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Preparation of *S*-Protected Cysteine-Containing Peptide Thioester and Its Use for the Synthesis of the Barnase-Like Domain in DNA-Directed RNA Polymerase II of *Saccharomyces cerevisiae*

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The Barnase-like Domain in DNA-Directed RNA Polymerase II of *Saccharomyces cerevisiae* containing three cysteine residues was synthesized by a thioester method. A partially-protected cysteine-containing peptide thioester was prepared via a peptide obtained by a solid-phase method using 3-nitro-2-pyridinesulfenyl amino acid derivatives. Four peptide segments covering 112 amino acid residues of the barnase-like domain were condensed from the *C*-terminal to the *N*-terminal one by one in the presence of silver ions and *N*-hydroxysuccinimide to give a full sequence peptide. RNase activity of the synthetic domain could not be detected because of its extreme low solubility in the neutral buffer.

The thioester method was successfully applied to protein syntheses.[1—4] However, cysteine-containing proteins could not be prepared using this method, because there was no known route to prepare an adequately protected cysteine-containing peptide segment. To expand this method to a general procedure for the synthesis of proteins with or without a cysteine residue(s), methods should be developed for the preparation of adequately *S*-protected cysteine-containing peptide thioesters.

In the present paper, we will describe the procedure for the preparation of partially-protected peptide thioester containing cysteine residues and its use for protein synthesis.

To demonstrate the usefulness of partially protected cysteine-containing peptide thioesters, the barnase-like domain (RPSc(299-410)), containing three cysteine residues, in the second largest subunit of RNA polymerase II of *Saccharomyces cerevisiae* (Fig. 1)[5] was synthesized.

Results and Discussion

Preparation of Peptide Segments. RPSc(299-410) was divided into four segments for synthetic purposes, as shown in Fig. 1 by arrows.

Peptide **1**, containing two cysteine residues, was prepared according to the scheme shown in Fig. 2. Starting from an acid labile resin (SAL),[6] peptide chain was elongated using 3-nitro-2-pyridinesulfenyl (Npys) amino acids according to the method described by Matsueda et al.[7,8] The side-chain functional groups of Npys-amino acids were blocked by the protecting groups which can be removed by trifluoroacetic acid (TFA) treatment. But the mercapto groups of cysteine residues were protected by the 2,4,6,-trimethylbenzyl (Tmb) group, which is stable under TFA treatment conditions and can be removed by HF treatment. Therefore, a peptide bearing Tmb groups on its cysteine residues was obtained when a protected peptide resin was treated with reagent K.[9] This peptide was easily purified on reversed-phase high performance liquid chromatography (RPHPLC). Thus [Cys (Tmb)^{302,317}]–RPSc(299-321)–$SC(CH_3)_2CH_2CONH_2$ was obtained in a 14% yield, based upon L-norleucine (Nle) in the starting resin. To *N*-terminal and side-chain amino groups of the purified peptide, *t*-butoxycarbonyl (Boc) groups were introduced with *N*-(*t*-butoxycarbonyloxy)succinimide (Boc–ONSu) in the presence of *N*-ethyldiisopropylamine (EDIA) to give peptide **1**.

According to this method, the desired peptide was directly obtained without reintroducing the Tmb group. In our previous paper,[10] we reported two methods. In one method, *S*-protected peptide thioester is obtained directly by selective cleavage of side chain protecting groups. This method is well applicable when the peptide has a few Arg residues. In the other method, protecting groups are reintroduced to free mercapto groups after all the side chain protecting groups are removed once. If a peptide is well soluble in *N*,*N*-dimethylformamide (DMF) or aqueous DMF, this method is widely applicable to the preparation of *S*-protected peptide thioester. But partial hydrolysis of thioester moiety and incomplete introduction of protecting groups were

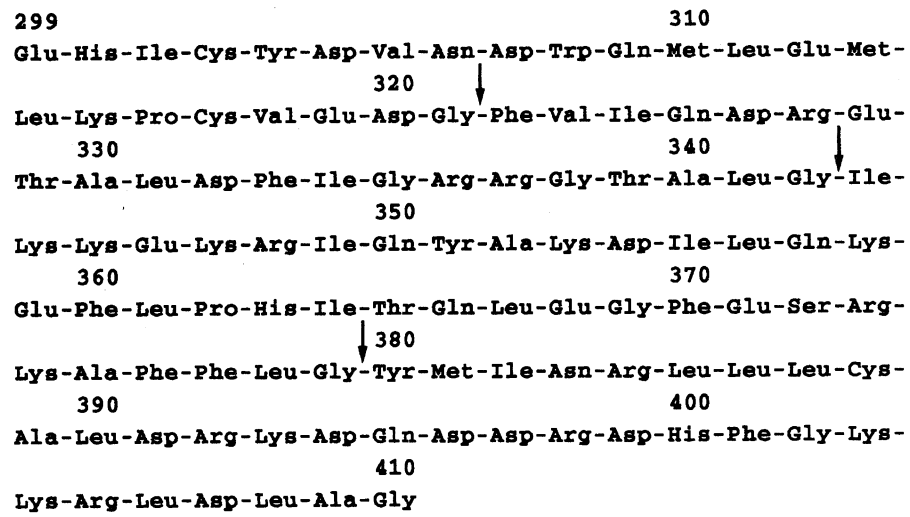

Fig. 1. Amino acid sequence of the barnase-like domain (RPSc(299-410)). The arrows indicate the sites of segment coupling.

