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Syntheses of Two Tyrosine-Sulphate Containing Peptides, Leucosulfakinin (LSK)-II and Cholecystokinin (CCK)-12, Using the O-p-(Methylsulphinyl)benzyl Serine for the Selective Sulphation of Tyrosine

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Key words *: tyrosine sulphate containing peptide ; selective sulphation on tyrosine ; p-(methylsulphinyl)benzyl protecting group for serine ; Fmoc-solid phase peptide synthesis*

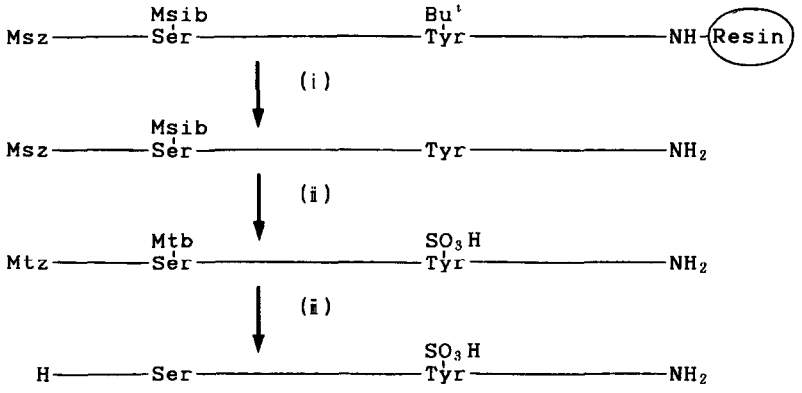
Abstracts *: A novel approach for the synthesis of tyrosine sulphate [Tyr(SO_3H)]-containing peptides was developed. In this approach, trifluoroacetic acid stable O-p-(methylsulphinyl)-benzyl group was used as a key protecting group for serine to achieve the selective sulphation of tyrosine.*

Various tyrosine sulphate [Tyr(SO_3H)]-containing peptides and proteins have been isolated from a wide variety of natural sources, and sulphate ester formation on Tyr residue in peptides and proteins has become to be recognized as one of the most general post-translational modifications [1]. The essential function of sulphate ester towards the organisms is still unclear, however, some biologically active peptides, such as CCK [2] and caerulein [3], require the sulphate ester on Tyr for the expression of their characteristic biological activities. From a synthetic point of view, the synthesis of Tyr(SO_3H)-containing peptides is a great challenge. Although many synthetic achievements have been reported [4], more efficient and convenient synthetic strategies are still being explored. One of the most crucial problems to be solved is the selection of a suitable protecting group for Ser and/or Thr, since the alcoholic hydroxy group

is known to be sulphated in preference to the phenolic hydroxy group of Tyr[4-7]. We report here a new approach for the synthesis of Tyr(SO_3H)-containing peptides, where Ser(Msib) [Msib=p-(methylsulphinyl)benzyl] was employed as a key protecting group for the selective sulphation of Tyr. In combination with the Fmoc-solid-phase method [5], we expected to obtain Tyr(SO_3H)-containing peptide easily.

The Msib group was originally introduced by Samanen and Brandeis as a carboxy protecting group [6] and has the unique property that a Msib ester is stable to TFA, but its reduced form, the Mtb ester [Mtb=p-(methylthio)benzyl] is readily removable with TFA. We expected that Ser(Msib) derivative has the similar chemical property to that of the reported Msib ester.

The outline of our synthetic approach is shown in Scheme 1. The peptide chain is conveniently constructed with Fmoc-solid-phase method, where Msib group is adopted as a side chain protecting group of Ser and the N-terminal and ε-amino group of Lys are protected with Msz [7, 8] groups [Msz=p-(methylsulphinyl)benzyloxycarbonyl]. After removal of all protecting groups except the Msib group on Ser and the Msz group on amino function with TFA treatment, sulphation of Tyr is conducted with DMF-SO_3 complex [9]. We have already shown that DMF-SO_3 complex has greater ability for sulphation than that of the commonly used pyridine-SO_3 complex. On addition of ethane-1,2-dithiol (EDT) to the DMF-SO_3 complex, reduction of the Msib and the Msz groups to the TFA-labile Mtb and Mtz[7, 8] [Mtz=p-(methylthio)benzyloxycarbonyl] groups, respectively, can be expected[10] simultaneously with the sulphation.



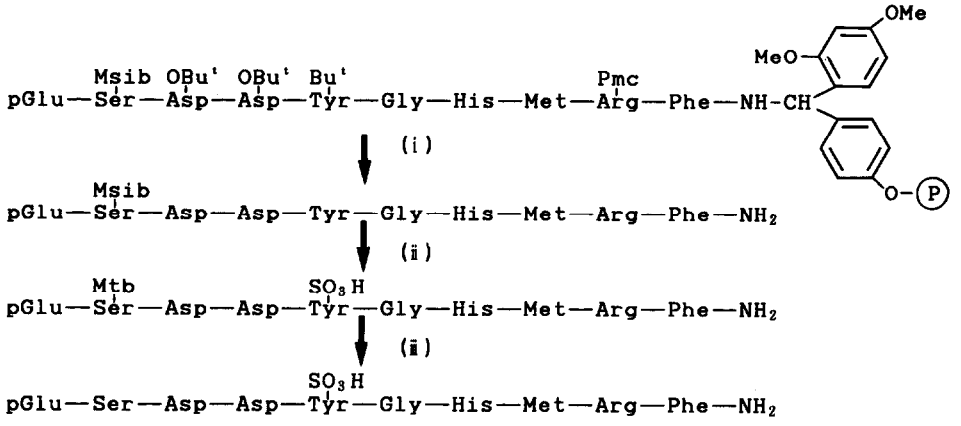
Scheme 1 . Synthetic Outline of a New Approach to the Tyr(SO_3H)-containing Peptide

(i) Detachment of peptide from the resin and removal of the Bu^t-type protecting groups by TFA; (ii) sulphation of Tyr and reduction of Msib and Msz groups by DMF-SO_3 complex in the presence of EDT; (iii) removal of Mtb and Mtz groups by TFA.

