Development of a Linker with an Enhanced Stability for the Preparation of Peptide Thioesters and Its Application to the Synthesis of a Stable-Isotope-Labelled HU-Type DNA-Binding Protein

Hironobu HOJO, Yeondae KWON, Yoshimitsu KAKUTA,† Sakae TSUDA,† Isao TANAKA,† Kunio HIKICHI,† and Saburo AIMOTO*

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

†Department of Polymer, Faculty of Science, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060

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An *S*-alkyl-thioester-moiety-containing linker with an enhanced stability on a resin has been developed. A linker containing *S*-alkyl thioester with a spacer group, $-CO-SC(CH_3)_2CH_2CO-Nle-$, was stable during the peptide chain elongation cycle. This thioester was stable under HF treatment conditions, and was rapidly activated by silver ions in the presence of *N*-hydroxysuccinimde (HONSu) to form a peptide bond. Using this linker, peptide segments covering the HU-type DNA-binding protein of *Bacillus stearothermophilus* (HBs), which was site-specifically labelled with ^2H, ^{13}C, and ^{15}N, were prepared. Using these peptide segments, multi-labelled HBs was synthesized. Distinct signals of ^2H, ^{13}C, and ^{15}N in HBs were detected by NMR spectrometry.

We previously demonstrated that partially protected peptide thioesters, which were prepared by a solid-phase method, were useful building blocks for polypeptide synthesis, referring to the syntheses of *c*-Myb (142-193) amide[1] and [(*methyl*-$^2H_3$) Met^{69}]-HU-type DNA-binding protein.[2]

In order to make the thioester building block method more widely applicable to the synthesis of proteins, especially those with a molecular weight above 10000, or with labelled amino acids, several processes require improvement. One of them is the low peptide thioester yield due to the instability of the linker containing a thioester moiety on a solid support. In our previous syntheses,[1,2] we used an *S*-*n*-alkyl thioester, where *n*-alkyl means normal alkyl, directly connected to *p*-methylbenzhydrylamine resin[3] (MBHA resin or NH_2-resin). The yield of the peptide thioester was almost half that prepared on the MBHA resin without the thioester moiety.

In this paper, we describe the development of an *S*-alkyl-thioester-moiety-containing linker with enhanced stability, preparation of thioester building blocks for the synthesis of HU-type DNA-binding protein of *Bacillus stearothermophilus* (HBs) labelled with ^2H, ^{13}C, and ^{15}N, the synthesis of multilabelled HBs and the NMR spectra of the product.

Results and Discussion

Comparison of Characteristics of Linkers Containing *S*-*n*-Alkyl, *S*-*s*-Alkyl or *S*-*t*-Alkyl Thioester: Pentapeptides with *S*-*n*-alkyl, *S*-*s*-alkyl or *S*-*t*-alkyl thioester, where *n*-alkyl, *s*-alkyl, and *t*-alkyl mean normal alkyl, 1-substituted alkyl, and 1,1-disubstituted alkyl, respectively, were prepared in order to estimate the stability of the thioester-containing linker during peptide chain elongation cycles according to the scheme shown in Fig. 1. Each crude product was analyzed by reversed-phase HPLC (RPHPLC) after an HF treatment. The results are summarized in Table 1. A

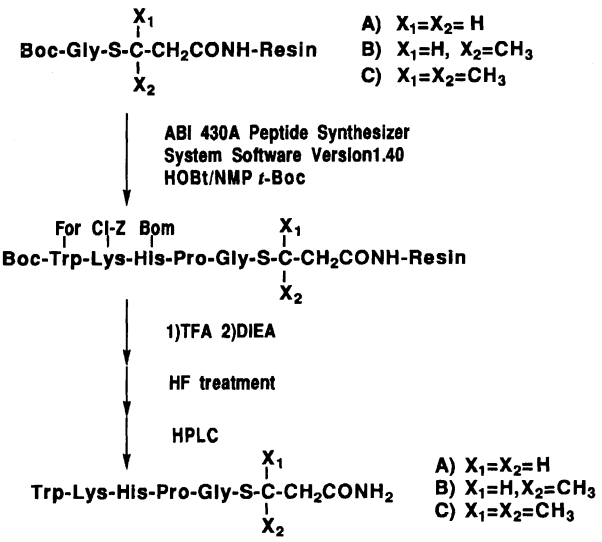

Fig. 1. Preparation of pentapeptide thioesters for a stability test of the thioester-containing linker.

pentapeptide with an *S*-*t*-alkyl thioester group was obtained in 35% yield, whereas the others were obtained in lower yields. The difference in the yield seems to be amplified by the 2,5-piperazinedione formation of a Pro–Gly sequence at the carboxyl terminal of the peptide. These results suggest that the linker containing the *S*-*t*-alkyl thioester has favorable characteristics for the preparation of the peptide thioester in regard to the yield and avoiding 2,5-piperazinedione formation. However, when an *S*-*t*-alkyl thioester-containing linker was applied to the preparation of HBs (16-39), no distinct difference in the yield was observed, compared to the peptide containing *S*-*n*-alkyl thioester, as shown in the upper 2 rows of Table 2. However, the amount of the by-products in the crude product was somewhat decreased, based on the elution profile on RPHPLC, when a linker containing *S*-*t*-alkyl thioester was used.

