Protein Synthesis Using *S*-Alkyl Thioester of Partially Protected Peptide Segments. Synthesis of DNA-Binding Protein of *Bacillus stearothermophilus*

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Using partially protected peptide thioesters as building blocks, we synthesized HU-type DNA-binding protein of *Bacillus stearothermophilus*. Four peptide segments, Boc–[Lys(Boc)^{3}]–HBs(1—15)–$SCH_2CH_2CONH_2$, *i*Noc–[Lys(Boc)^{18,19,23,38}]–HBs(16—39)–$SCH_2CH_2CONH_2$, *i*Noc–[Lys(Boc)^{41,59}]–HBs(40—60)–$SCH_2CH_2CONH_2$, [Lys(Boc)^{75,80,83,86,90}]–HBs(61—90) were prepared using peptides obtained by a solid-phase method. A partially protected peptide thioester was condensed to a peptide with a free amino group by converting the thioester to the corresponding active ester in the presence of silver ions and *N*-hydroxysuccinimide. Finally, highly pure synthetic HBs(1—90) was obtained.

In a previous paper[1] we reported that a partially protected peptide thioester was a promising building block for polypeptide synthesis. The developed method retained the benefits of the thiocarboxyl segment strategy,[2-7] such as selective activation of the carbonyl group and high solubility of an intermediate peptide segment in aqueous acetonitrile during purification. Furthermore, the method overcomes the weakness of the thiocarboxyl segment strategy, which mainly originates from the nucleophilic character and chemical instability of the thiol moiety of the thiocarboxyl group. These results suggested that proteins should be easily synthesized if partially protected peptide thioesters were used as building blocks.

In order to examine the efficiency of the thioester method in protein synthesis, we synthesized HU-type DNA-binding protein (HBs) of *Bacillus stearothermophilus* consisting of 90 amino acids, as shown in Fig. 1.

In this paper, we describe the preparation of a partially protected peptide thioester, segment coupling of the peptide and characterization of the synthetic HBs(1—90); we also discuss the efficiency of this process in protein synthesis.

Results and Discussion

The HBs(1—90) synthesized in this study was labelled with deuterium at the methyl group of Met^{69} in the flexible arm region where HBs is supposed to interact with DNA.[8] This deuterium-labelled methyl group will be used as a marker of synthetic HBs(1—90) as well as a probe of the HBs–DNA interaction.

Preparation of Peptide Segments. HBs was divided into four segments. The three arrows in Fig. 1 indicate the locations of segment coupling. The carboxyl terminals of the peptide thioesters were designed to be glycine in order to avoid the potential danger of racemization of the carboxyl-terminal amino acid residue during segment coupling.

A partially protected peptide thioester was prepared by the same procedure as described in a previous paper.[1] The terminal amino groups of peptides **2**, **3**, and **4** were blocked with an isonicotinoyloxycarbonyl (*i*Noc) group instead of the 2,2,2-trichloroethoxycarbonyl (Troc) group to estimate the chemical adaptability of the *i*Noc group to our method. A typical example of the preparation of a partially protected peptide thioester is shown in

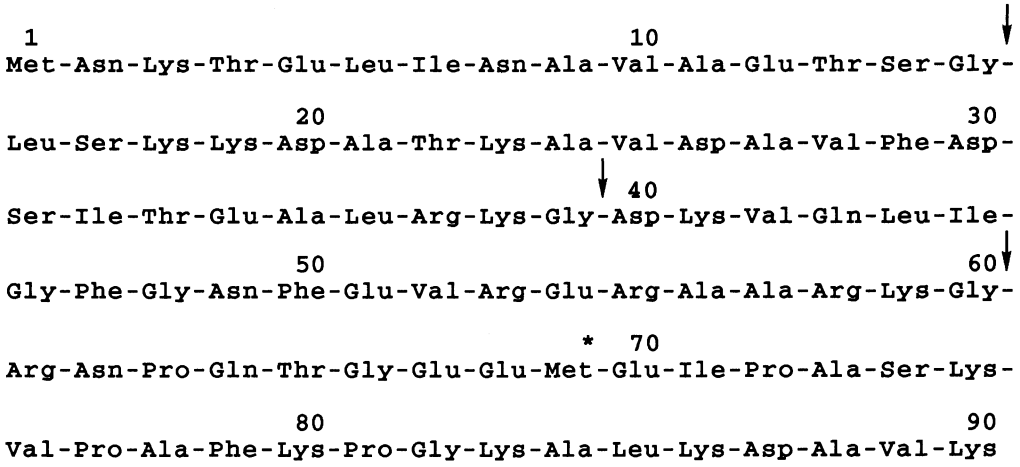

Fig. 1. Amino acid sequence of HBs. The arrows indicate the sites of segment coupling. Met* indicates methionine-*methyl*-d_3.

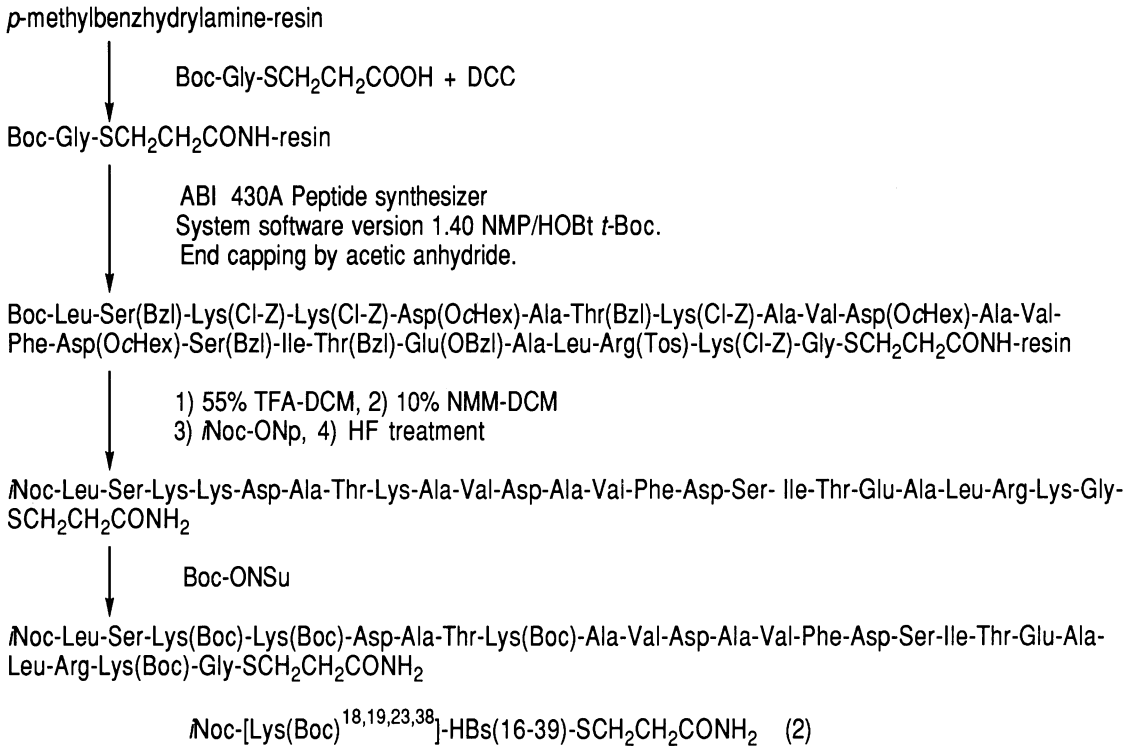

Fig. 2. Synthetic scheme of *i*Noc–[Lys(Boc)^{18,19,23,38}]–HBs(16—39)–$SCH_2CH_2CONH_2$.

