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Synthesis of a Phosphorylated Polypeptide by a Thioester Method

Toru Kawakami, Koki Hasegawa, and Saburo Aimoto*

Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871

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A method for the synthesis of phosphorylated polypeptides was investigated through the synthesis of the phosphorylated cAMP response element binding protein 1 (19-106) amide, [Thr(PO_3H_2)^{69,71}]-CRE BP1(19-106)-NH_2, as a model compound. Three partially protected peptide segments: Boc–[Lys(Boc)^{23,46,48,54}, Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2, Fmoc-[Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2, and [Lys(Boc)^{97,98,105,106}]–CRE BP1(84-106)–NH_2, were prepared by the Boc solid-phase method and were used as building blocks. The segment condensations were carried out sequentially by a thioester method in the presence of a silver compound, HOObt, and DIEA. The desired product was obtained in 19% yield, based on the C-terminal building block, after removal of all the protecting groups on the peptide. These results indicate that the thioester method is quite useful for the preparation of phosphorylated polypeptide even if it contains cysteine residues. One thing which remains to be improved is the efficiency of the method for the preparation of partially protected peptide thioesters, which contain phosphorylated amino acid residues.

Partially protected peptide thioester segments are useful building blocks for polypeptide syntheses.[1—4] In previous papers, we described the utility of silver chloride as a thioester activator in the condensation of Cys(Acm)-containing peptide segments.[5] Although silver chloride is only slightly soluble in DMSO, segment coupling proceeded at a reasonable rate while keeping the Acm groups intact, even for the case of a peptide which contained a quite labile Acm group as is located adjacent to a histidine residue. The utility of AgCl was demonstrated by synthesizing a reaper protein of 65 amino acid residues.[6] Based on these findings, conditions were investigated for the preparation of a phosphorylated polypeptide with a molecular weight of ten thousand via the thioester method, in which peptide thioesters were condensed sequentially. In the present study, we demonstrated the usefulness of the thioester method for the synthesis of phosphorylated polypeptide by synthesizing the phosphorylated cAMP response element binding protein 1 (19-106) amide, [Thr(PO_3H_2)^{69,71}]–CRE BP1(19-106)–NH_2, as shown in Fig. 1.[7] CRE BP1 is a member of a group of transcription factors, and is activated by the phosphorylation.[8] This peptide, which contains two phosphothreonine and three cysteine residues, represents a good target molecule to estimate the utility of the thioester method.

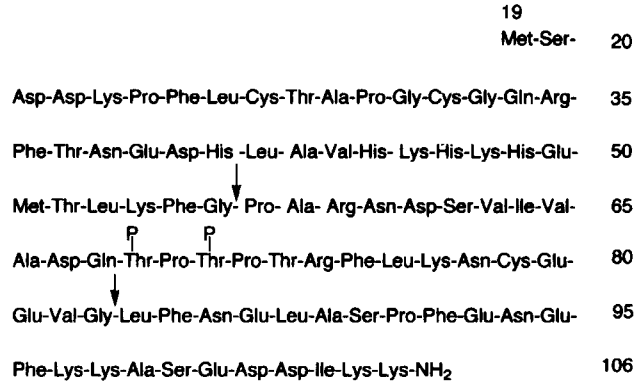

Fig. 1. The amino acid sequence of the phosphorylated cAMP response element binding protein 1 (19-106)–NH_2, [Thr(PO_3H_2)^{69,71}]–CRE BP1(19-106)–NH_2. Thr(P) denotes phosphothreonine. Arrows indicate the sites of segment coupling.

Results and Discussion

Preparation of Building Blocks. The [Thr(PO_3H_2)^{69,71}]–CRE BP1(19-106)–NH_2 was divided into three peptide segments for synthetic purposes, as shown in Fig. 1, and three partially protected peptide segments: Boc–Met–Ser–Asp–Asp–Lys(Boc)–Pro–Phe–Leu–Cys(Acm)–Thr–Ala–Pro–Gly–Cys(Acm)–Gly–Gln–Arg–Phe–Thr–Asn–Glu–Asp–His–Leu–Ala–Val–His–Lys(Boc)–His–Lys(Boc)–His–Glu–Met–Thr–Leu–Lys(Boc)–Phe–Gly–SCH_2CH_2CO–β–Ala–NH_2; Boc–[Lys(Boc)^{23,46,48,54}, Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β–Ala–NH_2 (**1**), Fmoc–Pro–Ala–Arg–Asn–Asp–Ser–Val–Ile–Val–Ala–Asp–Gln–Thr(PO_3H_2)–Pro–Thr(PO_3H_2)–Pro–Thr–Arg–Phe–Leu–Lys(Boc)–Asn–Cys(Acm)–Glu–Glu–Val–Gly–SCH_2CH_2CO–β–Ala–NH_2; Fmoc–[Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2 (**2**), and Leu–Phe–Asn–Glu–Leu–Ala–Ser–Pro–Phe–Glu–Asn–Glu–Phe–Lys(Boc)–Lys(Boc)–Ala–Ser–Glu–Asp–Asp–Ile–Lys(Boc)–Lys(Boc)–NH_2; [Lys(Boc)^{97,98,105,106}]–CRE BP1(84-106)–NH_2 (**3**), were prepared as building blocks via a Boc solid-phase method.