Stability Test of Boc–Gly–SC(CH$_3$)$_2$CH$_2$CO-

Table 1. Analysis of the Crude Products Obtained Using Three Thioester Moieties

	–SR	Yield/%	
		Trp–Lys–His–Pro–Gly–SR	Trp–Lys–His–SR
A)	$-SCH_2CH_2CONH_2$	16	8
B)	$-SCH(CH_3)CH_2CONH_2$	30	5
C)	$-SC(CH_3)_2CH_2CONH_2$	35	0

Table 2. Syntheses of the Peptide Thioester of Troc–HBs(16-39) Using 4 Resins with Different Thioester-Containing Linkers

Troc–Leu–Ser–Lys–Lys–Asp–Ala–Thr–Lys–Ala–Val–Asp–Ala–Val–Phe–Asp–Ser–Ile–Thr–Glu–Ala–Leu–Arg–Lys–Gly–SR	
–SR	Yield/%
$-SCH_2CH_2CONH_2$	15
$-SC(CH_3)_2CH_2CONH_2$	15
$-SC(CH_3)_2CH_2CO$–β-Ala–NH_2	26
$-SC(CH_3)_2CH_2CO$–Nle–NH_2	28

NH-Resin: In order to analyze the factors responsible for the low yield of the peptide *S-t*-alkyl thioester, Boc–Gly–$SC(CH_3)_2CH_2CONH$-resin was treated under the conditions used for peptide chain-elongation cycles; the materials liberated from the resin were analyzed using an amino acid analyzer and a fast atom bombardment (FAB) mass spectrometer. When Boc–Gly–$SC(CH_3)_2CH_2CONH$-resin was treated with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM), Gly–$SC(CH_3)_2CH_2CONH_2$ was liberated. The rate of decomposition was calculated to be 2 to 3% per one amino acid elongation cycle. Under the same conditions, Gly–NH_2 was removed from the Boc–Gly–NH-resin at a rate of 0.1% per cycle. On the other hand, the peptide thioester resin was rather stable in the presence of 5% *N,N*-diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF). These results suggest that a sulfur atom in the thioester might accelerate the cleavage of a peptide from the MBHA resin in the form of a peptide thioester amide. In this regard, the decomposition of the thioester, itself, was not mainly responsible for the loss of a peptide from the resin.

Effect of Spacer Groups on the Stability of the Linker Containing *S-t*-Alkyl Thioester: To estimate the effect of the sulfur atom upon the stability of the thioester-containing linker on the MBHA resin, Nle or β-Ala was inserted as a spacer between the thioester moiety and the MBHA resin. Boc–Nle or Boc–β-Ala, and then Boc–Gly–$SC(CH_3)_2CH_2COOH$, were successively introduced to the resin using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt), to obtain Boc–Gly–$SC(CH_3)_2CH_2CO$–Nle–(or β-Ala–)NH-resin. Using these resins, Troc–HBs (16-39) thioesters were synthesized. The yields were compared with those of peptide thioesters without spacer groups. The data in Table 2 show that the spacer groups, β-Ala and Nle, enhanced the stability of the linkers on the MBHA resin. The yield of peptide thioesters was almost equal to that of a peptide amide prepared on an MBHA resin without a thioester moiety.

Preparation of Peptide Segments of HBs: Using Boc–Gly–$SC(CH_3)_2CH_2CO$–Nle–NH-resin, peptide segments of multilabelled HBs were synthesized. The sequence of HBs and six amino acid residues, labelled with ^2H, ^{13}C, or ^{15}N, are shown in Fig. 2. The labelled amino acids used were (2-^{13}C) Phe for Phe^{47}, (1-^{13}C) Ala for Ala^{56}, (2-^{13}C) Gly for Gly^{60}, (*guanidino*-$N^{2,3}$-$^{15}N_2$) Arg for Arg^{61}, (*methyl*-$^2H_3$) Met for Met^{69}, (ε-^{15}N) Lys for Lys^{80}. These labelled amino acid residues are indicated by asterisks.

The same four peptide segments that were used in the previous synthesis were prepared except for the use of a 2,2,2-trichloroethoxycarbonyl (Troc) group instead of an 4-pyridylmethoxycarbonyl (iNoc) group and the carboxyl-terminal thioester portion. Starting from the Boc–Gly–$SC(CH_3)_2CH_2CO$–Nle–NH-resin, partially protected peptide segments **1**, **2**, **3**, and **4** (Table 3) were prepared by the same procedure described in a previous paper.[2] The yield of each peptide thioester almost doubled compared with the previous synthesis using the Boc–Gly–SCH_2CH_2CO–NH-resin. The yields obtained by this synthesis were almost comparable to that of the partially protected peptide **4** prepared using the Boc–Lys(Cl-Z)–OCH_2–$C_6H_4CH_2CONHCH_2$–C_6H_4-resin (Boc–Lys(Cl-Z)–OCH_2–PAM-resin).[4] The *S-t*-alkyl thioester was also stable during the purification of a peptide segment and the introduction of Boc groups.