Fig. 2.

Starting from Boc–Gly–SCH_2CH_2CONH-resin (0.968 g, 479 μmol of Gly), which was prepared from Boc–Gly–SCH_2CH_2COOH and *p*-methylbenzhydrylamine resin (MBHA-resin), the peptide chain was elongated using the double-coupling protocol of the benzotriazole-active ester method of system software version 1.40 NMP/HOBt *t*-Boc without any modifications. The end was capped by acetic anhydride after each amino acid introduction reaction. After completion of the chain assembly, an *i*Noc group was introduced to block the terminal amino group. The weight of the protected peptide resin was 1.52 g, the whole of which was treated by anhydrous hydrogen fluoride (HF) containing 10% *p*-cresol (vol/vol) at 0 °C for 90 min. The crude peptide (506 mg) was highly soluble in aqueous acetonitrile and easily purified by reversed-phase HPLC (RPHPLC) to give *i*Noc–HBs(16—39)–$SCH_2CH_2CONH_2$ (217 mg) at a yield of 11%, based on the glycine residue in the starting resin. This peptide was characterized by fast atom bombardment (FAB) mass spectrometry and amino acid analysis after acid hydrolysis. To the side-chain amino groups of the peptide, *t*-butoxycarbonyl (Boc) groups were introduced using *N*-(*t*-butoxycarbonyloxy)succinimide (Boc–ONSu) in dimethyl sulfoxide (DMSO) to yield *i*Noc–[Lys(Boc)^{18,19,23,38}]–HBs(16—39)–$SCH_2CH_2CONH_2$ (**2**) in 91%. The introduction reaction was easily monitored by RPHPLC and FAB mass measurements.

The other partially protected peptide segments were obtained in the same manner as listed in Fig. 3. Each partially protected peptide segment was obtained from a 4.5 to a 19% yield based on the carboxyl-terminal amino acid contents in the starting resin.

Segment Condensation. HBs(1—90) was synthesized by three different segment condensation methods in order to identify the most practical procedure. We also synthesized HBs by an all stepwise solid-phase method as a control experiment. The progress of the segment condensation reaction was monitored by RPHPLC after a TFA treatment of the reaction mixture.

Procedure A: HBs(1—90) was synthesized from peptides **1**, **2**, **3**, and **4** according to the scheme shown in Fig. 4. Segment condensation was carried out at a peptide concentration of about 2 to 10 mM[#] in the presence of *N*-hydroxysuccinimide (HONSu, about 100 mM), $AgNO_3$ (about 10 mM or a 1.8 to 3-fold molar excess of thioester groups) at room temperature. The thioester function was converted to the corresponding succinimide ester in situ by adding $AgNO_3$ to the solution. The condensation was completed within 1 to 2 days. In procedure A, the product was isolated by RPHPLC after every coupling reaction. Before isolation, the Boc groups in the side-chain amino groups were removed using trifluoroacetic acid (TFA) so as to avoid any undesirable adsorption of the peptides onto the RPHPLC column. Thus, the product had only one protecting *i*Noc group on the terminal amino group during RPHPLC isolation. Every peptide was soluble in aqueous acetonitrile and

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

		Yield
Boc-Met-Asn-Lys(Boc)-Thr-Glu-Leu-Ile-Asn-Ala-Val-Ala-Glu-Thr-Ser-Gly-$SCH_2CH_2CONH_2$		
Boc-[Lys(Boc)^{3}]-HBs(1-15)-$SCH_2CH_2CONH_2$	(1)	14%
*i*Noc-Leu-Ser-Lys(Boc)-Lys(Boc)-Asp-Ala-Thr-Lys(Boc)-Ala-Val-Asp-Ala-Val-Phe-Asp-Ser-Ile-Thr-Glu-Ala-Leu-Arg-Lys(Boc)-Gly-$SCH_2CH_2CONH_2$		
*i*Noc-[Lys(Boc)^{18,19,23,38}]-HBs(16-39)-$SCH_2CH_2CONH_2$	(2)	10%
*i*Noc-Asp-Lys(Boc)-Val-Gln-Leu-Ile-Gly-Phe-Gly-Asn-Phe-Glu-Val-Arg-Glu-Arg-Ala-Ala-Arg-Lys(Boc)-Gly-$SCH_2CH_2CONH_2$		
*i*Noc-[Lys(Boc)^{41,59}]-HBs(40-60)-$SCH_2CH_2CONH_2$	(3)	17%
Arg-Asn-Pro-Gln-Thr-Gly-Glu-Glu-Met-Glu-Ile-Pro-Ala-Ser-Lys(Boc)-Val-Pro-Ala-Phe-Lys(Boc)-Pro-Gly-Lys(Boc)-Ala-Leu-Lys(Boc)-Asp-Ala-Val-Lys(Boc)		
[Lys(Boc)^{75,80,83,86,90}]-HBs(61-90)	(4)	19%
Boc-Met-Asn-Lys(Boc)-Thr-Glu-Leu-Ile-Asn-Ala-Val-Ala-Glu-Thr-Ser-Gly-Leu-Ser-Lys(Boc)-Lys(Boc)-Asp-Ala-Thr-Lys(Boc)-Ala-Val-Asp-Ala-Val-Phe-Asp-Ser-Ile-Thr-Glu-Ala-Leu-Arg-Lys(Boc)-Gly-$SCH_2CH_2CONH_2$		
Boc-[Lys(Boc)^{3,18,19,23,38}]-HBs(1-39)-$SCH_2CH_2CONH_2$	(5)	4.5%

Fig. 3. Partially protected peptide segments prepared for HBs(1—90) synthesis. The yield was calculated based on the content of the carboxyl terminal amino acid in the resin.

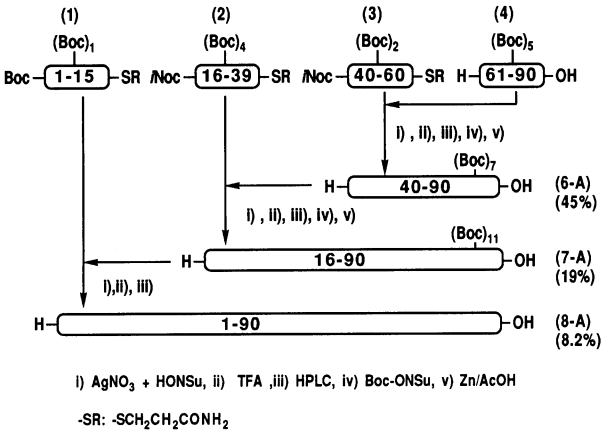

Fig. 4. Synthetic route of HBs(1—90) by segment coupling.

easily isolated by RPHPLC. The elution profile of each reaction mixture is shown in Fig. 5A, B, and C. After purification, the Boc group was reintroduced to the side-chain amino groups using Boc–ONSu (0.2 to 0.5 M) in the presence of triethylamine (TEA). The product was treated with zinc dust in the presence of acetic acid (50 mg ml^{-1}) to remove the *i*Noc group. The amino component peptide, thus obtained, was dialyzed against distilled water. The overall yield of HBs(1—90) after RPHPLC purification was 8.2% based on peptide **4**.

