Development of a Method for Protein Synthesis Using *S*-Alkyl Thioester of Partially Protected Peptide Segments

Hironobu Hojo

1994

Acknowledgements

This study was performed at the Research Center for Protein Engineering, Institute for Protein Research, Osaka University. I received various kinds of help and information from almost all members of the center and others, but I especially thank the following persons who made my work possible:

My supervisor Professor Saburo Aimoto for guidance, sharing his knowledge, encouraging support throughout this work, and for his great kindness and friendship.

The members of the center for much help and friendship: Ms. Shoko Yoshimura and Ms. Chieko Maegawa.

Professor Yasutsugu Shimonishi and Dr. Toshifumi Takao, for valuable suggestion and encouragement as well as for allowing me to measure mass spectrum.

Professor Fumio Sakiyama and Mr. Satoshi Kuroda for measuring the enzymatic activity of barnase and RPSc(299-410) as well as for valuable comments.

Professor Shoichi Kusumoto, Faculty of Science, Osaka University for valuable comments on this thesis.

Professor Yoshifumi Nishimura and Dr. Kazuhiro Ogata of Yokohama City University for their encouragement of the chemical synthesis of DNA binding domain of *c*-Myb protein in chapter I.

Professor Kunio Hikichi, Isao Tanaka, Dr. Sakae Tsuda, and Yoshimitsu Kakuta of Faculty of Science, Hokkaido University for NMR spectrum measurements of HBs in chapter II and III.

Professor Mitiko Gō of Faculty of Science, Nagoya University for her kind advice throughout the studies in chapter IV and V.

Dr. Haruhiko Tamaoki for his valuable suggestion, encouragement, and friendship.

My parents Nobumasa Hojo and Reiko Hojo for their encouragement and patience in all these years.

This study was partly supported by Grants-in-Aid for Scientific Research Nos. 62540407, 01740322, 02263101, and 03740287 from the Ministry of Education, Science and Culture.

Abbreviations

Bzl	benzyl
Boc	*t*-butoxycarbonyl
Boc-ONSu	*N*-(*t*-butoxycarbonyloxy)succinimide
Bom	benzyloxymethyl
Br-Z	2-bromobenzyloxycarbonyl
Bu^t	*t*-butyl
CHA	cyclohexylamine
Cl-Z	2-chlorobenzyloxycarbonyl
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DIEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
Fmoc-ONSu	*N*-(9-fluorenylmethoxycarbonyloxy)succinimide
For	formyl
HOBt	1-hydroxybenzotriazole
HONp	4-nitrophenol
HONSu	*N*-hydroxysuccinimide
*i*Noc	4-pyridylmethoxycarbonyl
*i*Noc-ONp	4-pyridylmethyl *p*-nitrophenyl carbonate
MBHA	4-methylbenzhydrylamine

MeBzl	4-methylbenzyl
Nle	L-norleucine
NMP	1-methyl-2-pyrrolidinone
NMM	4-methylmorpholine
Npys	3-nitro-2-pyridinesulfenyl
OcHex	cyclohexyl ester
OBzl	benzyl ester
OBu^t	*t*-butyl ester
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
RPHPLC	reversed-phase high performance liquid chromatography
TEA	triethylamine
TFA	trifluoroacetic acid
Tmb	2,4,6-trimethylbenzyl
Tos	tosyl
Troc	2,2,2-trichloroethoxycarbonyl
Troc-ONSu	*N*-(2,2,2-trichloroethoxycarbonyloxy)succinimide
Trt	triphenylmethyl
Z	benzyloxycarbonyl

CONTENTS

General Introduction

The purpose of the present study is to establish a facile method for chemical synthesis of protein. The development of a new synthetic strategy is essential to realize routine preparation of proteins by chemical means and protein studies using synthetic proteins. Its success will also open a way to create proteins with novel characteristics and functions, which will not be attained by a recombinant DNA technology.

The solution methods of peptide synthesis have been continuously improving over the past 90 years. Furthermore, based upon the accumulated knowledge obtained using solution methods, a new strategy of peptide synthesis, the solid-phase method, was invented by R.B. Merrifield in 1963.[1] Using these two methods, a tremendous number of complex peptides have been synthesized.

Although both methods have been successfully applied to the preparation of small peptides, most protein synthesis has not been performed by chemical means, but by recombinant DNA technology, because of intrinsic difficulties involved in both chemical methods.

In the solution method, the product has to be isolated and confirmed after every coupling reaction. Thus, this method of synthesis requires enormous numbers of experienced chemists. Nevertheless, synthesis is not always successful because of solubility and/or reactivity problems of protected intermediate peptides. In addition, the complete purification and confirmation of intermediates become more difficult as the chain length increases. Several groups have synthesized proteins by solution method, such as RNase A[2] and parathyroid hormone.[3] However, those procedures cannot form the general basis of protein synthesis because of the reasons mentioned above.

On the other hand, the solid-phase method realized simple and rapid preparation of peptides. Reagents are introduced to the reaction vessel containing a peptide on an insoluble

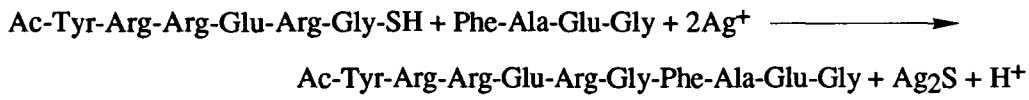
resin and mixed with it for a given time. After that, the resin is washed with appropriate solvents to remove excess reagents. Basic chemical processes, including the coupling conditions of each amino acid derivative, have been intensively studied and sophisticated protocols for chain elongation cycles have been developed for machine assisted synthesis.

Along with the development of related technologies such as reversed-phase high performance liquid chromatography (RPHPLC) and mass spectrometry, the solid-phase method became the major means of peptide synthesis. One can easily confirm the desired peptide in a crude product by the combination of RPHPLC and mass spectrometry and it can be efficiently isolated by RPHPLC.

The solid-phase method, however, also presents problems in the protein synthesis. For instance, inter-chain aggregation and secondary-structure formation occur more or less with the increase in peptide-chain length. This causes imperfect deprotection of an α-amino protecting group as well as incomplete incorporation of an amino-acid derivative. Mainly because of these intrinsic difficulties, the synthesis of a highly pure peptide with more than 50 amino acid residues is still difficult.

Thus, a new strategy has to be developed for the rapid synthesis of highly pure protein. Several groups are developing new methods, in which protected peptide segments are prepared by the solid-phase method and then used for segment condensation in solutions.[4-8] In their strategies, the protected peptide segments have to be designed taking the later condensation method in solution into account.

Among these methods, the thiocarboxyl segment condensation strategy[4] developed by J. Blake has an attractive feature that the selective activation of the carbonyl group of the terminal amino acid residue. He prepared a peptide with thiocarboxylic acid at the carboxyl terminus by the solid-phase method and realized the segment condensation with an amino component in the presence of silver ions and *N*-hydroxysuccinimide (HONSu) as shown below. In his method, no protecting group is required for the side

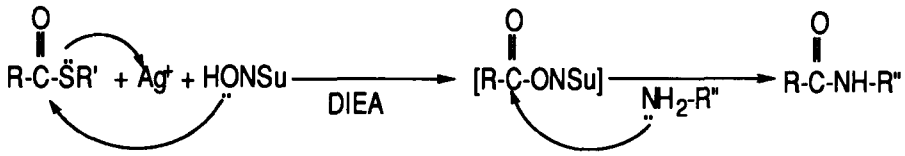


chain carboxyl group, for the thiocarboxyl group is selectively activated by silver ions. The protection of other side chain functional groups was not necessary except for amino groups. Using this strategy, he synthesized α-inhibin[9,10] which contains 92 amino-acid residues, β-lipotropin[11] and other polypeptides.[12-14] However, his method also has a limitation. Thiocarboxylic acid is easily decomposed by oxidation or hydrolysis. Furthermore, the amino protective reagent, such as *N*-(*t*-butoxycarbonyloxy)succinimide (Boc-ONSu), could not be used to protect side-chain amino groups, because the high nucleophilicity of the thiol moiety of the thiocarboxyl group causes a side reaction with the reagent. Only citraconic anhydride among known reagents could be used to introduce a citraconyl group into the side-chain amino groups of thiocarboxyl-group-containing peptides. Citraconyl is, however, unstable even under mild acidic conditions and it does not increase the solubility of the protected peptide even in a polar organic solvent such as dimethyl sulfoxide (DMSO). Furthermore, no route could be developed to prepare cysteine-containing proteins based upon the thiocarboxyl segment condensation procedure.

On the other hand, Aimoto et al. developed a method for polypeptide synthesis by a slightly different way.[5] They prepared a peptide segment using 3-nitro-2-pyridinesulfenyl (Npys) amino acid derivatives to construct an acidic amino acid-containing peptide segment by a solid-phase method, whose side-chain carboxyl group was protected as an ester. This realized the selective activation of the terminal carboxyl group. They used dicyclohexylcarbodiimide (DCC) for segment condensation. The segment without the acidic amino-acid residue was prepared using *t*-butoxycarbonyl (Boc) amino acid derivatives. Bovine pancreatic trypsin inhibitor (BPTI) was synthesized by means of this

method. However, increasing hydrophobic protecting groups such as cyclohexyl esters in aspartyl side chains decreases the solubility of a peptide segment in solvents such as aqueous acetonitrile. Consequently, the purification of this type of peptide segments by RPHPLC becomes difficult and the recovery yield tends to be lower. This is a potential disadvantage of their method.

To overcome the problems inherent in the above two methods, the author designed a new method for protein synthesis, in which the *S*-alkyl thioester of a partially protected peptide segment is used as a building block. The thioester group was expected to be activated by silver ions as shown below.



If so, the terminal thioester group will be selectively activated. Furthermore the thioester group is not as nucleophilic as the thiocarboxyl group. Hence various kinds of protecting groups can be introduced to the side-chain amino groups using protective reagents such as Boc-ONSu. If the *S*-alkyl thioester of a peptide segment could be synthesized by a solid-phase method in a high yield, a very promising novel method for protein synthesis would be developed.

In this thesis, the author describes the development of the method for protein synthesis according this idea using the *S*-alkyl thioester of partially protected peptide segments. (For simplicity the author calls the technique as "the thioester method" in this thesis.)

In chapter I, the basic strategy of the thioester method is described under the title of Polypeptide Synthesis Using the *S*-Alkyl Thioester of a Partially Protected Peptide Segment. *c*-Myb protein(142-193)-NH_2 was synthesized as a model compound (*Bull. Chem. Soc. Jpn.*, **64**, 111-117 (1991)).

In chapter II, the application of the thioester method to successive segment condensation is described under the title, Protein Synthesis Using *S*-Alkyl Thioesters of Partially Protected Peptide Segments. HU-type DNA-binding protein of *Bacillus stearothermophilus* (HBs) of 90 amino-acid residues was synthesized (*Bull. Chem. Soc. Jpn.*, **65**, 3055-3063 (1992)).

In chapter III, analysis of the chemical characteristics of the thioester moiety on the MBHA resin and the development of the thioester linker with enhanced stability are described. Using the new linker, peptide thioesters were prepared and used to synthesize HBs labelled with ^{13}C, ^{15}N and ^{2}H atoms (*Bull. Chem. Soc. Jpn.*, **66**, 2700-2706 (1993)).

In chapter IV, the synthesis of ^{13}C-labelled barnase, consisting of 110 amino-acid residues with ribonuclease activity, is described as an application of the thioester method (*Bull. Chem. Soc. Jpn.*, **66**, 3004-3008 (1993)).

In chapter V, attempts to extend this method to the synthesis of the cysteine-containing protein are described. Three methods were developed for the preparation of a cysteine-containing peptide thioester. Using peptide segments containing cysteine residue(s), the barnase-like domain of DNA-directed RNA-polymerase II of *Saccharomyces cerevisiae* was synthesized.

References

1) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).

2) H. Yajima and N. Fujii, *J. Chem. Soc., Chem. Commun.*, **1980**, 115.

3) T. Kimura, M. Takai, K. Yoshizawa, and S. Sakakibara, *Biochem. Biophys. Res. Commun.*, **114**, 493 (1983).

4) J. Blake, *Int. J. Peptide Protein Res.*, **17**, 273 (1981).

5) S. Aimoto, N. Mizoguchi, H. Hojo, and S. Yoshimura, *Bull. Chem. Soc. Jpn.*, **62**, 524 (1989).

6) E. T. Kaiser, H. Mihara, G. A. Laforet, J. W. Kelly, L. Walters, M. A. Findeis, and T. Sasaki, *Science*, **243**, 187 (1989).

7) J. M. Sabatier, M. Tessier-Rochat, C. Granier, J. V. Rietschoten, E. Pedrodo, A. Grandas, F. Albericio, and E. Giralt, *Tetrahedron*, **43**, 5973 (1987).

8) B. Riniker, A. Flörsheimer, H. Fretz, and B. Kamber, "Peptides 1992", ed. by C. H. Schneider and A. N. Eberle, ESCOM, Leiden (1993), p. 34.

9) J. Blake, D. Yamashiro, K. Ramasharma, and C. H. Li, *Int. J. Peptide Protein Res.*, **28**, 468 (1986).

10) D. Yamashiro and C. H. Li, *Int. J. Peptide Protein Res.*, **31**, 322 (1988).

11) J. Blake and C. H. Li, *Proc. Natl. Acad. Sci. USA*, **80**, 1556 (1983).

12) J. Blake and C. H. Li, *Proc. Natl. Acad. Sci. USA*, **78**, 4055 (1981).

13) J. Blake, M. Westphal, and C. H. Li, *Int. J. Peptide Protein Res.*, **24**, 498 (1984).

14) J. Blake, *Int. J. Peptide Protein Res.*, **27**, 191 (1986).