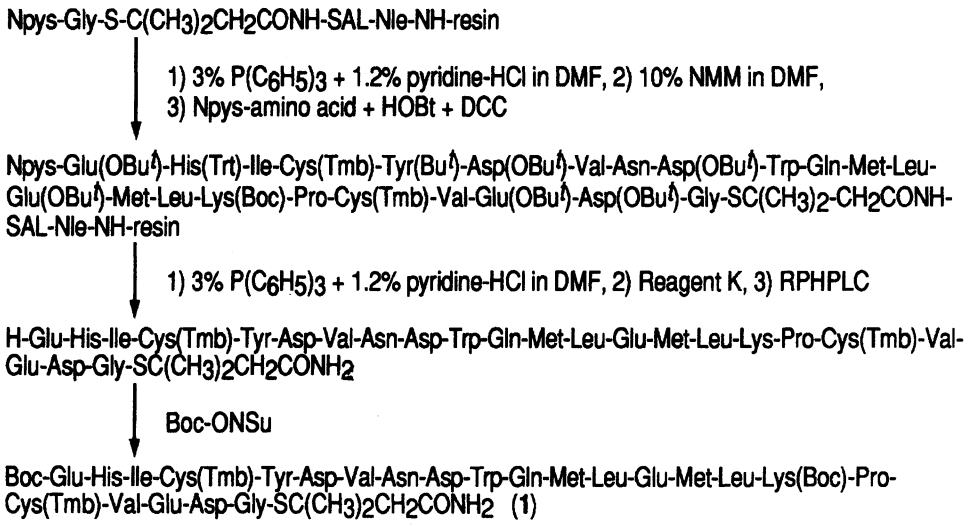

Fig. 2. Synthetic route of Boc–[Cys(Tmb)^{302,317}, Lys(Boc)^{315}]–RPSc(299-321)–SC $(CH_3)_2CH_2CONH_2$ (**1**).

sometimes observed in this method. The newly developed method avoids the problems involved in the previous methods, and will be widely applicable to the preparation of *S*-protected peptide thioesters regardless of the number of Cys, Arg, and other amino acid residues in the peptide.

Peptides **2** and **3**, in which no cysteine residue was contained, were prepared by a procedure similar to that described previously.[3,4] Starting from Boc–Gly–SC $(CH_3)_2CH_2$–CO–Nle–MBHA resin (MBHA resin: 4-methylbenzhydrylamine resin, NH_2-resin), Boc–amino acids were successively condensed. After the completion of the chain assembly, a 9-fluorenylmethoxycarbonyl (Fmoc) group was introduced to protect the terminal amino group using *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc–ONSu). The protected peptide resin was treated with HF and a crude product was purified by RPHPLC to give an Fmoc–peptide thioester. The side-chain amino groups of the peptide were protected by Boc groups with Boc–ONSu in the presence of EDIA to give partially protected peptide thioesters of **2** and **3**.

The *C*-terminal peptide segment, peptide **4**, contains one cysteine residue, but does not contain a thioester moiety. This peptide segment could be conveniently synthesized using Fmoc–amino acids[11] as shown in Fig. 3. The protected peptide resin corresponding to the sequence of RPSc(380-410) was synthesized on Wang resin.[12] The side-chain functional groups of Fmoc–amino acids were blocked by the protecting groups, which can be removed by TFA treatment. But the mercapto group of a cysteine residue was protected by a 4-methylbenzyl (MeBzl) group, which is stable under TFA treatment conditions and can be removed by HF treatment. Therefore, a peptide bearing a MeBzl group on a cysteine residue was obtained when a protected peptide resin was treated with reagent K. The peptide was purified on RPHPLC to give Fmoc–[Cys(MeBzl)^{388}]–RPSc

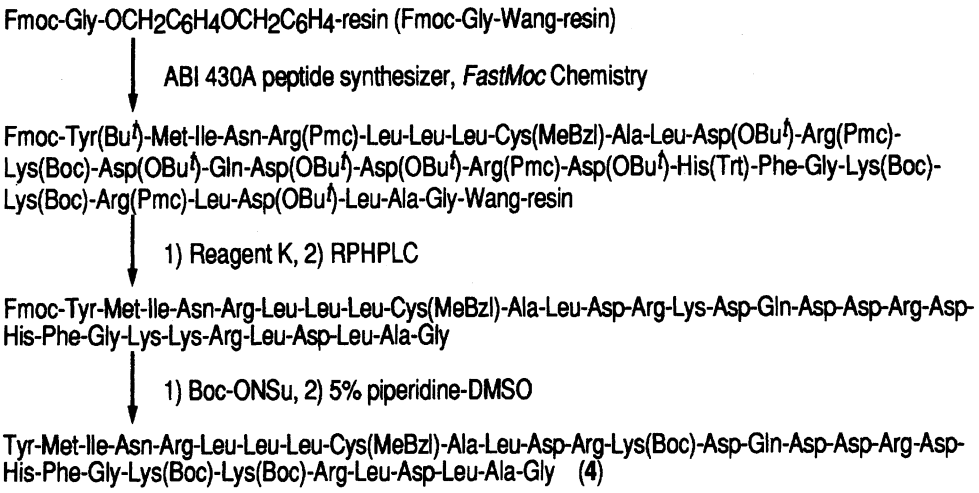

Fig. 3. Synthetic route of [Cys(MeBzl)^{388}, Lys(Boc)^{393,403,404}]–RPSc(380-410) (**4**).