Synthesis of Multilabelled HBs: Peptide segments from **1** to **4** were condensed according to the scheme shown in Fig. 3. As typical coupling conditions, peptides **3** (27 μmol) and **4** (27 μmol) were dissolved in dimethyl sulfoxide (DMSO) (1.4 ml) containing 4-methylmorpholine (NMM) (100 μmol) and HONSu (310 μmol). To this solution, $AgNO_3$ (47 μmol) was added. The *S-t*-alkyl thioester group was converted to a succinimide ester within 10 min, judging from the elution profile of RPHPLC. The condensation was completed within 1 d without any significant side reactions. A product was precipitated by adding distilled water, dissolved in a mixed solvent of 50% aqueous acetic acid (5 ml) and acetonitrile (1 ml) and treated with zinc dust (250 mg) under nitrogen to remove the Troc group. After zinc dust was removed, the reaction mixture was

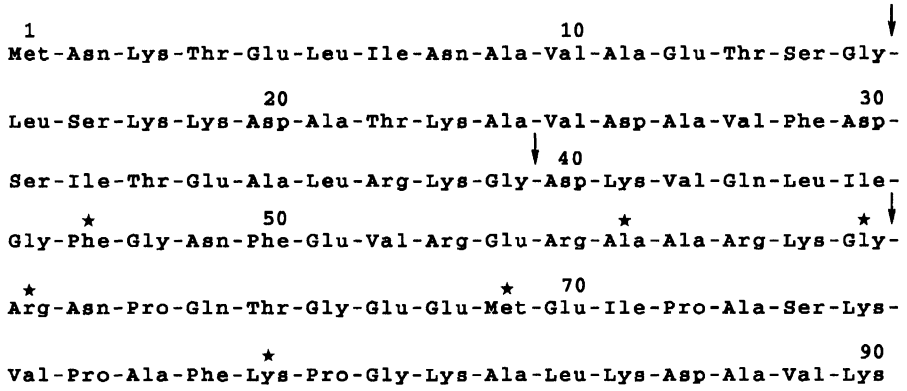

Fig. 2. Amino acid sequence of HBs. The arrows indicate the sites of segment coupling. The * indicates amino acids labelled with ^{2}H, ^{13}C, or ^{15}N; (2-^{13}C)Phe^{47}, (1-^{13}C)Ala^{56}, (2-^{13}C)Gly^{60}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^{2}H_3$)Met^{69} and (ε-^{15}N)Lys^{80}.

Table 3. Partially Protected Peptide Segments Prepared for Segment Coupling

Peptide segments		Yield/%[a]
Boc–[Lys(Boc)^{3}]–HBs(1-15)–SC$(CH_3)_2CH_2CO$–Nle–NH_2	(**1**)	22 (14)
Boc–Met–Asn–Lys(Boc)–Thr–Glu–Leu–Ile–Asn–Ala–Val–Ala–Glu–Thr–Ser–Gly–SC$(CH_3)_2CH_2CO$–Nle–NH_2		
Troc–[Lys(Boc)^{18,19,23,38}]–HBs(16-39)–SC$(CH_3)_2CH_2CO$–Nle–NH_2	(**2**)	22 (10)
Troc–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–SC$(CH_3)_2CH_2CO$–Nle–NH_2		
Troc–[Lys(Boc)^{41,59},(2-^{13}C)Phe^{47}, (1-^{13}C)Ala^{56}, (2-^{13}C)Gly^{60}]–HBs(40-60)–SC$(CH_3)_2CH_2CO$–Nle–NH_2	(**3**)	41 (17)
Troc–Asp–Lys(Boc)–Val–Gln–Leu–Ile–Gly–Phe*–Gly–Asn–Phe–Glu–Val–Arg–Glu–Arg–Ala*–Ala–Arg–Lys(Boc)–Gly*–SC$(CH_3)_2CH_2CO$–Nle–NH_2		
[Lys(Boc)^{75,80,83,86,90}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^{2}H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]–HBs(61-90)	(**4**)	20 (19)
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)		

a) The yields in parentheses are of previous syntheses using an $-SCH_2CH_2CONH_2$ group as the thioester linker and an *i*Noc group for terminal amino protection.[2]

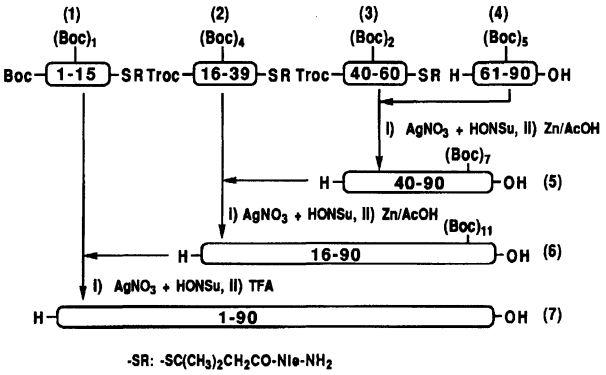

Fig. 3. Synthetic route of HBs(1-90).

dialyzed against distilled water and lyophilized to give a peptide mixture containing peptide **5**, which was used for the next coupling without purification. According to the same procedure, peptides **2** and **1** were then successively condensed. A product containing a peptide, which covered the entire sequence of HBs, was treated with TFA containing 10% 1,4-butanedithiol (v/v). The RPHPLC profile of crude HBs (1-90) (**7**) is shown in Fig. 4. HBs(1-90) was purified by RPHPLC using PROTEIN-RP, then further purified by ion-exchange chromatography on Pharmacia Mono S HR 5/5 and desalted by RPHPLC to give a highly-pure final product in 8.5% yield, based upon peptide **4**. In the previous synthesis, the overall yield was 7.2%, based on peptide **4** by method B.[2] In this synthesis, a high proportion of the by-product was formed up to 50% during deprotection of the Troc group.[5] Judging from the mass number of the by-product obtained by the zinc-dust treatment of Troc–[Lys(Boc)^{75,80,83,86,90}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^{2}H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]–HBs(61-90), the major by-products formed at every deprotection step were estimated to be 2,2-dichloroethoxycarbonyl derivatives, which were derived from the substitution of one chlorine atom of the Troc group by one hydrogen atom. During the synthesis of the *c*-Myb protein, the side reaction was not so serious.[1] A Troc group was not necessarily a good choice for the synthesis of HBs. The activation of the *S*-*t*-alkyl thioester was very rapid.