Peptide **1** was synthesized via a usual synthetic procedure, starting from Boc–Gly–SCH_2CH_2CO–β-Ala–NH_2.[3] [Cys

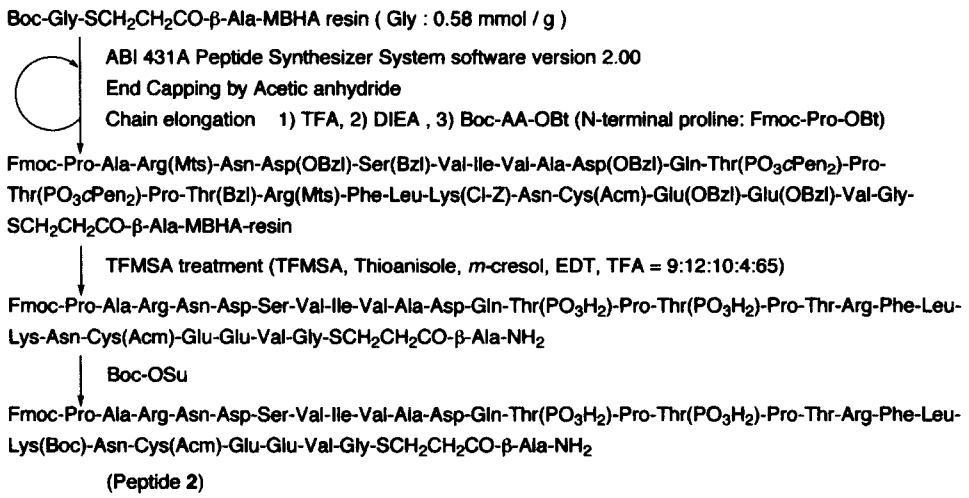

Fig. 2. The procedure for the synthesis of Fmoc–[Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2 via the Boc solid-phase method.

(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2 tended to aggregate in aqueous acetonitrile. Thus this crude product was dissolved in 8 M[#] urea and injected into RP-HPLC for purification. The peak containing [Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2 was efficiently separated from by-products. Peptide **1** was obtained after introduction of Boc groups to the side-chain amino groups in [Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2 in a 5.2% yield based on the Gly in the starting resin.

Peptide **2** was prepared according to the procedure shown in Fig. 2. Starting from the Boc–Gly–SCH_2CH_2CO–β–Ala–MBHA resin, Fmoc–Pro–Ala–Arg(Mts)–Asn–Asp(OBzl)–Ser(Bzl)–Val–Ile–Val–Ala–Asp(OBzl)–Gln–Thr(PO_3cPen$_2$)–Pro–Thr(PO_3cPen$_2$)–Pro–Thr(Bzl)–Arg(Mts)–Phe–Leu–Lys(Cl–Z)–Asn–Cys(Acm)–Glu(OBzl)–Glu(OBzl)–Val–Gly–SCH_2CH_2CO–β–Ala–MBHA resin was prepared. Two Thr(PO_3cPen$_2$)[9] residues were introduced manually. This fully protected peptide resin was treated with a reagent,[9,10] which contained TFMSA, thioanisole, *m*-cresol, EDT, and TFA, at 0 °C for 2 h. An elution profile of the crude product is shown in Fig. 3. The desired product, Fmoc–[Thr(PO_3H_2)^{69,71}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2, eluted as peak 4, was obtained in a 2.9% yield, based on Gly content in the starting resin, after isolation by RP-HPLC. To the purified Fmoc–[Thr(PO_3H_2)^{69,71}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2, a Boc group was introduced with no difficulty, to give peptide **2** in a 2.6% yield, based on the Gly in the starting resin.

Fmoc–CRE BP1(84-106)–NH_2 was obtained via the Boc solid-phase method in a yield of 18%, based on the amino group in the starting resin. Boc groups were introduced to its side-chain amino groups, and the obtained product was treated with piperidine to give peptide **3** in a 13% yield, based on the amino groups in the starting resin.

Analysis of Problems Encountered during the Preparation of Phosphopeptide Thioester. Synthesis of phosphorylated polypeptide using the thioester method has been reported, in which a phosphorylated peptide thioester was synthesized by using Boc–Ser(PO_3Bzl_2) to introduce a phosphoserine residue.[11] Though the exact yield was not clear, because it was not calculated based on a peptide content, but rather on a peptide weight, the yield of the phosphorylated peptide thioester was low. This low yield might be due to the undesirable removal of the benzyl groups from the Ser(PO_3Bzl_2) residue with TFA, which was used to deprotect Boc groups during the chain elongation cycle. Recently, cyclopentyl group was reported to be sufficiently stable to TFA as a protecting group of *O*-phosphono group and to be removed by TFMSA treatment. It can be used for the syn-

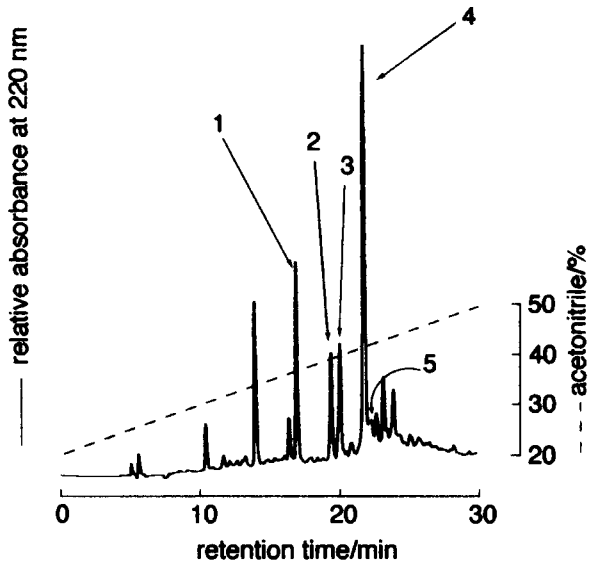

Fig. 3. HPLC elution profile of the crude product of Fmoc–[Thr(PO_3H_2)^{69,71}, Cys(Acm)^{79}]–CRE BP1(57-86)–SCH_2CH_2CO–β-Ala–NH_2. Peaks 1—3 and 5; see text. Peak 4 represents the desired product. column: Cosmosil 5C_{18}AR II (10×250 mm), eluent: 0.1% TFA in aqueous acetonitrile, 2.5 mL min^{-1}.

