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Preparation of phosphopeptide thioesters by Fmoc- and Fmoc(2-F)-solid phase synthesis

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Summary

Efficient methods for the preparation of phosphopeptide thioesters were examined, using Fmoc-based solid-phase method. Phosphopeptide thioesters were obtained in good yields by the use of 1-methylpyrrolidine, hexamethyleneimine and 1-hydroxybenzotriazole in a DMSO-DMF (1:1, v/v) solution for deblocking the Fmoc groups. Epimerization, which is often observed at the C-terminal amino acid, was effectively suppressed by shortening the time of deblocking process via the use of highly base sensitive Fmoc(2-F) groups for α-amino protection.

Abbreviations: Alko resin (Wang resin), 4-hydroxymethylphenoxymethylated copoly(styrene-1% divinylbenzene) resin; Boc, *t*-butoxycarbonyl; Bu^t, *t*-butyl; *c*Pen, cyclopentyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc(2-F), 9-(2-fluoro)fluorenylmethoxycarbonyl; GC, gas chromatography; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; $HOCH_2$-Pam, 4-(hydroxymethyl)phenylacetamidomethyl; HOObt, 3,4-dihydroxy-4-oxo-1,2,3-benzotriazine; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; NMP, 1-methylpyrrolidin-2-one; NMR, nuclear magnetic resonance; NH_2-SAL, 4-(α-amino-2,4-dimethoxybenzyl)phenoxymethyl; MBHA, 4-methylbenzhydrylamine; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; THF, tetrahydrofuran; Trt, triphenylmethyl; Tos, *p*-toluenesulfonyl; v/v, volume ratio; wt/v, weight by volume ratio.

Introduction

In 1991, Hojo and Aimoto reported on a method for polypeptide synthesis, which employed *S*-alkyl peptide thioesters (peptide thioesters) as building blocks [1]. Since then, improvements have been made to the thioester method, and it has been applied to the synthesis of a variety of polypeptides, including phosphopeptides [2–6]. Peptide thioesters are currently in use as intermediates not only in the thioester method, but also in the native chemical ligation method as well as related reactions used in polypeptide synthesis [7, 8]. However, a very basic problem that still remains to be solved involves the difficulty in the preparation of peptide thioesters. In particular, the synthetic yields of phosphopeptide thioesters are frequently low when they are prepared by a Boc solid-phase method using an MBHA resin [6]. Furthermore, as phosphorylated amino acid residues are often located in acid regions in proteins, there is the additional problem of aspartyl residue conversion to succinimide derivatives during peptide assembly [9, 10].

We wish to report herein on an improved method for the synthesis of a phosphopeptide thioester

based on Fmoc chemistry, adopting the thioester of [Ser(PO_3H_2)^{2,11}]-p21Max(1–13), Met-Ser(PO_3H_2)-Asp-Asn-Asp-Asp-Ile-Glu-Val-Glu-Ser(PO_3H_2)-Asp-Ala-SC$(CH_3)_2CH_2$CO-Leu, as a model compound.

Material and method

Fmoc-amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan). Fmoc-Ser[PO(OBzl)OH]-OH was purchased from Novabiochem (Läufelfingen, Switzerland). Fmoc-Leu Alko resin, NMP, DIEA, and TFA were purchased from Watanabe Chemical Ind. Ltd. (Hiroshima, Japan). 1-Methylpyrrolidine, hexamethyleneimine and 2-fluorofluorene were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The amino acid derivatives used in the solid phase peptide syntheses were of the L-configuration except for glycine. Reversed-phase HPLC (RP-HPLC) was performed using a linear increasing gradient of acetonitrile in 0.1% aqueous TFA. Amino acid compositions of peptides were analyzed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo) after hydrolysis with constant boiling point HCl (Nacalai Tesque, Kyoto, Japan) at 110 °C for 24 h in an evacuated sealed tube. Analyses of resin-bound amino acids were carried out after hydrolysis with a reagent containing 12 M HCl (50 μL) and propionic acid (50 μL) at 110 °C for 24 h in an evacuated sealed tube. All yields were calculated based on data obtained by amino acid analyses. Peptide molecular masses were determined by MALDI-TOF mass spectrometry using a Voyager^{TM} DE (PerSeptive Biosystems Inc., Framingham, MA). The matrices used were α-cyano-4-hydroxycinnamic acid or sinapinic acid. The composition of the D-Ala isomer was determined by using a G-3500 gas chromatograph (GC) (Hitachi Ltd., Tokyo) equipped an L-Val coated capillary, CHROMPACK^{TM} column (GL Sciences Inc., Netherlands).