Table 1. Partially Protected Peptide Segments Prepared for RPSc(299-410) Synthesis

Peptide segments		Yield/%
Boc–[Cys(Tmb)^{302,317},Lys(Boc)^{315}]–RPSc(299-321)–SC$(CH_3)_2CH_2CONH_2$	(**1**)	11
Boc–Glu–His–Ile–Cys(Tmb)–Tyr–Asp–Val–Asn–Asp–Trp–Gln–Met–Leu–Glu–Met–Leu–Lys(Boc)–Pro–Cys(Tmb)–Val–Glu–Asp–Gly–SC$(CH_3)_2CH_2CONH_2$		
Fmoc RPSc(322-342)–SC$(CH_3)_2CH_2CO$–Nle–NH_2	(**2**)	17
Fmoc–Phe–Val–Ile–Gln–Asp–Arg–Glu–Thr–Ala–Leu–Asp–Phe–Ile– Gly–Arg–Arg–Gly–Thr–Ala–Leu–Gly–SC$(CH_3)_2CH_2CO$–Nle—NH_2		
Fmoc–[Lys(Boc)^{344,345,347,353,358,374}]–RPSc(343-379)–SC$(CH_3)_2CH_2CO$–Nle–NH_2	(**3**)	6.4
Fmoc–Ile–Lys(Boc)–Lys(Boc)–Glu–Lys(Boc)–Arg–Ile–Gln–Tyr–Ala–Lys(Boc)–Asp–Ile–Leu–Gln–Lys(Boc)–Glu–Phe–Leu–Pro–His–Ile–Thr–Gln–Leu–Glu–Gly–Phe–Glu–Ser–Arg–Lys(Boc)–Ala–Phe–Phe–Leu–Gly–SC$(CH_3)_2CH_2CO$–Nle–NH_2		
[Cys(MeBzl)^{388},Lys(Boc)^{393,403,404}]–RPSc(380-410)	(**4**)	17
Tyr–Met–Ile–Asn–Arg–Leu–Leu–Leu–Cys(MeBzl)–Ala–Leu–Asp–Arg–Lys(Boc)–Asp–Gln–Asp–Asp–Arg–Asp–His–Phe–Gly–Lys(Boc)–Lys(Boc)–Arg–Leu–Asp–Leu–Ala–Gly		

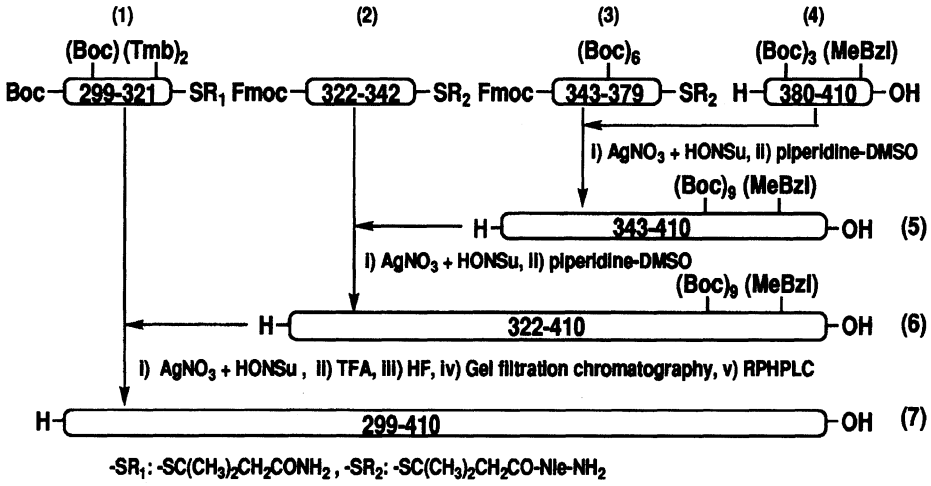

Fig. 4. Synthetic route of RPSc(299-410).

(380-410) in a yield of 20%.

All the peptide segments prepared for the synthesis of RPSc(299-410) are listed in Table 1. The yields of the peptide segments were calculated based upon the amino acids in the starting resins.

In this synthesis, MeBzl and Tmb groups were chosen to protect the thiol group of a cysteine residue, because both groups are stable toward silver ions under segment coupling conditions.