Chapter I

Polypeptide Synthesis Using the *S*-Alkyl Thioester of a Partially Protected Peptide Segment

I-1 Introduction

The thioester strategy consists of two steps; the preparation of a partially protected peptide thioester via a solid-phase method and segment condensation in the presence of silver ions in a homogeneous solution.

Thioester is a kind of active esters. Hence, its stability has to be confirmed throughout the preparation processes of partially protected peptide thioesters. Furthermore, the thioester moiety in a peptide segment has to be converted to the corresponding active ester efficiently in the presence of silver ions. All the processes involved in the thioester method were actually examined by synthesizing the DNA-binding domain of *c*-Myb protein, namely *c*-Myb protein(142-193)-NH_2.

In this chapter, the preparation of Boc-Gly-SCH_2CH_2CONH-resin, the synthesis of the *S*-alkyl thioester of a partially protected peptide segment, its chemical characteristics and the coupling of the segments are described and the efficiency of this method is discussed.

I-2 Results and Discussion

The amino acid sequence of the protooncogene product *c*-Myb protein was deduced from cDNA clones of murine *c-myb* mRNA.[1] This protein binds to DNA at a domain near its *N*-terminal region.[2] The binding domain has a 3 tandem repeats of an analogous primary structure. The peptide synthesized was the third part of the repeat corresponding to the amino acids from 142 to 193. The amino acid sequence is shown in

Fig. I-1. Two partially protected peptide segments corresponding to the sequences of 142-163 and 164-193 were prepared and were coupled to form the sequence 142-193.

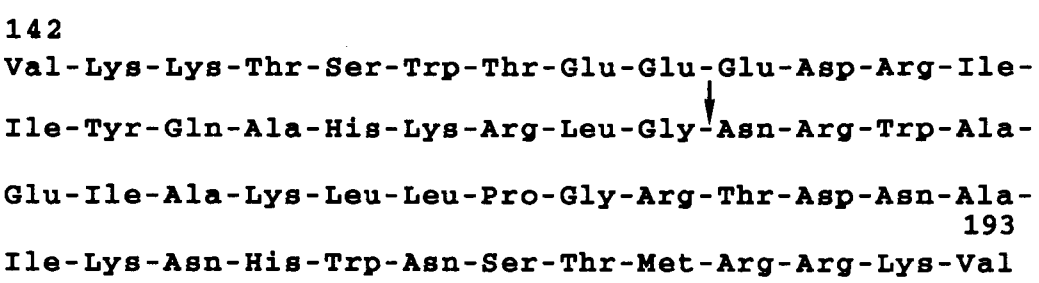


Fig. I-1. Primary sequence of *c*-Myb protein(142-193) deduced from a cDNA clone of murine *c-myb* mRNA.[1] Arrow indicates the site of segment coupling.

Preparation of Boc-Gly-SCH_2CH_2CONH-Resin.

To synthesize a peptide thioester using the solid-phase method, the resin, containing the thioester derivative of Boc-Gly, was prepared in 3 ways as shown in Fig. I-2. These resins were used to prepare the peptide thioester. The resin obtained by method 1 produced fine insoluble material, which blocked the filter at the bottom of the reaction vessel during chain elongation. In method 2, the introduction of an amino acid to the resin

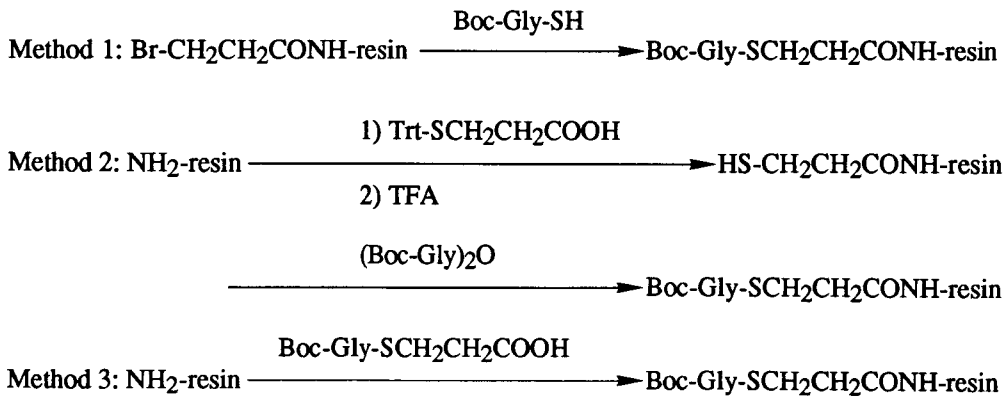


Fig. I-2. Synthetic route of Boc-Gly-SCH_2CH_2CONH-resin.

was not always complete. Unreacted thiol groups might cause undesirable side reactions during elongation of the peptide chain. In contrast, the resin prepared by method 3 was stable during peptide chain elongation by a synthesizer and yielded the product at an acceptable yield. Thus the resin obtained by method 3 was used to prepare the peptide thioester.

Preparation of Boc-[Lys(Boc)^{143,144,160}]-*c*-Myb Protein(142-163)-$SCH_2CH_2CONH_2$ (I-1).

The preparation of Peptide **I-1** is summarized in Fig. I-3. Starting from Boc-Gly-SCH_2CH_2CONH-resin (1.5 g, Gly: 0.50 mmol), a protected *c*-Myb protein(142-163)-SCH_2CH_2CONH-resin was prepared according to the protocol of the system software version 1.40 NMP/HOBt *t*-Boc without any modifications. End capping by acetic anhydride was carried out after each amino-acid introduction reaction. The resin weight increased by 1.4 g. This resin (500 mg from 2.9 g) was treated by anhydrous HF[3] in the presence of *p*-cresol and 1,4-butanedithiol at 0 °C for 90 min. Judging from the measurement of mass number of peptides separated by RPHPLC, the thioester was stable under HF-treatment. The crude peptide was purified by RPHPLC to yield 40 mg (14 μmol) of highly pure peptide **I-5**. The yield was 17% based on the glycine in the starting resin. The yield of peptide **I-5** was about one-half that of peptide amide **I-6** (30%) synthesized on 4-methylbenzhydrylamine (MBHA) resin as described later. This difference could be mainly due to partial aminolysis or hydrolysis of the thioester bond during the chain elongation reaction. The yield of peptide **I-5** was acceptable at the present early stage of the experiment, but further investigation of the chemical design as well as the preparation method of the thioester anchoring group is still required to increase the peptide thioester yield. The thioester in peptide **I-5** proved to be quite stable during purification on RPHPLC or during prolonged storage at 4 °C. The introduction reaction of Boc groups to

peptide **I-5** proceeded almost quantitatively and did not accompany any side reactions when Boc-ONSu was used as the protective reagent. The yield of peptide **I-1** was practically quantitative.

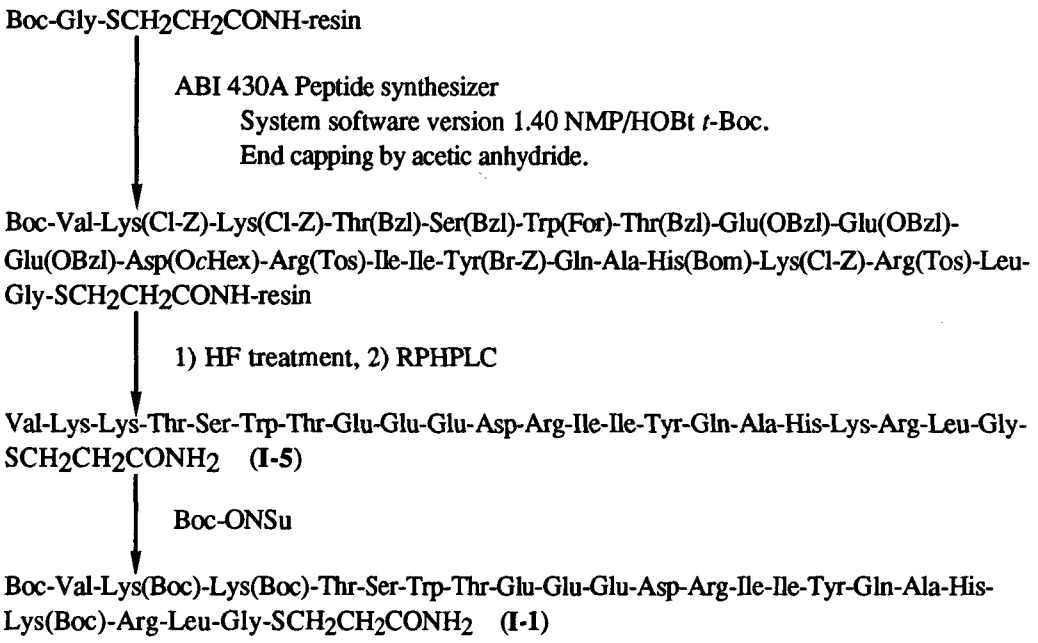


Fig. I-3. Synthetic scheme of Boc-[Lys(Boc)^{143,144,160}]-*c*-Myb protein(142-163)-$SCH_2CH_2CONH_2$ (**I-1**).

Preparation of [Lys(Boc)^{171,182,192}]-*c*-Myb Protein(164-193)-NH_2 (I-2).

The preparation of peptide amide **I-2** was carried out according to the scheme shown in Fig I-4. This procedure was proven to function well in our previous synthesis of BPTI.[4] A peptide chain of *c*-Myb protein(164-193) was assembled on 0.78 g of MBHA resin (NH_2: 0.50 mmol) according to the same protocol described for the synthesis of peptide **I-1**. After the completion of chain assembly, a 2,2,2-trichloroethoxycarbonyl (Troc) group was introduced to protect the terminal amino group using *N*-(2,2,2-trichloroethoxycarbonyloxy)succinimide (Troc-ONSu). The final weight of the protected

peptide resin was 3.2 g. An aliquot of the resin (0.8 g) was treated with HF in the presence of *p*-cresol and 1,4-butanedithiol at 0 °C for 90 min to yield a crude product (580 mg), which was purified on RPHPLC to give 140 mg of highly purified peptide amide **I-6**. The yield was 30% based on the amino group in the starting MBHA resin. Boc groups were introduced to peptide amide **I-6** according to the conditions described for the preparation of peptide **I-1**. The reaction proceeded completely to result in peptide amide **I-7** in a yield of 82%. The Troc group of peptide amide **I-7** was removed by zinc dust treatment in 50% aqueous acetic acid under nitrogen with mild sonication at room temperature for 1 h. Peptide amide **I-2** was obtained in a yield of 65% without any serious side reactions. But it must be noted that the almost total decomposition of peptide amide **I-7** occurred when the

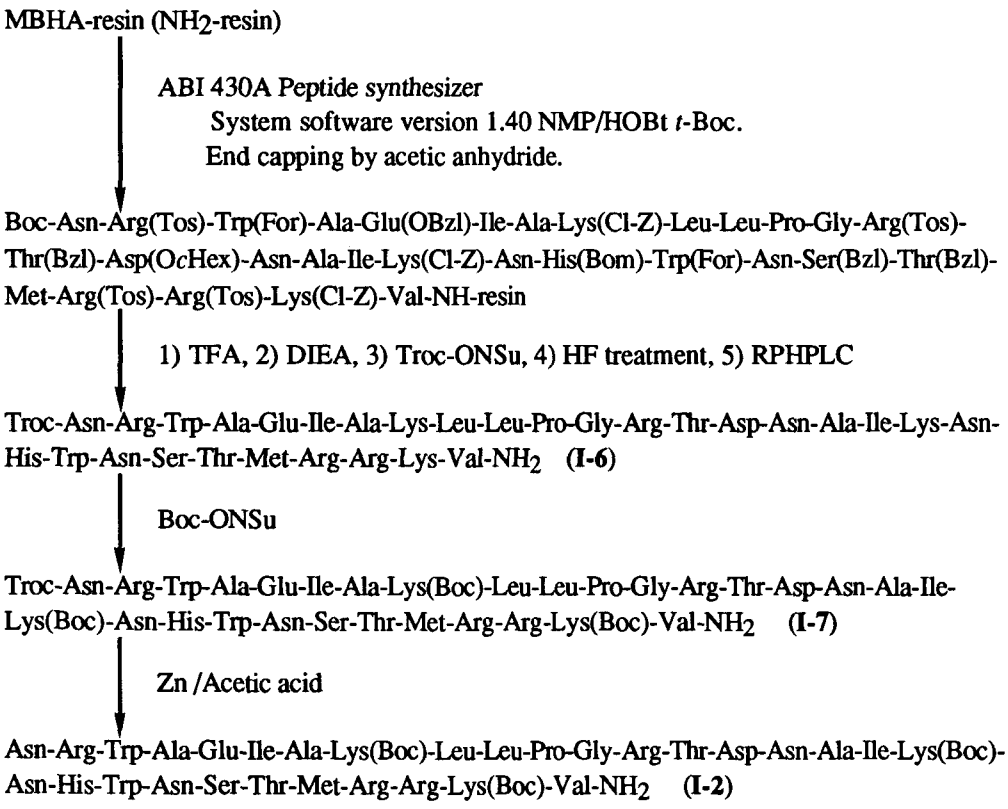


Fig. I-4. Synthetic scheme of [Lys(Boc)^{171,182,192}]-*c*-Myb protein(164-193)-NH_2 (**I-2**).

peptide solution was sonicated for 4 h without complete replacement of the atmosphere with nitrogen.

Preparation of *c*-Myb Protein(142-193)-NH_2 (I-4).