#1 M = 1 mol dm^{-3}

thesis of phosphorylated peptides.[9] Therefore, we used this protecting group for the synthesis of the phosphorylated peptide thioester. The yield of crude Fmoc–[Thr(PO_3H_2)^{69,71}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2, however, was low when this phosphorylated peptide thioester was prepared by using an MBHA resin. Then the resin, which was treated once with TFMSA, was further treated with anhydrous HF[12] to estimate any cleavable peptide still remaining in the MBHA resin. Although the *O*-phosphono group in a phosphothreonine residue is cleaved under HF treatment conditions, this treatment is useful to estimate the remaining peptide in the resin. Additional peptide was detected as a precipitate by adding ether to an HF treated sample. A protected peptide thioester with the same amino acid sequence was then prepared on Pam resin. The yield of the desired product increased to 7.8% after TFMSA treatment, followed by RP-HPLC purification. This difference in yield arose mainly from the difference in the efficiency of peptide cleavage from resins by TFMSA treatment at 0 °C for 2 h. Thus, the Pam resin is a more suitable choice than MBHA resin for the preparation of a phosphopeptide thioester if it is prepared via a Boc solid-phase method. Concerning the synthetic yield, the MBHA resin may not be good choice even for the synthesis of phosphopeptide amide, which must be cleaved from a resin with TFMSA or other acids of similar acidity.

In both syntheses, crude products, prepared by using MBHA or Pam resins, showed quite similar RP-HPLC elution patterns, containing several distinct peaks, which were derived from by-products. Mass and NMR analyses of compounds in peaks 1, 2, and 3 in Fig. 3 suggested that the by-products were the result of dephosphorylation reactions, followed by the addition of EDT (peaks 2 and 3) or acetic acid (peaks 1) (Data are not shown). In order to define the mechanism of by-product formation, detailed analyses will be required.

In the synthesis of peptide **2**, benzyl esters were employed for the side-chain protection of Asp residues. The mass number corresponding to the dehydrated product, which would be formed by succinimide formation, was observed as a small peak 5. This suggests that succinimide formation was a very minor side-reaction in this synthesis. We also synthesized peptide **2** by using Boc–Asp(OcHep)[13] on MBHA resin (Data are not shown). No improvement, however, was observed in the suppression of the formation of a dehydrated product. Therefore, in this synthesis, benzyl esters were superior to cycloheptyl esters in avoiding prolonged TFMSA treatment, which is required for the removal of cycloheptyl esters. In order to establish an efficient method for the synthesis of phosphopeptide thioesters, new amino acid derivatives having a protected *O*-phosphono group will be required, based on the detailed analyses of by-products.

Segment Condensation. The segment condensation was carried out according to the scheme shown in Fig. 4. Peptides **2** and **3** were condensed in the presence of $AgNO_3$, HOObt, and DIEA in DMSO with stirring for 24 h at room temperature. The elution profile of the final stage of the reaction mixture is shown in Fig. 5a. An arrow indicates the fraction containing Fmoc–[Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77,97,98,105,106}, Cys(Acm)^{79}]–CRE BP1(57-106)–NH_2. This product tended to adsorb irreversibly on RP-HPLC column, and hence its isolated yield was low. Therefore the product was isolated after removal of the Fmoc group. In order to avoid the undesirable decomposition of the Acm group during piperidine treatment, DTT was added to the reaction mixture to quench the activity of the silver ions, and piperidine was then added to remove Fmoc groups. The product **4**, [Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77,97,98,105,106}, Cys(Acm)^{79}]–CRE BP1(57-106)–NH_2, was isolated by RP-HPLC in a yield of 59% and, after freeze-drying, was used for the next coupling reaction. The peptides **1** and **4**, along with AgCl, HOObt, and DIEA, were dissolved in DMSO and the solution was stirred for 48 h (Fig. 5b). An arrow indicates the fraction containing a product, Boc–[Lys(Boc)^{23,46,48,54,77,97,98,105,106}, Cys(Acm)^{27,32,79},

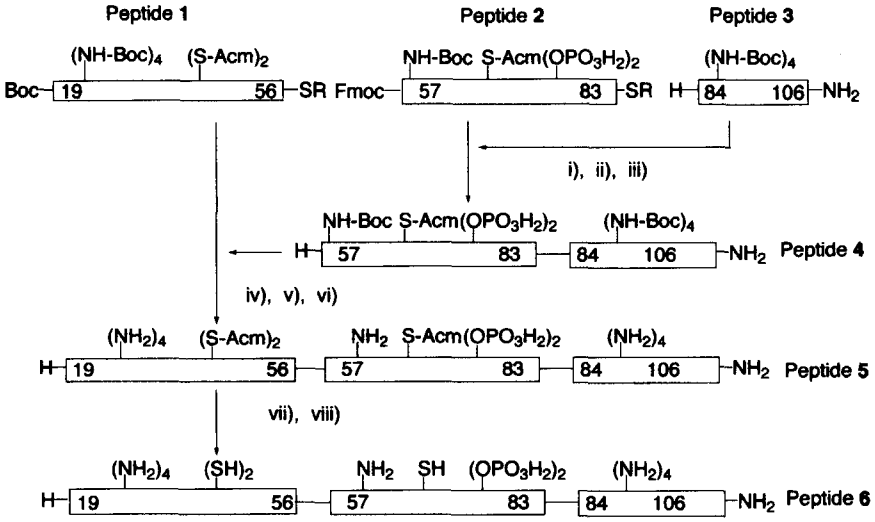

Fig. 4. The route for the preparation of [Thr(PO_3H_2)^{69,71}]–CRE BP1(19-106)–NH_2. i) $AgNO_3$ + HOObt + DIEA, ii) DTT, iii) piperidine, iv) AgCl + HOObt + DIEA, v) DTT, vi) 5% 1,4-butanedithiol/TFA, vii) $AgNO_3$ + DIEA + H_2O, viii) DTT + 1 M HCl.