Finally, the Mtb and Mtz groups are removed by the treatment with TFA to afford the desired sulphated peptide. With this approach, we expected to obtain the sulphated peptide easily, though Tyr(SO_3H) would be partially decomposed in the final TFA-treatment because of the instability of Tyr(SO_3H) to acids [11].

Chemical behaviours of Ser(Msib) and Ser(Mtb) were examined on TLC using model peptides, Z(OMe)-Ser(Msib)-Ala-NH_2 and Z(OMe)-Ser(Mtb)-Ala-NH_2. Z(OMe)-Ser(Msib)-OH was prepared from Z(OMe)-Ser-OH with Msib-Br[10-b] and NaH, and Z(OMe)-Ser(Mtb)-OH with Mtb-Br[10-b] and NaH, respectively, in basically the similar manner with the Ser(Bzl) derivative[12]. Each derivative was characterized as a cyclohexylamine salt. Then above two model dipeptides were prepared by the DCC plus N-hydroxybenzotriazole (HOBt)[13] or the mixed anhydride methods[14], respectively. When acid stability was examined, Ser(Msib) was found to have almost the same properties as those of the Msib ester and Msz group : Ser(Msib) is stable to TFA, 10% EDT in TFA, and 2% EDT-containing dimethylanisole (DMA) in TFA at 20 ℃ for 3 h, respectively, while its reduced form, Ser(Mtb), is cleavable with TFA, 90% aqueous TFA, and 10% EDT in TFA at 0 ℃ for 1 h. Also Ser(Msib) was readily reduced to Ser(Mtb) with DMF-SO_3 complex/ EDT system[10] (20 ℃, 1 h). With these model experiments, Ser(Msib) was confirmed to have the required chemical properties for our approach. We applied this approach for the synthesis of a cockroach-derived neuropeptide, leucosulfakinin (LSK)-II [15] (Scheme 2).

The peptide chain was constructed with Fmoc-solid-phase method. Fmoc-Ser(Msib)-OH was prepared from H-Ser(Msib)-OH and 9-fluorenylmethyl-N-succinimidylcarbonate (Fmoc-OSu) [16], the former of which was obtained by the TFA-treatment of Z(OMe)-Ser(Msib)-OH. Besides Ser(Msib), Fmoc-amino acid derivatives bearing TFA-labile protecting groups based on tert-butyl alcohol were employed, together with Arg(Pmc)[17] [Pmc=2,2,5,7,8-pentamethylchroman-6-sulphonyl]. N-terminal pGlu was incorporated as pGlu-OH instead of its Msz derivative since there would be no possibility for sulphamic acid formation at pGlu residue. TFA-labile trialkoxybenzhydrylamine-type resin[18] was used for the peptide anchor. The peptide chain was elongated manually with 2 equiv. of Fmoc-amino acid and BOP reagent [benzotriazolyl-N-oxytris(dimethylamino)phosphonium hexafluorophosphate][19] and 6 equiv. of N-methylmorpholine. The protected peptide resin thus obtained was treated with TFA in the presence of 10% EDT and 5% m-cresol at room temperature for 3 h to detach the peptide from the resin and to remove the protecting groups except the Msib on Ser [20]. Next, sulphation and reduction were simultaneously conducted on the partially protected peptide with DMF-SO_3 complex (50 equiv.) and EDT (50 equiv.) in DMF-

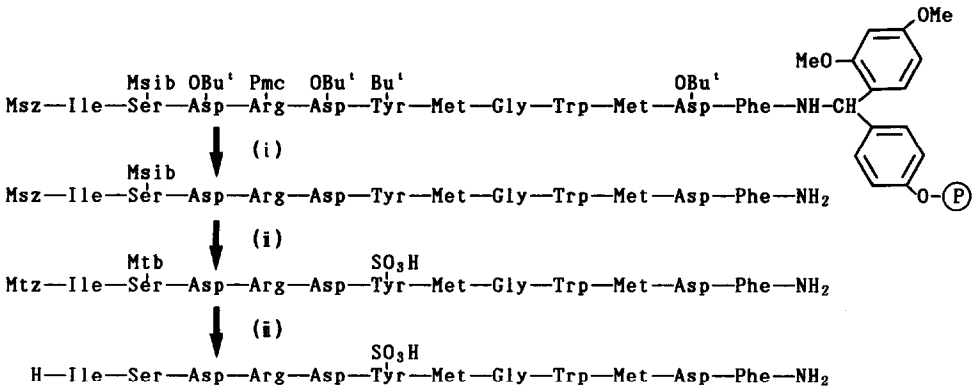


Scheme 2 . Synthetic Scheme to Leucosulfakinin-II

(i) TFA-10% EDT-5% m-cresol (room temp., 3 h), then Sephadex G-10; (ii) DMF-SO_3 complex/EDT (50 equiv. each, room temp., 36 h), then Sephadex LH-20; (iii) 90% aqueous TFA-2-methylindole (4 ℃, 1 h), then Sephadex G-10, then HPLC on YMC AM-312. ((P) =polystyrene resin.)

pyridine (4:1) at room temperature for 36 h. After gel-filtration on Sephadex LH-20, final deprotection of the Mtb group was conducted with 90% aqueous TFA in the presence of 2-methylindole at 4 ℃ for 1 h. This final deprotection system was previously recommended by Wünsch et al.[23] to minimize the decomposition of Tyr(SO_3H). Subsequent purification by gel-filtration on Sephadex G-10 followed by high performance liquid chromatography (HPLC) purification at neutral pH condition (Fig. 1-a) afforded pure LSK-II in 6% yield based on the initial introduction of Phe onto the resin. The structure of synthetic LSK-II was confirmed by amino acid analysis after $Ba(OH)_2$ hydrolysis[24], fast atom bombardment mass spectrometry (FABMS). In addition, the characteristic absorptions of the sulphate ester were observed in Fourie-transform infrared spectrometry (FTIR). On analytical HPLC, this sample exhibited a sharp single peak (Fig. 1-b and c) and coeluted with LSK-II prepared by the solution-phase method [9].