As shown in Fig. I-5 the thioester group in peptide **I-1** was converted to the corresponding active ester in the presence of 4-nitrophenol (HONp) and $AgNO_3$ in DMSO. To the DMSO solution, peptide amide **I-2** and 4-methylmorpholine (NMM) were added. The mixture, where the concentrations of peptides **I-1** and **I-2** were 16 mM and 10 mM, respectively, was stirred at room temperature. The progress of the coupling reaction was monitored by RPHPLC. After 3 d, the condensation reaction was almost complete (Fig. I-6). Silver ions were removed as AgCl. The product was precipitated by addition of ethyl acetate, collected by centrifugation, then suspended in dioxane and freeze-dried. The resultant powder was treated with trifluoroacetic acid (TFA) containing 1,4-butanedithiol at room temperature for 10 min. After removal of TFA, a product was isolated by RPHPLC (Fig. I-7). The yield was 1.1 μmol (50%). This yield is quite high, if the nonspecific adsorption of peptide amide **I-4** during the isolation process, which usually occurs in

Boc-[Lys(Boc)^{143,144,160}]-*c*-Myb protein(142-163)-$SCH_2CH_2CONH_2$ **(I-1)**

↓ $AgNO_3$ + HONp + NMM

Boc-[Lys(Boc)^{143,144,160}]-*c*-Myb protein(142-163)-ONp

[Lys(Boc)^{171,182,192}]-*c*-Myb protein(164-193)-NH_2 **(I-2)**
+ NMM

Boc-[Lys(Boc)^{143,144,160,171,182,192}]-*c*-Myb protein(142-193)-NH_2 **(I-3)**

↓ TFA containing $HS(CH_2)_4SH$ (5% v/v)

c-Myb protein(142-193)-NH_2 **(I-4)**

Fig. I-5. Synthetic route leading to *c*-Myb protein(142-193)-NH_2 (**I-4**) by segment coupling.

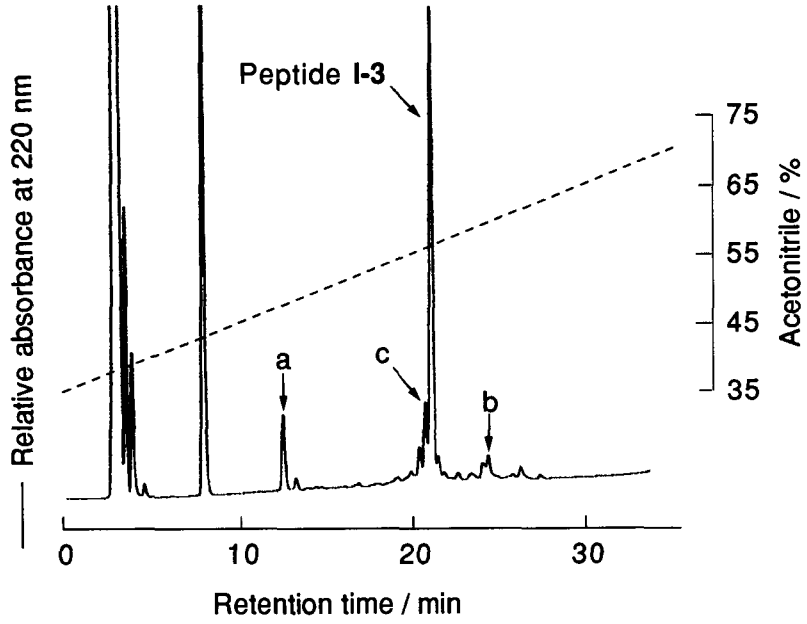


Fig. I-6. RPHPLC profile of the reaction mixture of peptide amide **I-3** after 3-day reaction. Column: Cosmosil $5C_{18}$ at a flow rate of 1 ml min^{-1} at 40 °C. Eluent: aqueous acetonitrile containing 0.1% TFA. Peak a: peptide amide **I-2**. Peak b: active ester of peptide **I-1**. Peak c: hydrolyzed product of peptide **I-1**.

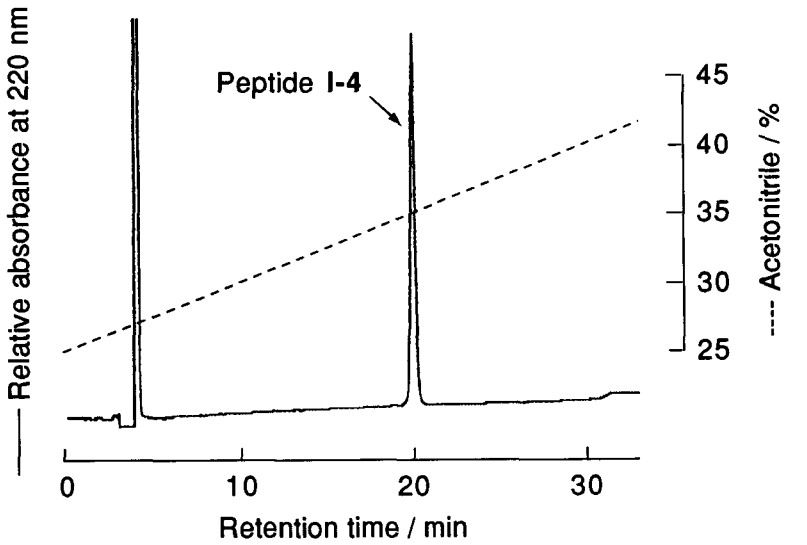


Fig. I-7. RPHPLC profile of synthetic *c*-Myb protein(142-193)-NH_2 (**I-4**) after preparative RPHPLC purification. Column: Cosmosil $5C_{18}$ at a flow rate of 1 ml min^{-1} at 40 °C. Eluent: aqueous acetonitrile containing 0.1% TFA.

peptide synthesis, is taken into account. The *N*-Hydroxysuccinimide ester of peptide **I-1** gave results as good as those of *p*-nitrophenyl ester with regard to the segment condensation reaction between peptides **I-1** and **I-2**. However in this experiment, the author employed *p*-nitrophenyl ester, whose reactivity was lower than that of *N*-hydroxysuccinimide ester but considerably more stable in the presence of moisture. The methionine was not damaged by silver ions under the reaction conditions employed. No other serious side reactions were observed during segment coupling under this minimum protection strategy either.

Confirmation of Synthetic *c*-Myb Protein(142-193)-NH_2 (I-4).

Peptide amide **I-4** was eluted as a symmetrical peak by ion-exchange chromatography (Fig. I-8). Its amino acid composition after RPHPLC purification is shown in Table I-1. These data suggest that highly pure product was obtained from this synthesis. Two-dimensional NMR spectroscopy also confirmed the purity of this peptide.[5)]

Evaluation of the Thioester Method.

Partially protected peptide segments **I-1** and **I-2** were successfully prepared and well characterized by amino acid analysis and fast atom bombardment (FAB) mass spectrometry. The thioester group of peptide **I-1** was activated by silver ions and converted to the corresponding active ester. The terminal carboxyl group was selectively activated by a thioester strategy exactly in the same manner as the thiocarboxyl strategy. As a result, protection of the side-chain carboxyl groups was unnecessary. Only the functional groups that required protection were amino groups in this synthesis. The thioester group did not react with Boc-ONSu though the thiol of the thiocarboxyl group was modified. Therefore, various kinds of protecting groups may be introduced in this way to the side

Table I-1. Amino Acid Composition of *c*-Myb Protein(142-193)-NH_2 (**I-4**)

	Peptide **I-4**	expected
Asp	6.08	6
Thr	3.93	4
Ser	1.78	2
Glu	5.13	5
Pro	0.93	1
Gly	2.15	2
Ala	4	4
Val	2.05	2
Met	1.09	1
Ile	3.19	4
Leu	3.03	3
Tyr	1.01	1
His	2.16	2
Lys	6.11	6
Trp	2.34	3
Arg	6.07	6

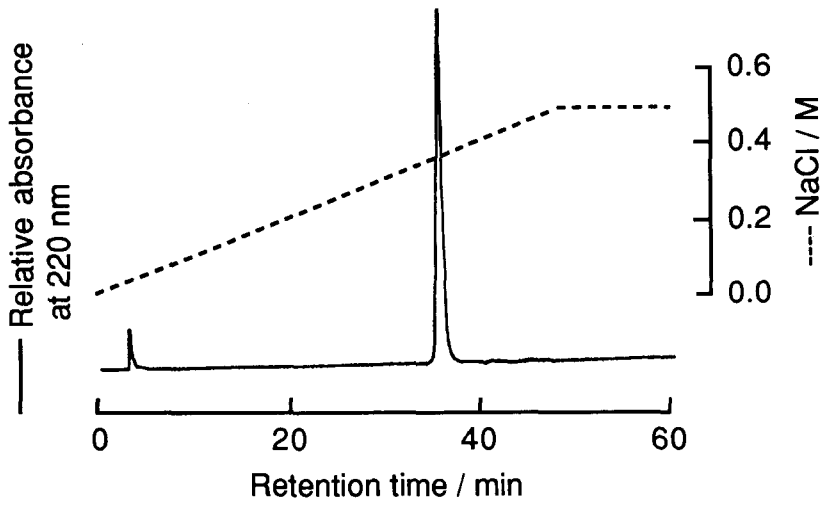


Fig. I-8. Ion-exchange chromatogram of synthetic *c*-Myb protein(142-193)-NH_2 on TSK gel CM-5PW (7.5X75 mm) at a flow rate of 1 ml min^{-1}. Broken line indicates the concentration of NaCl in buffer (pH 8.0).

chain, as well as α-amino groups after purification of a peptide segment on RPHPLC. Protecting groups such as the Boc group increases the solubility of a partially protected peptide segment in DMSO or *N*,*N*-dimethylformamide (DMF) used for the subsequent segment coupling step. This effect is much greater than the citraconyl group only which was used for side-chain amino groups in the thiocarboxyl segment strategy.[6,7]

Only one protecting group existed on peptide **I-5** or **I-6** when purified on RPHPLC. Consequently, purification was much more effectively compared with BPTI synthesis.[4] Therefore long peptide segments such as **I-5**, **I-6** were easily prepared. The yield of segment coupling was also satisfactory.

In conclusion, the partially protected peptide thioester is a promising building block for polypeptide synthesis and thus the thioester strategy is a versatile one.

I-3 Materials and Methods

All of the amino acids used were of the L-configuration, except for glycine. MBHA resin hydrochloride and Boc-amino acid derivatives were purchased from the Peptide Institute Inc. (Minoh, Osaka). Solvents and reagents used for solid-phase peptide synthesis were purchased from Applied Biosystems Japan (Tokyo). DMSO used for segment coupling was silylation grade (Pierce, Rockford, IL). Analytical RPHPLC was performed on Cosmosil $5C_{18}$-AR (4.6X250 mm) (Nacalai Tesque, Kyoto) and preparative RPHPLC was on YMC-Pack ODS-AM (10X250 mm or 25X250 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 ^{o}C for 24 h in an evacuated sealed tube. Peptide mass number was determined by FAB mass spectrometer using a JMS-HX100 (JEOL Ltd., Tokyo) equipped with a JMA-3100 mass data system. Peptide weight was calculated based upon the amino acid analysis data. Ultrasonication was carried out using a Branson Model B-220. Boc-Gly-SH was prepared according to the reference.[8]

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.) employing the 0.5 mmol scale standard protocol of the benzotriazole active ester method of the system software version 1.40 NMP/HOBt *t*-Boc. End capping by acetic anhydride was performed after each amino-acid introduction reaction.

Preparation of Trt-SCH_2CH_2COOH.

To a solution of triphenylmethyl (Trt) chloride (25 g, 90 mmol) in DMF (100 ml), 3-mercaptopropionic acid (7.5 ml, 86 mmol) was added. After the reaction mixture was stirred overnight, 10% sodium acetate was added. The precipitate formed was recrystallized from ethyl acetate to give Trt-SCH_2CH_2COOH (19 g, 55 mmol, 63%), mp 207.5-208.0 °C. Found: H, 5.80; C, 75.78%. Calcd for $C_{22}H_{20}O_2S$: H, 5.79; C, 75.83%.

Preparation of Boc-Gly-SCH_2CH_2COOH.

To a solution of Boc-Gly-ONp (1.5 g, 5 mmol) dissolved in DMF (50 ml), 3-mercaptopropionic acid (0.5 g, 5 mmol) and *N,N*-diisopropylethylamine (DIEA) (1.0 g, 7.5 mmol) were added with stirring at room temperature for 15 h. After evaporation of the solvent under reduced pressure, the product was dissolved in ethyl acetate. The ethyl acetate layer was washed with 0.1 M HCl (X2) and water saturated with NaCl (X5) and dried over sodium sulfate. The ethyl acetate solution was concentrated. An oil obtained was dissolved in ether. To the ethereal solution cyclohexylamine (CHA) (450 mg, 4.5 mmol) was added to give crystals, which were recrystallized from hot ethyl acetate: Boc-Gly-SCH_2CH_2COOH·CHA; 1.7 g, 94%, mp 122.5-123.2 °C. This CHA salt (1.7 g) was suspended in ethyl acetate and CHA was extracted by dilute aqueous citric acid. The

organic layer was washed with water saturated with NaCl (X3) and dried over sodium sulfate. After concentration of the solution, hexane was added to give Boc-Gly-SCH_2CH_2COOH (1.1 g, 92%), mp 104-106 °C. Found: H, 6.60; C, 45.49; N, 5.40%. Calcd for $C_{10}H_{17}O_5NS$: H, 6.51; C, 45.61; N, 5.32%.

Preparation of Boc-Gly-SCH_2CH_2CONH-Resin.

Method 1: MBHA resin hydrochloride (740 mg, NH_2: 0.50 meq) was washed with 5% DIEA/dichloromethane (DCM) (v/v, 5 minX2) and DCM (1 minX3), successively. Br-CH_2CH_2COOH (310 mg, 2.0 mmol) dissolved in DCM and a 0.5 M solution of DCC in DCM (4 ml) were successively added to the reaction vessel, which was shaken overnight. After washing, the resin was mixed with Boc-Gly-SH (290 mg, 1.5 mmol) in DMF in the presence of DIEA (400 µl) overnight to give Boc-Gly-SCH_2CH_2CONH-resin (800 mg, Gly: 0.42 mmol).