*Preparation of Fmoc-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (**1**)*

Fmoc-Leu Alko resin (1.0 g, Leu: 0.80 mmol g^{-1}) was treated with piperidine (20%) in NMP (1×5 min, 1×10min, 1×15 min). After washing the resin with NMP (3×2 min), an HSC$(CH_3)_2CH_2$COOBt solution, which was prepared by one-hour reaction of HSC$(CH_3)_2CH_2$COOH (0.19 g, 1.6 mmol), HOBt hydrate (0.28 g, 1.8 mmol) and DCC (0.33g, 1.6 mmol) in NMP (3.4 mL), was added to the resin. The resulting suspension was mixed for 3h and then washed with NMP (5×2 min). To the resin, an Fmoc-Ala-OBt solution, which was prepared by one hour reaction of Fmoc-Ala (530 mg, 1.6 mmol), HOBt hydrate (0.28 g, 1.8 mmol) and DCC (0.33 g, 1.6 mmol) in NMP (3.4 mL), was added in the presence of DIEA (0.56 ml, 3.2 mmol). The resulting suspension was mixed for 3 h and then washed with NMP (5×2 min). The resin was treated with an NMP solution (10 mL) containing acetic anhydride (1 mL, 9.5 mmol) and DIEA (0.5 mL, 3.0 mmol) for 10 min, and then successively washed with NMP (5×2 min) and MeOH (3×1 min), followed by drying under reduced pressure to give a resin, Fmoc-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (Ala: 0.47 mmol g^{-1}, 1.2 g).

*Synthesis of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13)-SC$(CH_3)_2CH_2$CO-Leu (**2**) using Fmoc-amino acid derivatives*

All peptide chain elongation cycles were carried out manually, as follows: The deprotection of Fmoc groups was carried out by treatment with a reagent, containing 1-methylpyrrolidine (25 mL), HOBt (4.8 g), hexamethylenimine (2 mL) in 100 mL of NMP-DMSO (1:1) solution, (reagent A), (1×3 min, 1×5 min, 1×7 min). The resin was washed with NMP (2×30 sec) and then with an NMP solution containing HOBt (5%, wt/v) (3×30 sec). To the resin, a solution containing the Fmoc-amino acid (4 eq.), HBTU (4.8 eq.), and HOBt (4.8 eq.) in a DMF solution was added. After the addition of DIEA (8 eq.) in NMP, the resulting suspension was mixed for 30 min and then washed with NMP (5×30 sec).

Starting from the Fmoc-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (**1**) (95 mg, Ala: 45 μmol), 142 mg of a peptide resin corresponding to the sequence of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13), Boc-Met-Ser(PO(OBzl)OH)- Asp(OBu^t)-Asn(Trt)-Asp(OBu^t)-Asp(OBu^t)-Ile-Glu(OBu^t)-Val-Glu(OBu^t)-Ser(PO(OBzl)OH)-Asp(OBu^t)-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin was obtained. An aliquot of this fully protected peptide resin (23 mg) was treated with a reagent (1 ml), composed of EDT (25 μL), water (25 μL), and TFA (0.95 mL), for 2 h. The reaction mixture was added to cold ether (20 mL). The resulting precipitate was washed with ether and collected by centrifugation. The precipitate was washed twice with ether and then dissolved in aqueous acetonitrile. The peptide solution was passed through a dispos-

able ODS cartridge and the product isolated by RP-HPLC. The fraction that contained the product was freeze-dried to give [Ser$(PO_3H_2)^{2,11}$]-p21Max(1-13)-SC$(CH_3)_2CH_2$CO-Leu (**2**) (2.1 mg, 14 % based on Ala in the starting resin): Found: 1830.7 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 1829.7 (average). Amino acid analysis: $Asp_{4.1}Ser_{1.3}Glu_{1.9}Ala_1Val_{0.85}Met_{1.0}Ile_{0.87}Leu_{0.90}$.