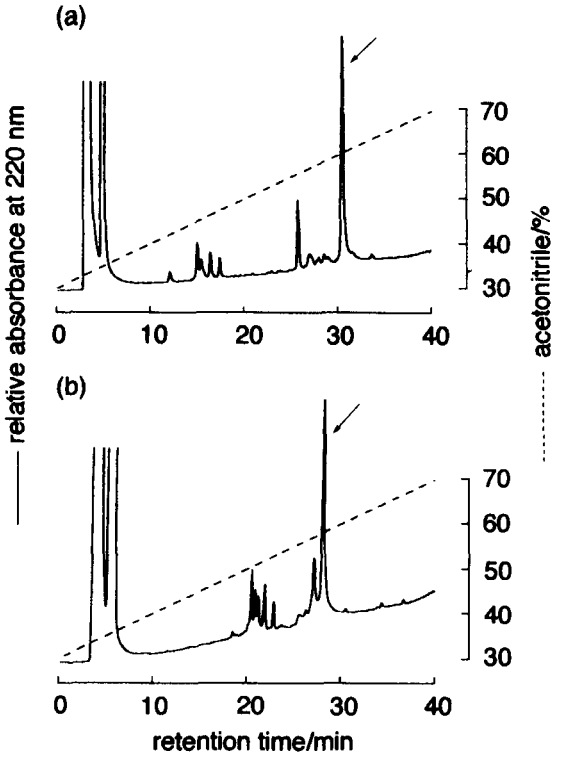

Fig. 5. The elution profiles of the reaction mixture of Fmoc-[Thr$(PO_3H_2)^{69,71}$, Lys(Boc)^{77,97,98,105,106}, Cys(Acm)^{79}]-CRE BP1(57-106)-NH_2 (A) and Boc-[Lys(Boc)^{23,46,48,54,77,97,98,105,106}, Cys(Acm)^{27,32,79}, Thr$(PO_3H_2)^{69,71}$]-CRE BP1(19-106)-NH_2 (B). Each arrow indicates the peak corresponding to the desired product. Column: Cosmosil $5C_{18}$ARII (4.6×250 mm). Eluent: 0.1% TFA in aqueous acetonitrile, 1.0 mL min^{-1}.

Thr$(PO_3H_2)^{69,71}$]-CRE BP1(19-106)-NH_2. After adding DTT to the reaction mixture to quench the activity of silver ions, ether was added to precipitate the crude product. The crude product was treated with TFA containing 1,4-butanedithiol, and then peptide **5**, [Cys(Acm)^{27,32,79}, Thr$(PO_3H_2)^{69,71}$]-CRE BP1(19-106)-NH_2, was isolated by RP-HPLC in a 56% yield, based on peptide **4**. Peptide **5** was treated with $AgNO_3$ and DIEA in a mixed solvent of water and 2,2,2-trifluoroethanol for 5 h at room temperature to remove the Acm groups.[3] DTT and then 1 M HCl were added to this solution, to decompose the silver thiolate. Peptide **6**, [Thr$(PO_3H_2)^{69,71}$]-CRE BP1(19-106)-NH_2, was obtained after purification by RP-HPLC in a 57% yield, based on peptide **5**. The RP-HPLC (Fig. 6) and mass spectrum indicated that the highly pure product **6** was obtained. The overall yield of peptide **6** was a 19%, based on peptide **3**.

At the first segment condensation, when silver nitrate was used as an activator of the thioester moiety, the Acm group was not decomposed during segment condensation. Silver chloride was used as an activator at the second segment condensation. Acm groups were intact during segment condensation, and the coupling reaction proceeded as efficiently as that of the first coupling. The undesirable cleavage of the Acm groups during the removal of Fmoc groups, which is

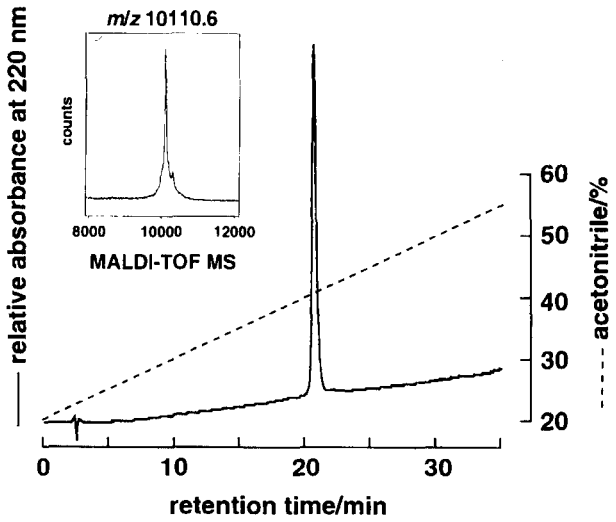

Fig. 6. RP-HPLC elution profile of the purified [Thr$(PO_3H_2)^{69,71}$]-CRE BP1(19-106)-NH_2. column: Cosmosil $5C_{18}$AR-300 (4.6×150 mm), eluent: 0.1% TFA in aqueous acetonitrile, 1.0 mL min^{-1}. Matrix-assisted laser desorption mass spectrum of [Thr$(PO_3H_2)^{69,71}$]-CRE BP1(19-106)-NH_2 is shown in a box.

caused by silver ions and piperidine as a nucleophile, could be efficiently suppressed by adding DTT to a reaction mixture before removal of the Fmoc group. DTT addition made the sequential condensation of Cys(Acm)-containing building blocks quite easy. The Acm groups on peptide **5** could be successfully removed in the presence of silver ions and water, followed by the treatment with 1 M HCl. No special problem was encountered during segment condensation of a building block containing phosphothreonine residues.

In conclusion, a phosphorylated polypeptide can be prepared via the thioester method even if it contains cysteine residues. Decomposition of the *S*-Acm groups during the removal of Fmoc groups and segment condensation can be avoided by controlling the activity of silver ions. One remaining issue which requires improvement is the method for the preparation of partially protected peptide thioesters containing phosphorylated amino acid residues.