Method 2: MBHA resin hydrochloride (1.0 g, NH_2: 0.64 meq) was washed with 5% DIEA/DCM (v/v, 5 minX2) and DCM (1 minX3), successively. The solution of Trt-SCH_2CH_2COOH (520 mg, 1.5 mmol) in DMF and 0.5 M DCC in DCM (3.0 ml) and 0.5 M 1-hydroxybenzotriazole (HOBt) in DMF (3.0 ml) was reacted with the resin for 1 h (X2). The resin thus obtained was treated with 50% TFA in DCM (v/v) for 5 and 15 min, followed by neutralization with 5% DIEA in DMF (v/v). $(Boc\text{-}Gly)_2O$, prepared by Boc-Gly (350 mg, 2 mmol) and 0.5 M DCC in DCM (4 ml), was added to the resin and shaken for 1 h (X2) to give Boc-Gly-SCH_2CH_2CONH-resin (1.1 g, Gly: 0.36 mmol).

Method 3: MBHA resin hydrochloride (3.4 g, NH_2: 1.6 meq) was washed with 5% DIEA/DCM (v/v, 5 minX2) and DCM (1 minX3), successively. Boc-Gly-SCH_2CH_2-COOH (720 mg, 2.7 mmol) dissolved in 20 ml of DCM and a 0.5 M solution of DCC in

DCM (4 ml) were successively added to the reaction vessel, which was shaken overnight. The resin obtained was treated with 5% acetic anhydride in DCM (v/v, 5 minX2) and dried after washing the resin with 2-propanol (2 minX3) and DCM (1 minX3) to give 3.8 g of a resin (Gly: 1.3 mmol).

c-Myb Protein(142-163)-$SCH_2CH_2CONH_2$ (I-5).

Starting from Boc-Gly-SCH_2CH_2CONH-resin (1.5 g), peptide chain elongation was carried out by a 430A peptide synthesizer to give 2.9 g of a protected peptide resin. An aliquot of the resin (500 mg) was treated with anhydrous HF (11 ml) containing *p*-cresol (0.5 ml) and 1,4-butanedithiol (1.7 ml) at 0 °C for 90 min. After evaporation of HF, the residual solid was washed with ether (X3). Crude peptide was extracted with 10% aqueous acetic acid and freeze-dried to give a powder (190 mg). This crude product was purified on RPHPLC to give peptide **I-5** (40 mg, 14 μmol, 17% based on Gly in the starting resin). Found m/z 2774.9 $(M+H)^+$. Calcd m/z 2774.6 $(M+H)^+$. Amino acid analysis of peptide **I-5**: $Asp_{1.04}Thr_{1.94}Ser_{0.92}Glu_{4.27}Gly_{1.04}Ala_1Val_{1.02}Ile_{1.23}Leu_{0.97}Tyr_{0.92}His_{1.08}Lys_{2.85}Trp_{0.65}Arg_{1.89}$.

Boc-[Lys(Boc)^{143,144,160}]-c-Myb Protein(142-163)-$SCH_2CH_2CONH_2$ (I-1).

Peptide **I-5** (25 mg, 9.0 μmol) was dissolved in DMSO (0.4 ml) containing triethylamine (TEA) (9.6 μl). Boc-ONSu (15 mg, 72 μmol) was added to the solution and stirred at room temperature for 2.5 h, after which ethyl acetate and ether were added. The precipitate was washed with ethyl acetate and lyophilized from a dioxane suspension to give peptide **I-1** (26 mg, 90%). Found m/z 3174.8 $(M+H)^+$. Calcd m/z 3174.8 $(M+H)^+$. Amino acid analysis of peptide **I-1**: $Asp_{1.00}Thr_{1.98}Ser_{0.90}Glu_{4.39}Gly_{1.07}Ala_1Val_{0.97}Ile_{1.25}Leu_{0.97}Tyr_{0.93}His_{1.08}Lys_{2.82}Trp_{0.63}Arg_{1.84}$.

Troc-*c*-Myb Protein(164-193)-NH_2 (I-6).

Starting from MBHA resin hydrochloride (0.78 g, NH_2: 0.50 meq), chain elongation reaction was carried out. After completion of a peptide chain elongation cycle, the peptide resin was treated with 55% TFA in DCM (v/v) for 5 and then 15 min, followed by neutralization with 5% DIEA in DCM (v/v) for 5 min twice. Troc-ONSu (440 mg, 1.5 mmol) was allowed to react with the terminal amino group at room temperature for 15 h. The peptide resin thus obtained was 3.2 g. An aliquot of the resin (0.8 g) was treated with anhydrous HF (17 ml) containing *p*-cresol (0.8 ml) and 1,4-butanedithiol (2.7 ml) at 0 °C for 90 min. After evaporation of HF, the residual solid was washed with ethyl acetate (X3) and with ether (X3). The peptide amide was extracted with 5% aqueous acetic acid and lyophilized to give a crude product (580 mg). This peptide was purified on RPHPLC to obtain peptide amide **I-6** (140 mg, 37 μmol, yield: 30% based on the amino groups in MBHA resin). Found m/z 3749.3 $(M+H)^+$. Calcd m/z 3749.1 $(M+H)^+$. Amino acid analysis of peptide amide **I-6**: $Asp_{5.10}Thr_{1.93}Ser_{0.79}Glu_{1.29}Pro_{0.75}Gly_{1.12}Ala_3Val_{1.10}$ $Met_{1.01}Ile_{1.94}Leu_{1.94}His_{1.07}Lys_{3.06}Trp_{1.75}Arg_{3.78}$.

Troc-[Lys(Boc)^{171,182,192}]-*c*-Myb Protein(164-193)-NH_2 (I-7).

Peptide amide **I-6** (24 mg, 6.5 μmol) was dissolved in DMSO (200 μl) containing TEA (7.8 μl) and Boc-ONSu (13 mg, 60 μmol) and stirred for 2.5 h. Peptide amide **I-7** was obtained according to the procedure of the preparation of peptide **I-1**: Yield 21 mg, 5.2 μmol, 80%. Found m/z 4050.0 $(M+H)^+$. Calcd m/z 4049.3 $(M+H)^+$. Amino acid analysis of peptide amide **I-7**: $Asp_{5.16}Thr_{1.93}Ser_{0.87}Glu_{1.15}Pro_{0.91}Gly_{1.09}Ala_3Val_{1.03}$ $Met_{1.12}Ile_{2.01}Leu_{1.97}His_{1.07}Lys_{2.85}Trp_{1.30}Arg_{3.81}$.

[Lys(Boc)^{171,182,192}]-*c*-Myb Protein(164-193)-NH_2 (I-2).

Peptide amide **I-7** (40 mg) was dissolved in a mixture of acetic acid (1 ml) and water (1 ml). Zinc (100 mg) was added under a nitrogen atmosphere and the solution was sonicated for 1 h at room temperature. After centrifugation of the reaction mixture, the supernatant was applied to an ODS column. The isolated product was freeze-dried to give peptide amide **I-2**: Yield 25 mg, 6.4 μmol, 65%. Found m/z 3875.5 $(M+H)^+$. Calcd m/z 3875.3 $(M+H)^+$. Amino acid analysis of peptide amide **I-2**: $Asp_{5.23}Thr_{1.95}Ser_{0.90}Glu_{1.37}Pro_{0.78}Gly_{1.25}Ala_3Val_{1.06}Met_{1.15}Ile_{2.05}Leu_{1.99}His_{1.12}Lys_{3.00}Trp_{1.39}Arg_{3.82}$.

_c_-Myb Protein(142-193)-NH_2 (I-4).

Peptide **I-1** (12 mg, 3.6 μmol) was dissolved in DMSO (220 μl) containing HONp (6.5 mg, 47 μmol) and NMM (0.3 μl, 2.7 μmol). $AgNO_3$ (2.4 mg, 14 μmol) was added and the solution was stirred at room temperature in the dark. After 2 h, peptide amide **I-2** (8.6 mg, 2.2 μmol) and NMM (0.6 μl, 5.5 μmol) were added. After stirring overnight, NMM (0.3 μl, 2.7 μmol) was added and the solution was stirred for 3 d. To the solution, solid NaCl (1.2 mg) was added. The precipitate formed was removed by centrifugation. Ethyl acetate was added to the supernatant. The precipitate formed was collected by centrifugation and washed with ethyl acetate. The peptide obtained was suspended in dioxane and freeze-dried to give a crude product of peptide amide **I-3**, which was treated with TFA (200 μl) containing 1,4-butanedithiol (10 μl) at room temperature for 10 min. TFA was removed by N_2 air flush. The residual solid was washed with dry ether (X2) and isolated by RPHPLC to give peptide amide **I-4** (6.9 mg, 1.1 μmol); yield: 50% based on peptide amide **I-2** used for coupling reaction. Amino acid analysis of peptide amide **I-4**: $Asp_{6.08}Thr_{3.93}Ser_{1.78}Glu_{5.13}Pro_{0.93}Gly_{2.15}Ala_4Val_{2.05}Met_{1.09}Ile_{3.19}Leu_{3.03}Tyr_{1.01}His_{2.16}Lys_{6.11}Trp_{2.34}Arg_{6.07}$.

Purity Check by Ion-Exchange Chromatography.

Peptide amide **I-4** purified on RPHPLC was subjected to a TSK gel CM-5PW column (TOSOH, Tokyo) (7.5X75 mm) equilibrated with 0.05 M sodium phosphate buffer (pH 8.0) and chromatographed by a linear 0 - 0.5 M NaCl gradient in buffer over 50 min at a flow rate of 1 ml min^{-1}.

References

1) T. J. Gonda, N. M. Gough, A. R. Dunn, and J. de Blaquiere, *EMBO J.*, **4**, 2003 (1985).

2) K-H. Klempnauer and A. E. Sippel, *EMBO J.*, **6**, 2719 (1987).

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

4) S. Aimoto, N. Mizoguchi, H. Hojo, and S. Yoshimura, *Bull. Chem. Soc. Jpn.*, **62**, 524 (1989).

5) K. Ogata, H. Hojo, S. Aimoto, T. Nakai, H. Nakamura, A. Sarai, S. Ishii, and Y. Nishimura, *Proc. Natl. Acad. Sci., USA*, **89**, 6428 (1992).

6) J. Blake, *Int. J. Peptide Protein Res.*, **27**, 191 (1986).

7) J. Blake, D. Yamashiro, K. Ramasharma, and C. H. Li, *Int. J. Peptide Protein Res.*, **28**, 468 (1986).

8) J. Blake and C. H. Li, *Proc. Natl. Acad. Sci. USA*, **78**, 4055 (1981).

Chapter II

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

II-1 Introduction

A partially protected peptide thioester was found to be a promising building block for polypeptide synthesis. To expand the thioester method to the synthesis of a protein, several peptide segments must be successively condensed. This could be realized if the terminal amino protecting group can be selectively removed after segment condensation. We protected the terminal amino group with an 4-pyridylmethoxycarbonyl (*i*Noc) group.[1] This protecting group is selectively removed by zinc dust in aqueous acetic acid, even if there are Boc groups for the side-chain amino protection. In order to examine the usefulness of this strategy, HU-type DNA-binding protein (HBs) of *Bacillus stearothermophilus*, consisting of 90 amino acids (Fig. II-1), was synthesized.

In this chapter, the preparation of partially protected peptide thioesters, the successive coupling of partially protected peptide segments and characterization of the final product of synthetic HBs(1-90) are discussed; the efficiency of this process in protein synthesis is also discussed.

II-2 Results and Discussion

Preparation of Peptide Segments.

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.[2]

This deuterium-labelled methyl group will be used as a marker of synthetic HBs(1-90) as well as a probe for study of the HBs-DNA interaction.

HBs was divided into four segments. The three arrows in Fig. II-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.

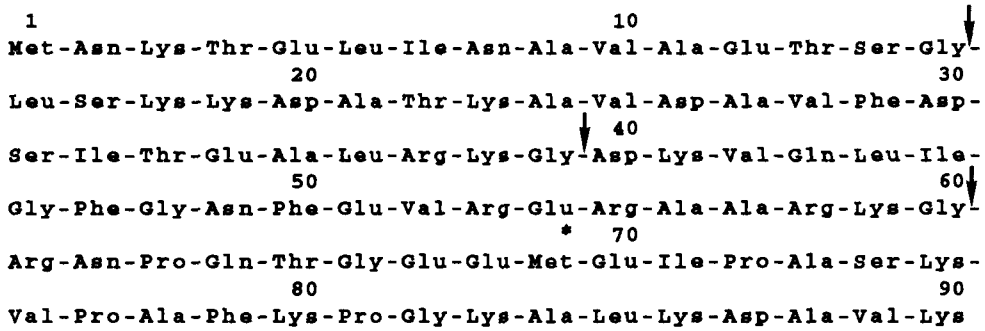


Fig. II-1. The amino acid sequence of HBs. The arrows indicate the sites of segment coupling. Met* indicates (*methyl*-$^2H_3$)methionine.

A partially protected peptide thioester was prepared by the same procedure as described in a previous chapter. The terminal amino groups of peptides **II-2**, **II-3**, and **II-4** were blocked with an *i*Noc group instead of the 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.II-2.