Procedure B: In this synthesis segment coupling was carried out practically under the same conditions as in procedure A. However, since none of the products were isolated after segment condensation, removal and reintroduction of Boc groups were unnecessary in this procedure. A peptide mixture containing the desired product as well as by-products and starting materials was treated with zinc dust to remove the *i*Noc group. This mixture was used for the next segment coupling. HBs(1—90) was easily isolated by RPHPLC after the final condensation reaction, as shown in Fig. 6. Furthermore, the yield of HBs(1—90) was improved from the 8.2% obtained using procedure A, to 16%. HBs(1—90) (**8-B**), thus obtained, was as pure as that from procedure A. This is discussed later.

Procedure C: Peptide **5** with 39 amino acids and peptide **6-A** with 51 amino acids were condensed using a 7-fold molar excess of peptide **5** to peptide **6-A** (4.2 mM) in the presence of $AgNO_3$ (40 mM) and *N*-methylmorpholine (NMM, 72 mM) at room temperature for 3 d. HBs(1—90) was obtained in a yield of 33% based on peptide **6-A** after TFA treatment followed by RPHPLC isolation. Although this yield was not so high, it is noteworthy that a 90 amino acid peptide was prepared by coupling 39 and 51 amino acid peptide segments.

Preparation of HBs(1—90) by All Stepwise Method. HBs(1—90) was synthesized by an all stepwise solid-phase method to compare the efficiency between segment

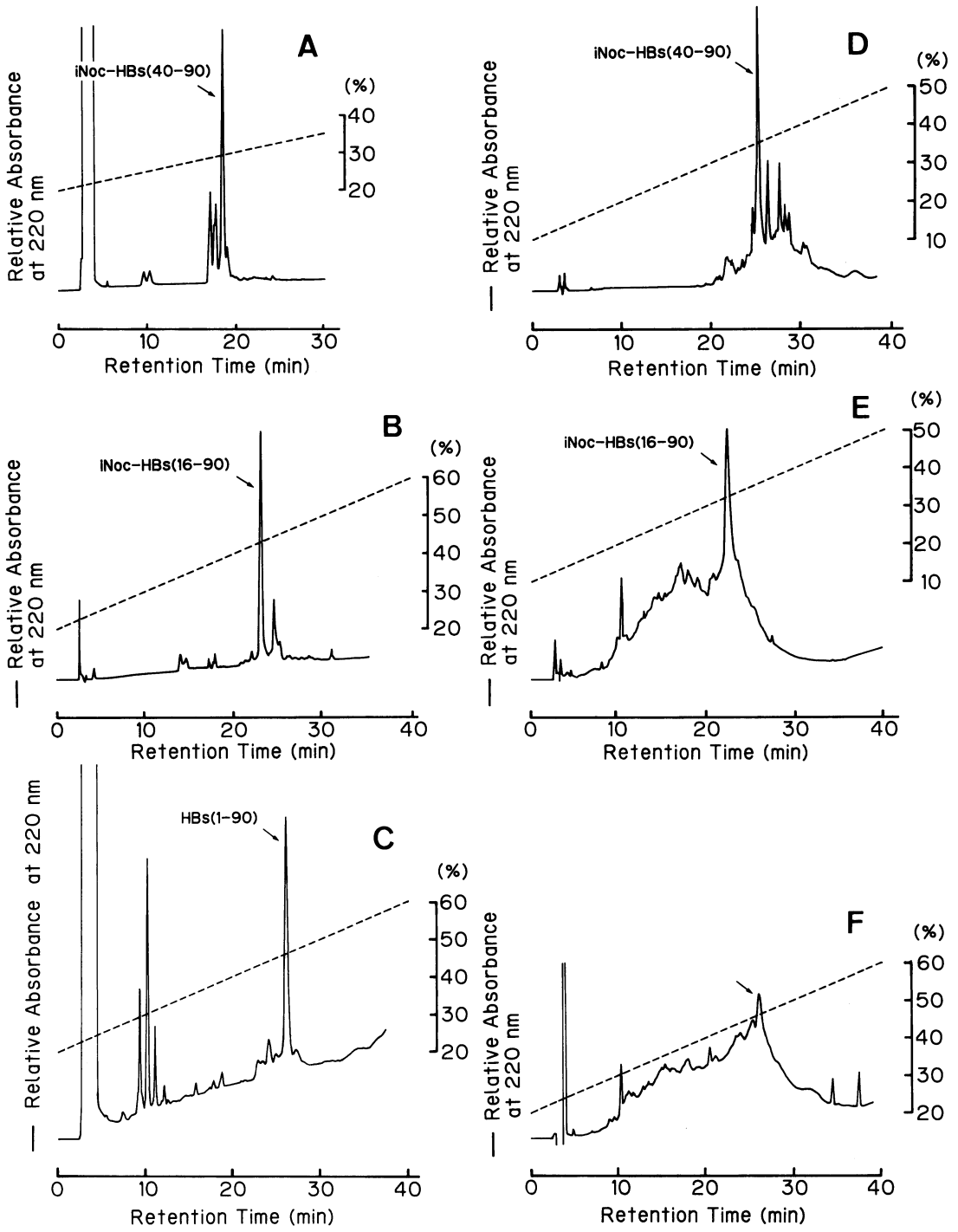

Fig. 5. RPHPLC elution profiles of the TFA treated reaction mixture of segment coupling (Panels A, B, C) and the crude products obtained by an all stepwise solid-phase synthesis after HF treatment (D, E, F); panel A: reaction mixture for the preparation for *i*Noc-HBs(40—90) (**6**), panel B: reaction mixture on the preparation of *i*Noc-HBs(16—90) (**7**), panel C: reaction mixture on the preparation for HBs(1—90) (**8-A**). Column: Cosmosil 5C_{18}-AR (4.6×250 mm) at a flow rate of 1 ml min^{-1} at 40°C.

condensation and stepwise solid-phase methods. The elution profiles of the peptides obtained from the all stepwise method is shown in Fig. 5D, E, and F. Comparing the corresponding products obtained using procedure A and the all stepwise solid-phase method, the advantage of procedure A is obvious regarding the preparation of HBs(16—90) and HBs(1—90). HBs(1—90) obtained by the stepwise method was very difficult to

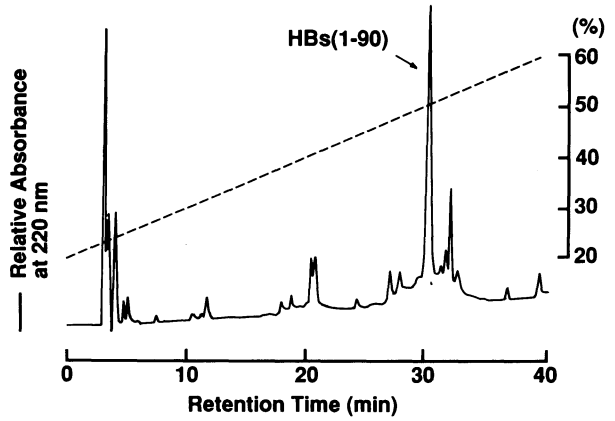

Fig. 6. RPHPLC profile of the crude product of peptide **8-B**.