Preparation of Fmoc(2-F)-amino acids

Fmoc(2-F)-OSu was derived from 2-fluorofluorene, using methods described in the literatures [11, 12]. To an aqueous solution containing an amino acid (2 mmol) and Na_2CO_3 (324 mg, 4 mmol) in water (20 mL) was added a solution of Fmoc(2-F)-OSu(710 mg, 2 mmol) in THF (20 mL). The reaction mixture was stirred for 30 min at room temperature, and then acidified with 5% aqueous citric acid solution. The product was extracted with three portions of ethyl acetate (30 mL). The combined organic layer was washed with 30 ml of saturated NaCl solution and 30 ml of water, followed by drying over Na_2SO_4. After the separation of the desiccant by filtration, the filtrate was concentrated under reduced pressure. The resulting solid or residual oil was crystallized from ethyl acetate/ hexane/ ether. The melting points of the amino acid derivatives used for this synthesis are as follows: Fmoc(2-F)-Ala (103-4 °C), Fmoc(2-F)-Val (163-5°C), Fmoc(2-F)-Leu (158-160°C), Fmoc(2-F)-Ile (160-2°C), Fmoc(2-F)-Ser(Bu^t) (138-140°C), Fmoc(2-F)-Asp(OBu^t) (124-5°C), Fmoc(2-F)-Glu(OBu^t) (51-54°C), Fmoc(2-F)-Ser (PO(OBzl)OH) (148-9°C).

*Preparation of Fmoc(2-F)-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (**3**)*

To the HSC$(CH_3)_2CH_2$CO-Leu-Alko resin that was prepared from Fmoc-Leu Alko resin (Leu: 0.80 mmol g^{-1}, 1.0 g) using the same procedure as used for the preparation of resin **1**, an Fmoc(2-F)-Ala-OBt solution, which was prepared by one hour reaction of Fmoc(2-F)-Ala (640 mg, 1.6 mmol), HOBt hydrate (0.28 g, 1.8 mmol), and DCC (0.33 g, 1.6 mmol) in NMP (3.4 mL), was added in the presence of DIEA (0.56 mL, 3.2 mmol). The resulting suspension was mixed for 1 h and then washed with NMP (5×2 min). The resin was treated with an NMP solution (10 mL) containing acetic anhydride (1 mL, 9.5 mmol) and DIEA (0.5 mL, 3.0 mmol) for 10 min and then successively washed with NMP (5×2 min) and MeOH (3×1 min), followed by drying under reduced pressure to obtain the resin, Fmoc(2-F)-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (**3**) (Ala: 0.28 mmolg^{-1}, 1.1 g).

*Synthesis of [Ser$(PO_3H_2)^{2,11}$]-p21Max(1-13)-SC$(CH_3)_2CH_2$CO-Leu (**4**) using Fmoc(2-F)-amino acid derivatives*