Segment Condensation: Segment condensation

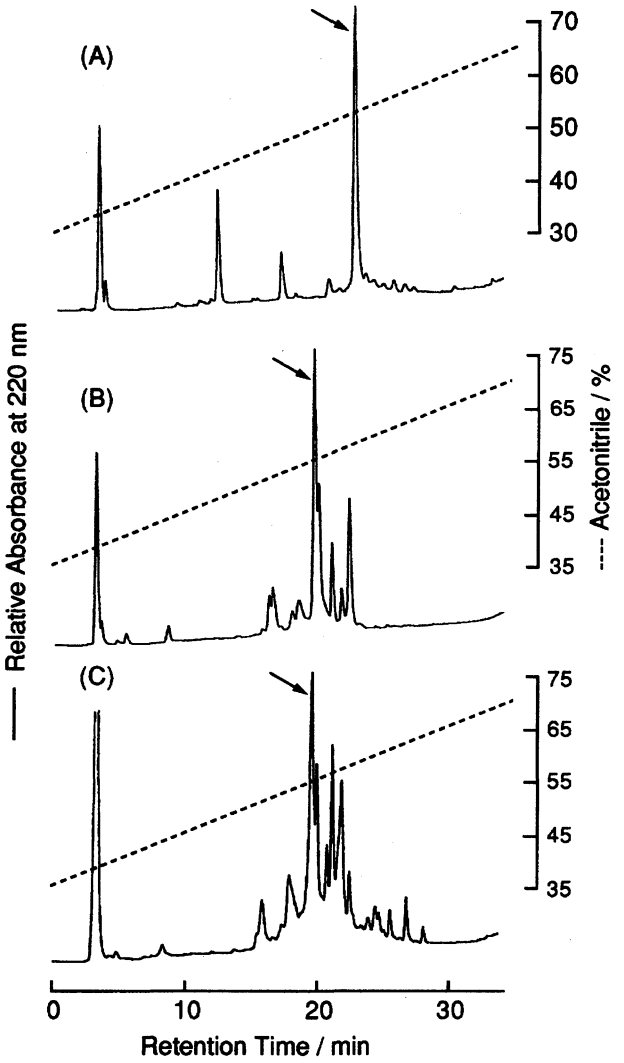

Fig. 5. RPHPLC elution profiles of the reaction mixtures of segment couplings after TFA treatment. Arrows in panel A, B, C indicate Fmoc–[Cys(MeBzl)^{388}]–RPSc(343-410), Fmoc–[Cys(MeBzl)^{388}]–RPSc(322-410), [Cys(Tmb)^{302,317}, Cys(MeBzl)^{388}]–RPSc(299-410), respectively. Column: YMC-Pack ODS-AM (4.6×250 mm) at a flow rate of 1 ml min^{-1} at 40 °C. The broken line indicates the acetonitrile concentration in 0.1% TFA.

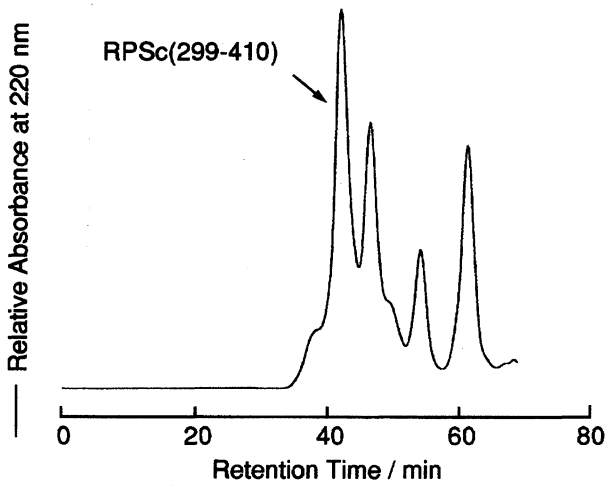

Fig. 6. Gel filtration chromatogram of crude RPSc (299-410). Column: TSKgel G3000SW (7.5×600 mm) at a flow rate of 0.3 ml min^{-1}. Eluent: 50% aqueous acetonitrile containing 0.1% TFA.

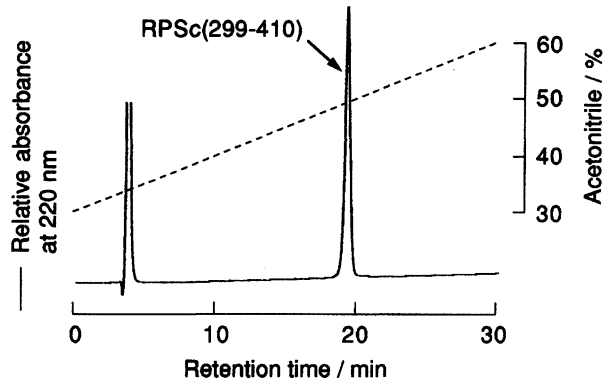

Fig. 7. RPHPLC profile of RPSc(299-410) (**7**) after gel filtration and RPHPLC purification. Column: YMC-Pack ODS-AM (4.6×250 mm) at a flow rate of 1 ml min^{-1} at 40 °C. The broken line indicates the acetonitrile concentration in 0.1% TFA.

proceeded according to the scheme shown in Fig. 4. The reaction was monitored by RPHPLC. When the coupling reaction was slow, extra EDIA was added. In the coupling between peptides **3** and **4**, the solution was stirred for 4 d. During this period, about 30% of the active ester were hydrolyzed and about 5% of the Fmoc group was removed from the product. The deprotected component was combined with the main product after the removal of Fmoc group from the product with 5% (v/v) piperidine in DMSO. Under these conditions the Fmoc group was cleaved without any serious side reactions within 10 min. The product was precipitated with ether and washed with distilled water containing 0.1% TFA. The mixture containing peptide **5** was obtained after lyophilization from distilled water. Following the same procedure, peptides **2** and **1** were successively condensed. After each coupling, an aliquot of the reaction mixture was treated with TFA and analyzed by RPHPLC, as shown in Fig. 5. The MeBzl and Tmb groups were quite stable during the segment condensation. Boc groups on the crude peptide covering the whole sequence of RPSc(299-410) were removed by the treatment with TFA containing 5% 1,4-butanedithiol (v/v) at room temperature for 10 min. This peptide was further treated with HF containing 7.5% (v/v) 1, 4-butanedithiol and 7.5% (v/v) anisole at 0 °C for 90 min to remove the Tmb and MeBzl groups. The crude peptide was purified by gel filtration chromatography using G3000SW, as shown in Fig. 6, followed by RPHPLC purification on a YMC-Pack ODS-AM type to give the final product of RPSc(299-410) at 12% yield based

upon peptide **4**. (Fig. 7) The structure of the product was confirmed by amino acid analysis and electrospray ionization (ESI) mass spectroscopy.

