Evaluation of the Final Deprotection System for the Solid-Phase Synthesis of Tyr(SO₃H)-Containing Peptides with 9-Fluorenymethyloxycarbonyl (Fmoc)-Strategy and Its Application to the Synthesis of Cholecystokinin (CCK)-12

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Acidolytic deprotection and cleavage conditions for an acid-labile Tyr(SO₃H)-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(SO₃H)Na in the peptide. The latter deprotection/cleavage system was applied to the 9-fluorenymethyloxycarbonyl (Fmoc)-based solid-phase synthesis of cholecystokinin (CCK)-12 on an acid-labile PAL-linked support (PAL = peptide amide linker), with Fmoc–Tyr(SO₃H)–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(SO₃H)] have been discovered and tyrosine sulfation is regarded as a widespread post-translational event in organisms. However, the roles of sulfate ester are poorly understood with the exception of cholecystokinin (CCK) and caerulein, the Tyr(SO₃H) 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(SO₃H)-containing peptides could serve as useful models. In this regard, much interest has been focused on the establishment of a facile synthetic method for Tyr(SO₃H) containing peptides, overcoming the acid-lability of Tyr(SO₃H).

In Fmoc-based solid-phase peptide synthesis, Fmoc = 9-fluorenymethyloxycarbonyl, 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. As tyrosine-O-sulfate derivatives, such as Tyr(SO₃H)Ba₁₂ or Tyr(SO₃H)Na₁₁, are relatively stable in TFA, the approach in which preparation of Tyr(SO₃H)-containing peptides is accomplished through the Fmoc-strategy using Fmoc–Tyr(SO₃H)–OH (X = Na, Ba₁₂, etc.) as a building block looks attractive. Here, the use of Fmoc–Tyr(SO₃H)–OH provides a practical approach because of its ease of preparation. Previously, Penke and Rivier 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 acetylation 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(SO₃H) in Tyr(SO₃H)-containing peptides and its application to the synthesis of CCK-dodecapeptide (CCK-12) on a recently developed acid-labile PAL-linked support by using the Fmoc-based solid-phase method.

First, the effects of several deprotection procedures currently used in Fmoc-based peptide synthesis upon Tyr(SO₃H)Na were examined by RP-HPLC using a model peptide substrate, Fmoc–Tyr(SO₃H)–Ala–OMe. Tyr(SO₃H)Na 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 of Fmoc–Tyr('Bu)–OH and H–Ala–OMe to give Fmoc–Tyr('Bu)–Ala–OMe, (2) subsequent deprotection of 'Bu group with TFA, and (3) sulfation of the resulting Fmoc–Tyr–Ala–OMe with DMF–SO₃ complex followed by neutralization with Na₂CO₃. Direct coupling of Fmoc–Tyr(SO₃H)–OH and H–Ala–OMe by the DCC–HOBt method resulted in a poor yield due to the lack of a suitable purification method.

The effects of various acids, temperature, and scavengers on Tyr(SO₃H)Na 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(SO₃H)Na at 4°C.](image-url)

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destructive acid for Tyr(SO₃Na), as already argued,¹⁰ whereas strong acids such as trimethylsilyl bromide (TMSBr)¹⁵ and trifluoromethanesulfonic acid (TFMSA)¹⁶ cannot be used for deprotection; (ii) temperature is a decisive determinant of the stability of Tyr(SO₃Na) 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 H₂O, anisole, m-cresol, and 2-methylindole have little effect on the decomposition of Tyr(SO₃Na).

Next, we examined the degradation of synthetic Tyr(SO₃H)-containing peptides, leucosulfakinin (LSK)-II¹⁷ 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(SO₃H)-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-methylindole²⁰ (in the case of Trp-containing peptides) as a final deprotection reagent for the synthesis of Tyr(SO₃H)-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(SO₃Na)-containing peptide is carried out through Fmoc-based solid-phase synthesis, in which Tyr(SO₃Na) is introduced by the use of Fmoc–Tyr(SO₃Na)–OH as a building block; (b) an acid-labile PAL-linked support [PAL = peptide amide linker, 5-(4Fmoc-aminomethyl-3',5'-dimethoxyphenyl)valeric acid]²⁰ 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(SO₃H)-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 reagent²² (3 eq) [BOP = benzotriazolyl tris(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 test²³ of the peptide-resin after each coupling step. The incorporation of Fmoc–Tyr(SO₃Na)–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)

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-methylnindole (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.