Next, we applied this approach to the synthesis of CCK-12. The synthetic outline is shown in Scheme 3. The peptide chain was constructed on trialkoxybenzhydrylamine-type resin with Fmoc-solid-phase method as described above. Here, in addition to the hydroxy group of Ser, N^{α}-amino function must be protected during sulphation to avoid possible sulphamic acid formation. Considering the one-step removal of protecting groups after sulphation, the N-terminal Ile residue was incorporated as Msz-Ile, which was easily prepared according to the method of Kiso et al [7]. Detachment of the peptide from the resin and removal of the protecting groups except the Msib



Scheme 3 . Synthetic Scheme to Cholecystokinin-12

(i) TFA-10% EDT-5% m-cresol (room temp., 3 h) ; (ii) DMF-SO_3 complex/EDT (100 equiv. each,, room temp., 36 h), then Sephadex LH-20 ; (iii) 90% aqueous TFA-m-cresol-2-methylindol (4 ℃, 1 h), then Sephadex G-10, then HPLC on YMC AM-312. ((P) =polystyrene resin.)

and Msz groups were conducted with TFA in the presence of 10% of EDT and 5% of m-cresol at room temperature for 3 h as in the case of LSK-II. The partially protected peptide thus obtained was treated with DMF-SO_3 complex (100 equiv.) and EDT (100 equiv.) in DMF-pyridine (4:1) at room temperature for 36 h, then gel-filtered on Sephadex LH-20. The Mtb and Mtz groups were finally removed with 90% aqueous TFA-m-cresol-2-methylindole [25] at 4 ℃ for 60 min. Subsequent purification by gel-filtration on Sephadex G-10 followed by HPLC purification at neutral pH condition (Fig. 2-a) afforded pure CCK-12 in 7% yield based on the initial introduction of Phe onto the resin. The structure of the synthetic CCK-12 was confirmed by leucine aminopeptidase (LAP) digestion [26], FABMS, and FTIR.

Finally, the degree of degradation of Tyr(SO_3H) under the final acidolytic condition was examined on HPLC using purified LSK-II and CCK-12, respectively, and loss of the sulphate ester was estimated to be 10-15%.

The characteristic feature of this method is to sulphate Tyr selectively with the aid of Msib-derived protecting groups. In addition, the peptide chain itself is constructed easily with Fmoc-solid-phase method. Through the syntheses of LSK-II and CCK-12 described here, we showed that Tyr(SO_3H)-containing peptides could be conveniently prepared with this approach. This approach will be applicable to the synthesis of other Tyr(SO_3H)-containing peptides.

EXPERIMENTAL

M.p.s were determined with a Yanagimoto micro apparatus and are uncorrected. Optical rotations were determined with a Union PM-201 polarimeter. HPLC was conducted with a Hitachi L-6200 model. Amino acid compositions in acid hydrolysates [12N HCl-propionic acid (1:1, v/v), 110 ℃, 22 h], $Ba(OH)_2$ hydrolysates [0.2M $Ba(OH)_2$, 110 ℃, 20 h], and LAP digests (Sigma, No. L-6007) were determined with a Hitachi 8500 model amino acid analyser. FAB mass spectra were recorded on a JEOL JMS-D 300 spectrometer. FTIR spectra were obtained on a Perkin Elmer 1720 spectrometer.

Thin layer chromatogram (TLC) was developed on pre-coated Silica gel $60F_{254}$ plate (Merck, 1 x 8 cm) and visualized by the following methods: UV light, I_2 vapor, ninhydrin, and Sakaguchi reagent. R_f Values on TLC refer to the following solvent systems : R_{f1} $CHCl_3$-MeOH-H_2O (8:3:1 v/v, lower phase), R_{f2} $CHCl_3$-MeOH-AcOH (9:1:0.5), R_{f3} n-BuOH-AcOH-AcOEt-H_2O (1:1:1:1).

DMF-SO_3 complex was purchased from Fluka, and 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin and Fmoc-Arg(Pmc)-OH•IPE were purchased from Novabiochem. Other Fmoc amino acid derivatives were purchased from MilliGen. DMF and pyridine, solvents used for the sulphation and reduction, were distilled and stored on freshly activated molecular sieves 4A. Deprotections with TFA and sulphation reactions were carried out under argon atmosphere.

Z(OMe)-Ser(Msib)-OH•CHA

NaH (60% oil suspension; 0.30 g, 7.77 mmol) was added to a solution of Z(OMe)-Ser-OH (0.95 g, 3.53 mmol) in distilled DMF (40 ml) at -20 ℃ in 30 min, after which Msib-Br (0.99 g, 4.24 mmol) was added. The mixture was stirred at ambient temperature for 3 h, then the solvent was evaporated off *in vacuo*. The residue was dissolved in 5% aqueous $NaHCO_3$ (25ml) and washed with ether. The aqueous layer was acidified with citric acid. The product was extracted with AcOEt (30 ml) and the extract was washed with water, dried (Na_2SO_4), and concentrated to 5 ml. Cyclohexylamine (0.35 ml) was added in one portion to this ice-chilled solution, then the solution was concentrated. The residue was treated with ether to afford a solid, which was recrystallized from MeOH with ether to afford crystals; yield 0.82 g (44%); m.p. 135-138 ℃; $[\alpha]_D^{25}$ +12.0° (c 1.0, MeOH); R_{f1} 0.31. *Anal*.Calcd for $C_{20}H_{23}NO_7S$•$C_6H_{13}N$•1/4H_2O : C,59.46; H,7.00; N,5.35%. Found : C,59.43; H,6.98; N,5.18%. FABMS, m/z 521.0 $(M+H)^+$.