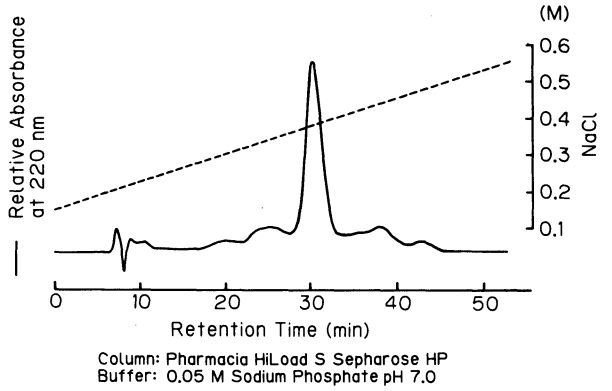

Fig. 7. Ion-exchange chromatogram of HBs(1—90) (**8-A**) isolated by RPHPLC on Pharmacia HiLoad S-Sepharose HP. The broken line indicates the NaCl concentration in a 0.05 M sodium phosphate buffer (pH 7.0).

purify.

Purification of Synthetic HBs(1—90) by Ion-Exchange Chromatography as the Native Form. The HBs(1—90) isolated from a reaction mixture by RPHPLC was unfolded, judging from the chemical shift of the ^{1}H NMR spectrum of the aromatic protons of phenylalanine residues. However, synthetic HBs(1—90) spontaneously assumed the native structure, judging from the ^{1}H NMR spectrum, when unfolded synthetic HBs(1—90) was dissolved in a 50 mM sodium phosphate buffer (pH 7.0). RPHPLC-purified HBs(1—90), (peptide **8-A** or **8-B**) was then converted to the native form by dissolving the peptide in this buffer; it was further purified by ion-exchange chromatography, as shown in Fig. 7. The synthetic HBs(1—90), thus obtained, showed the same ^{1}H NMR spectra as that of HBs extracted from *B. stearothermophilus*, except for Met^{69}-*methyl-d*$_3$, as shown in Fig. 8. The difference in the ^{1}H NMR spectra between native HBs and synthetic HBs(1—90) is shown in the small box in Fig. 8. Comparing both ^{1}H NMR spectra, the chemical and three-dimensional structure of synthetic HBs(1—90) should be quite similar to that of the native protein. Consequently, synthetic HBs(1—90) can be used for an ^{1}H NMR spectroscopic study of the interaction between HBs(1—90) and DNA. Peptides **8-A** and **8-B** had practically the same elution profile and gave the same quality of synthetic HBs(1—90) at yields of 50 and 45% based on peptides **8-A** and **8-B**, respectively. Peptide **8-C** also gave the same elution profile as those of peptides **8-A** and **8-B**. HBs(1—90) was obtained in a yield of 50% based on peptide **8-C**.

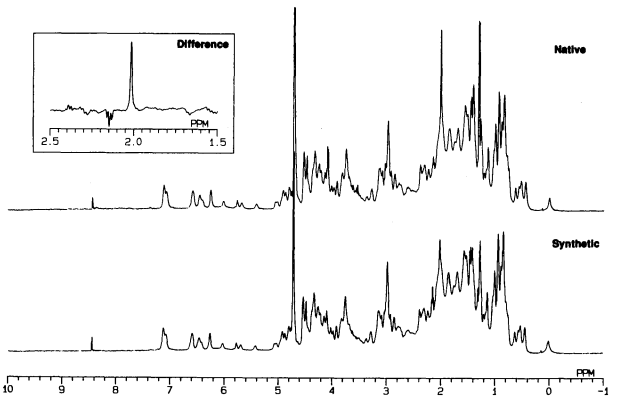

Fig. 8. 500 MHz ^{1}H NMR spectra of native and synthetic HBs.

Evaluation of the Method for Protein Synthesis. All of the partially protected peptide thioesters (peptides **1**, **2**, **3**, and **5**) were similarly prepared according to the previously described method.[1] The *i*Noc group was used instead of the Troc group to protect the terminal amino group. Removal of the *i*Noc group with zinc was not accompanied by any serious side reactions like that observed during the removal of the Troc group, but tended to take longer than that of the Troc group. More consideration is required concerning the selection of the terminal amino protecting group. A thioester group was efficiently converted to an active ester, and segment condensation proceeded without any serious side reactions. Procedure B gave a good overall yield of HBs(1—90) compared with procedure A. This means that purification of intermediate peptides is not essential if a large peptide segment is used for coupling and the segment condensation is not accompanied by any side reactions. In procedures A and B, HBs(1—90) was eluted as a separated peak on RPHPLC, suggesting that a polypeptide, with neither standard material nor a specific feature such as enzymatic activity, can be synthesized using this method. On the contrary, the product obtained by the all stepwise solid-phase method gave a broad peak (Fig. 5-F). It was difficult to distinguish the fraction that contained HBs(1—90). A partially protected peptide thioester is a useful building block for protein synthesis. This strategy has not yet been applied to the preparation of cysteine-containing proteins, but

will be expanded to cover the synthesis of cysteine-containing proteins in the near future.

Materials and Methods

Boc-amino acid derivatives and *p*-methylbenzhydrylamine (MBHA) resin were purchased from the Peptide Institute Inc. (Osaka). The solvents and reagents used for solid-phase peptide synthesis were purchased from Watanabe Chemical Ind., Ltd. (Hiroshima). The dimethyl sulfoxide used for segment coupling was silylation grade (Pierce, Rockford, IL). Analytical RPHPLC was performed on YMC A-803 S-5 120 Å C4 (YMC, Kyoto) or Cosmosil 5C_{18}-AR (4.6×250 mm) (Nacalai Tesque, Kyoto) and preparative RPHPLC was on YMC-Gel ODS S-5 AM-type (10×250 mm or 20×250 mm) (YMC, Kyoto). Amino acids were analyzed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo) after hydrolysis by 4 M methanesulfonic acid at 110°C for 48 h in an evacuated sealed tube. The peptide mass number was determined by fast atom bombardment mass spectrometry using a JMS-HX100 (JEOL Ltd.,Tokyo) equipped with a JMA-3100 mass data system. Though the peptide weight was an observed value, the yield was calculated based upon the amino acid analysis data. Sonication was carried out using a Branson Model B-220.