Enzymatic Activity: This RPSc domain shows sequence similarity with the bacterial RNase, barnase. All of the catalytic sites of barnase are conserved in the domain.[5] Thus this domain is expected to have an RNase activity. But enzymatic activity of RPSc-(299-410) was not detected under same conditions with barnase. This could be mainly due to the low solubility of this protein in the buffer. As RPSc(299-410) is a part of the sequence of RNA polymerase II, the hydrophobic parts of RPSc(299-410), which interact with other domains of the whole protein, would be exposed to the solvent.

Evaluation of the Method: Partially protected cysteine-containing peptide segments could be prepared by a solid-phase method. These segments could be condensed by silver ions without any loss of the MeBzl or Tmb groups. The desired product was isolated as a distinct peak by RPHPLC. Thus, the thioester method has been proven useful even for the preparation of cysteine-containing proteins.

As a terminal amino protecting group, Fmoc group was easier to remove than 2,2,2-trichloroethoxycarbonyl (Troc) or 4-pyridylmethoxycarbonyl (*i*Noc) groups. Products were obtained as precipitates only by adding ether to the reaction mixture.

Experimental

Materials and Methods. Fmoc-amino acids and *p*-[(*R,S*)-α-(fluoren-9-ylmethoxycarbonylamino)-2,4-dimethoxybenzyl]phenoxyacetic acid (Fmoc–NH–SAL resin linker) were purchased from Watanabe Chemical Ind. Ltd. (Hiroshima). Npys–amino acids were purchased from Kokusan Chemical Works, Ltd. (Tokyo). Fmoc–Gly–OCH_2–C_6H_4–OCH_2–C_6H_4-resin (Fmoc–Gly–OCH_2–Wang-resin) was purchased from Advanced ChemTech (Louisville, KY.). Reagent K was prepared according to the published reference.[9] ESI mass spectrum of Fmoc–RPSc (343-379)–$SC(CH_3)_2CH_2CO$–Nle–NH_2 and peptides **7** were measured by JMS-SX102 mass spectrometer (JEOL Ltd., Tokyo) equipped with an ESI ion source (Analytica of Branford, Conn.). Boc–amino acid derivatives used were of L-configuration. Additional abbreviations: Bzl, benzyl; Bom, benzyloxymethyl; Br-Z, 2-bromobenzyloxycarbonyl; Bu^t, *t*-butyl; Cl-Z, 2-chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; HOBt, 1-hydroxybenzotriazole; NMP, 1-methyl-2-pyrrolidinone; NMM, 4-methylmorpholine; O*c*Hex, cyclohexyl ester; OBzl, benzyl ester; OBu^t, *t*-butyl ester; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tos, tosyl; Trt, triphenylmethyl.

Synthesis. Peptide Chain Elongation on a Solid-Support. Solid-phase syntheses of peptide segments were performed on a peptide synthesizer 430A (Applied Biosystems Inc., Foster City, CA.). Peptides **2** and **3** were prepared using the 0.5 mmol scale single or double coupling protocol of the benzotriazole active ester method of the system software version 1.40 NMP/HOBt *t*-Boc. Peptide **4** was prepared according to the 0.25 mmol scale standard protocol of the Fmoc/NMP chemistry of the system software version 1.40 *Fast Moc.*

Npys–Cys(Tmb). Cys(Tmb) (1.27 g, 5.0 mmol) was dissolved in a mixture of 1 M# NaOH (5 ml) and dioxane (2.5 ml). Npys–Cl (1.05 g, 5.5 mmol) dissolved in dioxane (10 ml) and 2 M NaOH (3 ml) were added dropwise to the solution at 10 °C over the period of 30 min. The solution was further stirred for 3 h at room temperature. After concentration of the solution, a product was extracted with ethyl acetate by adding aq citric acid. An organic layer was dried over Na_2SO_4 and concentrated to give crystals of Npys–Cys (Tmb) (1.75 g, 86%), mp 151—152 °C. Found: C, 52.51; H, 5.09; N, 10.27%. Calcd for $C_{18}H_{21}N_3O_4S_2$: C, 53.05; H, 5.19; N, 10.31%.

Npys–Gly–ONSu. To a solution of Npys–Gly (690 mg, 3 mmol) and HONSu (380 mg, 3.3 mmol) in a mixture of dioxane and ethyl acetate (1 : 1, 6 ml), DCC (620 mg, 3.3 mmol) was added at 0 °C. After the solution was stirred for 4 h at room temperature, 1,3-dicyclohexylurea was removed by filtration; then the solvent was removed in vacuo. The residual oil was crystallized from 2-propanol to give Npys–Gly–ONSu (907 mg, 93%), mp 151—152 °C. Found: C, 40.70; H, 3.24; N, 17.53%. Calcd for $C_{11}H_{10}N_4O_6S$: C, 40.30; H, 3.10; N, 17.23%.