NMR Spectra of Labelled HBs: The final goal

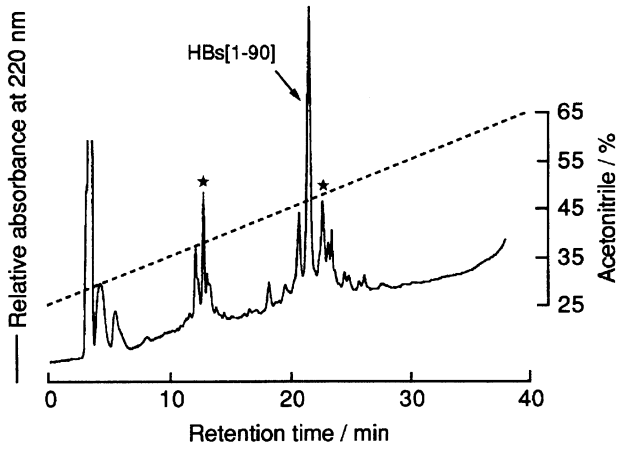

Fig. 4. HPLC elution profile of a crude product of HBs(1-90) after treating the reaction mixture with TFA. The * indicates the peaks derived from by-products during deprotection of the Troc group.

of this synthesis was to obtain HBs labelled with stable isotopes, which can be used to analyze the local flexibility of HBs, as well as the mode of interaction between HBs and DNA. This is the first chemically synthesized protein, which is site-specifically labelled with stable isotopes for the analysis of protein functions. In order to confirm the usefulness of this molecule, its ^{2}H, ^{13}C, and ^{15}N NMR spectra were measured. Synthetic HBs showed the same ^{1}H NMR spectrum as that of the native HBs, except for methyl-$^{2}H_3$ of Met^{69} (Data not shown). These results indicate that the synthetic HBs has the same three-dimensional structure as that of native HBs. The ^{13}C and ^{15}N signals of the HBs were also apparent by ^{13}C and ^{15}N NMR spectroscopy using this sample at a concentration of 20 mg ml^{-1}, as shown in Fig. 5. Thus, multilabelled HBs should yield appropriate information. Detailed analysis data will be published elsewhere.

Experimental

Materials and Methods. All of the amino acids used were of the L-configuration, except for glycine. Boc-amino acid derivatives and the MBHA resin were purchased from the Peptide Institute Inc. (Osaka). Boc–Lys(Cl-Z)–OCH_2–PAM-resin was purchased from Applied Biosystems Inc. (Foster City, CA.). (2-^{13}C)Phe, (1-^{13}C)-Ala, (2-^{13}C)Gly and (*methyl*-$^{2}H_3$)Met were purchased from Isotec Inc. (Miamisburg, OH.). (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg and (ε-^{15}N)Lys were synthesized in house. The solvents and reagents used for solid-phase peptide synthesis were purchased from Watanabe Chemical Ind. Ltd. (Hiroshima). Analytical RPHPLC was performed on YMC-Pack ODS-AM (4.6×250 mm), and preparative RPHPLC was performed on YMC-Pack ODS-AM or PROTEIN-RP (20×250 mm) (YMC, Kyoto). The amino acids were analyzed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo) after hydrolysis with 4 M# methanesulfonic acid at 110 °C for 24 h in an evacuated sealed tube. The peptide mass number was determined by FAB mass spectrometry using a JMS-HX100 (JEOL Ltd., Tokyo) equipped with a JMA-3100 mass data system. Although the peptide weight was the observed value, the yield was calculated based upon the amino acid analysis data. Sonication was carried out using a Branson Model B-220.

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

Peptide Chain Elongation on a Solid Support. The solid-phase synthesis of a peptide segment was performed using a peptide synthesizer (model 430A (Applied Biosystems Inc.)) on 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. Boc–Gly–SCH_2CH_2COOH was prepared form Boc–Gly–ONp and HS–CH_2CH_2COOH.[1] The side-chain-protecting groups of the Boc-amino acids were *o*-chlorobenzyloxycarbonyl (Cl-Z) for the N^{ε} of Lys, benzyl for the alcoholic OH of Thr and Ser, cyclohexyl ester for the β-carboxyl group of Asp, benzyl ester for the γ-carboxyl group of Glu, tosyl (Tos) for the N^{g} of Arg, benzyloxymethyl (Bom) for N^{π} of His, and formyl (For) for N^{i} of Trp.