Z(OMe)-Ser(Mtb)-OH•CHA

NaH (60% oil suspension; 0.16 g, 4.10 mmol) was added to a solution

of Z(OMe)-Ser-OH (0.50 g, 1.86 mmol) in distilled DMF (20 ml) at -20 ℃ in 30 min, after which Mtb-Br (0.48 g, 2.20mmol) was added. After the mixture was stirred at room temperature for 3 h, the product was isolated and characterized as a CHA salt in the similar manner described for the preparation of Z(OMe)-Ser(Msib)-OH·CHA. Recrystallization from MeOH with ether afforded crystals; yield 0.51 g (54%); m.p. 113-116 ℃; $[\alpha]_D^{25}$ +11.9° (c 1.0, MeOH); R_{f1} 0.67. Anal.Calcd for $C_{20}H_{23}NO_6S \cdot C_6H_{13}N \cdot 1/4H_2O$: C,61.33; H,7.23; N,5.50%. Found : C,61.47; H,7.39; N,5.52%. FABMS, m/z 505.0 $(M+H)^+$.

Z(OMe)-Ser(Msib)-Ala-NH_2

Z(OMe)-Ala-NH_2 (63 mg, 0.25 mmol) was treated with TFA (0.5 ml) in the presence of anisole (0.2 ml) in an ice-bath for 1.5 h, then TFA was evaporated in vacuo. After the residue was washed with n-hexane, and dried over KOH pellets for 2 h, then dissolved in DMF (5 ml) containing TEA (35 µl, 0.25 mmol). To an ice-chilled solution of Z(OMe)-Ser(Msib)-OH [prepared from its CHA salt (0.13 g, 0.25 mmol)] in DMF (5 ml), DCC (52 mg, 0.25 mmol), HOBt (38 mg, 0.25 mmol) and above prepared solution of H-Ala-NH_2 were added. After the mixture was stirred at room temperature for 24 h, the formed dicyclohexylurea was removed by filtration. The filtrate was concentrated, and the residue was treated with 5% aqueous citric acid. The resulting solid was collected by filtration and washed with 5% aqueous citric acid, 5% aqueous $NaHCO_3$, and water in a batchwise manner. Recrystallization from DMF with ether afforded crystals; yield 60 mg (49%); m.p. 168-170 ℃; $[\alpha]_D^{25}$ +14.5° (c 1.0, DMF); R_{f1} 0.55; R_{f2} 0.30. Anal.Calcd for $C_{23}H_{29}N_3O_7S \cdot 1/4H_2O$: C,55.68; H,5.99; N,8.47%. Found : C,55.36; H, 5.90; N, 8.37%.

Z(OMe)-Ser(Mtb)-Ala-NH_2

Z(OMe)-Ala-NH_2 (0.28 g, 1.13 mmol) was treated with TFA (1 ml)-anisole (0.5 ml) for 1.5 h with ice-cooling. The product was dissolved in DMF (10 ml) containing NMM (0.12 ml, 1.13 mmol). Z(OMe)-Ser(Mtb)-OH [prepared from its CHA salt (0.38 g, 0.94 mmol)] was dissolved in DMF (10 ml) and, to this solution, TEA (0.13 ml, 0.94 mmol) and isobutylchloroformate (0.15 ml, 1.13 mmol) were added at -30 ℃. After 15 min, the above prepared solution of H-Ala-NH_2 was added to this solution. The mixture was stirred for 3 h at 0 ℃, the solvent was removed by evaporation in vacuo. The residue was dissolved in AcOEt (50 ml) and the AcOEt layer was successively washed with 5% aqueous citric acid, 5% aqueous $NaHCO_3$, and water, then dried (Na_2SO_4). The solvent was concentrated and the residue was triturated with ether to afford a solid. Recrystallization from MeOH with ether afforded crystals;

yield 0.38 g (85%); m.p. 126-129 ℃; $[\alpha]_D^{25}$ +12.5° (c 1.0, DMF); R_{f1} 0.57; R_{f2} 0.54. Anal.Calcd for $C_{28}H_{29}N_3O_6S$: C,58.09; H,6.15; N,8.84%. Found : C,58.26; H,6.39; N,8.59%.

Z(OMe)-Ser-Ala-NH_2

Z(OMe)-Ala-NH_2 (2.0 g, 8.00 mmol) was treated with TFA (10 ml)-anisole (1 ml) for 1.5 h with ice-cooling. The product was dissolved in DMF (50ml) containing TEA (2.52 ml, 18.0 mmol). Z(OMe)-Ser-$NHNH_2$ (2.72 g, 9.60mmol) was dissolved in DMF (20 ml) containing 4N HCl/DMF (4.8 ml, 19.2mmol) and solution was cooled to -20 ℃, then isoamyl nitrite (1.35 ml, 10.0 mmol) was added. The mixture was stirred for 20 min below 0 ℃, then TEA (2.70 ml, 19.2 mmol) and the above prepared solution of H-Ala-NH_2 were added. After the mixture was stirred for 24 h at 4 ℃, the solvent was removed by evaporation in vacuo. The product was isolated by the extraction method (n-BuOH was used as an extraction solvent) in the similar manner described for the preparation of Z(OMe)-Ser(Mtb)-Ala-NH_2. Recrystallization from DMF with ether afforded crystals; yield 2.65 g (98%); m.p. 168-170 ℃; $[\alpha]_D^{25}$ +28.7° (c 1.0, DMF); R_{f1} 0.50. Anal.Calcd for $C_{15}H_{21}N_3O_6$: C, 53.09; H,6.24; N,12.38%. Found : C,52.78; H,6.26; N,12.37%.