Npys–Gly–$SC(CH_3)_2CH_2COOH$. Npys–Gly–ONSu (910 mg, 2.8 mmol) and 3-methyl-3-mercaptobutyric acid (360 mg, 2.7 mmol) were dissolved in DMF (6 ml). EDIA (580 mg, 4.5 mmol) was added and the solution was stirred for 3 h at room temperature. Extra EDIA (130 mg, 1.0 mmol) was added and the solution was kept at room temperature for 1 h. After the solvent was removed in vacuo, the residue was dissolved in ethyl acetate, washed with aqueous citric acid and distilled water (×5) and dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified on RPHPLC using aqueous acetonitrile containing 0.1% TFA as an eluent, to give Npys–Gly–$SC(CH_3)_2CH_2COOH$ (190 mg, 550 μmol, 20%), mp 107.0—107.5 °C. Found: C, 41.66; H, 4.36; N, 12.16%. Calcd for $C_{12}H_{15}N_3O_5S_2$: C, 41.73; H, 4.38; N,12.17%.

Fmoc–NH–SAL–Nle–NH-resin. Boc–Nle (1.1 g, 4.7 mmol) was mixed with 1 M HOBt in NMP (4.7 ml) and 1 M DCC in NMP (4.7 ml). After stirring for 30 min, the solution was mixed with neutralized MBHA resin (5 g, NH_2: 3.2 mmol) and shaken for 3 h. After washing with DMF, the resin was treated with 5% acetic anhydride in DMF (v/v) for 15 min. After washing, the resin was treated with 55% TFA in DCM (v/v) for 5 and 15 min followed by 5% EDIA in DMF (v/v) for 5 min (×2). The Fmoc–NH–SAL resin linker (2.5 g, 4.7 mmol) was introduced in a similar manner to give Fmoc–NH–SAL–Nle–NH-resin (6.8 g, Nle: 2.8 mmol).

Boc–[Cys(Tmb)^{302,317}, Lys(Boc)^{315}]–RPSc(299-321)–$SC(CH_3)_2CH_2CONH_2$ (1). Fmoc–NH–SAL–Nle–NH-resin (240 mg, Nle: 100 μmol) was treated with 50% piperidine in DMF (v/v) for 1 and 5 min, followed by washing with DMF for 1 min (×6). This resin was mixed with Npys–Gly–$SC(CH_3)_2CH_2COOBt$, prepared by mixing with Npys–Gly–$SC(CH_3)_2CH_2COOH$ (42 mg, 110 μmol), HOBt (15 mg, 110 μmol), and DCC (23 mg, 110 μmol) in DMF

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

for 40 min, and shaken overnight. The resin was treated with 10% acetic anhydride in DCM (v/v) for 10 min to give Npys–Gly–SC$(CH_3)_2CH_2CO$–NH–SAL–Nle–NH-resin. Using this resin, the protected peptide resin corresponding to the sequence of RPSc(299-321), H–Glu(OBu^t)–His (Trt)–Ile–Cys(Tmb)–Tyr(Bu^t)–Asp(OBu^t)–Val– Asn–Asp (OBu^t)– Trp– Gln– Met– Leu– Glu(OBu^t)– Met– Leu– Lys (Boc)–Pro–Cys(Tmb)–Val–Glu(OBu^t)–Asp(OBu^t)–Gly–SC $(CH_3)_2CH_2CONH$–SAL–Nle–NH-resin (460 mg), was synthesized according to the following procedure: (1) 3% triphenylphosphine and 1.2% pyridine hydrochloride/DMF (v/v) (2 min, 20 min×3); (2) DMF wash (1 min×3); (3) 10% NMM/DMF (v/v) (1 and 3 min); (4) Npys–amino acid HOBt ester (0.4 mmol) (3 h); (5) DMF wash (1 min×3). An aliquot of the resin (220 mg) was treated with reagent K (5 ml) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with ether. The residual mass was dissolved in 50% aqueous acetonitrile containing 0.1% TFA and the resin was removed by filtration. The solution was lyophilized to give 88 mg of crude product. This peptide was purified by RPHPLC to give [Cys(Tmb)^{302,317}]–RPSc (299-321)–SC$(CH_3)_2CH_2CONH_2$ (26 mg, 6.9 μmol, 14% based on Nle in the starting resin). Found: m/z 3146.3 $(M+H)^+$. Calcd for: $(M+H)^+$, 3146. 4. Amino acid composition: $Asp_{3.82}Glu_{3.99}Pro_{0.77}Gly_{1.01}$1/2Cystine$_{0.76}Val_{2.07}$ $Met_{1.66}Ile_{0.74}Leu_2Tyr_{0.97}Lys_{1.01}His_{0.97}Trp_{0.54}$.

[Cys(Tmb)^{302,317}]– RPSc(299- 321)– SC$(CH_3)_2CH_2CON$ H_2 (26 mg, 6.9 μmol) dissolved in DMSO (150 μl) was reacted with Boc–ONSu (6.9 mg, 32 μmol) in the presence of EDIA (5.6 μl, 32 μmol). The resulting solution was stirred for 6 h. Ether was added to the reaction mixture to precipitate the product which was collected by centrifugation and freeze-dried from a dioxane suspension to give 29 mg (5.1 μmol, 11% based on Nle in the starting resin) of peptide **1**. Found: m/z 3346.7 $(M+H)^+$. Calcd for: $(M+H)^+$, 3346.5. Amino acid analysis of peptide **1**: $Asp_{3.89}$ $Glu_{4.16}$ $Pro_{0.96}$ $Gly_{1.32}$ 1/2 Cystine$_{0.64}$ $Val_{1.98}$ $Met_{1.91}$ $Ile_{0.65}Leu_2Tyr_{0.94}Lys_{1.19}His_{1.17}Trp_{0.50}$.

