMSBr/TFA, (3) 1 M TFMSA/TFA, (4) 1 M MSA/TFA, (5) 5 N HCl/DMF, and (6) AcOH (at 30 °C). At intervals (1, 2, and 4 h), aliquots of 50 μl were withdrawn and diluted with CH_3CN–0.1 M $AcONH_4$ (30 : 70, 1 ml). Then 50 μl of the solution was analyzed by RP-HPLC [Nucleosil $7C_{18}$ (4.6 × 250 mm); gradient system, CH_3CN/0.1 M $AcONH_4$ (pH 6.5) 30—75% in 30 min; flow rate, 1 ml/min; detected at 300 nm]; t_R for Fmoc–Tyr(SO_3-Na)–Ala–OMe 14.86 min and Fmoc–Tyr–Ala–OMe 21.86 min.

(ii) Effect of Temperature: Fmoc–Tyr(SO_3Na)–Ala–OMe (5 mg) was treated with TFA (1 ml) at (1) 4 °C, (2) 18 °C, and (3) 30 °C, respectively. The degradation rate was determined by means of RP-HPLC as described in (i).

(iii) Effect of Scavenger: Fmoc–Tyr(SO_3Na)–Ala–OMe (5 mg) was treated with the following reagent systems (1 ml) at 4 °C; (1) 1 M EDT/TFA, (2) 1 M thioanisole/TFA, (3) 1 M anisole/TFA, (4) 1 M 2-methylindole/TFA, (5) 1 M dimethylsulfide/TFA, (6) 1 M *m*-cresol/TFA, and (7) 90% aqueous TFA. Aliquots of 50 μl were diluted with an initial elution buffer (500 μl) and 50 μl of the solution was analyzed by RP-HPLC in the same manner as described in (i). In 1 M thioanisole/TFA, decrease of Fmoc–Tyr(SO_3Na)–Ala–OMe was determined by using the same HPLC conditions.

Degradation Studies of Tyr(SO_3H) Using Synthetic LSK-II and CCK-12 (i) Degradation of LSK-II: Synthetic LSK-II (100 μg) was dissolved in the following reagents (300 μl), which contained *m*-cresol (50 eq) and/or 2-methylindole (25 eq) with respect to the peptide, as indicated, with ice-cooling to 4 °C; (1) 90% aqueous TFA, (2) 90% aqueous TFA/*m*-cresol, (3) 90% aqueous TFA/2-methylindole, and (4) 90% aqueous TFA/*m*-cresol/2-methylindole. At intervals (1, 2, and 4 h), aliquots of 100 μl were withdrawn and diluted with 15% CH_3CN/H_2O (200 μl), and 100 μl was analyzed by RP-HPLC [Daiso SP-300-5-C_{18} (4.6 × 250 mm); gradient system, CH_3CN/0.1% TFA 15—35% in 20 min; flow rate, 1 ml/min; detected at 215 nm]; t_R for LSK-II 11.13 min and LSK-II non-sulfate 13.24 min.

(ii) Degradation of CCK-12: Synthetic CCK-12 (150 μg) was dissolved in the following reagents (300 μl) with ice-cooling to 4 °C; (1) 90% aqueous TFA, (2) 90% aqueous TFA/*m*-cresol, (3) 90% aqueous TFA/2-methylindole, and (4) 90% aqueous TFA/*m*-cresol/2-methylindole. At intervals, aliquots of 50 μl were withdrawn and diluted with 25% CH_3CN/H_2O (200 μl), and 100 μl was analyzed by RP-HPLC [Daiso SP-300-5-C_{18} (4.6 × 250 mm); gradient system, CH_3CN/0.1% TFA 25—45% in 20 min; flow rate, 1 ml/min; detected at 275 nm]; t_R for CCK-12 13.30 min and CCK-12 non-sulfate 14.50 min.

Fmoc–Tyr–OH Fmoc–OSu (3.0 g, 8.9 mmol) dissolved in tetrahydrofuran (THF) (25 ml) was added to a solution of H–Tyr–OH (2.42 g, 13.3 mmol) in H_2O (50 ml) containing Et_3N (1.95 ml, 14.0 mmol) and the reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated off *in vacuo*, and the residue was partitioned between AcOEt (50 ml) and H_2O (50 ml). The aqueous layer was pooled and acidified with 1 N HCl, and extracted with AcOEt (100 ml). The organic layer was washed with 5% citric acid, brine, and H_2O successively, and dried over Na_2SO_4. The AcOEt was evaporated off *in vacuo*, and the residue was triturated with *n*-hexane to afford a solid, which was recrystallized from AcOEt–ether to give crystals; yield 2.80 g (78%), mp 184—186 °C, $[\alpha]_D^{15}$ −19.9° ($c=0.6$, DMF), Rf_1 0.28. *Anal.* Calcd for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.47. Found: C, 71.09; H, 5.28; N, 3.32.