Experimental

Materials and Methods. Boc-amino acid derivatives and MBHA resin were purchased from the Peptide Institute Inc. (Osaka). Amino acid derivatives used were of the L-configuration except for glycine. Peptide syntheses were carried out by using a peptide synthesizer 431A version 2.00 (Applied Biosystems Inc., Foster City, CA.) employing the Boc (Standard) [0.5 mmol] double coupling protocol with an acetic anhydride capping module. The *N*-terminal Fmoc groups were introduced by using Fmoc-amino acid derivatives instead of Boc-amino acid derivatives in cases of the syntheses of peptides **2**, **3**, and **8**. The DMSO used for the segment coupling reaction was silylation grade (Pierce, Rockford, IL). The ether used for the precipitation and washing of peptides was free of peroxide (Nacalai Tesque, Kyoto). Reversed-phase HPLC (RP-HPLC) was performed on Cosmosil $5C_{18}$-AR II (10×250 mm) (Nacalai Tesque, Kyoto), unless otherwise specified, using a linear increasing gradient of acetonitrile in 0.1% aqueous TFA. Amino

acid compositions in peptides were analyzed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo) after hydrolysis with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce, Rockford, IL) or with constant boiling point HCl for amino acid analysis (Nacalai Tesque, Kyoto) at 110 °C for 24 h in an evacuated sealed tube. Amino acid analyses of resin-bound compounds 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 the data obtained by amino acid analyses. Peptide mass numbers were determined by MALDI-TOF mass spectrometry using a Voyager™DE (PerSeptive Biosystems, Inc., Framingham, MA). The matrices used were α-cyano-4-hydroxycinnamic acid or sinapinic acid.

Abbreviations used are Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; Boc-OSu, *N*-*t*-butoxycarbonyloxysuccinimide; Bom, benzyloxymethyl; Bzl, benzyl; Cl-Z, 2-chlorobenzyloxycarbonyl; cHep, cycloheptyl; cHex, cyclohexyl; cPen, cyclopentyl; CRE BP1, cAMP response element binding protein 1; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HOObt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MBHA, 4-methylbenzhydrylamine; Mts, mesitylene-2-sulfonyl; NMP, 1-methylpyrolidin-2-one; $HOCH_2$-Pam, 4-(hydroxymethyl)phenylacetamidomethyl; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; Tos, tosyl; TFMSA, trifluoromethanesulfonic acid.

Boc–Thr(PO_3cPen$_2$). Boc–Thr(PO_3cPen$_2$) was prepared according to the method described by Wakamiya et al.[9] The over-all yield of Boc–Thr(PO_3cPen$_2$) was 78% based on Boc–Thr–OPac; mp 122 °C.

Boc–[Lys(Boc)^{23,46,48,54}, Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2 (1). Starting from the Boc–Gly–SCH_2CH_2CO–β-Ala–MBHA-resin (Gly: 0.58 mmol g^{-1}, 0.92 g), which was prepared by the procedure described in the literature,[3] 2.1 g of a protected peptide resin corresponding to the sequence of CRE BP1(19-56), Boc–Met–Ser(Bzl)–Asp(OcHex)–Asp(OcHex)–Lys(Cl-Z)–Pro–Phe–Leu–Cys(Acm)–Thr(Bzl)–Ala–Pro–Gly–Cys(Acm)–Gly–Gln–Arg(Tos)–Phe–Thr(Bzl)–Asn–Glu(OBzl)–Asp(OcHex)–His(Bom)–Leu–Ala–Val–His(Bom)–Lys(Cl-Z)–His(Bom)–Lys(Cl-Z)–His(Bom)–Glu(OBzl)–Met–Thr(Bzl)–Leu–Lys(Cl-Z)–Phe–Gly–SCH_2CH_2CO–β-Ala–MBHA resin, was obtained. An aliquot of the resin (0.50 g) was treated with a reagent containing anhydrous HF (17 mL), anisole (1.5 mL), and 1,4-butanedithiol (1.5 mL) with stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether (10 mL) was added to the mixture, and the resulting precipitate was washed with ether (10 mL×2), dissolved in aqueous acetonitrile containing 5% acetic acid. A peptide solution was passed through a disposable ODS cartridge, TOYOPACK ODS M (Tosoh, Tokyo), and the filtrate was freeze-dried to give a powder (215 mg), which was dissolved in 8 M urea. This peptide solution was subjected to a RP-HPLC column (Cosmosil 5C_4-AR-300, 10×250 mm (Nacalai Tesque, Kyoto)). The fraction containing the desired peptide was collected and the solution was freeze-dried to give a powder, [Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2. (50 mg, 7.8 μmol, 6.7% based on Gly content in the starting resin): MS Found: *m/z* 4629.1 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 4629.3 (average). Amino acid analysis: $Asp_{3.8}Thr_{2.8}Ser_{0.94}Glu_{2.8}Pro_{1.6}$ $Ala_{2.2}Gly_{3.0}$ 1/2 $Cys_{0.62}Val_{0.84}Met_{1.8}Leu_3$(Phe + βf/eqn >- Ala)$_{3.3}Lys_{3.8}His_{3.5}$ $Arg_{1.0}$.

To a solution of [Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2-CO–β-Ala–NH_2 (22.5 mg, 3.3 μmol) in DMSO (0.80 mL) were added Boc–OSu (26 mg, 0.12 mmol) and DIEA (24 μL). After stirring for 3 h at room temperature, ether was added to precipitate a peptide. The precipitate was washed with ether three times to give Boc–[Lys(Boc)^{23,46,48,54}, Cys(Acm)^{27,32}]–CRE BP1(19-56)–SCH_2CH_2CO–β-Ala–NH_2 (**1**) as a freeze-dried powder (26 mg, 2.6 μmol, in 4.8% yield based on the Gly content of the starting resin): MS Found: *m/z* 5131.6 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 5129.9 (average). Amino acid analysis: $Asp_{4.0}Thr_{3.0}Ser_{1.0}Glu_{3.1}Pro_{2.0}Gly_{3.2}Ala_{2.3}Pro_{1.5}$ 1/2$Cys_{0.36}Val_{1.0}$ $Met_{1.7}Leu_3$(Phe+β-Ala)$_{3.2}Lys_{3.8}$ $His_{3.8}Arg_{0.98}$.