Starting from Boc-Gly-SCH_2CH_2CONH-resin (0.97 g, Gly: 480 μmol), which was prepared from Boc-Gly-SCH_2CH_2COOH and 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

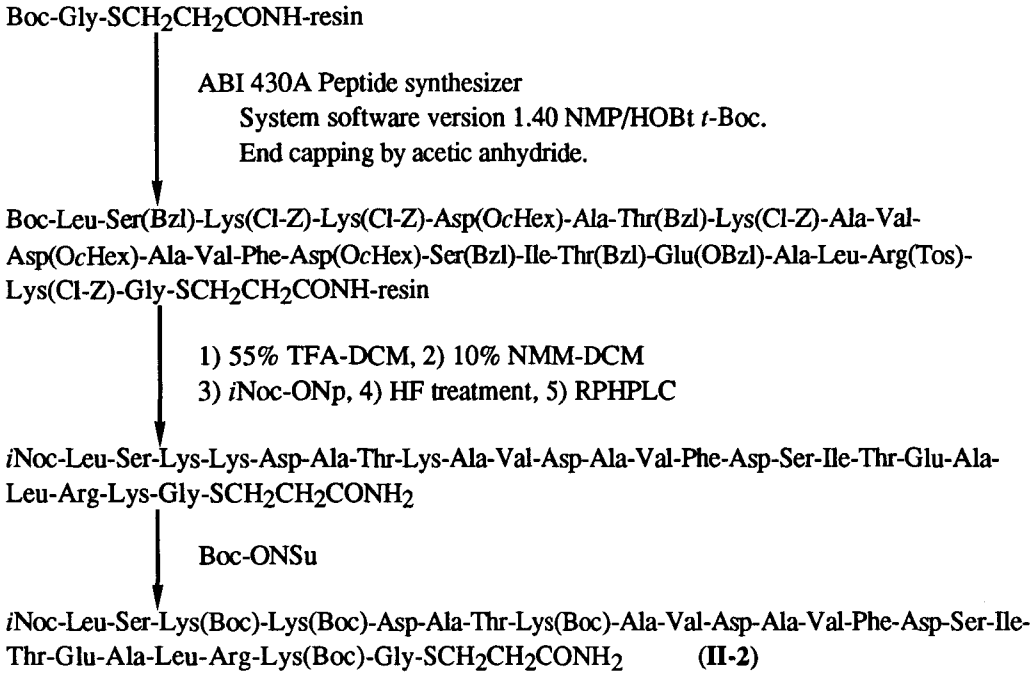


Fig. II-2. Synthetic scheme of *i*Noc-[Lys(Boc)^{18,19,23,38}]-HBs(16-39)-$SCH_2CH_2CONH_2$ (**II-2**).

the chain assembly, an *i*Noc group was introduced to block the terminal amino group. The weight of the protected peptide resin was 1.5 g, the whole of which was treated by anhydrous HF containing 10% *p*-cresol (v/v) at 0 ^{o}C for 90 min. The crude peptide (510 mg) was highly soluble in aqueous acetonitrile and easily purified by RPHPLC to give *i*Noc-HBs(16-39)-$SCH_2CH_2CONH_2$ (220 mg) in a yield of 11%, based on the glycine residue in the starting resin. This peptide was characterized by FAB mass spectrometry and amino acid analysis after acid hydrolysis. To the side-chain amino groups of the peptide, Boc groups were introduced using Boc-ONSu in DMSO to yield *i*Noc-[Lys(Boc)^{18,19,23,38}]-HBs(16-39)-$SCH_2CH_2CONH_2$ (**II-2**) in 91%. The progress of the reaction was readily monitored by RPHPLC and FAB mass measurements.

The other partially protected peptide segments were obtained in the same manner as listed in Table II-1. Each partially protected peptide segment was obtained from a 4.5 to a

Table II-1. Partially Protected Peptide Segments Prepared for HBs(1-90) Synthesis

Peptide segments		Yield/%[a]
Boc-[Lys(Boc)^{3}]-HBs(1-15)-$SCH_2CH_2CONH_2$	**(II-1)**	14
Boc-Met-Asn-Lys(Boc)-Thr-Glu-Leu-Ile-Asn-Ala-Val-Ala-Glu-Thr-Ser-Gly-$SCH_2CH_2CONH_2$		
*i*Noc-[Lys(Boc)^{18,19,23,38}]-HBs(16-39)-$SCH_2CH_2CONH_2$	**(II-2)**	10
*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)^{41,59}]-HBs(40-60)-$SCH_2CH_2CONH_2$	**(II-3)**	17
*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-$SCH_2CH_2CONH_2$		
[Lys(Boc)^{75,80,83,86,90}, (*methyl*-$^{2}H_3$)Met^{69}]-HBs(61-90)	**(II-4)**	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)		
Boc-[Lys(Boc)^{3,18,19,23,38}]-HBs(1-39)-$SCH_2CH_2CONH_2$	**(II-5)**	4.5
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-$SCH_2CH_2CONH_2$		

a) The yield was calculated based on the content of the carboxyl terminal amino acid in the resin.

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 practically advantageous procedure. The author 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 **II-1**, **II-2**, **II-3**, and **II-4** according to the scheme shown in Fig. II-3. Segment condensation was carried out at a peptide concentration of about 2 to 10 mM in the presence of 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. The product was isolated by RPHPLC after every coupling reaction. Before isolation, the Boc groups in the side-chain amino groups were removed using 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 easily isolated by

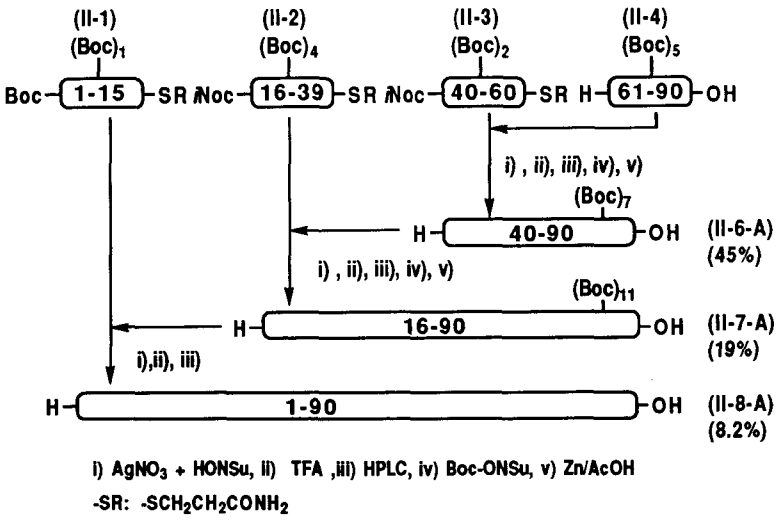


Fig. II-3. Synthetic route of HBs(1-90) by segment coupling.

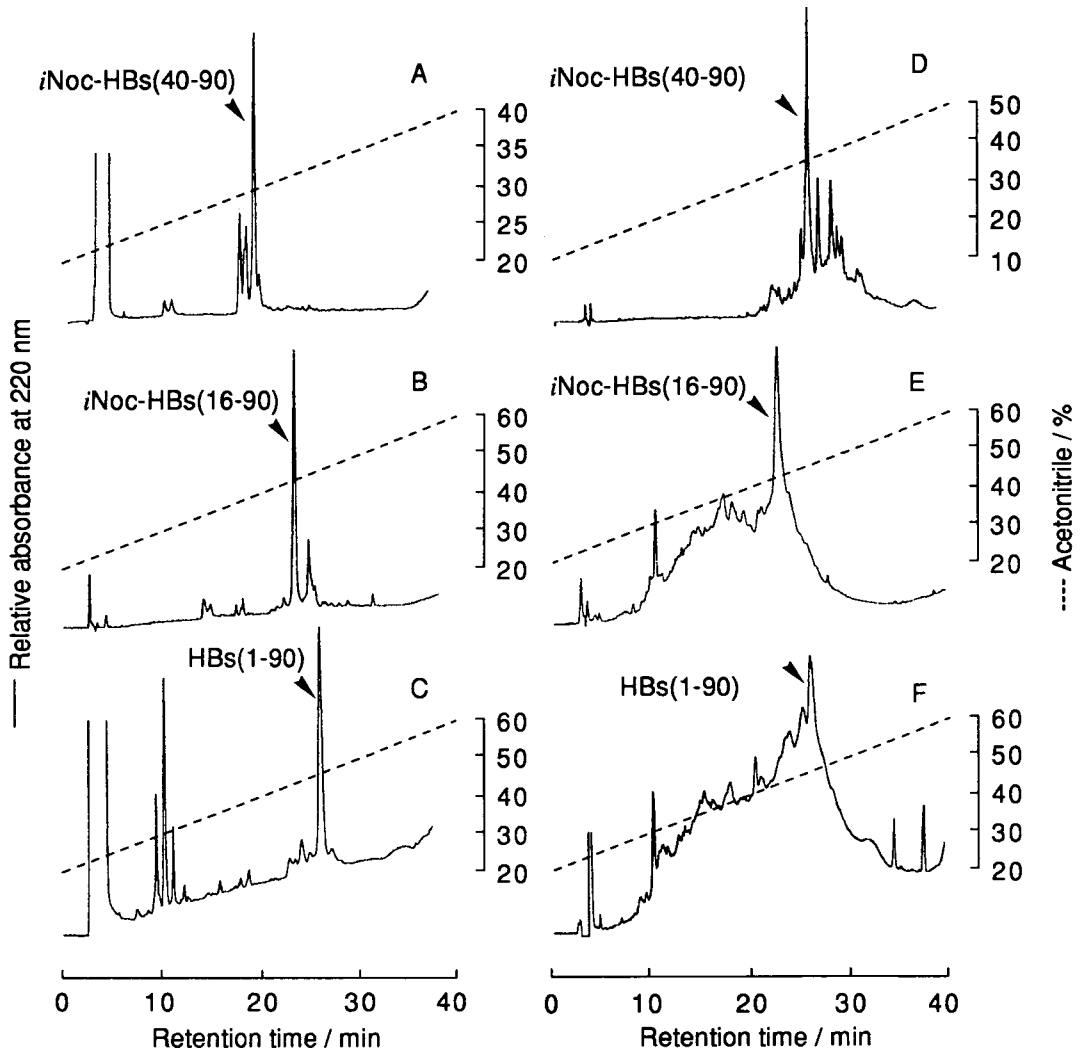


Fig. II-4. RPHPLC elution profiles of the TFA treated reaction mixtures of the segment coupling according to procedure A (Panels A, B, C) and the crude products obtained by an all stepwise solid-phase synthesis after HF treatment (D, E, F); panel A: the reaction mixture for the preparation for *i*Noc-HBs(40-90) (**II-6**), panel B: the reaction mixture on the preparation of *i*Noc-HBs(16-90) (**II-7**), panel C: the reaction mixture on the preparation for HBs(1-90) (**II-8-A**). Column: Cosmosil $5C_{18}$-AR (4.6X250 mm) at a flow rate of 1 ml min^{-1} at 40 ^{o}C. Eluent: aqueous acetonitrile containing 0.1% TFA.

RPHPLC. The elution profile of each reaction mixture is shown in Fig. II-4A, 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 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) (**II-8-A**) after RPHPLC purification was 8.2% based on peptide **II-4**.

Procedure B: In this synthesis segment coupling was carried out practically under the same conditions as in procedure A. However, none of the products were isolated after each segment condensation. Removal and reintroduction of Boc groups were hence 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. II-5. Furthermore, the yield of HBs(1-90) was improved to 16% from 8.2% as obtained in procedure A. HBs(1-90) (**II-8-B**), thus obtained, was as pure as that from procedure A.

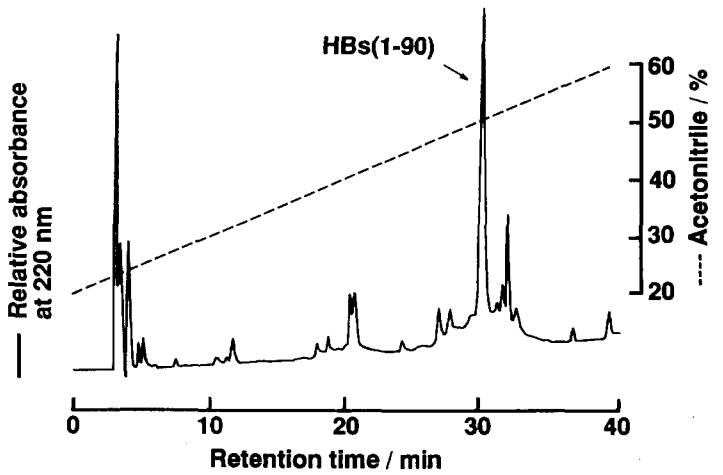


Fig. II-5. RPHPLC profile of the crude product of HBs(1-90) (**II-8-B**).

Procedure C: Peptide **II-5** with 39 amino acid residues and peptide **II-6-A** with 51 amino acid residues were condensed using a 7-fold molar excess of peptide **II-5** to peptide **II-6-A** (4.2 mM) in the presence of $AgNO_3$ (40 mM) and NMM (72 mM) at room temperature for 3 d. HBs(1-90) (**II-8-C**) was obtained in a yield of 33% based on peptide **II-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 of two large peptide segments containing as many as 39 and 51 amino acid residues, respectively, by the present thioester strategy.

Preparation of HBs(1-90) by All Stepwise Method.

HBs(1-90) was synthesized by a conventional all stepwise solid-phase method to compare the efficiency between segment condensation and stepwise solid-phase methods. The elution profiles of the peptides obtained from the all stepwise method is shown in Fig. II-4D, E, and F. As judged from comparison of 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 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 ^1H NMR spectrum of the aromatic protons of phenylalanine residues. However, when unfolded synthetic HBs(1-90) was dissolved in a 50 mM sodium phosphate buffer (pH 7.0), the peptide spontaneously assumed the native structure, as judged from the ^1H NMR spectrum. RPHPLC-purified HBs(1-90), (peptide **II-8-A** or **II-8-B**) was thus converted to the native form by dissolving in this buffer; the

peptide was further purified by ion-exchange chromatography as shown in Fig. II-6. The synthetic HBs(1-90), thus obtained, showed identical ^1H NMR spectra to that of HBs extracted from *B. stearothermophilus*, except for (*methyl*-$^2H_3$)Met^{69}, as shown in Fig. II-7. The difference in the ^1H NMR spectra between native HBs and synthetic HBs(1-90) is shown in the small box in Fig. II-7. Comparing both ^1H 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 ^1H NMR spectroscopic study of the interaction between HBs(1-90) and DNA. Peptides **II-8-A** and **II-8-B** had practically the same elution profile and gave the same quality of synthetic HBs(1-90) (**II-8**) in yields of 50 and 45% based on peptides **II-8-A** and **II-8-B**, respectively. Peptide **II-8-C** also gave the same elution profile as those of peptides **II-8-A** and **II-8-B**. HBs(1-90) (**II-8**) was obtained in a yield of 50% based on peptide **II-8-C**.