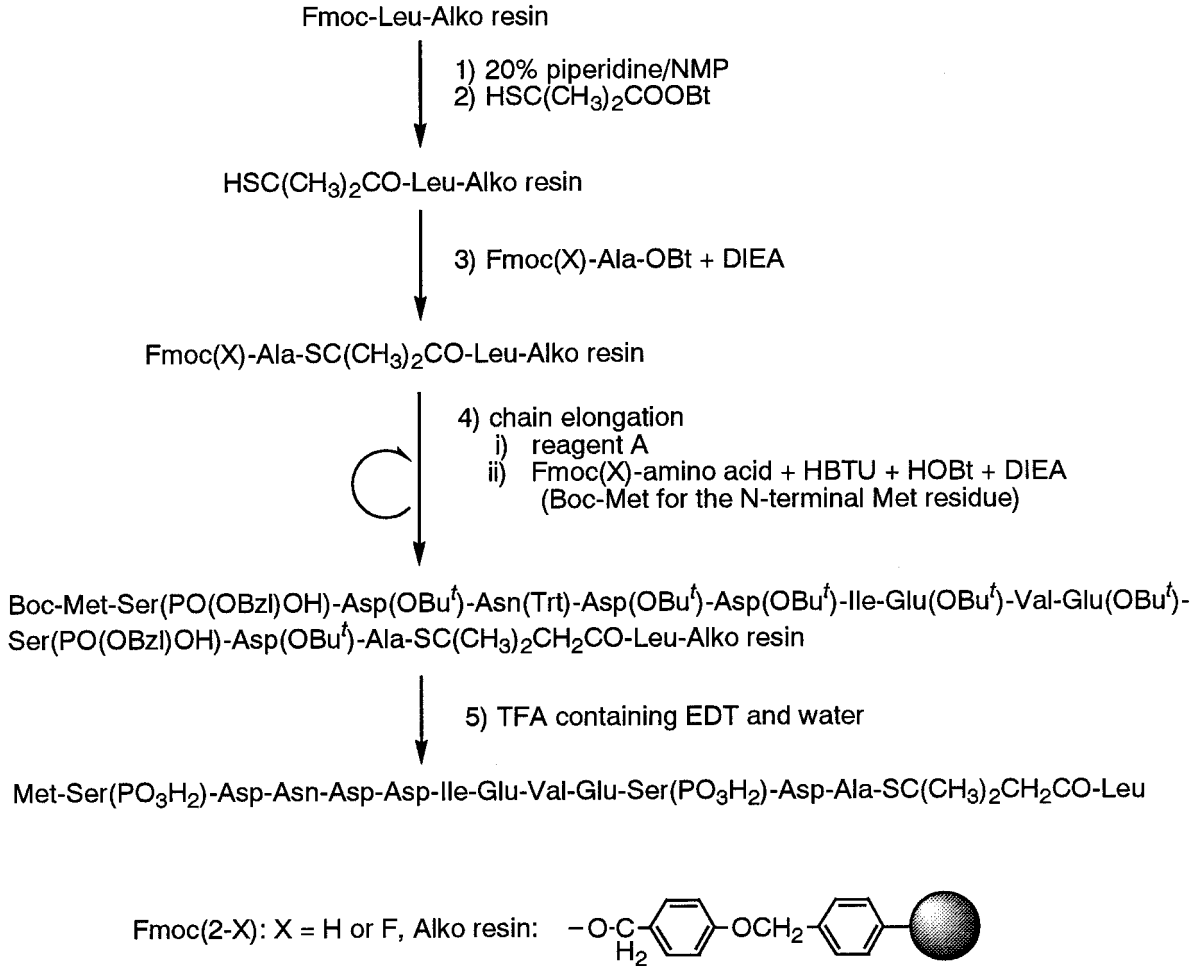
Peptide chain elongation cycles were carried out manually as described for the synthesis of peptide thioester **2** except for the deblocking time used for the Fmoc(2-F) groups. Deprotection of the Fmoc(2-F) groups was carried out by treatment with reagent A (2×1 min, 1×2 min). Starting from the Fmoc(2-F)-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (**3**) (32 mg, Ala; 9.0μmol), 45.9 mg of a peptide resin corresponding to the sequence of [Ser$(PO_3H_2)^{2,11}$]-p21Max(1-13), Boc-Met-Ser(PO(OBzl)OH)-Asp(OBu^t)-Asn(Trt)- Asp(OBu^t)-Asp(OBu^t)-Ile-Glu(OBu^t)-Val-Glu(OBu^t)-Ser(PO(O-Bzl)OH)-Asp(OBu^t)-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin was obtained. After post-treatment of the resin (15.1 mg)) using the same cleavage method as above, followed by RP-HPLC purification, product **4** (2.0 mg) was obtained in 20% yield based on the Ala content of the starting resin: Found: 1830.3 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 1829.7 (average). Amino acid analysis: $Asp_{4.4}Ser_{1.5}Glu_{1.9}Ala_1Val_{0.79}Met_{1.2}Ile_{0.83}Leu_{0.99}$.