Fmoc– RPSc(322- 342)– SC$(CH_3)_2CH_2CO$– Nle– NH_2 (2). Starting from Boc–Gly–SC$(CH_3)_2CH_2CO$–Nle–NH- resin (900 mg, Gly: 490 μmol), a protected peptide resin corresponding to the sequence of RPSc(322-342): Boc–Phe–Val–Ile–Gln–Asp(OcHex)–Arg(Tos)–Glu (OBzl)–Thr(Bzl)–Ala–Leu–Asp (OcHex)–Phe–Ile–Gly–Arg (Tos)– Arg(Tos)– Gly– Thr(Bzl)– Ala– Leu– Gly– SC$(CH_3)_2$ CH_2CO–Nle–NH-resin was prepared by the synthesizer using double coupling protocol. After the completion of the chain assembly, a peptide resin was treated 55% TFA in DCM (v/v) for 5 and 15 min, and then with 5% EDIA in DMF (v/v) for 5 min (×2). This resin was mixed with Fmoc–ONSu (670 mg, 2 mmol) in DMF (20 ml) overnight to give a protected peptide resin (1.8 g). An aliguot of the resin (740 mg) was treated with HF (10 ml) containing anisole (1 ml) at 0 °C for 90 min to give 340 mg of a crude product. This product was purified on RPHPLC to obtain Fmoc–RPSc(322-342)–SC$(CH_3)_2CH_2CO$–Nle–NH_2 (120 mg, 34 μmol, 17% based on the Gly in the starting resin). Found: m/z 2785.2 $(M+H)^+$. Calcd for: $(M+H)^+$, 2785.5. Amino acid analysis of peptide **2**: $Asp_{1.92}Thr_{2.05}Glu_{2.14}Gly_{3.34}Ala_{1.95}Val_{0.52}Ile_{1.23}Leu_2Nle_{1.15}$ $Phe_{1.52}Arg_{3.09}$.

Fmoc– [Lys(Boc)^{344,345,347,353,358,374}]– RPSc(343-379)–SC$(CH_3)_2CH_2$–CO–Nle–NH_2 (3). Starting from Boc–Gly–SC$(CH_3)_2CH_2CO$–Nle–NH-resin (900 mg, Gly: 490 μmol), Fmoc–Ile–Lys(Cl-Z)–Lys(Cl-Z)–Glu(OBzl) –Lys(Cl- Z)– Arg(Tos)– Ile– Gln– Try(Br- Z)– Ala– Lys(Cl- Z) –Asp(OcHex)–Ile–Leu–Gln–Lys(Cl-Z)–Glu(OBzl)–Phe–Leu –Pro–His(π-Bom)–Ile–Thr(Bzl)–Gln–Leu–Glu(OBzl)–Gly–Phe–Glu(OBzl)–Ser(Bzl)–Arg(Tos)–Lys(Cl-Z)–Ala–Phe–Phe –Leu–Gly–SC$(CH_3)_2CH_2CO$–Nle–NH-resin (3.3 g) was prepared by the method described for the synthesis of peptide **2**. An aliquot of the resin 0.7 g was treated with HF (15 ml) containing anisole (1.5 ml) at 0 °C for 90 min to give 380 mg of a crude product. This peptide was purified on RPHPLC to yield 41 mg (6.6 μmol, 6.4% based on Gly in the starting resin) of Fmoc–RPSc(343-379)–SC$(CH_3)_2CH_2CO$–Nle–NH_2. Found: 4901.5 daltons. Calcd: (average) 4901.9 daltons. Amino acid analysis: $Asp_{0.95}Thr_{0.94}Ser_{0.97}Glu_{6.79}Pro_{0.70}Gly_2Ala_{1.77}Ile_{3.29}Leu_{3.91}$ $(Tyr+Nle)_{2.30}Phe_{4.02}Lys_{5.48}His_{1.01}Arg_{1.84}$.

To the solution of Fmoc–RPSc(343-379)–SC$(CH_3)_2CH_2$-CO–Nle–NH_2 (41 mg, 6.6 μmol) dissolved in DMSO (200 μl), Boc–ONSu (22 mg, 100 μmol) and EDIA (18 μl, 100 μmol) were added and the solution was stirred for 2.5 h to give peptide **3** (47 mg, 6.6 μmol, 6.4% based on Gly in the starting resin) as described for the synthesis of peptide **1**. Amino acid analysis of peptide **3**: $Asp_{0.93}Thr_{0.93}Ser_{0.93}Glu_{6.80}$ $Pro_{0.97}Gly_2Ala_{1.93}Ile_{3.15}Leu_{3.86}(Tyr+Nle)_{2.03}Phe_{3.93}Lys_{4.73}$ $His_{0.86}Arg_{1.88}$.