Boc–Gly–SCH(CH_3)CH_2COOH. Crotonic acid (3.7 g, 43 mmol) and phenylmethanethiol (5 ml, 43 mmol) were heated in piperidine (6.4 ml) at 150 °C for 12 h. The reaction mixture was acidified with HCl and extracted with ether. The products in the ether phases were extracted with concentrated $NaHCO_3$. After acidification of the aqueous phase, the product was extracted with ether, dried over Na_2SO_4 and concentrated under reduced pressure to give an oil (5.3 g, 25 mmol), 3-(benzylthio)butyric acid, which was confirmed by ^{1}H NMR. All of this oil was dissolved in liquid ammonia (200 ml) and treated with Na metal (1.2 g) until a blue color persisted for 1 min. After NH_3 was removed by a nitrogen stream, the residue was acidified with HCl, extracted 3 times with AcOEt and dried over Na_2SO_4. AcOEt layers were combined and concentrated under reduced pressure to give an oil (3.2 g), 3-mercaptobutyric acid. After this, the oil (3.0 g) and Boc–Gly–ONSu (6.1 g, 22 mmol) were dissolved in DMF (50 ml), DIEA (5.9 ml, 34 mmol) was then added. The reaction mixture was stirred overnight under nitrogen. After DMF was evaporated in vacuo, the residual oil was dissolved in AcOEt and washed successively with water containing 10% citric acid and NaCl. After concentrating the organic layer, the residual oil was purified on Wako gel (45×160 mm) using chloroform–methanol–acetic acid (95 : 1 : 3, v/v) as the eluent. The fraction containing the product was collected and concentrated in vacuo. Crystallization from hexane gave Boc–Gly–SCH(CH_3)CH_2COOH (4.1 g, 15 mmol), mp 109.0—111.2 °C. Found: C, 47.49; H, 6.91; N, 5.08%. Calcd for $C_{11}H_{19}O_5N_1S_1$: C, 47.64; H, 6.91; N, 5.05%.

Boc–Gly–SC(CH_3)$_2CH_2$COOH. 3-Methyl-3-(benzylthio)butyric acid (24 g, 110 mmol)[6] was dissolved in liquid NH_3 (1 dm^3) and treated with Na metal (5.0 g). According to the procedure described for the preparation of 3-mercaptobutyric acid, 3-methyl-3-mercaptobutyric acid (16 g) was obtained as an oil. Boc–Gly–ONSu (36 g, 130 mmol) and 3-methyl-3-mercaptobutyric acid (16 g) were dissolved in DMF (350 ml) containing DIEA (20 ml, 120 mmol); the reaction mixture was stirred for 5 h. The oil obtained by the same procedure described for the synthesis of Boc–Gly–SCH(CH_3)CH_2COOH was purified by RPHPLC using aque-

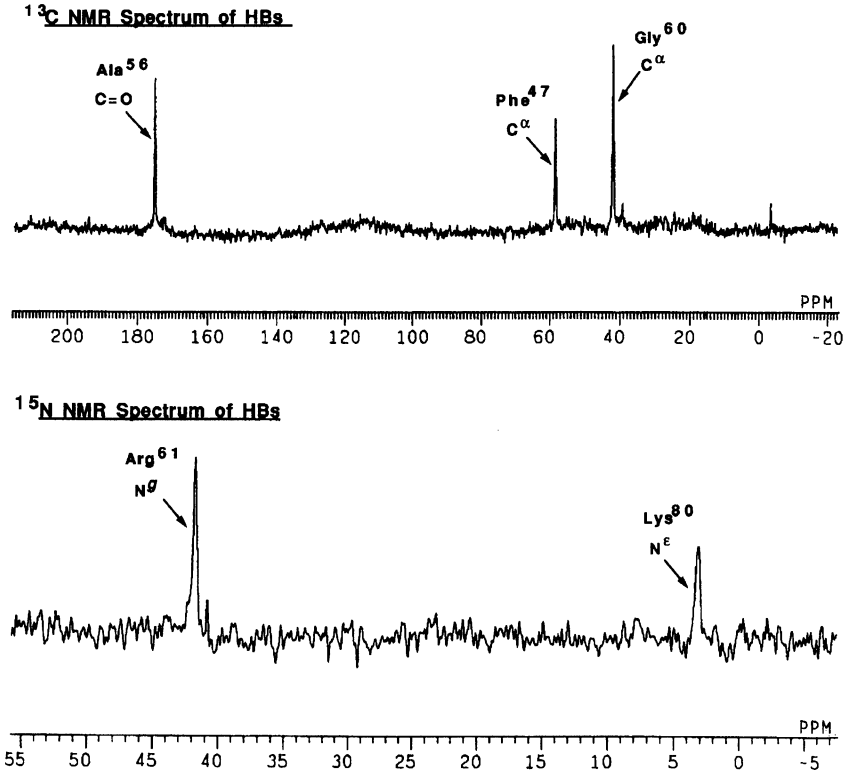

Fig. 5. ^{13}C and ^{15}N signals obtained using ^{13}C and ^{15}N NMR spectroscopy, respectively. N^g means *guanidino-*$N^{2,3}$.

ous acetonitrile containing 0.1% TFA as the eluent to obtain Boc–Gly–$SC(CH_3)_2CH_2COOH$ (27 g, 93 mmol), mp 51.0—53.2 °C. Found: C, 49.20; H, 7.17; N, 4.67; S, 11.01%. Calcd for $C_{12}H_{21}O_5N_1S_1$: C, 49.47; H, 7.27; N, 4.81; S, 11.01%.