H-Ser-Ala-NH_2·TFA

Z(OMe)-Ser-Ala-NH_2 (100 mg, 0.30mmol) was treated with TFA (2 ml) in the presence of anisole (0.2 ml) in an ice-bath for 1 h, after which TFA was removed by evaporation in vacuo. Trituration of the residue with ether afforded a powder, which was recrystallized from MeOH with ether to afford crystals; yield 42 mg (82%); m.p. 173-175 ℃; $[\alpha]_D^{25}$ +27.6° (c 1.0, DMF); R_{f3} 0.26. Anal.Calcd for $C_6H_{13}N_3O_3 \cdot CF_3COOH$: C, 33.22; H,4.88; N,14.53%. Found : C,33.33; H,5.06; N,14.72%.

H-Ser(Msib)-Ala-NH_2·HCl

Z(OMe)-Ser(Msib)-Ala-NH_2 (100 mg,0.20 mmol) was treated with TFA (1 ml) in the presence of anisole (0.2 ml) in the similar manner described above. TFA was removed by evaporation in vacuo, then the residue was partitioned between AcOEt (2 ml) and water (5 ml). The aqueous layer was lyophilized and the residue was again dissolved in water (10 ml) containing 1N HCl (0.2 ml) and lyophilized to afford a hygroscopic powder; yield 49 mg (67%); R_{f3} 0.33. Anal.Calcd for $C_{14}H_{21}N_3O_4S \cdot HCl \cdot 1.5H_2O$: C,43.02; H,6.45; N,10.75%. Found : C,42.63; H,6.14; N,10.58%. FABMS, m/z 328.0 $(M+H)^+$.

Acid Stabilities of Ser(Msib) and Ser(Mtb)

Acid stabilities of Ser(Msib) and Ser(Mtb) were examined using the

model peptides, Z(OMe)-Ser(Msib)-Ala-NH_2 or Z(OMe)-Ser(Mtb)-Ala-NH_2, on TLC. When Z(OMe)-Ser(Msib)-Ala-NH_2 (10 mg, R_{f1} 0.55, R_{f2} 0.30) was treated with (i) TFA (0.5 ml), (ii) TFA-10% EDT (0.5 ml), and (iii) TFA-2% EDT containing DMA (3:1 v/v, 0.5 ml) at 20 ℃ for 3 h, the spot on TLC was identical with that of H-Ser(Msib)-Ala-NH_2 (R_{f3} 0.33). No other spots including H-Ser-Ala-NH_2 (R_{f3} 0.26) were observed. When Z(OMe)-Ser(Mtb)-Ala-NH_2 (10 mg, R_{f1} 0.57, R_{f2} 0.54) was treated with the above reagents, (i) - (iii), and (iv) 90% aqueous TFA (0.5 ml) at 0 ℃ for 1 h, respectively, the only spot observed was identical with that of H-Ser-Ala-NH_2 (R_{f3} 0.26).

Reduction of Ser(Msib) by DMF-SO_3 Complex/EDT System

Z(OMe)-Ser(Msib)-Ala-NH_2 (10 mg) was dissolved in DMF-pyridine (4:1, 1 ml), in which DMF-SO_3 complex (31 mg, 10 equiv.) and EDT (19 µl, 10 equiv.) were added. The mixture was stirred at 25 ℃ for 1 h, the starting material (R_{f1} 0.55, R_{f2} 0.30) disappeared (TLC), having been fully converted into Z(OMe)-Ser(Mtb)-Ala-NH_2 (R_{f1} 0.57, R_{f2} 0.54).

Mtz-Ile-OH

To an ice-chilled solution of H-Ile-OH (0.43 g, 3.20 mmol) in H_2O (20 ml) containing TEA (0.45 ml, 3.20 mmol), Mtz-OSu [prepared from p-(methylthio)benzyl alcohol (0.50 g, 3.20 mmol), disuccinimidyl-carbonate (0.90 g, 3.50 mmol), and TEA (0.45 ml, 3.20 mmol) in THF-CH_3CN (15 ml-15 ml)] was added. The mixture was stirred at room temperature for 15 h, after which the solvent was removed by evaporation. The residue was dissolved in water (20 ml) and washed with ether twice. The aqueous layer was acidified with 0.5M HCl. The product was extracted with AcOEt (25 ml) and the extract was washed with water, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was triturated with n-hexane to afford a solid, which was recrystallized from MeOH with n-hexane to afford crystals; yield 0.70 g (69%); m.p. 77-79 ℃; $[\alpha]_D^{25}$ +5.0° (c 1, MeOH); R_{f1} 0.53; R_{f2} 0.61. Anal. Calcd for $C_{15}H_{21}NO_4S$:C,57.85; H,6.80; N,4.50%. Found : C,57.76; H,6.94; N, 4.35%. FABMS, m/z 312.0 $(M+H)^+$.