Synthesis

Peptide Chain Elongation on a Solid Support. Solid-phase synthesis of a peptide segment was carried out on a peptide synthesizer (430A; Applied Biosystems Inc., Foster City, CA.) using the 0.5 mmol-scale double-coupling protocol of the benzotriazole active ester method of the system software version 1.40 NMP/HOBt *t*-Boc. The ends were capped by acetic anhydride after each amino acid introduction reaction. The side-chain protecting groups of Boc-amino acids used were *o*-chlorobenzyloxycarbonyl (Cl-Z) for the N^{ε} of Lys, benzyl (Bzl) for the alcoholic OH of Thr and Ser, cyclohexyl ester (O*c*Hex) for the β-carboxyl group of Asp, benzyl ester (OBzl) for the γ-carboxyl group of Glu and tosyl (Tos) for the N^{g} of Arg. Boc–Gly–SCH_2CH_2CONH-resin was prepared according to a previously described method.[1)]

Boc-[Lys(Boc)^{3}]-HBs(1—15)-$SCH_2CH_2CONH_2$ (1). Boc–Met–Asn–Lys(Cl-Z)–Thr(Bzl)–Glu(OBzl)–Leu–Ile–Asn–Ala–Val–Ala–Glu(OBzl)–Thr(Bzl)–Ser(Bzl)–Gly–SCH_2CH_2-CONH-resin was prepared from Boc–Gly–SCH_2CH_2CONH-resin (1.22 g, 312 μmol). The *N*-terminal Boc group was removed by 55% TFA in dichloromethane (DCM) for 5 and 15 min. A protected peptide resin (1.55 g) was obtained. An aliquot of the resin (1.51 g) was treated with anhydrous HF (20 ml) containing *p*-cresol (2 ml) at 0°C for 90 min. After complete evaporation of HF under a high vacuum, a residual solid was washed with ether (×2) and ethyl acetate (×2). A peptide was extracted with 40% aqueous acetonitrile containing 10% acetic acid. The extract was freeze-dried to give 390 mg of a crude product. This was purified on RPHPLC to give 130 mg (46.4 μmol, 15%) of HBs(1—15)-$SCH_2CH_2CONH_2$. Found: m/z 1664.5 $(M+H)^+$. Calcd: m/z 1664.8 $(M+H)^+$. Amino acid composition: $Asp_{2.04}Thr_{1.90}Ser_{0.88}Glu_{2.11}Gly_{1.03}Ala_2Val_{0.97}Met_{0.98}Ile_{1.00}Leu_{0.99}Lys_{0.98}$.

HBs(1—15)-$SCH_2CH_2CONH_2$ (99.0 mg, 35.4 μmol) dissolved in DMSO (1.0 ml) was reacted with Boc-ONSu (49.0 mg, 228 μmol) in the presence of TEA (32 μl, 230 μmol). The resulting solution was stirred for 4 h. A mixed solvent of ether and ethyl acetate was added to the reaction mixture to precipitate the product which was collected by centrifugation and freeze-dried from a dioxane suspension to give 103 mg of peptide **1** (33.1 μmol, 94%). Found: m/z 1864.6 $(M+H)^+$. Calcd: m/z 1864.9 $(M+H)^+$. Amino acid analysis of peptide **1**: $Asp_{2.07}Thr_{1.93}Ser_{0.92}Glu_{2.31}Gly_{1.06}Ala_2Val_{1.00}Met_{1.02}Ile_{1.02}Leu_{1.01}Lys_{1.02}$.

***i*Noc-[Lys(Boc)^{18,19,23,38}]-HBs(16—39)-$SCH_2CH_2CONH_2$ (2).** Boc–Leu–Ser(Bzl)–Lys(Cl-Z)–Lys(Cl-Z)–Asp(O*c*Hex)–Ala–Thr(Bzl)–Lys(Cl-Z)–Ala–Val–Asp(O*c*Hex)–Ala–Val–Phe–Asp(O*c*Hex)–Ser(Bzl)–Ile–Thr(Bzl)–Glu(OBzl)–Ala–Leu–Arg(Tos)–Lys(Cl-Z)–Gly–SCH_2CH_2CONH-resin was prepared from Boc–Gly–SCH_2CH_2CONH-resin (0.968 g, 479 μmol). After removing the *N*-treminal Boc group by treatment with 55% TFA in DCM (vol/vol) for 5 and 15 min followed by neutralization by 5% *N*,*N*-diisopropylethylamine (DIEA) in *N*,*N*-dimethlyformamide (DMF) (vol/vol), the peptide resin was treated with *i*Noc–ONp (411 mg, 1.50 mmol) in DMF overnight to give 1.52 g of protected peptide resin. All of the resin was treated with HF (15 ml) containing 10% *p*-cresol (vol/vol) at 0°C for 90 min to give 506 mg of a crude product. This product was purified on RPHPLC to obtain 217 mg (53.0 μmol, 11%) of *i*Noc–HBs(16—39)-$SCH_2CH_2CONH_2$. Found: m/z 2785.2 $(M+H)^+$. Calcd: m/z 2785.5 $(M+H)^+$. Amino acid composition: $Asp_{2.98}Thr_{1.81}Ser_{1.63}Glu_{1.03}Gly_{1.00}Ala_4Val_{1.70}Ile_{0.91}Leu_{1.92}Phe_{0.80}Lys_{3.79}Arg_{0.94}$.

To the solution of *i*Noc–HBs(16—39)-$SCH_2CH_2CONH_2$ (217 mg, 53.0 μmol) dissolved in DMSO (2.3 ml), Boc-ONSu (134 mg, 623 μmol) and TEA (87 μl, 620 μmol) were added and the resulting solution stirred for 5 h. A mixed solvent of ether and ethyl acetate was added to the reaction mixture. The formed precipitate was collected by centrifugation and freeze-dried from a dioxane suspension to give 201 mg of peptide **2** (48.0 μmol, 91%). Found: m/z 3186.3 $(M+H)^+$. Calcd: m/z 3185.7 $(M+H)^+$. Amino acid analysis of peptide **2**: $Asp_{3.04}Thr_{1.84}Ser_{1.81}Glu_{1.19}Gly_{1.11}Ala_4Val_{1.93}Ile_{0.97}Leu_{1.81}Phe_{1.03}Lys_{3.83}Arg_{1.03}$.

***i*Noc-[Lys(Boc)^{41,59}]-HBs(40—60)-$SCH_2CH_2CONH_2$ (3).** *i*Noc–Asp(O*c*Hex)–Lys(Cl-Z)–Val–Gln–Leu–Ile–Gly–Phe–Gly–Asn–Phe–Glu(OBzl)–Val–Arg(Tos)–Glu(OBzl)–Arg(Tos)–Ala–Ala–Arg(Tos)–Lys(Cl-Z)–Gly–SCH_2CH_2CONH-resin (2.18 g) was prepared from Boc–Gly–SCH_2CH_2CONH-resin (1.30 g, 299 μmol) as described for the synthesis of peptide **2**. An aliquot of the resin (1.98 g) was treated with HF (20 ml) containing 15% anisole (vol/vol) at 0°C for 90 min to give 623 mg of a crude product. This was purified on RPHPLC to obtain 204 mg (55.8 μmol, 21%) of *i*Noc–HBs(40—60)-$SCH_2CH_2CONH_2$. Found: m/z 2612.3 $(M+H)^+$. Calcd: m/z 2612.4 $(M+H)^+$. Amino acid composition: $Asp_{2.07}Glu_{2.94}Gly_{2.97}Ala_2Val_{2.09}Ile_{0.99}Leu_{1.05}Phe_{2.05}Lys_{2.11}Arg_{3.00}$.

Boc-ONSu (67.0 mg, 311 μmol) and TEA (42 μl, 300 μmol) were added to a solution of *i*Noc–HBs(40—60)-$SCH_2CH_2CONH_2$ (204 mg, 55.8 μmol) in DMSO (2.0 ml); the solution was stirred for 5 h at room temperature. Peptide **3** was obtained at a yield of 82% (233 mg, 45.7 μmol) according to the same procedure as described in peptide **1**. Found: m/z 2813.1 $(M+H)^+$. Calcd: m/z 2812.5 $(M+H)^+$. Amino acid analysis of peptide **3**: $Asp_{2.01}Glu_{2.87}Gly_{2.90}Ala_2Val_{2.04}Ile_{0.94}Leu_{0.99}Phe_{2.04}Lys_{1.97}Arg_{2.87}$.