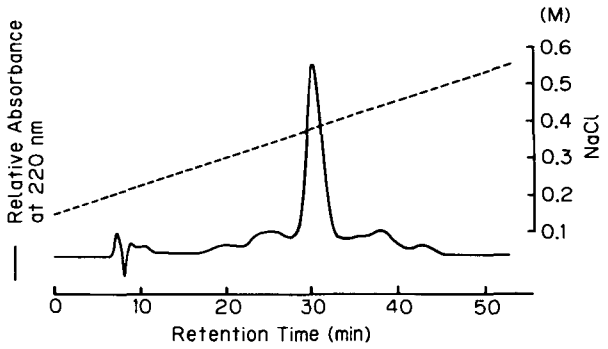


Fig. II-6. Ion-exchange chromatogram of HBs(1-90) (**II-8-A**) isolated by RPHPLC after equilibration in a sodium phosphate buffer on Pharmacia HiLoad S-Sepharose HP at a flow rate of 2.5 ml min^{-1}. The broken line indicates the NaCl concentration in a 0.05 M sodium phosphate buffer (pH 7.0).

Evaluation of the Thioester Method for Protein Synthesis.

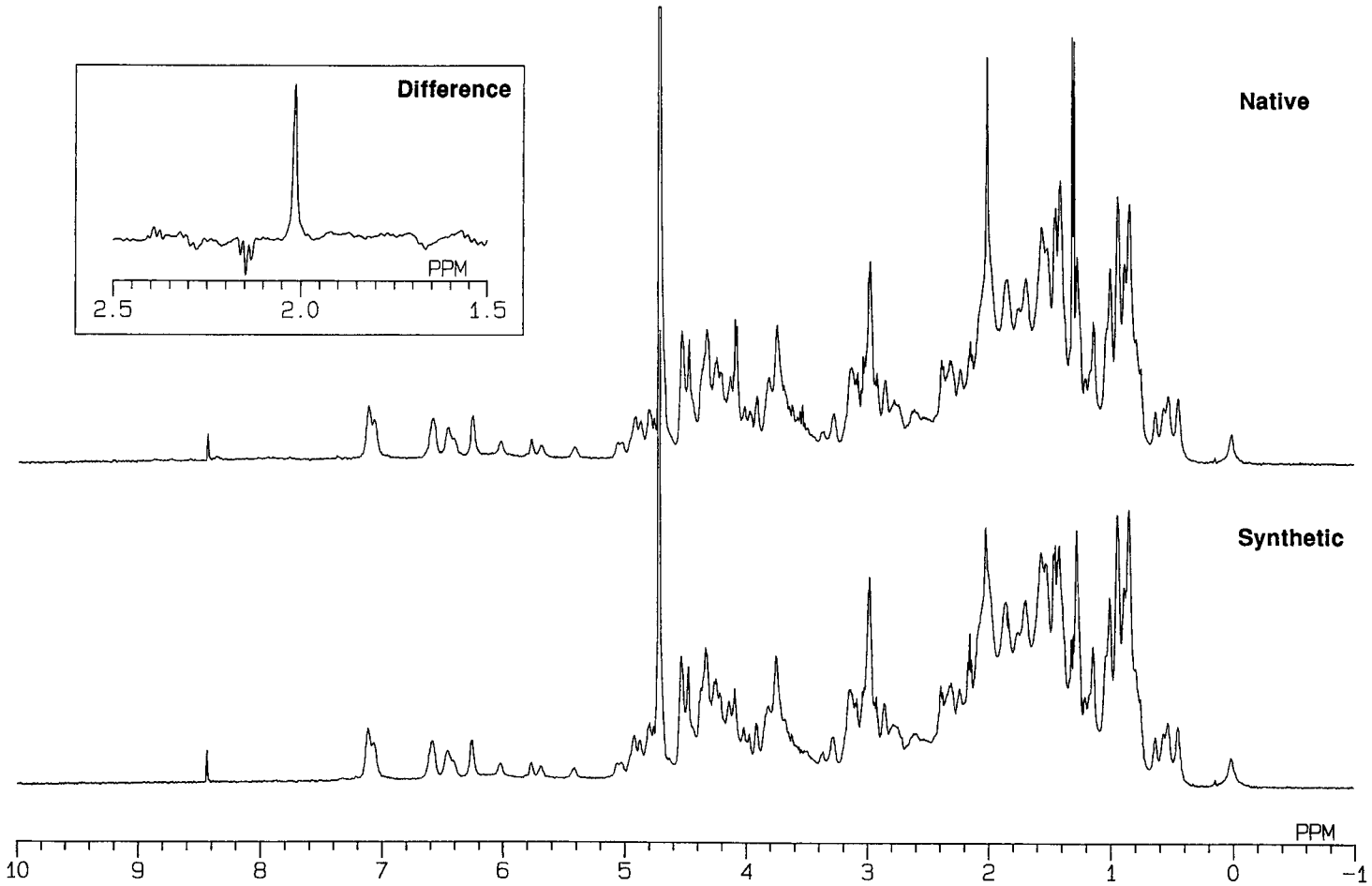


Fig. II-7. 500 MHz ^{1}H NMR spectra of native and synthetic HBs.

All of the partially protected peptide thioesters (peptides **II-1**, **II-2**, **II-3**, and **II-5**) were similarly prepared according to the method described in the previous chapter. 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 to improve the effectiveness of the procedure. 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 an authentic material nor a specific feature such as enzymatic activity for the identification, can be synthesized using this method. A partially protected peptide thioester was thus proved to be a useful building block for protein synthesis by the use of the present procedure. On the contrary, the product obtained by the conventional all stepwise solid-phase method gave a broad peak (Fig II-4F). It was difficult to distinguish the fraction that contained HBs(1-90).

II-3 Materials and Methods

Boc-Lys(Cl-Z)-OCH_2-$C_6H_4CH_2CONHCH_2$-C_6H_4-resin (Boc-Lys(Cl-Z)-OCH_2-PAM-resin)[3] was purchased from Applied Biosystems Inc. (Foster City, CA.). Analytical RPHPLC was performed on YMC-Pack C_4 (4.6X250 mm) (YMC, Kyoto) or Cosmosil 5C_{18}-AR (4.6X250 mm) (Nacalai Tesque, Kyoto) and preparative RPHPLC was on YMC-Pack ODS-AM (10X250 mm or 20X250 mm) (YMC, Kyoto). Though the

peptide weight was an observed value, the yield was calculated based upon the amino acid analysis data. Dialysis was carried out using a Spectrapor 6 membrane (M. W. cut off 1000).

Peptide Chain Elongation on a Solid Support.

Solid-phase synthesis of a peptide segment was carried out according to the procedure described in the preceding chapter.

Boc-[Lys(Boc)^3]-HBs(1-15)-$SCH_2CH_2CONH_2$ (II-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_2CONH-resin was prepared from Boc-Gly-SCH_2CH_2CONH-resin (1.2 g, Gly: 310 μmol). The *N*-terminal Boc group was removed by 55% TFA in DCM (v/v) for 5 and 15 min. A protected peptide resin (1.6 g) was obtained. An aliquot of the resin (1.5 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 (X2) and ethyl acetate (X2). 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 μmol, 15% based on Gly in the starting resin) 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 mg, 35 μmol) dissolved in DMSO (1.0 ml) was reacted with Boc-ONSu (49 mg, 230 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 100 mg of peptide **II-1**

(33 μmol, 14% based on Gly in the starting resin). Found: m/z 1864.6 $(M+H)^+$. Calcd: m/z 1864.9 $(M+H)^+$. Amino acid analysis of peptide **II-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)-$SCH_2CH_2CONH_2$ (II-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_2-CH_2CONH-resin (0.97 g, Gly: 480 μmol). After removing the *N*-terminal Boc group by treatment with 55% TFA in DCM (v/v) for 5 and 15 min followed by neutralization by 5% DIEA in DMF (v/v), the peptide resin was treated with 4-pyridylmethyl *p*-nitrophenyl carbonate (*i*Noc-ONp) (410 mg, 1.5 mmol) in DMF overnight to give 1.5 g of protected peptide resin. All of the resin was treated with HF (15 ml) containing 10% *p*-cresol (v/v) at 0 °C for 90 min to give 510 mg of a crude product. This product was purified on RPHPLC to obtain 220 mg (53 μmol, 11% based on Gly in the starting resin) 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)-$SCH_2CH_2CONH_2$ (220 mg, 53 μmol) dissolved in DMSO (2.3 ml), Boc-ONSu (130 mg, 620 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 200 mg of peptide **II-2** (48 μmol, 10% based on Gly in the starting resin). Found: m/z 3186.3 $(M+H)^+$. Calcd: m/z 3185.7 $(M+H)^+$. Amino acid analysis of peptide **II-2**: $Asp_{3.04}Thr_{1.84}Ser_{1.81}Glu_{1.19}Gly_{1.11}Ala_4Val_{1.93}Ile_{0.97}Leu_{1.81}Phe_{1.03}Lys_{3.83}Arg_{1.03}$.

iNoc-[Lys(Boc)^{41,59}]-HBs(40-60)-$SCH_2CH_2CONH_2$ (II-3).

iNoc-Asp(OcHex)-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 (2.2 g) was prepared from Boc-Gly-SCH_2CH_2CONH-resin (1.3 g, Gly: 300 μmol) as described for the synthesis of peptide **II-2**. An aliquot of the resin (2.0 g) was treated with HF (20 ml) containing 15% anisole (v/v) at 0 °C for 90 min to give 620 mg of a crude product. This was purified on RPHPLC to obtain 200 mg (56 μmol, 21% based on Gly in the starting resin) of iNoc-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 mg, 310 μmol) and TEA (42 μl, 300 μmol) were added to a solution of iNoc-HBs(40-60)-$SCH_2CH_2CONH_2$ (200 mg, 56 μmol) in DMSO (2.0 ml); the solution was stirred for 5 h at room temperature. Peptide **II-3** was obtained in a yield of 17% (230 mg, 46 μmol) based on Gly in the starting resin according to the same procedure as described in peptide **II-1**. Found: m/z 2813.1 $(M+H)^+$. Calcd: m/z 2812.5 $(M+H)^+$. Amino acid analysis of peptide **II-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}, (*methyl*-$^2H_3$)Met^{69}]-HBs(61-90) (II-4).

iNoc-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(OcHex)-Ala-Val-Lys(Cl-Z)-OCH_2-PAM-resin was prepared from Boc-Lys(Cl-Z)-OCH_2-PAM-resin (0.78 g, Lys: 500 μmol) using a 430A synthesizer. The Met^{69} residue was introduced manually using Boc-(*methyl*-$^2H_3$)Met (380 mg, 1.5 mmol) in DMF and a 0.5 M solution of DCC in DCM (3.0 ml, 1.5 mmol). The iNoc group was incorporated as described regarding the synthesis of peptide **II-2**. An aliquot of the

obtained resin (0.98 g out of 2.7 g) was treated under low-HF conditions (HF-dimethyl sulfide-*p*-cresol, 25:65:10 (v/v), 10 ml) at 0 °C for 2 h, followed by high-HF conditions (HF-*p*-cresol, 9:1 (v/v), 10 ml) at 0 °C for 1 h;[4] a crude peptide (840 mg) was obtained by the procedure described regarding peptide **II-1**. The crude product (840 mg) was applied on a Pharmacia HiLoad S-Sepharose HP (16X100 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-[(*methyl*-$^2H_3$)Met^{69}]-HBs(61-90) (310 mg, 48 μmol, 26% based on Lys in the starting resin). 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-[(*methyl*-$^2H_3$)Met^{69}]-HBs(61-90) (290 mg, 43 μmol) and Boc-ONSu (140 mg, 650 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-[$Lys(Boc)^{75,80,83,86,90}$, (*methyl*-$^2H_3$)$Met^{69}$]-HBs(61-90) (300 mg, 37 μmol) as described in the preparation of peptide **II-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-[$Lys(Boc)^{75,80,83,86,90}$, (*methyl*-$^2H_3$)$Met^{69}$]-HBs(61-90) (300 mg, 37 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 (1 lX3) and freeze-dried to give powdered peptide **II-4** (190 mg, 31 μmol, 19% based on Lys in the starting resin). Found: m/z 3739.4 $(M+H)^+$. Calcd: m/z 3740.0 $(M+H)^+$. Amino acid analysis of peptide **II-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)-$SCH_2CH_2CONH_2$ (II-5).

Starting from Boc-Gly-SCH_2CH_2CONH-resin (1.0 g, Gly: 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 (2.9 g) was obtained using the same amino acid derivatives. An aliquot of the resin (770 mg) was treated with HF (10 ml) containing 10% anisole (v/v) at 0 °C for 90 min. The crude peptide obtained was purified on RPHPLC to give 30 mg (5.7 μmol, 6.4% based on Gly in the starting resin) 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 mg, 3.2 μmol) and Boc-ONSu (11 mg, 49 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 **II-5** (19 mg, 2.3 μmol, 4.5% based on Gly in the starting resin) after the same posttreatment as described in the preparation of peptide **II-1**. Amino acid analysis of peptide **II-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 [(*methyl*-$^2H_3$)Met^{69}]-HBs(1-90) (II-8-A) by Procedure A.