Results and discussion

The α-thioester of [Ser$(PO_3H_2)^{2,11}$]-p21Max(1-13) was used as a model since this peptide thioester contains two Ser(PO_3H_2) and four Asp residues. This represents an ideal model compound for use in estimating the utility of the methods developed. The outline of the synthetic route that we examined is summarized in Scheme 1.

Preparation of starting resins

The *S*-tertiary alkyl thioester type linker was used to suppress the undesirable cyclization of the growing peptides [13]. Fmoc-Ala-SC$(CH_3)_2CH_2$CO-Leu-Alko resin (**1**) was prepared by a stepwise method. HSC$(CH_3)_2CH_2$COOH was introduced to the Leu-Alko resin (Leu: 0.80 mmolg^{-1}) in the presence of HOBt hydrate and DCC without added DIEA. To the resin, an Fmoc-Ala-OBt solution was added in the presence of DIEA. The resulting suspension was mixed for 3 h and then treated with an NMP solution



Scheme 1. The synthetic route of phosphorylated peptide thioester by using Fmoc- or Fmoc(2-F) amino acids.

containing acetic anhydride and DIEA to give Fmoc-Ala-$SC(CH_3)_2CH_2CO$-Leu-Alko resin **1** (Ala: 0.47 mmol g^{-1}). Judging from the amino acid analysis, the percentage of Ala introduced to Leu was 70%.

An Fmoc(2-F)-Ala-$SC(CH_3)_2CH_2CO$-Leu-Alko resin **(3)** was prepared using the same procedure as used for the preparation of resin **1** except for the introduction time used for the Fmoc(2-F)-Ala. The time was shortened to 1 h in order to avoid the undesirable cleavage of Fmoc(2-F) groups by DIEA. Resin **3**, whose substitution by Ala to Leu was 39%, was obtained.

*Syntheses of the α-thioesters of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13) (**2**) using Fmoc-amino acids*

The α-thioester of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13), Met-Ser(PO_3H_2)-Asp-Asn-Asp-Asp-Ile-Glu-Val-Glu-Ser(PO_3H_2)-Asp-Ala-$SC(CH_3)_2CH_2CO$-Leu (**2**) was prepared using Fmoc-amino acid derivatives and a deblocking reagent (reagent A) that contained 1-methylpyrrolidine, HOBt and hexamethylenimine in the solution of NMP-DMSO (1:1, v/v), which was slightly modified from the original composition [14]. The desired product **2** (peak **a**) was obtained in 14.4% yield after RP-HPLC purification as shown in Fig. 1. This synthesis, however, accompanied a product (peak b), whose C-terminal Ala residue was epimerized, in 5.3% yield.