Msz-Ile-OH

Above prepared Mtz-Ile-OH (100 mg, 0.32 mmol) dissolved in AcOH (5 ml) was treated with 30% H_2O_2 (170 µl, 0.48 mmol) at room temperature for 5 h. The reaction mixture was poured into AcOEt (20 ml) and the AcOEt layer was washed with 1M HCl and water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was triturated with a mixture of n-hexane/ether (3:1, 20 ml) to afford a solid, which was recrystallized from MeOH with a mixture of n-hexane/ether (3:1) to afford crystals; yield

95 mg (90%); m.p. 119-121 ℃; $[\alpha]_D^{25}$ +13.4° (c 1, MeOH); R_{f1} 0.42; R_{f2} 0.32. Anal.Calcd for $C_{15}H_{21}NO_5S \cdot 1/4H_2O$: C,54.28; H,6.53; N,4.22%. Found : C,54.65; H,6.55; N,4.03%. FABMS, m/z 328.0 $(M+H)^+$.

Acid Stability of Msz-Ile-OH and Mtz-Ile-OH

Msz-Ile-OH (10 mg) was dissolved in 10% EDT in TFA (1 ml) and the mixture was stirred at 20 ℃ for 3 h. No change was observed on TLC. When Mtz-Ile-OH (10 mg) was treated with TFA (1 ml), 90% aqueous TFA (1 ml), or 10% EDT in TFA (1 ml) at 0 ℃ for 1 h, the spot corresponding to Mtz-Ile-OH (R_{f1} 0.53) disappeared, having been fully converted into the spot corresponding to H-Ile-OH (R_{f3} 0.51).

Reduction of Msz-Ile-OH by DMF-SO_3 Complex/EDT System

Msz-Ile-OH (10 mg) was dissolved in DMF-pyridine (4:1, 1 ml), in which DMF-SO_3 complex (48 mg, 10 equiv.) and EDT (28 µl, 10 equiv.) were added. The mixture was stirred at 20 ℃ for 1 h, starting material (R_{f1} 0.42, R_{f2} 0.32) dissappeared (TLC), having been fully converted into Mtz-Ile-OH (R_{f1} 0.53, R_{f2} 0.61).

H-Ser(Msib)-OH

Z(OMe)-Ser(Msib)-OH [prepared from its CHA salt (100 mg, 0.20 mmol)] was treated with TFA (2 ml) in the presence of anisole (0.4 ml) in an ice-bath for 1 h, after which TFA was removed by evaporation. Trituration of the residue with ether afforded a powder, which was recrystallized from MeOH with ether in the presence of a few drops of TEA to afford crystals; yield 30 mg (61%); m.p. 139-141 ℃; $[\alpha]_D^{25}$ +14.8° (c 1, AcOH); R_{f3} 0.33. Anal.Calcd for $C_{11}H_{15}NO_4S \cdot 1/2H_2O$: C, 49.60; H,6.06; N,5.26%. Found : C,49.80; H,5.72; N,5.31%.

Fmoc-Ser(Msib)-OH

To an ice-chilled solution of H-Ser(Msib)-OH (0.78 g, 1.92 mmol) in DMF (20 ml), Fmoc-OSu (0.71 g, 2.11 mmol) was added and the pH of the solution was maintained at 8 - 9 with TEA. After the mixture was stirred for 2 h, the solution was diluted with AcOEt (100 ml). The AcOEt layer was washed with 0.5M HCl and water, dried (Na_2SO_4), and concentrated. Trituration of the residue with n-hexane afforded a solid, which was recrystallized from AcOEt to afford crystals; yield 0.80 g (85%); m.p. 134-135 ℃; $[\alpha]_D^{25}$ -1.9° (c 1, DMF); R_{f1} 0.41. Anal.Calcd for $C_{26}H_{25}NO_6S$: C,65.12; H,5.26; N,2.92%. Found : C,65.04; H, 5.25; N,2.82%. FABMS, m/z 480.0 $(M+H)^+$.

Peptide Chain Assembly of LSK-II by Solid-phase Synthesis

Solid-phase synthesis was carried out manually, basically according to

the method of Atherton et al[27]. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (substitution level; 0.57 mmol/g resin) was used for the peptide anchor. After deprotection of Fmoc group attached on the resin with 20% piperidine in DMF, Fmoc-Phe-OH (2.0 equiv.) was introduced with BOP-reagent (2.0 equiv.) in the presence of NMM (6.0 equiv.). The manipuration in each elongation step were summarized in Table. The following Fmoc-amino acid derivatives were introduced successively ; Arg(Pmc), Met, His(Fmoc), Gly, Tyr(Bu^t), Asp(OBu^t), Ser(Msib). The N-terminal pGlu was introduced in the form of pGlu-OH. Every condensation reaction was continued until the resin became negative to the Kaiser test[28]. Starting with 351 mg (0.20 mmol) of the resin, 687 mg of the protected peptide resin was obtained. Amino acid analysis of an acid hydrolysate gave the following ratios : Asp 1.90 (2), Ser 0.59(1), Glu 0.85 (1), Gly 1.00 (1), Met 0.67 (1), Tyr 0.74 (1), Phe 0.90(1), His 0.85 (1), Arg 0.80 (1).

Preparation of LSK-II

The protected LSK-II resin (100 mg) was treated with TFA-EDT-m-cresol (85:10:5 v/v, 3 ml) at room temperature for 3 h. The resin was removed by filtration and washed with cold TFA (3 ml) twice. The filtrate and washing were combined and concentrated at 30 ℃, then dry ether (50 ml) was added. The formed precipitate was collected by centrifugation and the residue was dried over KOH pellets in vacuo, 40 mg. The dried material was dissolved in DMF-pyridine (4:1, 2 ml) and treated with DMF-SO_3 complex (225 mg, 50 equiv.) and EDT (135 µl, 50 equiv.) at room temperature for 36 h. The solution was applied to a column of

Manipuration	Reagent	Solvent	Time/Repeat
Deprotection	20% piperidine/DMF		5 min x 1 20 min x 1
Washing		DMF	1 min x 6
Coupling	Fmoc-A.A.-OH (2 equiv.) BOP-reagent (2 equiv.) NMM (6 equiv.)	DMF	120 min x 1
Washing		DMF	1 min x 6
Kaiser test[28]			

Table. Manual Schedule for the Fmoc-Solid Phase Synthesis. (A.A.=amino acid.)