[Lys(Boc)^{75,80,83,86,90}, Met^{69}-*methyl-d*$_3$]-HBs(61—90) (4). *i*Noc–Arg(Tos)–Asn–Pro–Gln–Thr(Bzl)–Gly–Glu(OBzl)–Glu(OBzl)–Met–Glu(OBzl)–Ile–Pro–Ala–Ser(Bzl)–Lys(Cl-Z)–Val–Pro–Ala–Phe–Lys(Cl-Z)–Pro–Gly–Lys(Cl-Z)–Ala–Leu–

Lys(Cl-Z)-Asp(O*c*Hex)-Ala-Val-Lys(Cl-Z)-OCH_2-Pam-resin was prepared from Boc-Lys(Cl-Z)-OCH_2-Pam-resin (0.78 g, 500 μmol) using a 430A synthesizer. The Met^{69} residue was introduced manually using Boc-Met-*methyl-*d_3 (379 mg, 1.50 mmol) in DMF and a 0.5 M solution of dicyclohexylcarbodiimide (DCC) in DCM (3.0 ml, 1.5 mmol). The *i*Noc group was incorporated as described regarding the synthesis of peptide **2**. An aliquot of the obtained resin (0.984 g out of 2.70 g) was treated under low-HF conditions (HF–dimethyl sulfide–*p*-cresol, 25 : 65 : 10 (vol/vol), 10 ml) at 0°C for 2 h, followed by high-HF conditions (HF–*p*-cresol, 9 : 1 (vol/vol), 10 ml) at 0°C for 1 h;[9] a crude peptide (837 mg) was obtained by the procedure described regarding peptide **1**. The crude product (837 mg) was applied on a Pharmacia HiLoad S-Sepharose HP (16×100 mm), which was equilibrated with a 0.05 M sodium phosphate buffer (pH 6.0) and eluted with a NaCl concentration gradient in a buffer from 0 to 35 mM, over 27 min at a flow rate of 2.5 ml min^{-1}. The purified peptide was desalted by RPHPLC to give *i*Noc-HBs(61—90)-[Met^{69}-*methyl-*d_3] (314 mg, 47.5 μmol, 26%). Found: *m/z* 3375.3 $(M+H)^+$. Calcd: *m/z* 3375.0 $(M+H)^+$. Amino acid composition: $Asp_{2.01}Thr_{0.97}Ser_{0.90}Glu_{4.10}Pro_{4.24}Gly_{2.00}Ala_4Val_{1.99}Met_{1.15}Ile_{1.00}Leu_{1.01}Phe_{1.03}Lys_{4.93}Arg_{0.85}$.

The *i*Noc-[Met^{69}-*methyl-*d_3]-HBs(61—90) (285 mg, 43.2 μmol) and Boc-ONSu (139 mg, 646 μmol) were dissolved in DMSO (1.5 ml). The solution was stirred for 4 h after adding TEA (65 μl, 470 μmol) to give *i*Noc-[Lys(Boc)^{75,80,83,86,90}, Met^{69}-*methyl-*d_3]-HBs(61—90) (296 mg, 37.4 μmol, 87%) as described in the preparation of peptide **1**. Amino acid composition: $Asp_{2.05}Thr_{0.99}Ser_{0.93}Glu_{4.23}Pro_{4.21}Gly_{2.06}Ala_4Val_{2.07}Met_{0.89}Ile_{1.05}Leu_{1.06}Phe_{1.04}Lys_{4.94}Arg_{0.85}$.

The *i*Noc-[Lys(Boc)^{75,80,83,86,90}, Met^{69}-*methyl-*d_3]-HBs(61—90) (296 mg, 37.4 μmol) was sonicated with zinc dust (450 mg) in 50% aqueous acetic acid (9.0 ml) under a nitrogen atmosphere for 3.5 h. Zinc dust was removed by centrifugation. The solution was packed in a Spectrapor 6 membrane (M.W. cut off 1000), dialyzed against distilled water (11×3) and freeze-dried to give powdered peptide **4** (192 mg, 31.4 μmol, 84%). Found: *m/z* 3739.4 $(M+H)^+$. Calcd: *m/z* 3740.0 $(M+H)^+$. Amino acid analysis of peptide **4**: $Asp_{1.99}Thr_{0.94}Ser_{0.88}Glu_{3.97}Pro_{4.11}Gly_{1.97}Ala_4Val_{2.14}Met_{0.85}Ile_{0.95}Leu_{1.01}Phe_{1.10}Lys_{5.14}Arg_{0.98}$.

Boc-[Lys(Boc)^{3,18,19,23,38}]-HBs(1—39)-$SCH_2CH_2CONH_2$ (5). Starting from Boc-Gly-SCH_2CH_2CONH-resin (1.00 g, 340 μmol), Boc-Met-Asn-Lys(Cl-Z)-Thr(Bzl)-Glu(OBzl)-Leu-Ile-Asn-Ala-Val-Ala-Glu(OBzl)-Thr(Bzl)-Ser(Bzl)-Gly-Leu-Ser(Bzl)-Lys(Cl-Z)-Lys(Cl-Z)-Asp(O*c*Hex)-Ala-Thr(Bzl)-Lys(Cl-Z)-Ala-Val-Asp(O*c*Hex)-Ala-Val-Phe-Asp(O*c*Hex)-Ser(Bzl)-Ile-Thr(Bzl)-Glu(OBzl)-Ala-Leu-Arg(Tos)-Lys(Cl-Z)-Gly-SCH_2CH_2CONH-resin (2.94 g) was obtained using the same amino acid derivatives. An aliquot of the resin (774 mg) was treated with HF (10 ml) containing 10% anisole (vol/vol) at 0°C for 90 min. The crude peptide obtained was purified on RPHPLC to give 30.0 mg (5.70 μmol, 6.4%) of HBs(1—39)-$SCH_2CH_2CONH_2$. Amino acid composition: $Asp_{5.12}Thr_{3.95}Ser_{2.82}Glu_{3.19}Gly_{2.02}Ala_6Val_{2.84}Met_{0.95}Ile_{1.95}Leu_{3.02}Phe_{1.07}Lys_{4.73}Arg_{1.02}$.

HBs(1—39)-$SCH_2CH_2CONH_2$ (17.0 mg, 3.23 μmol) and Boc-ONSu (10.5 mg, 48.8 μmol) were dissolved in DMSO (100 μl). The solution was stirred for 6 h at room temperature after adding TEA (6.6 μl, 47 μmol) to give peptide **5** (19.0 mg, 2.30 μmol, 71%) after the same posttreatment as described in the preparation of peptide **1**. Amino acid analysis of peptide **5**: $Asp_{5.05}Thr_{3.76}Ser_{2.86}Glu_{3.17}Gly_{2.02}Ala_6Val_{2.77}Met_{0.80}Ile_{1.94}Leu_{2.98}Phe_{1.05}Lys_{4.61}Arg_{1.03}$.