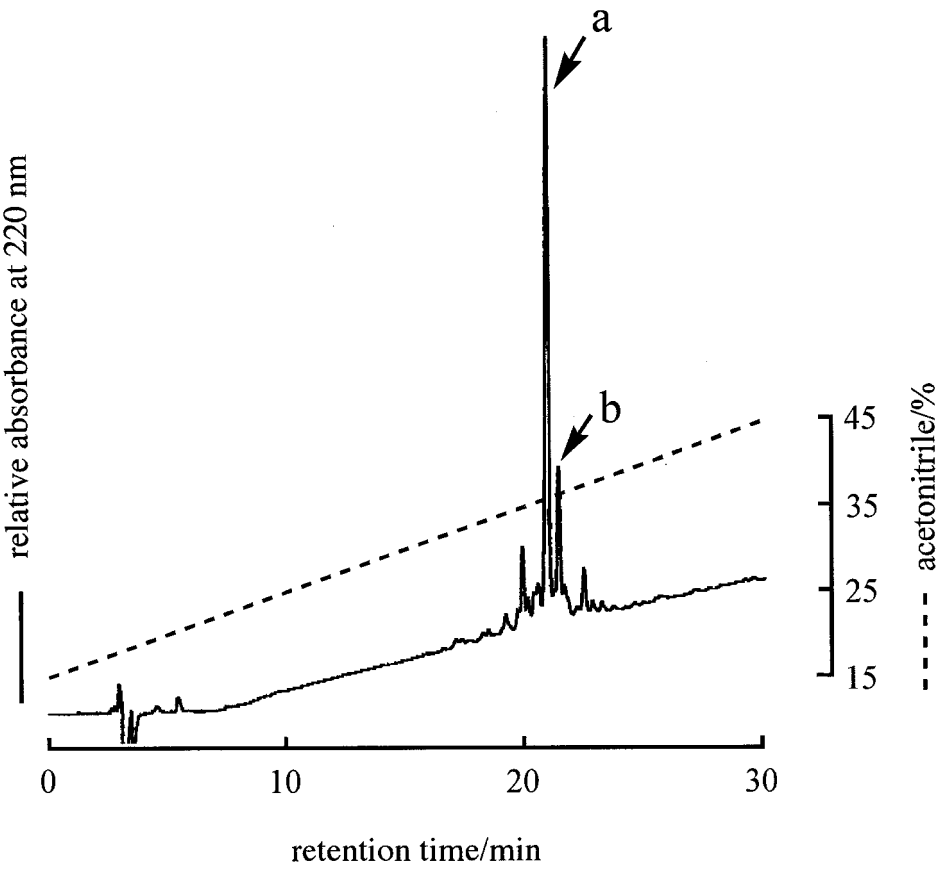


Figure 1. RP-HPLC elution profile of a crude preparation of [Ser(PO_3H_2)^{2,11}]-p21Max (1-13)-SC(CH_3)$_2$$CH_2$CO-Leu (**2**) prepared by N^{α}-Fmoc protection. The product was analyzed using a linear gradient of acetonitrile concentration using a Cosmosil 5C18AR II column (4.6 × 250 mm) at a flow rate of 1.0 ml/min. Peaks **a** and **b** indicate the desired product and the epimerized product, respectively.

*Syntheses of the α-thioesters of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13) (**4**) using Fmoc(2-F)-amino acids*

Fmoc(2-F)-amino acid derivatives were obtained without any problems via the use of Fmoc(2-F)-OSu. Using these Fmoc(2-F)-amino acids, the α-thioester of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13) (**4**) was prepared using the same procedure as was used in the synthesis of peptide thioester **2** except for the time for reagent A treatment. The deblocking of Fmoc(2-F) groups was performed by two 1-min and one 2-min treatments with the reagent A. In this synthesis, the desired product **4** (peak c) was obtained in 20% yield and the epimer (peak d), containing D-Ala, in 2.0% yield as shown in Fig. 2.

Comparison of synthetic methods for the α-thioesters of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13)

The α-thioester of [Ser(PO_3H_2)^{2,11}]-p21Max(1-13) was prepared under three different sets of conditions. The first synthesis was carried out by a Boc solid phase method using Boc-Ser[PO_3(*c*Pen)$_2$] [15] for the introduction of Ser(PO_3H_2) residues. The [Ser(PO_3H_2)^{2,11}]-p21Max(1-13)-SCH_2CH_2CO-Leu was obtained in 11% yield (peak e in Fig. **3**) from Boc-Met-Ser[PO_3(*c*Pen)$_2$]-Asp(OBzl)-Asn-Asp(OBzl)-Asp(OBzl)-Ile-Glu(OBzl)-Val-Glu(OBzl)-Ser[PO_3(*c*Pen)$_2$]-Asp(OBzl)-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin after TFMSA treatment at 0 °C for 3 h, followed by HPLC purification [16]. In this preparation, the RP-HPLC-purified sample still contained a dehydrated peptide that was separated after the introduction of a Boc group to the terminal amino group. Thus the actual yield of