Fmoc–Tyr(SO_3Na)–OH Fmoc–Tyr–OH (1.30 g, 3.22 mmol) was dissolved in a mixture of DMF–pyridine–dioxane (8 ml–2 ml–1 ml) and DMF–SO_3 complex (1.48 g, 9.66 mmol) was added in one portion. The reaction mixture was stirred overnight at room temperature, the solvent was evaporated off *in vacuo*, and the residue was dissolved in ice-chilled H_2O (20 ml). The pH of the solution was adjusted at 6.5 with the addition of 5% Na_2CO_3 over 1 h and the remaining solution was lyophilized. The residue was taken up in DMF (20 ml) and the insoluble material was filtered off, then the DMF was evaporated off *in vacuo*. Trituration of the residue with EtOH gave a solid, which was reprecipitated from MeOH with ether twice. For further purification, this powder was dissolved in a mixture of $CHCl_3$–MeOH–AcOH (9 : 3 : 0.5, v/v, 10 ml) and the solution was applied to a column of silica gel (3.3 × 6 cm), which was eluted with the same solvent system. The fractions containing the desired material (Rf_2 0.23) were pooled. The solvent was removed by evaporation *in vacuo* and the residue was triturated with ether to afford a powder; yield 1.41 g (87%), mp 225 °C (dec.), $[\alpha]_D^{18}$ +27.9° ($c=1$, MeOH), Rf_2 0.23, Rf_3 0.60. *Anal.* Calcd for $C_{24}H_{19}NNaO_8S \cdot 3/2H_2O$: C, 54.13; H, 4.16; N, 2.63. Found: C, 53.87; H, 4.11; N, 2.59. FAB-MS *m/z*: 506 $(M+H)^+$, 528 $(M+Na)^+$. FT-IR (KBr): 1055, 1250 cm^{-1}. RP-HPLC: [Nucleosil $7C_{18}$ (4.6 × 250 mm); gradient system, CH_3CN/0.1 M $AcONH_4$ (pH 6.5) 25—55% in 30 min; flow rate, 1 ml/min; detected at 300 nm]; t_R 9.82 min.

Deprotection of Pmc Protecting Group on Arg with 90% Aqueous TFA Fmoc–Arg(Pmc)–OH · IPE (10 mg) was treated with 90% aqueous TFA (1 ml) in the presence of *m*-cresol (50 eq) at 4 °C. Aliquots of 25 μl were withdrawn and diluted with CH_3CN/0.1% aqueous TFA (50 : 50, 300 μl), and 25 μl of this solution was analyzed by RP-HPLC [Nucleosil $7C_{18}$ (4.6 × 250 mm); gradient system, CH_3CN/0.1% aqueous TFA 35—80% in 30 min; flow rate, 1 ml/min; detected at 300 nm]; t_R for Fmoc–Arg–OH 9.48 min and Fmoc–Arg(Pmc)–OH 24.22 min. After 8 h at 4 °C, 95% of the Pmc protecting group had been removed.

Solid-Phase Synthesis of CCK-12 Solid-phase synthesis was carried out manually on a PAL-linked support (substitution level: 0.33 meq/g, 453 mg, 0.15 mmol), which was treated with 20% piperidine/DMF (5 ml) for 30 min, then washed with DMF (5 ml, 10 times). Fmoc–Phe–OH (3 eq) was introduced with BOP reagent (0.45 mmol, 3 eq) in the presence of NMM (1.35 mmol, 9 eq) in DMF (5 ml) for 90 min. The peptide chain was elongated in a similar manner; the manipulations in each elongation cycle consisted of (i) deprotection of the Fmoc group with 20% piperidine/DMF (2 min and 20 min), (ii) washing with DMF (5 ml, 10 times), (iii) coupling of each amino acid derivative in DMF (5 ml) for 90 min, (iv) washing with DMF (5 ml, 10 times). All couplings were monitored by means of the Kaiser test.[23] The following Fmoc-amino acid derivatives were successively introduced: Asp(O^{t}Bu), Met, Trp, Gly, Met, Tyr(SO_3Na), Asp(O^{t}Bu), Arg(Pmc), Asp(O^{t}Bu), Ser(^{t}Bu), and Ile. No double coupling was needed in this synthesis. After final incorporation of Fmoc–Ile–OH, the Fmoc group was removed by using 20% piperidine/DMF and washed with DMF (5 times), MeOH (5 times), and ether (5 times), then dried over KOH pellets *in vacuo*; yield 660 mg. Amino acid analysis after acid hydrolysis gave the following ratios (numbers in parentheses are theoretical values): Asp(3) 2.87, Ser(1) 0.56, Gly(1) 1.00, Met(2) 1.77, Ile(1) 0.85, Tyr(1) 0.68, Phe(1) 1.06, Trp(1) not determined, Arg(1) 0.85.