Synthesis of [Met^{69}-*methyl-*d_3]-HBs(1—90) by Procedure A. [Lys(Boc)^{41,59,75,80,83,86,90}, Met^{69}-*methyl-*d_3]-HBs(40—90) (6-A): Peptides **3** (46.5 mg, 10.5 μmol) and **4** (44.1 mg, 10.1 μmol) were dissolved in DMSO (1.0 ml) containing HONSu (11.9 mg, 103 μmol) and NMM (1.1 μl, 10 μmol). The solution was stirred at room temperature for 3 h in the dark after adding $AgNO_3$ (2.10 mg, 12.4 μmol). $AgNO_3$ (1.10 mg, 6.47 μmol) and NMM (0.5 μl, 4.6 μmol) were further added to the solution and stirred again overnight. Ethyl acetate was then added. The precipitated peptide was collected by centrifugation, washed with ethyl acetate (×2) and dried in vacuo. The obtained powder was treated with TFA (1.0 ml) containing 1,4-butanedithiol (100 μl) at room temperature for 10 min. TFA was removed by flashing with nitrogen gas. The residual oil was triturated with ether and dried in vacuo. This product was purified on RPHPLC to give *i*Noc-[Met^{69}-*methyl-*d_3]-HBs(40—90) (Peptide **6**, 49.0 mg, 5.15 μmol, 51%). Amino acid analysis of peptide **6**: $Asp_{4.13}Thr_{1.01}Ser_{1.00}Glu_{7.10}Pro_{4.24}Gly_{5.12}Ala_6Val_{4.17}Met_{0.78}Ile_{1.95}Leu_{2.01}Phe_{2.98}Lys_{7.17}Arg_{4.04}$.

Peptide **6** (25.0 mg, 2.63 μmol) and Boc-ONSu (13.4 mg, 62.3 μmol) were dissolved in DMSO (300 μl) containing TEA (6.2 μl, 45 μmol). The solution was stirred for 8 h at room temperature. *i*Noc-[Lys(Boc)^{41,59,75,80,83,86,90}, Met^{69}-*methyl-*d_3]-HBs(40—90) (24.0 mg, 2.33 μmol) was obtained according to the procedure described in the preparation of peptide **1**. Amino acid composition: $Asp_{4.11}Thr_{0.97}Ser_{0.91}Glu_{7.27}Pro_{4.28}Gly_{5.12}Ala_6Val_{4.10}Met_{0.90}Ile_{1.99}Leu_{2.04}Phe_{3.21}Lys_{7.19}Arg_{4.10}$.

This peptide (24.0 mg, 2.33 μmol) was dissolved in 75% acetic acid (400 μl) and zinc dust (20.0 mg) was added under nitrogen; the solution was sonicated for 2 h at room temperature. The workup of the reaction mixture followed the same procedure described regarding the preparation of peptide **4**, resulting in peptide **6-A** (18.4 mg, 2.30 μmol, 45% yield based on peptide **4**). Amino acid analysis of peptide **6-A**: $Asp_{4.29}Thr_{1.13}Ser_{1.05}Glu_{7.11}Pro_{3.92}Gly_{5.04}Ala_6Val_{3.38}Met_{0.64}Ile_{1.96}Leu_{2.01}Phe_{3.00}Lys_{6.45}Arg_{3.74}$.

[Lys(Boc)^{18,19,23,38,41,59,75,80,83,86,90}, Met^{69}-*methyl-*d_3]-HBs(16—90) (7-A): Peptide **2** (19.8 mg, 4.00 μmol) and peptide **6-A** (18.4 mg, 2.30 μmol) were dissolved in DMSO (300 μl) containing HONSu (6.90 mg, 60.0 μmol) and NMM (0.66 μl, 6.0 μmol). $AgNO_3$ (1.20 mg, 7.06 μmol) was added to the solution, which was stirred for 5 h in the dark. More $AgNO_3$ (300 μg, 1.77 μmol) was added, and the reaction mixture was stirred again overnight. The crude product (38.9 mg) obtained by the same procedure described regarding peptide **6-A** was treated with TFA (400 μl) containing 10% 1,4-butanedithiol (vol/vol) for 10 min and purified on RPHPLC followed by elution through TSKgel G3000SW (7.5×600 mm) 50% aq acetonitrile containing 0.1% TFA, at a flow rate of 0.3 ml min^{-1}. Thus, 19.3 mg of *i*Noc-[Met^{69}-*methyl-*d_3]-HBs(16—90) (Peptide **7**) were obtained (1.34 μmol, 58%). Amino acid analysis of peptide **7**: $Asp_{7.21}Thr_{2.91}Ser_{2.81}Glu_{8.33}Pro_{3.89}Gly_{6.04}Ala_{10}Val_{5.66}Met_{0.92}Ile_{2.91}Leu_{3.87}Phe_{3.85}Lys_{10.35}Arg_{4.74}$.

Peptide **7** (18.3 mg, 1.27 μmol) was dissolved in DMSO (100 μl) containing Boc-ONSu (10.4 mg, 48.3 μmol) and TEA (6.7 μl, 48 μmol). The solution was stirred for 10 h at room temperature. *i*Noc-[Lys(Boc)^{18,19,23,38,41,59,75,80,83,86,90}, Met^{69}-*methyl-*d_3]-HBs(16—90) (17.3 mg, 1.08 μmol) was obtained as described in the preparation of peptide **6-A**. Amino acid composition: $Asp_{7.29}Thr_{2.98}Ser_{2.73}Glu_{8.45}Pro_{4.42}Gly_{6.12}Ala_{10}Val_{6.48}Met_{0.98}Ile_{3.11}Leu_{4.16}Phe_{4.25}Lys_{10.99}Arg_{5.19}$.

This peptide (17.3 mg, 1.08 μmol) was dissolved in 85% acetic acid (1.4 ml) and sonicated in the presence of zinc (70.0 mg) under nitrogen at room temperature for 3 h. Peptide **7-A** (14.8 mg, 920 nmol, 42% yield based on peptide **6-A**) was obtained according to a procedure similar to that described for the preparation of peptide **4**. Amino acid analysis of peptide **7-A**: $Asp_{7.58}Thr_{2.81}Ser_{2.74}Glu_{8.55}Pro_{4.08}Gly_{6.13}Ala_{10}Val_{5.37}Met_{1.26}Ile_{2.81}Leu_{3.86}Phe_{4.10}Lys_{10.54}Arg_{4.87}$.

[Met^{69}-*methyl-d*$_3$]-HBs(1—90) (8-A): Peptides **1** (7.90 mg, 1.80 μmol) and **7-A** (14.8 mg, 920 nmol) were dissolved in DMSO (500 μl) containing HONSu (4.90 mg, 42.6 μmol) and NMM (0.56 μl, 5.1 μmol). The solution was stirred overnight in the dark after adding $AgNO_3$ (860 μg, 5.06 μmol). Peptide **1** (3.00 mg, 680 nmol), HONSu (1.90 mg, 16.5 μmol), NMM (0.21 μl, 1.9 μmol) and more $AgNO_3$ (330 μg, 1.94 μmol) were added and the solution was stirred for 24 h. The workup of the reaction mixture was performed as described for peptide **7-A**. The peptide obtained was treated with TFA (500 μl, vol/vol) containing 10% 1,4-butanedithiol for 15 min at 0°C. TFA was removed by a stream of nitrogen gas and ether was added to the residual oil. The precipitate that formed was dried in vacuo and purified on RPHPLC to give peptide **8-A** (7.30 mg, 399 nmol, 43% based on peptide **7-A**). Amino acid analysis of peptide **8-A**: $Asp_{9.31}Thr_{4.92}Ser_{3.64}Glu_{10.43}Pro_{3.65}Gly_{6.97}Ala_{12}Val_{6.87}Met_{2.13}Ile_{3.97}Leu_{4.92}Phe_{4.07}Lys_{11.82}Arg_{4.95}$.