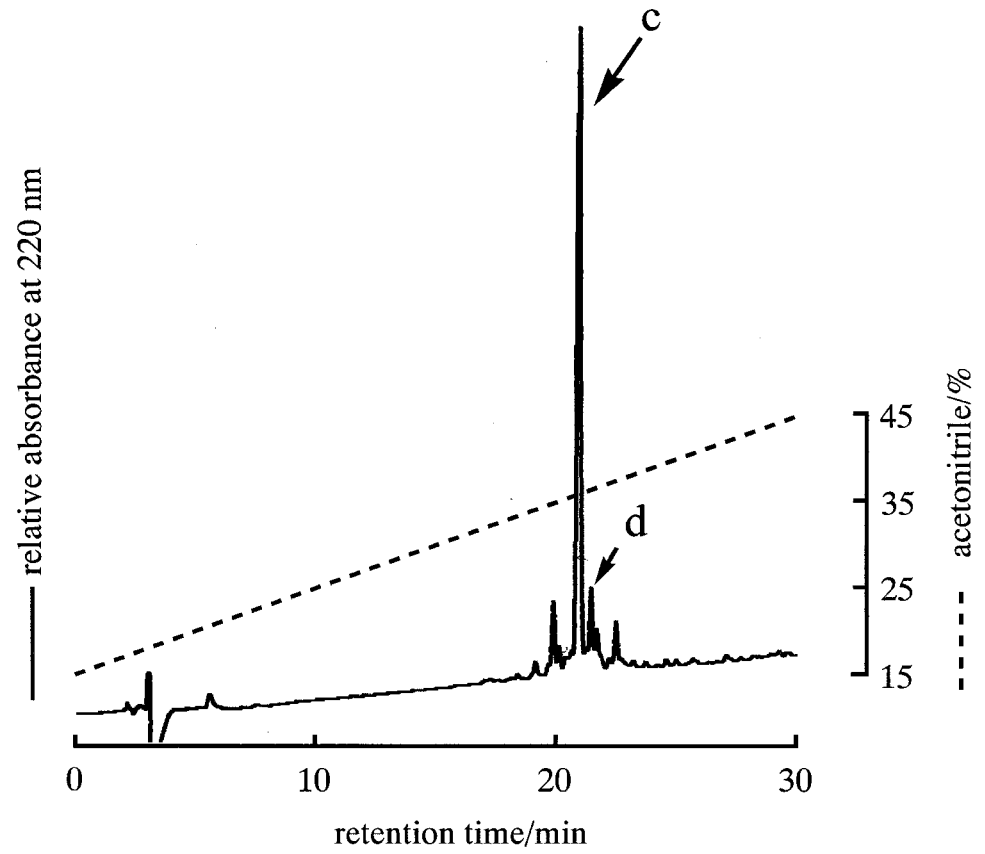


Figure 2. RP-HPLC elution profile of a crude preparation of [Ser(PO_3H_2)^{2,11}]-p21Max (1-13)-SC(CH_3)$_2$$CH_2$CO-Leu (**4**) prepared by N^{α}-Fmoc(2-F) protection. The product was analyzed using a linear gradient of acetonitrile concentration using a Cosmosil 5C18AR II column (4.6 × 250 mm) at a flow rate of 1.0 ml/min. Peaks **c** and **d** indicate the desired product and the epimerized product, respectively.

the desired product, [Ser(PO_3H_2)^{2,11}]-p21Max(1-13)-SCH_2CH_2CO-Leu, was estimated to be in the vicinity of 9%. Judging from the mass number of peak f, the benzyl group was not completely removed. Peak g indicates the mass number of the1,2-ethanedithiol adduct, the predicted product of peptide bond cleavage at the N-terminal side of the Ser(PO_3H_2) residue at the second amino acid residue [17]. No epimer peak was detectable by RP-HPLC.

Since phosphopeptides are generally prepared by an Fmoc solid phase method in better yields than by the Boc solid phase method, we applied the Fmoc solid phase method, in which a modified deblocking reagent [14] and Fmoc-Ser(PO(OBzl)OH) [15] are used for the preparation of phosphopeptide thioesters. As expected, the desired product was prepared in higher yield by the Fmoc solid phase method than by the Boc solid phase method. The by-product derived from the phophorylated amino acid was not observed and the HOBt in the reagent A appeared to suppress succinimide formation of the Asp(OBu^t) residues during the removal of the Fmoc groups [18, 19]. One problem that arose in this preparation was the epimerization of the Ala residue adjacent to the thioester. Although the epimer could be easily separated by RP-HPLC in this synthesis, the overall synthesis would be simpler if this side reaction were to be suppressed. The other problem was the relatively low yield of peptide thioester. The major loss of peptide from a resin during chain elongation cycles occurres during deprotection of Fmoc- or Fmoc(2-F)-group on the first and second amino acid residues from the thioester terminal. Usually 70 to 80% of peptide is removed during this process. However, no significant peptide loss was observed after the introduction of the third amino acid residue. The weight increase of a protected peptide resin well agreed with an isolated peptide yield and its RP-HPLC elution profile of a crude product.