[Cys(MeBzl)^{388}, Lys(Boc)^{393,403,404}]– RPSc(380-410) (4). Starting from Fmoc–Gly–OCH_2–Wang-resin (260 mg, 200 μmol), Fmoc–Tyr(Bu^t)–Met–Ile–Asn (Trt)–Arg(Pmc)–Leu–Leu–Leu–Cys(MeBzl)–Ala–Leu–Asp (Bu^t)– Arg(Pmc)– Lys(Boc)– Asp(OBu^t)– Gln(Trt)– Asp (OBu^t)–Asp(OBu^t)–Arg (Pmc)–Asp(OBu^t)–His(Trt)–Phe–Gly–Lys(Boc)–Lys(Boc)–Arg(Pmc)–Leu–Asp(OBu^t)–Leu–Ala–Gly–OCH_2–Wang resin (1.3 g) was obtained using the synthesizer. An aliquot of the resin (630 mg) was treated with reagent K (10 ml) at room temperature for 60 min. The peptide (370 mg) precipitated with ether was purified on RPHLC to give 110 mg (20 μmol, 20% based on Gly in the starting resin) of Fmoc–[Cys(MeBzl)^{388}]–RPSc(380-410). Found: m/z 4015.1 $(M+H)^+$. Calcd: 4015.1 $(M+H)^+$, Amino acid composition: $Asp_{7.15}Glu_{1.46}Gly_{2.22}Ala_{1.94}Met_{0.56}Ile_{0.90}Leu_{5.63}Tyr_{0.72}Phe_1$ $Lys_{3.13}His_{1.08}(Arg+Cys(MeBzl))_{4.57}$.

Fmoc–[Cys(MeBzl)^{388}]–RPSc(380-410) (110 mg, 20 μmol) and Boc–ONSu (47 mg, 220 μmol) were dissolved in DMSO (770 μl). The solution was stirred for 3 h after adding EDIA (38 μl, 220 μmol) to give Fmoc–[Cys (MeBzl)^{388}, Lys(Boc)^{393,403,404}]–RPSc(380-410) (130 mg, 17 μmol, 17% based on Gly in the starting resin) as the procedure described for peptide **1**. Amino acid composition: $Asp_{6.98}Glu_{1.01}Gly_{1.97}Ala_{1.93}Met_{0.67}Ile_{0.86}Leu_{5.71}Tyr_{0.68}Phe_1$ $Lys_{2.96}His_{1.00}(Arg+Cys(MeBzl))_{4.72}$.

Fmoc–[Cys(MeBzl)^{388}, Lys(Boc)^{393,403,404}]– RPSc(380-410) (130 mg, 17 μmol) was dissolved in DMSO (770 μl) and piperidine (41 μl) was added. The solution was stirred for 15 min at room temperature. Ether added to precipitate the product, which was dissolved in DMSO (400 μl) and precipitated by distilled water containing 0.1% TFA. The precipitate was lyophilized from distilled water to give peptide **4** (110 mg, 17 μmol, 17% based on Gly

in the starting resin). Amino acid analysis of peptide **4**: $Asp_{7.15}Glu_{1.10}Gly_{1.97}Ala_{1.93}Met_{0.83}Ile_{0.95}Leu_{5.94}Tyr_{0.97}Phe_1$ $Lys_{2.67}His_{1.02}(Arg+Cys(MeBzl))_{4.93}$.

Synthesis of RPSc(299-410) (7). Peptides **3** (7.7 mg, 1.1 μmol), **4** (5.7 mg 0.9 μmol), and HONSu (1.6 mg, 14 μmol) were dissolved in DMSO (150 μl) containing EDIA (0.37 μl, 2.1 μmol). $AgNO_3$ (0.37 mg, 2.1 μmol) was then added and the mixture was stirred overnight at room temperature. EDIA (0.1 μl, 0.6 μmol) was added and the solution was stirred for another 3 d. Piperidine (8 μl) was added and the solution was stirred for 30 min. Ether was added to form a precipitate; this was washed with distilled water containing 0.1% TFA (×2). A mixture containing peptide **5** (13 mg) was obtained after lyophilization. Following the same procedure, peptides **2** (7.8 mg, 2.3 μmol) and **1** (7.9 mg, 1.4 μmol) were successively condensed. The crude peptide (19 mg) was treated with TFA (180 μl) containing 5% 1,4-butanedithiol (v/v) at room temperature for 10 min. The TFA was removed under a nitrogen stream and the peptide was precipitated with ether. This peptide was treated with HF (5.0 ml) containing 1,4-butanedithiol (0.38 ml) and anisole (0.38 ml) at 0 °C for 90 min. This peptide was purified by gel filtration on G3000SW (7.5×600 mm, Tosoh, Tokyo) at a flow rate of 0.3 ml min^{-1}, using 50% aqueous acetonitrile containing 0.1% TFA as an eluent to give partially purified RPSc(299-410) (5.5 mg, 310 nmol). This peptide was further purified by RPHPLC on YMC-Pack ODS-AM to give RPSc(299-410) (**7**) (2.0 mg, 110 nmol, 12% based on peptide **4**). Found: 13193.9 daltons. Calcd: (average) 13191.2 daltons. Amino acid analysis of peptide **7**: $Asp_{14.40}Thr_{2.74}Ser_{0.95}Glu_{13.15}Pro_{1.95}Gly_8Ala_{6.04}$ $1/2Cys_{0.86}Val_{2.28}Met_{2.65}Ile_{5.28}Leu_{12.86}Tyr_{2.81}Phe_{6.05}$ $Lys_{8.76}His_{2.49}Trp_{0.64}Arg_{8.24}$.

Measurement of RNase Activity. RNase activity of RPSc(299-410) was measured according to the procedure described by Rushizky[13] with some modification.[4]

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