Fmoc–[Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2 (2). Starting from the Boc–Gly–SCH_2CH_2CO–β-Ala–MBHA resin (Gly: 0.58 mmol g^{-1}, 1.11 g), 1.90 g of the protected peptide resin corresponding to the sequence of CRE BP1(57-83), Fmoc–Pro–Ala–Arg(Mts)–Asn–Asp(OBzl)–Ser(Bzl)–Val–Ile–Val–Ala–Asp(OBzl)–Gln–Thr(PO_3cPen$_2$)–Pro–Thr(PO_3cPen$_2$)–Pro–Thr(Bzl)–Arg(Mts)–Phe–Leu–Lys(Cl-Z)–Asn–Cys(Acm)–Glu(OBzl)–Glu(OBzl)–Val–Gly–SCH_2CH_2CO–β-Ala–MBHA resin, was obtained. Each Thr(PO_3cPen$_2$) residue was introduced manually by mixing for 3 h with a peptide resin, which had free α-amino groups, and Boc–Thr(PO_3cPen$_2$)–OBt, which was prepared by mixing Boc–Thr(PO_3cPen$_2$) (0.40g, 0.92 mmol), HOBt hydrate (0.13 g, 0.83 mmol) in NMP solution (0.83 mL), and DCC (0.15 g, 0.75 mmol) in NMP solution (0.75 mL) for 40 min at room temperature.

An aliquot of the resin (200 mg) was placed in a solution (12 mL) which contained TFMSA (1.08 mL), thioanisole (1.44 mL), *m*-cresol (1.2 mL), EDT (0.48 mL), and TFA (7.7 mL) and stirred at 0 °C for 2 h. The reaction mixture was added to cold ether (200 mL) and resulting precipitate was washed with ether and collected by centrifugation. The precipitate was washed twice with ether and dissolved in aqueous acetonitrile. The peptide solution was passed through a disposable ODS cartridge. The filtrate was injected to RP-HPLC column and the isolated peak was freeze-dried to yield Fmoc–[Thr(PO_3H_2)^{69,71}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2 (12 mg, 3.4 μmol, 2.9% based on Gly content in the starting resin): Found: 3572.3 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 3570.8 (average). Amino acid analysis: $Asp_{3.6}Thr_{1.7}Ser_{0.93}Glu_{2.7}Pro_{2.1}$ $Gly_1Ala_{2.0}$ 1/2$Cys_{n.d.}Val_{2.3}Ile_{0.54}Leu_{1.0}$(Phe+β-Ala)$_{1.3}Lys_{0.94}Arg_{1.7}$.

To a solution of Fmoc–[Thr(PO_3H_2)^{69,71}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2 (17 mg, 4.7 μmol) in DMSO (0.50 mL) were added Boc–OSu (5.0 mg, 23 μmol) and DIEA (6.0 μL). After stirring for 3 h at room temperature, the peptide was isolated by RP-HPLC to give Fmoc–[Thr(PO_3H_2)^{69,71}, Lys(Boc)^{77}, Cys(Acm)^{79}]–CRE BP1(57-83)–SCH_2CH_2CO–β-Ala–NH_2 (**2**) (18 mg, 4.2 μmol, 2.6% yield based on Gly content in the starting resin): Found: *m/z* 3669.7 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 3670.9 (average). Amino acid analysis: $Asp_{3.9}Thr_{2.1}Ser_{1.0}Glu_{2.8}Pro_{2.2}Gly_{0.94}Ala_2Cys_{n.d.}Val_{3.0}Ile_{0.64}Leu_{1.0}$ (Phe+β-Ala)$_{1.3}Lys_{0.93}Arg_{1.7}$.

[Lys(Boc)^{97,98,105,106}]–CRE BP1(84-106)–NH_2 (3). Starting from MBHA resin hydrochloride (0.95 g, NH_2: 0.41 mmol), the protected peptide resin (2.31 g) corresponding to the sequence CRE BP1(84-106), Fmoc–Leu–Phe–Asn–Glu(OBzl)–Leu–Ala–Ser(Bzl)–Pro–Phe–Glu(OBzl)–Asn–Glu(OBzl)–Phe–Lys(Cl-Z)–Lys(Cl-Z)–Ala–Ser(Bzl)–Glu(OBzl)–Asp(OcHex)–Asp(OcHex)–Ile–Lys(Cl-Z)–Lys(Cl-Z)–MBHA resin, was obtained. An aliquot of the resin (53 mg) was treated with a solution of anhydrous HF

(10 mL) containing anisole (1.0 mL) at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to wash the residual solid. The solid was washed twice more with ether and dissolved in 50% aqueous acetonitrile containing 5% acetic acid. A peptide solution was passed through a disposable ODS cartridge, and freeze-dried to give a powder (21 mg). This crude material was purified by RP-HPLC to yield Fmoc–CRE BP1(84-106)–NH_2 (7.0 mg, 1.7 mmol, 19% based on the amino group in the starting resin): Found: 2922.4 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 2922.2 (average). Amino acid analysis: $Asp_{3.9}Ser_{1.6}Glu_{3.5}Pro_{0.94}Ala_{1.9}Ile_{0.94}Leu_2Phe_{2.8}Lys_{3.9}$.