Synthesis of Boc–Gly–$SCH_{(2-n)}(CH_3)_nCH_2CO$–NH-Resin. ($n$=0,1,2). MBHA resin hydrochloride (1.0 g, 0.39 mequiv of NH_2) was washed with 5% DIEA in DMF for 5 min (×2), then with DCM for 1 min (×3). Boc–Gly–SCH_2CH_2COOH (200 mg, 0.76 mmol) in DCM (8 ml) and 0.5 M DCC in DCM (1.5 ml) were added to the resin and the reaction mixture was shaken for 5 h. The resulting resin was mixed with 5% acetic anhydride in DCM for 15 min to give Boc–Gly–SCH_2CH_2CONH-resin (1.1 g, Gly: 0.33 mmol g^{-1}). Boc–Gly–$SCH(CH_3)CH_2CONH$-resin (1.1 g, Gly: 0.34 mmol g^{-1}) and Boc–Gly–$SC(CH_3)_2CH_2CO$–NH-resin (0.79 g, Gly: 0.30 mmol g^{-1}) were synthesized by the same procedure.

Synthesis of Trp–Lys–His–Pro–Gly–$SCH_{(2-n)}(CH_3)_nCH_2CONH_2$ (n=0, 1,2). Starting from Boc–Gly–SCH_2CH_2CONH-resin (500 mg, 170 μmol), Boc–Trp(For)–Lys(Cl-Z)–His(Bom)–Pro–Gly–SCH_2CH_2CONH-resin was prepared by means of a synthesizer using a single coupling protocol. An aliquot of the resin (94 mg out of 560 mg) was treated with HF (2.7 ml) containing *p*-cresol (100 μl) and 1,4-butanedithiol (350 μl) at 0 °C for 1.5 h. The crude peptide was extracted with 25% aqueous acetonitrile containing 0.1% TFA (10 ml), washed with ether (3 ml) (×3) and lyophilized to give a powder (18 mg), which was analyzed by RPHPLC. The isolated fractions were hydrolyzed and the peptide structure and contents were estimated. From this synthesis, 4.4 μmol of the desired product were obtained. In the same manner, pentapeptide thioesters were synthesized on Boc–Gly–$SCH(CH_3)CH_2CONH$-resin and Boc–Gly–$SC(CH_3)_2CH_2CONH$-resin, and the products were analyzed. The data are given in Table 1.

Stability of the Boc–Gly–$SC(CH_3)_2CH_2CONH$-Resin. Boc–Gly–$SC(CH_3)_2CH_2CONH$-resin was stirred with 50% TFA in DCM. At various intervals, aliquots of the solvent were withdrawn and analyzed on an amino acid analyzer after acid hydrolysis. After overnight stirring, the resin was filtered off and the filtrate was concentrated in vacuo. The residual mass was treated with 0.1 M *N*-(*t*-butoxycarbonyloxy)succinimide (Boc–ONSu) in DMF in the presence of triethylamine (TEA) for 5 h. The reaction mixture was analyzed on RPHPLC and the main product was analyzed by FAB mass spectrometry. Found: m/z 291.1 $(M+H)^+$. Calcd for Boc–Gly–$SC(CH_3)_2CH_2CONH_2$: m/z 291.1 $(M+H)^+$.

Boc–Gly–$SC(CH_3)_2CH_2CO$–Nle–NH-Resin. Boc–Nle (170 mg, 0.75 mmol) was mixed with 1 M HOBt in 1-methyl-2-pyrrolidinone (NMP) (0.75 ml) and 1 M DCC in NMP (0.75 ml). After stirring for 30 min, the reaction mixture was mixed with neutralized MBHA resin (810 mg, 0.51 mequiv of NH_2) and shaken for 4 h. After washing with DCM (×3), 50% methanol in DCM (×3) and DCM (×3), the resin was treated with 55% TFA in DCM for 5 and 15 min, followed by neutralization with 5% DIEA in DMF for 5 min (×2). Boc–Gly–$SC(CH_3)_2CH_2COOH$ (220 mg, 0.75 mmol) was introduced to the Nle–NH-resin in the same manner to give Boc–Gly–$SC(CH_3)_2CH_2CO$–Nle–NH-resin (980 mg, Gly: 0.46 mmol).

Boc–Gly–$SC(CH_3)_2CH_2CO$–β-Ala–NH-Resin. Boc–Gly–$SC(CH_3)_2CH_2CO$–β-Ala–NH-resin (980 mg, Gly: 0.47 mmol) was prepared by the same procedure

as described regarding the preparation of Boc–Gly–S-$C(CH_3)_2CH_2CO$–Nle–NH-resin using Boc–β-Ala.