[Lys(Boc)^{41,59,75,80,83,86,90}, (*methyl*-$^2H_3$)Met^{69}]-HBs(40-90) (II-6-A): Peptides **II-3** (47 mg, 11 μmol) and **II-4** (44 mg, 10 μmol) were dissolved in DMSO (1.0 ml) containing HONSu (12 mg, 100 μmol) and NMM (1.1 μl, 10 μmol). The solution was stirred at room temperature for 3 h in the dark after adding $AgNO_3$ (2.1 mg, 12 μmol). $AgNO_3$ (1.1 mg, 6.5 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 (X2) 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-[(*methyl*-$^2H_3$)Met^{69}]-HBs(40-90) (Peptide **II-6**, 49 mg, 5.2 µmol, 51% based on peptide **II-4**). Amino acid analysis of peptide **II-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 **II-6** (25 mg, 2.6 µmol) and Boc-ONSu (13 mg, 62 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}, (*methyl*-$^2H_3$)Met^{69}]-HBs(40-90) (24 mg, 2.3 µmol) was obtained according to the procedure described in the preparation of peptide **II-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 mg, 2.3 µmol) was dissolved in 75% aqueous acetic acid (400 µl) and zinc dust (20 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 **II-4**, resulting in peptide **II-6-A** (18 mg, 2.3 µmol, 45% yield based on peptide **II-4**). Amino acid analysis of peptide **II-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}, (*methyl*-$^2H_3$)Met^{69}]-HBs(16-90) (II-7-A): Peptide **II-2** (20 mg, 4.0 µmol) and peptide **II-6-A** (18 mg, 2.3 µmol) were dissolved in DMSO (300 µl) containing HONSu (6.9 mg, 60 µmol) and NMM (0.66 µl, 6.0 µmol). $AgNO_3$ (1.2 mg, 7.1 µmol) was added to the solution, which was stirred for 5 h in the dark. More $AgNO_3$ (300 µg, 1.8 µmol) was added, and the reaction mixture

was stirred again overnight. The crude product (39 mg) obtained by the same procedure described regarding peptide **II-6-A** was treated with TFA (400 µl) containing 10% 1,4-butanedithiol (v/v) for 10 min and purified on RPHPLC followed by elution through TSKgel G3000SW (7.5X600 mm) 50% aqueous acetonitrile containing 0.1% TFA, at a flow rate of 0.3 ml min^{-1}. Thus, 19 mg of *i*Noc-[(*methyl*-$^2H_3$)Met^{69}]-HBs(16-90) (Peptide **II-7**) were obtained (1.3 µmol, 58% based on peptide **II-6-A**). Amino acid analysis of peptide **II-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 **II-7** (18 mg, 1.3 µmol) was dissolved in DMSO (100 µl) containing Boc-ONSu (10 mg, 48 µmol) and TEA (6.7 µl, 48 µmol). The solution was stirred for 10 h at room temperature. *i*Noc-[$Lys(Boc)^{18,19,23,38,41,59,75,80,83,86,90}$, (*methyl*-$^2H_3$)$Met^{69}$]-HBs(16-90) (17 mg, 1.1 µmol) was obtained as described in the preparation of peptide **II-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 mg, 1.1 µmol) was dissolved in 85% aqueous acetic acid (1.4 ml) and sonicated in the presence of zinc (70 mg) under nitrogen at room temperature for 3 h. Peptide **II-7-A** (15 mg, 920 nmol, 42% yield based on peptide **II-6-A**) was obtained according to a procedure similar to that described for the preparation of peptide **II-4**. Amino acid analysis of peptide **II-7-A**: $Asp_{7.58}Thr_{2.81}Ser_{2.74}Glu_{8.55}Pro_{4.08}Gly_{6.13}$ $Ala_{10}Val_{5.37}Met_{1.26}Ile_{2.81}Leu_{3.86}Phe_{4.10}Lys_{10.54}Arg_{4.87}$.

[(*methyl*-$^2H_3$)Met^{69}]-HBs(1-90) (II-8-A): Peptides **II-1** (7.9 mg, 1.8 µmol) and **II-7-A** (15 mg, 920 nmol) were dissolved in DMSO (500 µl) containing HONSu (4.9 mg, 43 µmol) and NMM (0.56 µl, 5.1 µmol). The solution was stirred overnight in the dark after adding $AgNO_3$ (860 µg, 5.1 µmol). Peptide **II-1** (3.0 mg, 680 nmol), HONSu (1.9 mg, 17 µmol), NMM (0.21 µl, 1.9 µmol) and more $AgNO_3$ (330 µg, 1.9 µmol) were

added and the solution was stirred for 24h. The workup of the reaction mixture was performed as described for peptide **II-7-A**. The peptide obtained was treated with TFA (500 µl, v/v) containing 10% 1,4-butanedithiol for 15 min at 0 ^{o}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 **II-8-A** (7.3 mg, 400 nmol, 43% based on peptide **II-7-A**). Amino acid analysis of peptide **II-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 [(*methyl*-$^{2}H_3$)Met^{69}]-HBs(1-90) (II-8-B) by procedure B.

[Lys(Boc)^{41,59,75,80,83,86,90}, (*methyl*-$^{2}H_3$)Met^{69}]-HBs(40-90) (II-6'): Peptides **II-3** (150 mg, 34 µmol), **II-4** (140 mg, 32 µmol) and HONSu (38 mg, 330 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 $AgNO_3$ (6.8 mg, 40 µmol). The solution was stirred overnight after adding more $AgNO_3$ (3.4 mg, 20 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% aqueous acetic acid (2.6 ml) under nitrogen for 3 h at room temperature. Peptide mixture **II-6'** (230 mg) containing peptide **II-6-B** was obtained after dialysis followed by freeze-drying, as described regarding the preparation of peptide **II-4**.

[Lys(Boc)^{18,19,23,38,41,59,75,80,83,86,90}, (*methyl*-$^{2}H_3$)Met^{69}]-HBs(16-90) crude (II-7'): Peptides **II-2** (170 mg, 36 µmol) and **II-6'** (230 mg), HONSu (42 mg, 370 µmol), NMM (4.8 µl, 45 µmol) were dissolved in DMSO (3.7 ml). $AgNO_3$ (7.5

mg, 44 μmol) was added then the solution was stirred overnight in the dark. $AgNO_3$ (1.4 mg, 8.0 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 (480 mg). This peptide was sonicated with zinc dust (450 mg) in 80% aqueous acetic acid (9.0 ml) for 8 h at room temperature. Peptide mixture **II-7'** (340 mg) containing peptide **II-7-B** was obtained as described in the preparation of peptide **II-4**.

[(*methyl*-$^2H_3$)Met^{69}]-HBs(1-90) (II-8-B): Peptides **II-1** (100 mg, 33 μmol) and **II-7'** (300 mg), HONSu (63 mg, 550 μmol) and NMM (4.6 μl, 42 μmol) were dissolved in DMSO (7.0 ml). After the addition of $AgNO_3$ (7.1 mg, 42 μmol), the solution was stirred for 4.5 h in the dark at room temperature. $AgNO_3$ (2.0 mg, 12 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 **II-1** (60 mg, 20 μmol), HONSu (37 mg, 320 μmol), NMM (2.6 μl, 24 μmol) and $AgNO_3$ (4.2 mg, 25 μmol). The reaction mixture was posttreated as described regarding the preparation of peptide **II-8-A**. The crude powder (470 mg) was treated with TFA (4.5 ml) containing 10% 1,4-butanedithiol (v/v) at 0 °C for 15 min. The thus-obtained material was purified on RPHPLC to yield powdered peptide **II-8-B** (92 mg, 4.7 μmol, 16% yield based on peptide **II-4**) after freeze-drying. Amino acid analysis of peptide **II-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 [(*methyl*-$^2H_3$)Met^{69}]-HBs(1-90) (II-8-C) by Procedure C.

Peptides **II-5** (3.7 mg, 680 nmol) and **II-6-A** (4.9 mg, 420 nmol) were dissolved in DMSO (100 μl) containing HONSu (900 μg, 7.8 μmol) and NMM (0.17 μl, 1.6 μmol). After adding $AgNO_3$ (150 μg, 860 nmol), the solution was stirred overnight; then, peptide

II-5 (13 mg, 2.4 μmol), $AgNO_3$ (510 μg, 3.0 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 (v/v) 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 **II-8-C** (140 nmol, 33% based on peptide **II-6-A**). Amino acid analysis of peptide **II-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 [(*methyl*-$^2H_3$)Met^{69}]-HBs(1-90) (II-8).

Peptide **II-8-A** (7.3 mg, 400 nmol) was dissolved in a 0.05 M sodium phosphate buffer (pH 7.0) and loaded onto Pharmacia HiLoad S-Sepharose HP (16X100 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 [(*methyl*-$^2H_3$)Met^{69}]-HBs(1-90) (200 nmol). Amino acid analysis of peptide **II-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 **II-8-B** (93 mg, 4.7 μmol) was purified to yield peptide **II-8** (78 mg, 2.1 μmol).

^1H 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 ^{o}C.

References

1) D. F. Veber, W. J. Paleveda, Jr., Y. C. Lee, and R. Hirschmann, *J. Org. Chem.*, **42**, 3286 (1977).

2) I. Tanaka, K. Appelt, J. Dijk, S. W. White, and K. S. Wilson, *Nature*, **310**, 376 (1984).

3) A. R. Mitchell, S. B. H. Kent, M. Engelhard, and R. B. Merrifield, *J. Org. Chem.*, **43**, 2845 (1978).

4) J. P. Tam, W. F. Heath, and R. B. Merrifield, *J. Am. Chem. Soc.*, **105**, 6442 (1983).

Chapter III

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

III-1 Introduction

In order to make the thioester method more effective for the synthesis of proteins, especially those with a molecular weight above 10000 or with labelled amino acids, improvements are required of several points. One of the major drawbacks is the low yields of peptide thioesters due to the instability of the linker containing a thioester moiety on a solid support. In the previous chapters, the author used an *S*-*n*-alkyl thioester, where *n*-alkyl means normal alkyl, directly connected to MBHA resin. The yield of the peptide thioester was almost half of the values obtained on the MBHA resin without the thioester moiety.

In this chapter are described the development of a linker containing an *S*-alkyl-thioester-moiety with enhanced stability, the preparation of thioester building blocks for the synthesis of HU-type DNA-binding protein of *Bacillus stearothermophilus* (HBs) labelled with ^{2}H, ^{13}C and ^{15}N and the synthesis of multilabelled HBs. NMR spectral analysis of the product is also discussed.

III-2 Results and Discussion

Comparison of Chemical 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 represent normal alkyl, 1-substituted alkyl, and 1,1-disubstituted alkyl,

respectively, were prepared according to the scheme shown in Fig. III-1 in order to estimate the stability of the thioester-containing linker during peptide chain elongation cycles. Each crude product was analyzed by RPHPLC after an HF treatment. The results are summarized in Table III-1. A 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.

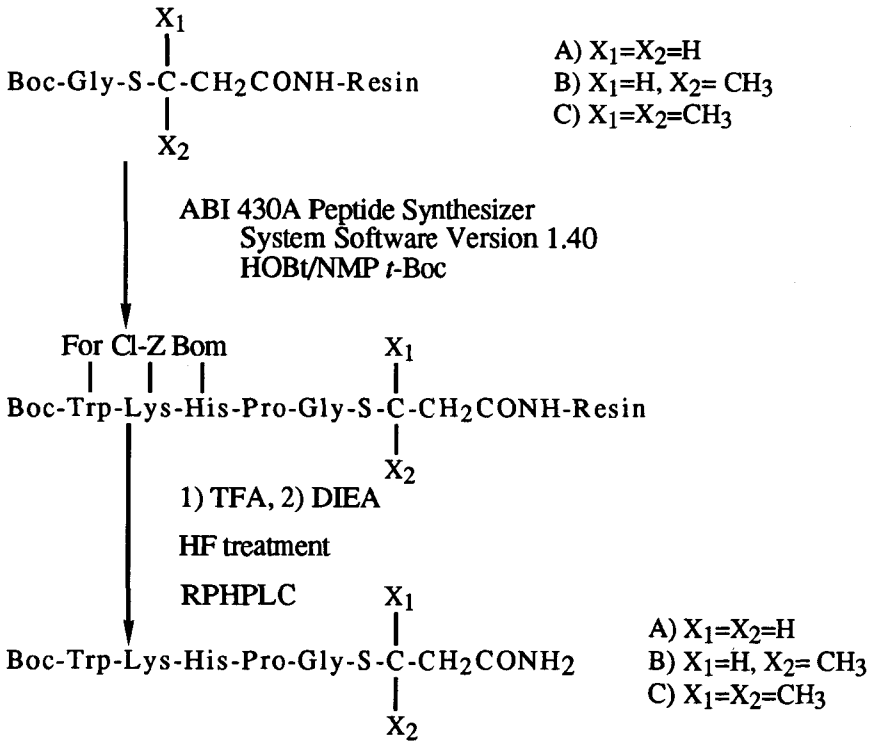


Fig. III-1. Preparation of pentapeptide thioesters for a stability test of the thioester-containing linker.

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 III-2. However, the amount of the by-products in the crude product was somewhat decreased, as judged from the elution profile on RPHPLC, when a linker containing *S*-*t*-alkyl thioester was used.

Table III-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

Stability Test of Boc-Gly-$SC(CH_3)_2CH_2CONH$-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 FAB mass spectrometer. When Boc-Gly-$SC(CH_3)_2CH_2$-CONH-resin was treated with 50% TFA in DCM (v/v), 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% DIEA in DMF (v/v). 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 DCC and 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 III-2 show that the presence of the spacer groups, β-Ala and Nle, enhanced the stability of the linkers on the MBHA resin. The yields of the peptide thioesters with these spacers were almost equal to that of a peptide amide prepared on an MBHA resin without a thioester moiety.