To a solution of Fmoc–CRE BP1(84-106)–NH_2 (28 mg, 6.6 μmol) in DMSO (0.81 mL) were added Boc-OSu (40 mg, 0.19 mmol) and DIEA (16 μL). After stirring for 2 h, ether was added to the reaction mixture. The resulting precipitate was washed with ether (7 mL×3), and then dissolved in DMSO (0.30 mL). Piperidine (81 μL) was added to the solution, followed by stirring for 1 h at room temperature. After the addition of acetic acid (81 μL) to the reaction mixture, the product was isolated by RP-HPLC to give [$Lys(Boc)^{97,98,105,106}$]–CRE BP1(84-106)–NH_2 (**3**) (20 mg, 4.7 μmol, 13% based on the amino group in the starting resin) as a freeze-dried powder: Found: 3121.5 $(M+Na)^+$ (average). Calcd for $(M+Na)^+$, 3122.4 (average). Amino acid analysis: $Asp_{4.1}Ser_{2.1}Glu_{4.1}Pro_{1.2}Ala_{2.0}Ile_{0.9}Leu_2Phe_{2.8}Lys_{3.8}$.

[$Thr(PO_3H_2)^{69,71}$, $Lys(Boc)^{77,97,98,105,106}$, $Cys(Acm)^{79}$]–CRE BP1(57-106)–NH_2 (4). Peptide **2** (2.2 mg, 0.51 μmol), peptide **3** (1.6 mg, 0.41 μmol), and HOOBt (2.5 mg, 15 μmol) were dissolved in DMSO (50 μL). DIEA (1.8 μL, 10 μmol) and $AgNO_3$ (0.26 mg, 1.5 μmol) were then added and, after stirring at room temperature for 24 h, DTT (2.0 mg, 13 μmol) and DMSO (45 μL) were added. The resulting solution was stirred for a further 30 min, following which piperidine (10 μL) was added to the solution. After stirring for 1 h, 85% phosphoric acid (20 μL) was added to the solution. This solution was subjected to RP-HPLC to give [$Thr(PO_3H_2)^{69,71}$, $Lys(Boc)^{77,97,98,105,106}$, $Cys(Acm)^{79}$]–CRE BP1(57-106)–NH_2 (**4**) (2.0 mg, 0.24 μmol, 59% yield, based on peptide **3**) after freeze-drying: Found: *m/z* 6375.7 $(M+H)^+$ (average). Calcd for $(M+H)^+$: 6371.9 (average). TFA-treated peptide **4**, [$Cys(Acm)^{79}$, $Thr(PO_3H_2)^{69,71}$]–CRE BP1(57-106)–NH_2: Found: *m/z* 5872.5 $(M+H)^+$ (average). Calcd for $(M+H)^+$: 5871.3 (average). Amino acid analysis: $Asp_{7.8}Thr_{2.0}Ser_{2.7}Glu_{7.0}Pro_{3.4}Gly_{1.1}Ala_{3.9}$ 1/2$Cys_{n.d.}Val_{2.1}Ile_{1.4}$ $Leu_3Phe_{4.0}Lys_{4.4}Arg_{1.8}$.

[$Thr(PO_3H_2)^{69,71}$, $Cys(Acm)^{27,32,76}$]–CRE BP1(19-106)–NH_2 (5). Peptide **4** (4.4 mg, 0.50 μmol), peptide **1** (5.0 mg, 0.76 μmol), AgCl (0.30 mg, 2.1 μmol), HOOBt (2.9 mg, 18 μmol), and DIEA (2.1 μL, 12 μmol) were mixed in DMSO (120 μL) and the solution was stirred for 48 h. After adding DTT (2.5 mg) to the reaction mixture, ether was added to precipitate the crude product. The crude product was treated with a solution of TFA (100 μL) containing 1,4-butanedithiol (5%) for 30 min. Ether was then added to the reaction mixture to precipitate the product. The product, [$Cys(Acm)^{27,32,79}$, $Thr(PO_3H_2)^{69,71}$]–CRE BP1(19-106)–NH_2 (**5**), was washed 3 times with ether and isolated by RP-HPLC in a yield of 56%, based on peptide **4**. Found: *m/z* 10322.5 $(M+H)^+$ (average). Calcd for $(M+H)^+$: 101323.4 (average). Amino acid analysis: $Asp_{12}Thr_{5.2}Ser_{3.6}Glu_{9.8}Pro_{4.6}Gly_4Ala_{6.6}$ 1/2$Cys_{2.8}Val_{3.5}Met_{2.0}$ $Ile_{1.7}Leu_{6.5}$ $Phe_{6.9}$ $Lys_{8.8}His_{3.8}Arg_{3.0}$.

[$Thr(PO_3H_2)^{69,71}$]–CRE BP1(19-106)–NH_2 (6). Peptide **5** (3.6 mg, 0.28 μmol) was treated with $AgNO_3$ (0.47 mg, 2.7 μmol) and DIEA (0.94 μL, 5.4 μmol) in a mixed solvent of water (110 μL) and 2,2,2-trifluoroethanol (340 μL) for 5 h at room temperature. DTT (5.0 mg) and then 0.5 M HCl (50 μL) were added to the solution. Peptide **6** (2.0 mg, 0.16 μmol) was isolated by RP-HPLC in a yield of 57%, based on peptide **5**. Found: 10110.6 $(M+H)^+$ (average). Calcd for $(M+H)^+$: 10110.1 (average). Amino acid analysis: $Asp_{12}Thr_{4.9}Ser_{3.7}$ Glu_{10} $Pro_{4.6}$ $Gly_4Ala_{6.2}$ 1/2$Cys_{2.2}Val_{3.4}Met_{1.8}Ile_{1.6}Leu_{6.3}Phe_{6.9}Lys_{8.5}His_{3.6}Arg_{3.0}$.