Preparation of CCK-12 The peptide resin (104 mg) was treated with a pre-cooled deprotection reagent [90% aqueous TFA (3.0 ml)/*m*-cresol (125 μl)/2-methylindole (62 mg)][24] with ice-cooling for 30 min, then at 4 °C for 15 h under an argon atmosphere. The resin was removed by filtration and washed with cold TFA (5 ml). The combined filtrate was concentrated *in vacuo* at 4 °C, then dry ether (50 ml) was added. The formed precipitate was collected by centrifugation, washed again with dry ether, then dried over KOH pellets *in vacuo*. The dried powder was dissolved in 0.05 M NH_4HCO_3 (5 ml) and lyophilized to afford a fluffy powder. This sample was dissolved in 0.025 M NH_4HCO_3 (3 ml) and applied to a column of

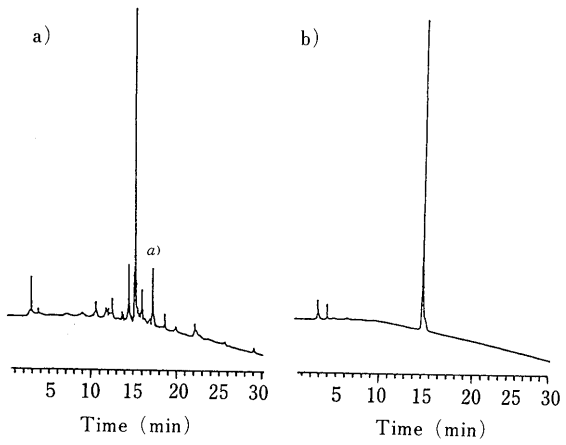

Fig. 6. HPLC Profile of the Synthetic Cholecystokinin-12

(a) Crude CCK-12 after gel-filtration (*a*) shows CCK-12 non-sulfate) and (b) HPLC-purified CCK-12. Column, YMC AM-312 (6 × 150 mm); gradient system, CH_3CN/0.1 M $AcONH_4$ (pH 6.5) 20–45% in 30 min; flow rate, 1 ml/min; detected at 220 nm.

Sephadex G-10 (2 × 40 cm), which was eluted with the same solvent. The eluate was monitored by UV absorption measurement at 280 nm and the fractions of the first main peak were combined. The solvent was removed by lyophilization to afford a fluffy powder; yield 17.0 mg (37% cleavage yield based on the peptide content of the residual resin). This sample (Fig. 6a) was purified by HPLC on a column of Cosmosil $5C_{18}$ AR (10 × 250 mm). The crude sample obtained above was dissolved in 0.1 M $AcONH_4$ (1 ml) and a portion of the solution (containing *ca.* 1 mg) was applied to the column, which was eluted with gradient of CH_3CN (25—50% in 30 min) in 0.1 M $AcONH_4$ (pH 6.5) at a flow rate of 2 ml/min. The eluate corresponding to the main peak (t_R = 11.38 min, monitored by measuring UV absorbance at 275 nm) was collected and subjected to repeated lyophilization to afford a white fluffy powder; 3.37 mg, $[\alpha]_D^{18}$ −20.7° (c = 0.4, MeOH), Rf_3 0.40. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical values): Asp(3) 2.59, Ser(1) 0.94, Gly(1) 0.94, Met(2) 1.78, Ile(1) 0.91, Tyr(SO_3H) (1) 0.93, Phe(1), 1.00, Trp(1) 0.94, Arg(1) 0.89 (recovery of Phe, 78%). FT-IR (KBr): 1050, 1238 cm^{-1}. RP-HPLC: [YMC AM-312 (6 × 150 mm); gradient system, CH_3CN/0.1 M $AcONH_4$ (pH 6.5) 20—45% in 30 min; flow rate, 1 ml/min; detected at 275 nm]; t_R 15.33 min.

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References and Notes

1) Amino acids used in this work are of L-configuration. The following abbreviations are used: Fmoc = 9-fluorenylmethyloxycarbonyl, ^{t}Bu = *tert*-butyl, Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, TFA = trifluoroacetic acid, TMSBr = trimethylsilyl bromide, TFMSA = trifluoromethanesulfonic acid, MSA = methanesulfonic acid, AcOH = acetic acid, NMM = *N*-methylmorpholine, DMF = dimethylformamide, AcOEt = ethyl acetate, MeOH = methanol, *n*-BuOH = *n*-butanol, DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, LAP = leucine aminopeptidase.
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