Sephadex LH-20 (2.4 x 78 cm) and eluted with DMF. The first main peak (tube Nos.21-30, 6 ml each, detected by UV absorption measurement at 275 nm) was combined and the solvent was removed by evaporation *in vacuo* at 30 ℃. The residue was treated with dry ether to afford a powder (40 mg). The dried powder thus obtained was finally treated with 90% aqueous TFA (1 ml) in the presence of 2-methylindole (94 mg, 25 equiv.) at 4 ℃ for 60 min, then dry ether (50 ml) was added. The resulting precipitate was collected by centrifugation and washed with dry ether three times. The residue was dissolved in 5% AcOH (2 ml) and applied to a column of Sephadex G-10 (2.0 x 53 cm), which was eluted with 5% AcOH. The first main peak (tube Nos. 11-19, 5 ml each, detected by UV absorption measurement at 265 nm) was combined and the solvent was removed by lyophilization to afford a white fluffy powder; 21 mg (Fig. 1-a). Subsequent purification on HPLC was carried out on a YMC AM-312 column (6 x 150 mm), which was eluted with a gradient of CH_3CN (12-25% in 30 min) in 0.1M $AcONH_4$ (pH 6.5) at a flow rate of 1 ml/min. Portion of the above obtained powder (*ca*.1.5 mg each) in 0.1M $AcONH_4$ (2 ml) was applied to the column, and eluate corresponding to the main peak (monitored by UV absorption measurement at 265 nm) was collected. The solvent was removed by repeated lyophilization to afford a white fluffy powder (1.9 mg combined yield, 6% from the initial Phe introduction onto the resin). R_{f3} 0.25. HPLC: R_t 15.2 min (Fig.1-b), R_t 16.8 min (Fig.1-c), conditions are described in the

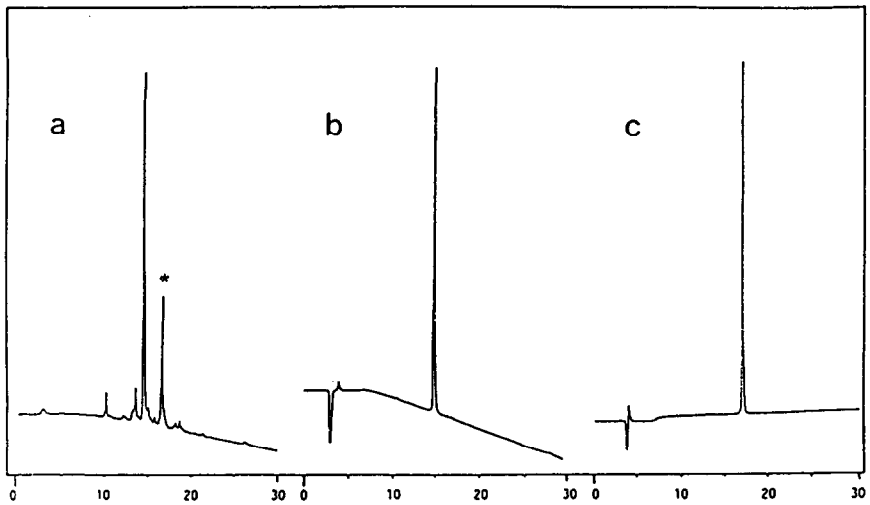


Figure 1 . HPLC of the Synthetic Leucosulfakinin (LSK)-II.

a) Gel-filtered sample (Asterisk shows LSK-II non-sulphate); b) and c) HPLC-purified sample. [Column: Cosmosil $5C_{18}$-AR (6 x 150 mm). Elution: for a) and b), gradient elution with CH_3CN (10-40% in 30 min) in 0.1M $AcONH_4$ (pH 6.5); for c), gradient elution with CH_3CN (10-40% in 30 min) in 0.1% aqueous TFA. Flow rate: 1 ml/min. Detection: 215 nm.].

legend of Fig.1. The purified sample was coeluted on HPLC with the LSK-II prepared before[9]. Amino acid ratios after 0.2M $Ba(OH)_2$ hydrolysis : Asp 1.82 (2), Ser 0.43 (1), Glu 1.03 (1), Gly 1.09 (1), Met 0.90 (1), Phe 1.00 (1), Tyr(SO_3H) 0.87 (1), His 0.71 (1), Arg N.D. (1), (recovery of Phe, 87%). FABMS, m/z 1317.0 $(M+H)^+$; FTIR, 1047 and 1259 cm^{-1} (sulphate ester).

Peptide Chain Assembly of CCK-12 by Solid-Phase Synthesis

Solid-phase synthesis was carried out basically in the same manner described in LSK-II. The N-terminal Ile was introduced as Msz-Ile-OH. Starting with 476 mg (0.20 mmol) of the resin (substitution level; 0.42 mmol/g resin), 927 mg of the protected peptide resin for CCK-12 was obtained. Amino acid analysis of an acid hydrolysate gave the following ratios : Asp 3.10 (3), Ser 0.59 (1), Gly 1.00 (1), Met 1.80 (2), Ile 1.01 (1), Tyr 1.00 (1), Phe 1.00 (1), Trp N.D.(1), Arg 0.94 (1).