Synthesis of [Met^{69}-*methyl-d*$_3$]-HBs(1—90) (8-B) by Procedure B. [Lys(Boc)^{41,59,75,80,83,86,90}, Met^{69}-*methyl-d*$_3$]-HBs(40—90) (6′): Peptides **3** (149 mg, 33.7 μmol), **4** (141 mg, 32.2 μmol) and HONSu (38.2 mg, 332 μmol) were dissolved in DMSO (3.2 ml) containing NMM (3.6 μl, 33 μmol) and stirred for 3 h at room temperature in the dark after adding $AgNO_3$ (6.80 mg, 40.0 μmol). The solution was stirred overnight after adding more $AgNO_3$ (3.40 mg, 20.0 μmol) dissolved in DMSO (34 μl) and NMM (1.8 μl, 16 μmol). A mixture of ethyl acetate and ether was added to the solution to obtain a precipitate, which was washed with the same mixed solvent. The precipitate was freeze-dried from a dioxane suspension to give a powder (330 mg). This peptide was sonicated with zinc dust (140 mg) in 75% acetic acid (2.6 ml) under nitrogen for 3 h at room temperature. Peptide mixture **6′** (226 mg) containing peptide **6-B** was obtained after dialysis followed by freeze-drying, as described regarding the preparation of peptide **4**.

[Lys(Boc)^{18,19,23,38,41,59,75,80,83,86,90}, Met^{69}-*methyl-d*$_3$]-HBs(16—90) Crude (7′): Peptides **2** (173 mg, 36.4 μmol) and **6′** (226 mg), HONSu (42.0 mg, 365 μmol), NMM (4.8 μl, 45 μmol) were dissolved in DMSO (3.7 ml). $AgNO_3$ (7.50 mg, 44.1 μmol) was added then the solution was stirred overnight in the dark. $AgNO_3$ (1.36 mg, 8.03 μmol), NMM (0.9 μl, 8.2 μmol) and DMSO (2.0 ml) were added and the mixture was stirred again overnight. The peptide precipitated by adding water was first collected by centrifugation, then lyophilized to give a powder (481 mg). This peptide was sonicated with zinc dust (450 mg) in 80% acetic acid (9.0 ml) for 8 h at room temperature. Peptide mixture **7′** (335 mg) containing peptide **7-B** was obtained as described in the preparation of peptide **4**.

[Met^{69}-*methyl-d*$_3$]-HBs(1—90) (8-B): Peptides **1** (102 mg, 33.1 μmol) and **7′** (300 mg), HONSu (63.0 mg, 547 μmol) and NMM (4.6 μl, 42 μmol) were dissolved in DMSO (7.0 ml). After the addition of $AgNO_3$ (7.10 mg, 41.8 μmol), the solution was stirred for 4.5 h in the dark at room temperature. $AgNO_3$ (2.00 mg, 11.8 μmol) and NMM (1.0 μl, 9.1 μmol) were then added. After the mixture was first stirred overnight, and then for an additional 24 h after adding peptide **1** (60.0 mg, 19.5 μmol), HONSu (37.0 mg, 322 μmol), NMM (2.6 μl, 24 μmol) and $AgNO_3$ (4.20 mg, 24.7 μmol). The reaction mixture was posttreated as described regarding the preparation of peptide **8-A**. The crude powder (471 mg) was treated with TFA (4.5 ml) containing 10% 1,4-butanedithiol (vol/vol) at 0°C for 15 min. The thus-obtained material was purified on RPHPLC to yield powdered peptide **8-B** (92.3 mg, 4.71 μmol, 16% yield based on peptide **4**) after freeze-drying. Amino acid analysis of peptide **8-B**: $Asp_{9.32}Thr_{4.97}Ser_{3.76}Glu_{10.39}Pro_{3.65}Gly_{6.99}Ala_{12}Val_{6.46}Met_{2.10}Ile_{3.92}Leu_{4.98}Phe_{4.12}Lys_{11.72}Arg_{4.75}$.

Synthesis of HBs(1—90) by Procedure C. Peptides **5** (3.70 mg, 680 nmol) and **6-A** (4.92 mg, 420 nmol) were dissolved in DMSO (100 μl) containing HONSu (900 μg, 7.82 μmol) and NMM (0.17 μl, 1.6 μmol). After adding $AgNO_3$ (146 μg, 860 nmol), the solution was stirred overnight; then, peptide **5** (13.0 mg, 2.39 μmol), $AgNO_3$ (513 μg, 3.02 μmol) and NMM (0.61 μl, 5.6 μmol) were added. The solution was stirred for 48 h. The peptide was precipitated with ethyl acetate and washed with the same solvent twice to give a powder, which was treated with TFA (100 μl) containing 10% 1,4-butanedithiol (vol/vol) at 0°C for 15 min. TFA was removed by a nitrogen flush. The obtained oil was powderized by adding ether, then dried. The crude peptide was purified on RPHPLC to give peptide **8-C** (140 nmol). Amino acid analysis of peptide **8-C**: $Asp_{9.82}Thr_{4.65}Ser_{3.76}Glu_{9.51}Pro_{3.02}Gly_{6.76}Ala_{12}Val_{5.84}Met_{2.22}Ile_{3.18}Leu_{4.88}Phe_{4.00}Lys_{9.74}Arg_{4.30}$.

Ion-Exchange Chromatography of [Met^{69}-*methyl-d*$_3$]-HBs (1—90) (8). Peptide **8-A** (7.30 mg, 399 nmol) was dissolved in a 0.05 M sodium phosphate buffer (pH 7.0) and loaded onto Pharmacia HiLoad S-Sepharose HP (16×100 mm), which was equilibrated with the same buffer and eluted with a NaCl gradient in a buffer increasing from 0.1 to 0.35 M over 50 min, at a flow rate of 2.5 ml min^{-1}. The elution of the peptide was monitored by absorbance at 220 nm. The main fraction was collected and dialyzed against 1 l of distilled water three times in a Spectrapor 6 membrane (M.W. cut off 1000) and freeze-dried to give folded [Met^{69}-*methyl-d*$_3$]-HBs(1-90) (201 nmol). Amino acid analysis of peptide **8**: $Asp_{9.10}Thr_{4.84}Ser_{4.06}Glu_{10.16}Pro_{3.56}Gly_{7.16}Ala_{12}Val_{6.54}Met_{2.03}Ile_{3.95}Leu_{5.07}Phe_{4.17}Lys_{11.11}Arg_{4.56}$.

According to the same procedure, peptide **8-B** (92.7 mg, 4.71 μmol) was purified to yield peptide **8** (77.7 mg, 2.11 μmol).

^{1}H NMR Spectroscopy. Synthetic or native HBs was dissolved at a concentration of 0.8 mg per 0.5 ml of 99.96% D_2O containing 10 mM sodium phosphate (pH 7.0) after exchanging all amide protons. The spectra were recorded at 500 MHz on a JEOL GX-500 spectrometer with a DEC 11-73 computer. NMR data were recorded at 30°C.

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