Troc–[Lys(Boc)^{18,19,23,38}]–HBs(16-39)–SC$(CH_3)_2CH_2CO$–Nle–NH_2 (2). Starting from Boc–Gly–S-$C(CH_3)_2CH_2CO$–Nle–NH-resin (980 mg, Gly: 0.46 mmol), the protected peptide resin corresponding to the HBs sequence (16-39) was prepared on a synthesizer using a double coupling protocol. After completion of a peptide chain assembly followed by a TFA treatment, the resin was mixed with *N*-(2,2,2-trichloroethoxycarbonyloxy)succinimide (Troc–ONSu) (440 mg, 1.5 mmol) in DCM for 4 h to give a protected peptide resin (2.4 g). The resin (1.0 g) was treated with HF (10 ml) containing *p*-cresol (1 ml) at 0 °C for 90 min. The crude peptide (500 mg) obtained was purified on RPHPLC to give Troc–HBs(16-39)–S-$C(CH_3)_2CH_2CO$–Nle–NH_2 (200 mg, 55 μmol, 28% based on Gly in the starting resin). Found: m/z 2965.4 $(M+H)^+$. Calcd: m/z 2965.4 $(M+H)^+$. Amino acid composition: $Asp_{2.70}Thr_{1.68}Ser_{1.60}Glu_{0.97}Gly_{0.94}Ala_4Val_{1.93}Ile_{0.91}Leu_{1.55}Nle_{0.85}Phe_{0.94}Lys_{3.50}Arg_{0.94}$. Troc–HBs(16-39)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$ (200 mg, 55 μmol) and Boc–ONSu (100 mg, 460 μmol) were dissolved in DMSO (1 ml). The solution was stirred for 3 h after adding TEA (73 μl, 520 μmol). A mixed solvent of ether and ethyl acetate was added to the reaction mixture to precipitate a product which was collected by centrifugation and freeze-dried from a dioxane suspension to give peptide **2** (170 mg, 43 μmol, 22% based on Gly in the starting resin). Found: m/z 3365.5 $(M+H)^+$. Calcd: m/z 3365.7 $(M+H)^+$. Amino acid analysis of peptide **2**: $Asp_{2.99}Thr_{1.89}Ser_{1.80}Glu_{1.10}Gly_{1.08}Ala_4Val_{2.03}Ile_{0.97}Leu_{1.61}Nle_{0.98}Phe_{0.99}Lys_{3.84}Arg_{1.04}$.

Troc–HBs(16-39)–$SCH_2CH_2CONH_2$. Starting from Boc–Gly–SCH_2CH_2CONH-resin (970 mg, Gly: 0.48 mmol), this peptide was prepared by the same method described for the synthesis of peptide **2**. Yield: 15% based upon Gly in the starting resin. Found: m/z 2824.5 $(M+H)^+$. Calcd: m/z 2824.3 $(M+H)^+$. Amino acid composition: $Asp_{3.03}Thr_{1.85}Ser_{1.81}Glu_{1.10}Gly_{1.13}Ala_4Val_{2.13}Ile_{1.09}Leu_{1.79}Phe_{1.12}Lys_{4.04}Arg_{0.98}$.

Troc–HBs(16-39)–SC$(CH_3)_2CH_2CONH_2$. Starting from Boc–Gly–SC$(CH_3)_2CH_2CONH$-resin (900 mg, Gly: 0.46 mmol), this peptide was prepared by the same method described for the synthesis of peptide **2**. Yield: 15% based upon Gly in the starting resin. Found: m/z 2852.4 $(M+H)^+$. Calcd: m/z 2852.4 $(M+H)^+$. Amino acid composition: $Asp_{2.98}Thr_{1.84}Ser_{1.82}Glu_{1.02}Gly_{1.06}Ala_4Val_{1.95}Ile_{1.07}Leu_{1.61}Phe_{0.90}Lys_{3.91}Arg_{0.95}$.

Troc–HBs(16-39)–SC$(CH_3)_2CH_2CO$–β–Ala–NH_2. Starting from Boc–Gly–SC$(CH_3)_2CH_2CO$–β–Ala–NH-resin (980 mg, Gly: 0.47 mmol), this peptide was prepared as described for the synthesis of peptide **2**. Yield: 26% based on Gly in the starting resin. Found: m/z 2923.5 $(M+H)^+$. Calcd: m/z 2923.4 $(M+H)^+$. Amino acid composition: $Asp_{3.15}Thr_{1.95}Ser_{1.92}Glu_{1.06}Gly_{1.04}Ala_4Val_{1.84}Ile_{1.07}Leu_{1.84}(Phe+β\text{-}Ala)_{1.32}Lys_{4.17}Arg_{0.80}$.

Boc–[Lys(Boc)^3]–HBs(1-15)–SC$(CH_3)_2CH_2CO$–Nle–NH_2 (1). This peptide was prepared using the procedure described for the synthesis of peptide **2**. Yield: 22% based on Gly in the starting resin. Found: m/z 2005.6 $(M+H)^+$. Calcd: m/z 2006.0 $(M+H)^+$. Amino acid analysis of peptide **1**: $Asp_{1.94}Thr_{1.81}Ser_{0.85}Glu_{1.96}Gly_{0.95}Ala_2Val_{0.97}Met_{0.85}Ile_{0.83}Leu_{0.89}Nle_{0.96}Lys_{1.02}$.

Troc–[Lys(Boc)^{41,59}, (2-^{13}C)Phe^{47}, (1-^{13}C)Ala^{56}, (2-^{13}C)Gly^{60}]–HBs(40-60)–SC$(CH_3)_2CH_2CO$–Nle–NH_2 (3). This peptide was prepared according to the procedure described for peptide **2**. Boc–(2-^{13}C)Gly–S-$C(CH_3)_2CH_2CO$–Nle–NH-resin was obtained by the same procedure as described for the preparation of the corresponding nonlabelled resin. Ala^{56} and Phe^{47} were introduced manually by mixing with 0.75 mmol of Boc–(1-^{13}C)Ala or Boc–(2-^{13}C)Phe, 1 M DCC in NMP (0.75 ml) and 1 M HOBt in NMP (0.75 ml) for 4 h. Yield of peptide **3**: 41% based upon Gly in the starting resin. Found: m/z 2995.5 $(M+H)^+$. Calcd: m/z 2995.5 $(M+H)^+$. Amino acid analysis of peptide **3**: $Asp_{1.90}Glu_{2.91}Gly_{2.91}Ala_2Val_{1.86}Ile_{0.92}Leu_{0.96}Nle_{0.94}Phe_{1.99}Lys_{1.87}Arg_{2.87}$.