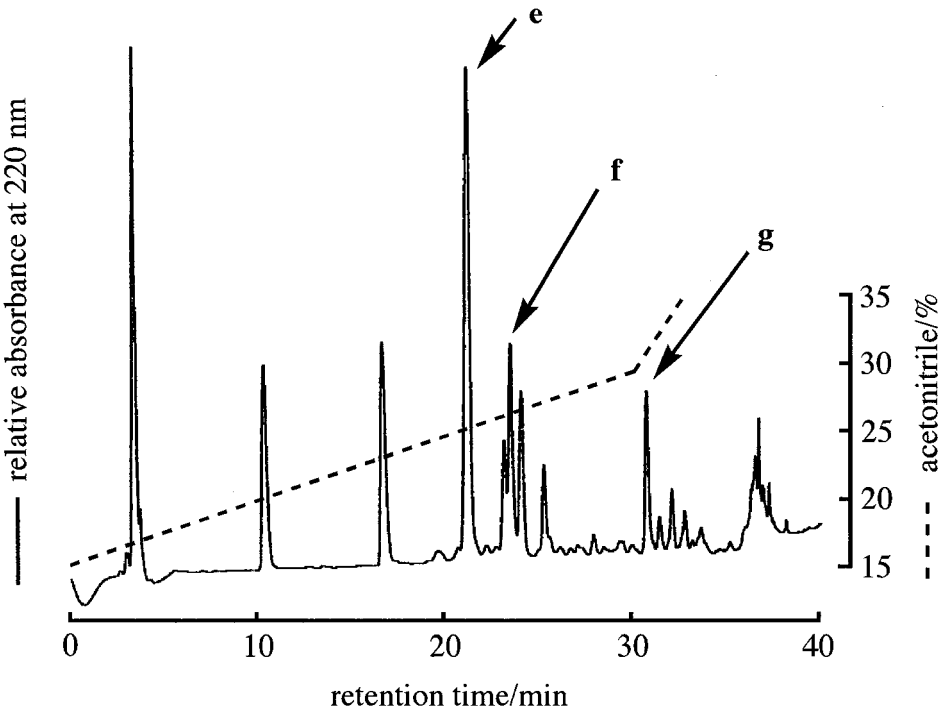


Figure 3. Analytical HPLC elution profile of a crude preparation of [Ser$(PO_3H_2)^{2,11}$]-p21Max (1-13)-SCH_2CH_2CO-Leu prepared by the Boc solid phase method [16]. Peptides were analyzed by linear gradient of acetonitrile concentration using a Cosmosil 5C18AR II column (4.6 × 250 mm) at a flow rate of 1.0 ml/min. Peak e indicates the desired product. Peak f contained a product with a higher mass number than the desired product by 90 and peak g was estimated to be a 1,2-ethaneduthiol adduct from its mass number.

As a result, we examined the use of an Fmoc(2-F) group, which would be expected to be a more base-sensitive protecting group than an Fmoc group [20], as an α-amino protecting group. The Fmoc(2-F) groups in the peptide thioester resin could be removed within 4 min by treatment with reagent A. Thus, peptide thioester **4** was obtained in 20% yield, suppressing the generation of the epimer to a 2.0% yield.

Conclusion

A peptide thioester can be prepared by the Fmoc-based solid phase method by using reagent A that contains 1-methylpyrrolidine, HOBt, hexamethylenimine in an NMP-DMSO solution. This reagent enables the de-protection of Fmoc groups while keeping the thioester largely intact. The HOBt in the reagent A suppresses succinimide formation with respect to Asp(OBu^t) residues. This constitutes one of the useful feature of reagent A for the preparation of phosphopeptide thioesters, since phosphorylated amino acid residues are often surrounded by Asp residues. No side reaction derived from Ser(PO(OBzl)OH) was observed in the Fmoc-based preparation.

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