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.

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 obtained on a JEOL JMS-D300 spectrometer. FT-IR spectra were obtained on a Perkin Elmer 1720 spectrophotometer. Amino acid compositions in acid hydrolysates (2N 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 F254 (1 × 8 cm, 0.25 mm thickness (Merck)) with the following solvent systems (v/v): RF1, CHCl3:MeOH:H2O (8:3:1, lower layer); RF2, CHCl3:MeOH:AcOH (9:3:0.5); RF3, n-BuOH:MeOH:AcOH:AcOEt:H2O (1:1:1:1:1). For detection, ninhydrin, UV light, and I2 vapor were used.

DMF-SO3 complex was purchased from Fluka, and Fmoc-Arg(Pmc)-OH-PE was purchased from Novabiochem. Other Fmoc amino acid derivatives and PAL-linked support were purchased from Milligen.

Fmoc-Tyr(Bu)-Ala-OMe Fmoc-Tyr(Bu)-OH (1.00 g, 2.18 mmol) was dissolved in DMF (10 ml) containing Et3N (0.3 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 Et3N (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 H2O (20 ml). The organic phase was washed with 5% Na2CO3 (x 2), 5% citric acid (x 3), and H2O (x 3), then dried over Na2SO4. 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 (90%), mp 176-178°C, [α]D 6.6° (c = 0.6, DMF), RF 0.42. Anal. Calcd for C17H17NO4: 1/4H2O: C, 59.99; H, 6.70; N, 5.10. Found: C, 70.04; H, 6.82; N, 4.95.

Fmoc-Tyr-Ala-OMe Fmoc-Tyr(Bu)-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 (x 3) and H2O (x 3), then dried over Na2SO4. 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, [α]D 16.7° (c = 0.6, DMF), RF 0.80. Anal. Calcd for C19H19NO4: 1/2H2O: C, 67.59; H, 5.98; N, 5.63. Found: C, 67.93; H, 5.90; N, 5.63.

Fmoc-Tyr(SO2Na)-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-SO3 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 pOH of the solution was adjusted to 7 with 5% Na2CO3. After evaporation of the solvent, the gelatineous 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 CHCl3-MeOH:H2O (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; 0.85) was eluted and the fractions containing the desired material (RF; 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. [α]D 7.4 to 7.7° (c = 1.0, MeOH), RF; 0.34, RF; 0.52.

Analytical Data: C31H42N2O10S2; C:45.45; H:3.64; N:10.10; S:10.98. FT-IR (KBr): 1501, 1320 cm⁻¹.

Examination of Stability of Tyr(SO₄)Na Using Fmoc–Tyrosine (SO₄)–Ala–Ome

(i) Effect of Acid: Fmoc–Tyr(SO₄)Na–Ala–Ome (10 mg) was treated with the following reagents (1 ml) at 4°C: (1) TFA (90%), (2) 1 M TMSBr/TFA, (3) 1 M TMFMSA/TFA, (4) 1 M MSA/TFA, (5) 5% HCl/DMF, and (6) AcOH (at 30°C). At intervals (1, 2, and 4 h), aliquots of 50 µl were withdrawn and diluted with CH₃CN–0.1 M AcONa (30:70, 1 ml). Then 50 µl of the solution was then analyzed by RP-HPLC [Nucleosil 7C₅ (4.6 x 250 mm)] gradient system, CH₃CN/0.1 M AcONa/0.05 M TFA (pH 5.6) 30–75% in 30 min; flow rate, 1 ml/min; detected at 205 nm); t₁/₂ for Fmoc–Tyr(SO₄)Na–Ala–Ome 14.86 min and Fmoc–Tyrosine–Ala–Ome 21.86 min.

(ii) Effect of Temperature: Fmoc–Tyr(SO₄)Na–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(SO₄)Na–Ala–Ome (5 mg) was treated with the following reagents (1 ml) at 4°C: (1) 1 M EDT/TFA, (2) 1 M thioanisole/TFA, (3) 1 M anisole/TFA, (4) 1 M 2-methylinole/TFA, (5) 1 M 2-benzyl mercaptan/TFA, (6) 1 M 2-nitroanilin/TFA, and (7) 90% aqueous TFA. Aliquots of 50 µl were diluted with an internal standard solution (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(SO₄)Na–Ala–Ome was determined by using the same HPLC conditions.