[Lys(Boc)^{75,80,83,86,90}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^2H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]–HBs(61-90) (4). This peptide was synthesized following the same procedure described regarding the preparation of the corresponding peptide used in the previous synthesis,[2] except for the use of a Troc group for the temporary protection of the terminal amino group. Lys^{80}, Met^{69}, and Arg^{61} residues were incorporated manually by mixing with 0.75 mmol of Boc–(ε-^{15}N)Lys(Cl-Z), Boc–(*methyl*-$^2H_3$)Met or Boc–(*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg(Tos), 1 M DCC in NMP (0.75 ml) and 1 M HOBt in NMP (0.75 ml) for 4 h. Yield of peptide **4**: 20% based upon Lys in the starting resin. Found: m/z 3743.4 $(M+H)^+$. Calcd: m/z 3743.0 $(M+H)^+$. Amino acid analysis of peptide **4**: $Asp_{1.95}Thr_{0.96}Ser_{0.93}Glu_{3.91}Pro_{3.83}Gly_{1.99}Ala_4Val_{1.95}Met_{0.72}Ile_{0.97}Leu_{1.03}Phe_{1.01}Lys_{4.75}Arg_{0.96}$.

During the zinc- dust treatment of Troc–[Lys(Boc)^{75,80,83,86,90}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^2H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]–HBs(61-90) in aqueous acetic acid, 12% of by-product were detected on RPHPLC. This peptide was treated with TFA for 1 min, purified on RPHPLC and analyzed by FAB mass spectrometry. Found: m/z 3382.4 $(M+H)^+$. Calcd for $CHCl_2CH_2OCO$–[(*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^2H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]–HBs(61-90): m/z 3382.7 $(M+H)^+$.

Synthesis of [(2-^{13}C)Phe^{47}, (1-^{13}C)Ala^{56}, (2-^{13}C)Gly^{60}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^2H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]–HBs(1-90) (7). Peptides **3** (100 mg, 27 μmol), **4** (96 mg, 27 μmol) and HONSu (36 mg, 310 μmol) were dissolved in DMSO (1.4 ml) containing NMM (11 μl, 100 μmol). After adding $AgNO_3$ (8.0 mg, 47 μmol), the solution was stirred overnight at room temperature in the dark. Distilled water was added to the solution to precipitate a product which was freeze-dried from a dioxane suspension to give a powder (190 mg). This peptide was sonicated with zinc dust (250 mg) in a mixture of 50% aqueous acetic acid (5 ml) and acetonitrile (1 ml) under nitrogen for 10 min at room temperature. A mixture (160 mg) containing peptide **5** was obtained after dialysis, followed by freeze-drying. Following the same procedure, peptide **2** (110 mg, 27 μmol) was coupled with crude peptide **5** to obtain a mixture containing peptide **6**, which was further condensed with peptide **1** (120 mg, 33 μmol) to obtain a crude product **7** (350 mg). This peptide was mixed with TFA (4.5 ml) containing 10% 1,4-butanedithiol (v/v) at room temperature for 10 min. TFA was removed by a nitrogen stream and

the peptide was precipitated with ether. This powder was purified on RPHPLC to yield partly purified peptide **7** (56 mg, 2.9 μmol) after freeze-drying. This was further purified by ion-exchange chromatography on Pharmacia Mono-S HR 5/5 (5×50 mm), which was equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol and eluted with a 0.05 to 0.2 M NaCl gradient in the buffer over 30 min at a flow rate of 1 ml min^{-1}. The major fraction was desalted by RPHPLC to give purified peptide **7** (2.3 μmol, 8.5% based upon peptide **4**). Amino acid analysis of peptide **7**: $Asp_{8.87}Thr_{4.62}Ser_{3.41}Glu_{10.05}Pro_{4.10}Gly_{7.01}Ala_{12}Val_{6.47}Met_{1.87}Ile_{3.74}Leu_{5.06}Phe_{3.85}Lys_{11.13}Arg_{4.62}$.

NMR Spectroscopy. The concentration of HBs was 20 mg ml^{-1}. The pH was adjusted to 7.5 by adding NaOD in a sodium phosphate buffer. The ^{13}C and ^{15}N NMR spectra were measured at 38 °C and at 125 and 50 MHz respectively, using a JEOL JNM-GX500 spectrometer. The chemical shifts were measured relative to the external standards, dioxane (67 ppm) and formamide (85 ppm).

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References

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

2) H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **65**, 3055 (1992).

3) K. Channabasavaiah and J. M. Stewart, *Biochem. Biophys. Res. Commun.*, **86**, 1266 (1979).

4) A. R. Mitchell, B. W. Erickson, M. N. Ryabtsev, R. S. Hodges, and R. B. Merrifield, *J. Am. Chem. Soc.*, **98**, 7357 (1976).

5) G. Hancock, I. J. Galpin, and B. A. Morgan, *Tetrahedron Lett.*, **23**, 249 (1982).

6) H. Schultz and V. du Vigneaud, *J. Med. Chem.*, **9**, 647 (1966).