Preparation of CCK-12

The protected CCK-12 resin (100 mg) was treated with TFA-EDT-*m*-cresol (85:10:5 v/v, 3 ml) at room temperature for 3 h. The resin was removed by filtration and washed with ice-chilled TFA (5 ml). The filtrate and the washing were combined and TFA was concentrated by evaporation at 30 ℃, then dry ether (50 ml) was added. The formed precipitate was collected by centrifugation and washed with dry ether (50 ml) three times and dried over KOH pellets *in vacuo*. The dried material (20 mg) was dissolved in DMF-pyridine (4:1, 2 ml) and treated with DMF-SO_3 complex (182 mg, 100 equiv.) and EDT (110 µl, 100 equiv.) at room temperature for 36 h. The solution was applied to a column of Sephadex LH-20 (2.4 x 78 cm) and eluted with DMF. The first main peak (tube Nos.21-26, 5 ml each, detected by UV absorption measurement at 275 nm) was combined and the solvent was removed by evaporation *in vacuo* at 30 ℃. The residue thus obtained was treated with 90% aqueous TFA (1.0 ml) in the presence of *m*-cresol (100 µl) and 2-methylindole (35 mg, 25 equiv.) at 4 ℃ for 1 h. Dry ether (50 ml) was added to this solution, and the formed precipitate was collected by centrifugation. The residue was washed with dry ether three times, then dissolved in 5% NH_4HCO_3 (5 ml). This solution was applied to a column of Sephadex G-10 (2.0 x 53 cm), which was eluted with 5% NH_4HCO_3 and the eluate was monitored by UV absorption measurement at 280 nm. The fractions of first main peak (tube Nos.11-25, 5 ml each) were combined and the solvent was removed by lyophilization to afford a white powder (9.3 mg). Finally, the crude peptide thus obtained (Fig.2-a) was purified by HPLC. Portions of the sample (*ca*. 1.5 mg each) in 0.1M $AcONH_4$ (2 ml) was applied to a YMC AM-312 column (6 x 150 mm), which was eluted

with a gradient of CH_3CN (25-45% in 30 min) in 0.1M $AcONH_4$ (pH 6.5) at a flow rate of 1 ml/min. The eluate corresponding to the main peak (monitored by UV absorption measurement at 265 nm) was collected and the solvent was removed by repeated lyophilization to afford a white fluffy powder (2.3 mg combined yield, 7% from initial Phe introduction onto the resin). R_{f3} 0.33. HPLC: R_t 18.6 min (Fig.2-b), R_t 14.8 min (Fig.2-c), conditions are described in the legend of Fig.2. Amino acid ratios after LAP digestion: Asp 2.76 (3), Ser 0.96 (1), Gly 0.97 (1), Met 1.86 (2), Ile 0.97 (1), Phe 1.00 (1), Tyr(SO_3H) 0.90 (1), Trp 0.87 (1), Arg 0.92 (1) (recovery of Phe, 93%). FABMS, m/z 1614.5 $(M+H)^+$; FTIR, 1051 and 1276 cm^{-1} (sulphate ester).

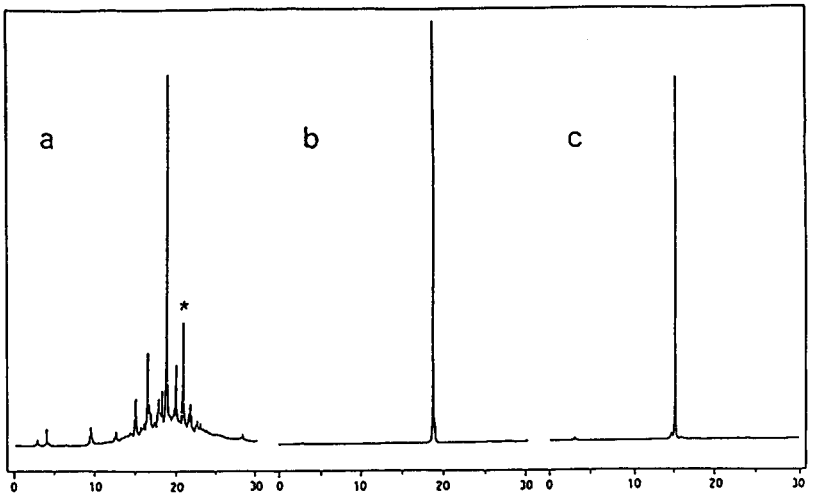


Figure 2. HPLC of the Synthetic Cholecystokinin (CCK)-12.
a) Gel-filtered sample (Asterisk shows CCK-12 non-sulphate); b) and c) HPLC-purified sample. [Column: YMC AM-312 (6 x 150 mm). Elution: for a) and b), gradient elution with CH_3CN (20-45% in 30 min) in 0.1M $AcONH_4$ (pH 6.5); for c), gradient elution with CH_3CN (25-50% in 30 min) in 0.1% aqueous TFA. Flow rate: 1 ml/min. Detection: 265 nm.].

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Abbreviations

All amino acids used in this study are of L-configuration. Abbreviations according to IUPAC-IUB joint commission on Biochemical Nomenclature (Eur. J. Biochem., 1984, 138, 9) were used. Z(OMe)=p-methoxybenzyloxycarbonyl, Bzl=benzyl, Bu^t=tert-butyl, Su=N-hydroxysuccinimidyl, HOBT=N-hydroxybenzotriazole, TFA=trifluoroacetic acid, EDT=ethane-1,2-dithiol, DMA=3,5-dimethylanisole, AcOH=acetic acid, NMM=N-methylmorpholine, TEA=triethylamine, CHA=cyclohexylamine, DMF=

N,N-dimethylformamide, AcOEt=ethyl acetate, MeOH=methanol, n-BuOH=n-butanol, IPE=isopropylether, $AcONH_4$=ammonium acetate, LAP=leucine aminopeptidase, pGlu=pyroglutamic acid.

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