Degradation Studies of Tyr(SO₄)H Using Synthetic LSK-II and CCK-12

(i) Degradation of LSK-I: Synthetic LSK-I (100 µg) was dissolved in the following reagents (300 µl), which contained m-cresol (50 µl) or 2-methylisothiazole (25 eq) with respect to the peptide, as indicated, with ice-cooling to 4°C: (10% aqueous TFA, (20% aqueous TFA/m-cresol, (3) 90% aqueous TFA/2-methylisothiazole, and (4) 90% aqueous TFA/m-cresol/2-methylisothiazole. At intervals, aliquots of 100 µl were withdrawn after dilution with CH₃CN (250 µl), and 100 µl was analyzed by RP-HPLC [Nucleosil 7C₅ (4.6 x 250 mm)] gradient system, CH₃CN/0.1% TFA 15–35% in 20 min; flow rate, 1 ml/min; detected at 215 nm); t₁/₂ for LSK-I 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-methylisothiazole, and (4) 90% aqueous TFA/m-cresol/2-methylisothiazole. At intervals, aliquots of 50 µl were withdrawn and diluted with 25% CH₃CN/H₂O (200 µl), and 100 µl was analyzed by RP-HPLC [Nucleosil 7C₅ (4.6 x 250 mm)] gradient system, CH₃CN/0.1% TFA 25–45% in 20 min; flow rate, 1 ml/min; detected at 275 nm); t₁/₂ for CCK-12 13.30 min and CCK-12 non-sulfate 14.50 min.

Fmoc–Tyrosine–OH Fmoc–Osu (3.0 g, 8.9 mmol) dissolved in tetrahydrofuran (THF) (25 ml) was added to a solution of H–Tyrosine–OH (2.42 g, 13.3 mmol) in H₂O (50 ml) containing EtN (15.9 ml, 140 mmol) and the reaction mixture stirred 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 H₂O (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 H₂O successively, and dried over Na₂SO₄. The AcOEt was evaporated off in vacuo, and the residue was triturated with n-hexane to a solid, which was recrystallized from AcOEt–ether to give crystals; yield 2.80 g (78%), mp 185–186°C. [α]D 19° (c = 0.6, DMF). RF: 0.28. Analytical Data: C₃₁H₄₂N₂O₁₀S₂; C: 45.45; H: 3.64; N: 10.10; S: 10.98. FT-IR (KBr): 1501, 1320 cm⁻¹.

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, YM-AM-312 (6 x 150 mm); gradient system, CH₃CN/0.1 M AcONa, (pH 6.5) 20–45% in 30 min; flow rate, 1 ml/min; detected at 220 nm.
Sephadex G-10 (2 x 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). The sample (Fig. 6a) was purified by HPLC on a column of Cosmosil 5C18-RP (10 x 250 mm). The crude sample obtained above was dissolved in 0.1 M AcONH4 (1 ml) and a portion of the solution (containing ca. 1 mg) was applied to the column, which was eluted with gradient of CH3CN (25-50% in 30 min) in 0.1 M AcONH4 (pH 6.5) at a flow rate of 2 ml/min. The eluate corresponding to the main peak (tR = 11.38 min, measured by monitoring UV absorbance at 275 nm); tR = 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, Boc = tert-butyloxycarbonyl, Pmc = 2.2.5.7.8-pentamethyloxycamphor-6-sulfonyl, TFSA = trifluoroacetic acid, TMSBr = trimethylsilyl bromide, TFMSA = trifluoromethanesulfonic acid, MSM = methanesulfonic acid, AcOEt = ethyl acetate, MeOH = methanol, i-PrOH = isopropanol, DCC = dicyclohexylcarbodiimide, HOBT = 1-hydroxybenzotriazole, LAP = leucine aminopeptidase.


20) 2-Methylindole is reported to be effective to suppress alkylation on the indole moiety of Trp; E. Wünsch, A. Fontana and F. Dreeze, Z. Naturforsch., 22b, 607 (1967). 2-Methylindole is also effective to minimize deterioration of the Tyr(SO3H) residue in Tyr(SO3H)-containing peptides; G. Borin, A. Calderan, P. Ruzza, L. Moroder, W. Göhring, G. Bovermann and E. Wünsch, Biol. Chem. Hoppe-Seyler, 368, 1368 (1987).


24) In practice, we used the reagent system consisting of 50 eq of m-cresol and 25 eq of 2-methylindole with respect to the peptide.