Boc–Gly–SCH_2CH_2CO–Ala–OCH_2–Pam Resin (7). Boc–Ala–OCH_2–Pam resin (Ala: 0.74 mmol g^{-1}, 1.5 g) was mixed with 50% TFA in DCM (1×5 min, 1×20 min), washed with DCM (3×1 min) and NMP (3×1 min), and then mixed with 5% DIEA in NMP (3×2 min). To this resin, DIEA (0.26 mL, 1.5 mmol) and a solution, which was prepared by a one hour reaction of Trt–SCH_2CH_2COOH (1.5 g, 4.5 mmol), HOBt hydrate (0.77 g, 5 mmol), and DCC (0.93 g, 4.5 mmol) in an NMP solution (9.5 mL), were added. The resulting suspension was mixed for 1 h and then washed with NMP (5×2 min) and DCM (3×1 min). The obtained resin was treated with TFA containing 5% EDT for 2 min, 3 min, and 30 min, successively, and washed with DCM (3×1 min) and then NMP (3×1 min). To this resin, DIEA (0.26 mL, 1.5 mmol) and a solution, which was prepared by a one hour reaction of Boc–Gly (0.78 g, 4.4 mmol), HOBt hydrate (0.75 g, 4.9 mmol), and DCC (0.90 g, 4.4 mmol) in NMP solution (8.3 mL), were added. The resulting suspension was stirred for 1 h. A resin was washed with NMP (3×2 min), and treated with an NMP solution (10 mL) containing acetic anhydride (1.0 mL, 9.5 mmol) and DIEA (0.5 mL, 3.0 mmol) for 10 min. This resin was washed with NMP (3×1 min) and methanol (3×1 min), and dried under reduced pressure to obtain resin **7** (1.7 g). Amino acid analysis: Gly, 0.64 mmol g^{-1}; Ala, 0.65 mmol g^{-1}).

Fmoc–[$Thr(PO_3H_2)^{69,71}$, $Lys(Boc)^{77}$, $Cys(Acm)^{79}$]–CRE BP1(57-83)–SCH_2CH_2CO–Ala–OH (8). Starting from resin **7** (0.50 g), a protected peptide resin (1.5 g) corresponding to the sequence of CRE BP1(57-83), Fmoc–Pro–Ala–Arg(Mts)–Asn–Asp (OBzl)– Ser(Bzl)– Val– Ile– Val– Ala– Asp(OBzl)– Gln– $Thr(PO_3cPen_2)$ –Pro–$Thr(PO_3cPen_2)$–Pro–Thr(Bzl)–Arg(Mts)–Phe–Leu–Lys(Cl- Z)– Asn– Cys(Acm)– Glu(OBzl)– Glu(OBzl)– Val– Gly–SCH_2CH_2CO–Ala–OCH_2–Pam resin, was obtained. Each Thr (PO_3cPen_2) residue was introduced manually in a manner which was the same as that described for the synthesis of peptide **2**.

An aliquot of the resin (100 mg) was stirred in a reagent which contained TFMSA (0.45 mL), thioanisole (0.60 mL), *m*-cresol (0.56 mL), EDT (0.20 mL), and TFA (3.25 mL) at 0 °C for 2 h. The reaction mixture was added to cold ether (200 mL) and the resulting precipitate was washed with ether and collected by centrifugation. The precipitate was washed twice with ether and dissolved in aqueous acetonitrile. The peptide solution was passed through a disposable ODS cartridge. The crude product (74 mg) was obtained after freeze-drying of the solution. An aqueous acetonitrile solution of the crude product (10 mg) was injected to RP-HPLC column and the isolated peak was freeze-dried to yield peptide **8**. (0.90 mg, 0.22 mmol, 7.8% yield based on Gly content in the starting resin): Found: *m/z* 3570.2 $(M+H)^+$ (average). Calcd for $(M+H)^+$, 3571.8 (average). Amino acid analysis: $Asp_{3.7}Thr_{2.0}Ser_{0.94}Glu_{3.2}Pro_{2.6}Gly_{1.1}Ala_{3.0}$ 1/2$Cys_{0.2}Val_{2.4}Ile_{0.6}Leu_1$ $Phe_{1.1}Lys_{0.93}Arg_{1.9}$.

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References

1 H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **64**, 111 (1991).

2 H. Hojo, S. Yoshimura, M. Go, and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **68**, 330 (1995).

3 T. Kawakami, S. Kogure, and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **69**, 3331 (1996).

4 M. Mizuno, K. Haneda, R. Iguchi, I. Muramoto, T. Kawakami, S. Aimoto, K. Yamamoto, and T. Inazu, *J. Am. Chem. Soc.*, **121**, 284 (1999).

5 T. Kawakami and S. Aimoto, *Chem. Lett.*, **1997**, 1157.

6 T. Kawakami, S. Yoshimura, and S. Aimoto, *Tetrahedron Lett.*, **39**, 7901 (1997).

7 T. Maekawa, H. Sakura, C. K. Ishii, T. Sudo, T. Yoshimura, J. Fujisawa, M. Yoshida, and S. Ishii, *EMBO J.*, **8**, 2023 (1989).

8 S. Gupta, D. Campbell, B. Derijard, and R. J. Davis, *Science*, **267**, 389 (1995).

9 T. Wakamiya, R. Togashi, T. Nishida, K. Saruta, J. Yasuoka, S. Kusumoto, S. Aimoto, K. Y. Kumagaye, K. Nakajima, and K. Nagata, *Bioorg. Med. Chem.*, **5**, 135 (1997).

10 K. Akaji, N. Fujii, H. Yajima, and D. Pearson, *Chem. Pharm. Bull.*, **30**, 349 (1982).

11 H. Sakamoto, H. Kodama, Y. Higashimoto, M. Kondo, M. S. Lewis, C. W. Anderson, E. Appella, and K. Sakaguchi, *Int. J. Peptide Protein Res.*, **48**, 429 (1996).

12 S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).

13 N. Fujii, M. Nomizu, S. Futaki, A. Otaka, S. Funakoshi, K. Akaji, K. Watanabe, and H. Yajima, *Chem. Pharm. Bull.*, **34**, 864 (1986).