Table III-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

Preparation of Peptide Segments of HBs.

Using Boc-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH-resin, peptide segments of multi-labelled HBs were synthesized. The sequence of HBs and six amino acid residues, labelled with ^2H, ^{13}C or ^{15}N, are shown in Fig. III-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*-$^{2}H_3$)Met for Met^{69}, (ε-^{15}N)Lys for Lys^{80}. These labelled amino acid residues are indicated by asterisks in Fig. III-2.

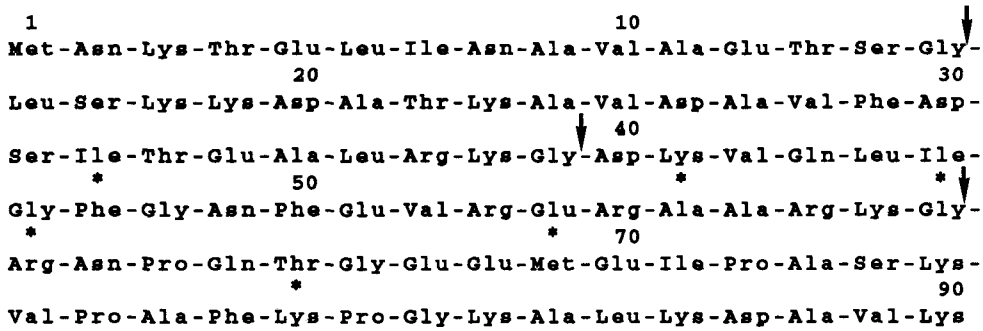


Fig. III-2. The amino acid sequence of HBs. The arrows indicate the sites of segment coupling. The asterisks indicate amino acids labelled with ^{2}H, ^{13}C or ^{15}N; (2-^{13}C)Phe^{47}, (1-^{13}C)Ala^{56}, (2-^{13}C)Gly^{60}, (*guanidino*-$N^{2,3}$-$^{15}N_2$)Arg^{61}, (*methyl*-$^{2}H_3$)Met^{69}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

The same four peptide segments that were used in the previous synthesis (chapter II) were prepared except for the use of a Troc group instead of an *i*Noc group and the carboxyl-terminal thioester portion. Starting from the Boc-Gly-SC(CH$_3$)$_2$CH$_2$CO-Nle-NH-resin, partially protected peptide segments **III-1**, **III-2**, and **III-3** (Table III-3) were prepared by the same procedure described in the previous chapter. The yield of each peptide thioester almost doubled compared with the previous synthesis using the Boc-Gly-SCH$_2$CH$_2$CO-NH-resin. The yields obtained by this synthesis were almost comparable to that of the partially protected peptide **III-4** prepared using the Boc-Lys(Cl-Z)-OCH$_2$-PAM-resin. The *S*-*t*-alkyl thioester with the spacer was also stable during the purification of a peptide segment and the introduction of Boc groups.

Synthesis of Multilabelled HBs.

Peptide segments from **III-1** to **III-4** were condensed according to the scheme

Table III-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	**(III-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	**(III-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$(CH_3)_2CH_2$-CO-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	**(III-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)	**(III-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.

shown in Fig. III-3. As typical coupling conditions, peptides **III-3** (27 μmol) and **III-4** (27 μmol) were dissolved in DMSO (1.4 ml) containing NMM (100 μmol) and HONSu (310 μmol). To this solution, $AgNO_3$ (47 μmol) was added. The *S-t*-alkyl thioester with the spacer 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 atmosphere to remove the Troc group. After zinc dust was removed, the reaction mixture was dialyzed against distilled water and lyophilized to give a

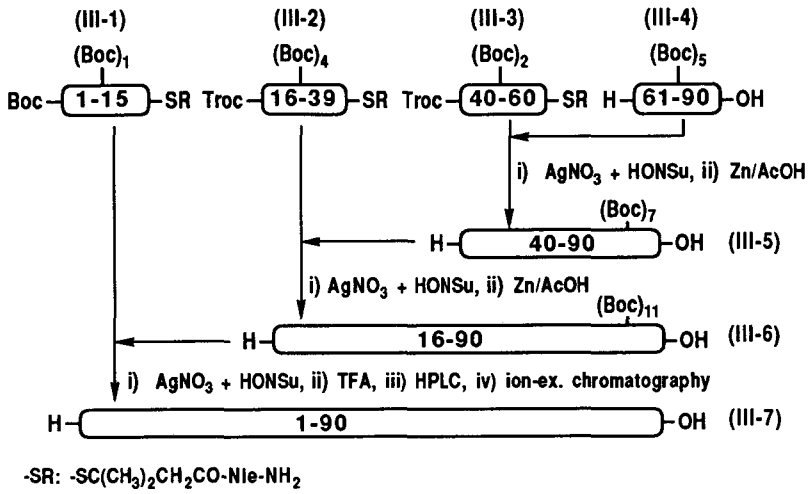


Fig. III-3. Synthetic route of HBs(1-90).

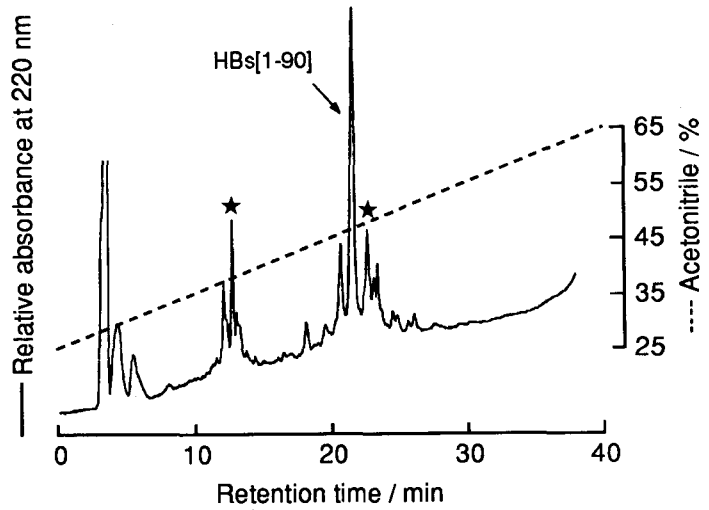


Fig. III-4. RPHPLC elution profile of a crude product of HBs(1-90) after treating the reaction mixture with TFA. The asterisks indicate the peaks derived from by-products during deprotection of the Troc group. Column: YMC-Pack ODS-AM (4.6X250 mm) at a flow rate of 1 ml min^{-1}. Eluent: aqueous acetonitrile containing 0.1% TFA.

peptide mixture containing peptide **III-5**, which was used for the next coupling without purification. According to the same procedure, peptides **III-2** and **III-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) (**III-7**) is shown in Fig. III-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 **III-4**. In the previous synthesis, the overall yield was 7.2%, based on peptide **II-4** by method B. In the present synthesis, a high proportion of the by-product was formed up to 50% during deprotection of the Troc group.[1)] 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. A Troc group was not necessarily a good choice for the synthesis of HBs. It has to be noted, however, that during the synthesis of the *c*-Myb protein, the side reaction was not so serious.

The activation of the *S*-*t*-alkyl thioester with the spacer was completed within 10 min.

NMR Spectra of Labelled HBs.

The final goal reached by this synthesis was preparation of 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. III-5. Thus, multilabelled HBs should yield significant information. Detailed analysis data will be published elsewhere.

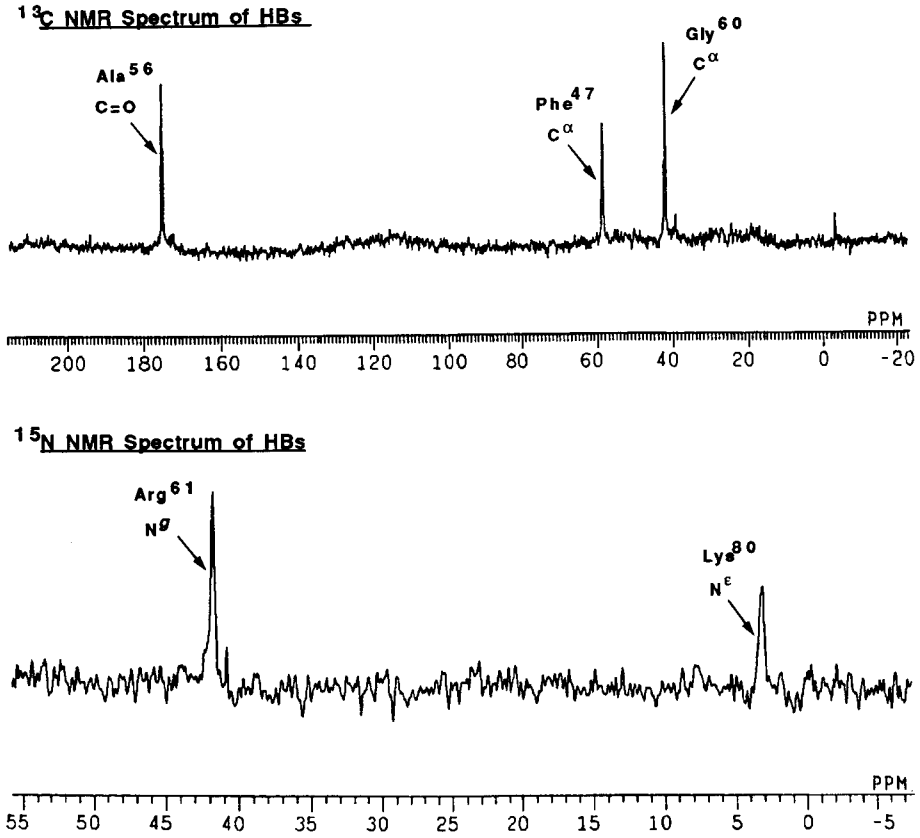


Fig. III-5. ^{13}C and ^{15}N signals obtained using ^{13}C and ^{15}N NMR spectroscopy, respectively. N^{g} means *guanidino-*$N^{2,3}$.

III-3 Materials and Methods

(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. Analytical RPHPLC was performed on YMC-Pack ODS-AM (4.6X250 mm), and preparative RPHPLC was performed on YMC-Pack ODS-AM or PROTEIN-RP (20X250 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 ^{o}C for 24 h in an evacuated sealed tube.

Peptide Chain Elongation on a Solid Support.

The solid-phase synthesis of a peptide segment was performed as the same procedure described in the preceding chapter.

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 ^{o}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 x 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)[2] was dissolved in liquid NH_3 (1 l) 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 hr. The oil obtained by the same procedure described for the synthesis of Boc-Gly-SCH(CH_3)CH_2COOH was purified by RPHPLC using aqueous acetonitrile containing 0.1% TFA as the eluent to obtain Boc-Gly-SC(CH_3)$_2CH_2$COOH (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)$_n$$CH_2$CONH-Resin (n=0, 1, 2).

MBHA resin hydrochloride (1.0 g, NH_2: 0.39 meq) was washed with 5% DIEA in DMF (v/v) for 5 min (X2), then with DCM for 1 min (X3). Boc-Gly-SCH$_2$CH$_2$COOH (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 (v/v) 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-$SC(CH_3)_2CH_2CONH$-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) (X3) 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-$SC(CH_3)_2$-CH_2CONH-resin, and the products were analyzed. The data are given in Table III-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 (v/v). 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 Boc-ONSu in DMF in the presence of 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, NH_2: 0.51 meq) and shaken for 4 h. After washing with DCM (X3), 50% methanol in DCM (v/v, X3) and DCM (X3), the resin was treated with 55% TFA in DCM (v/v) for 5 and 15 min, followed by neutralization with 5% DIEA in DMF (v/v) for 5 min (X2). 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-$SC(CH_3)_2$-CH_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-$SC(CH_3)_2CH_2CO$-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(III-2).

Starting from Boc-Gly-$SC(CH_3)_2CH_2CO$-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 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)-$SC(CH_3)_2CH_2CO$-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 **III-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 **III-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 **III-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 **III-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 **III-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 (III-1).

Starting from Boc-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH-resin (1.0 g, Gly: 0.44 mmol), the protected peptide resin corresponding to the sequence of HBs(1-15) was prepared. After the removal of *N*-terminal Boc group by TFA treatment, 1.8 g of a protected peptide resin was obtained. The resin (800 mg) was treated with HF (10 ml) containing *p*-cresol (1 ml) at 0 °C for 90 min to give 330 mg of crude product. This was purified on RPHPLC to obtain 120 mg (53 μmol, 26% based on Gly in the starting resin) of HBs(1-15)-$SC(CH_3)_2CH_2CO$-Nle-NH_2. Found: m/z 1805.9 $(M+H)^+$. Calcd: m/z 1805.9 $(M+H)^+$. Amino acid composition: $Asp_{2.05}Thr_{1.99}Ser_{0.97}Glu_{2.08}Gly_{1.04}Ala_2Val_{1.10}Met_{0.93}Ile_{0.97}Leu_{0.98}Nle_{0.93}Lys_{1.08}$.

Boc-ONSu (56 mg, 260 μmol) and TEA (36 μl, 260 μmol) were added to a solution of HBs(1-15)-$SC(CH_3)_2CH_2CO$-Nle-NH_2 (120 mg, 53 μmol) in DMSO (1.4 ml). The solution was stirred for 5 h at room temperature. Peptide **III-1** (130 mg, 44 μmol, 22% based on Gly in the starting resin) was obtained according to the same procedure as described in peptide **III-2**. Found: m/z 2005.6 $(M+H)^+$. Calcd: m/z 2006.0

$(M+H)^+$. Amino acid analysis of peptide **III-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$(CH_3)_2CH_2$CO-Nle-NH_2 (III-3).

Starting from Boc-(2-^{13}C)Gly-SC$(CH_3)_2CH_2$CO-Nle-NH-resin (1.0 g, Gly: 0.4 mmol), obtained by the same procedure as described for the preparation of the corresponding nonlabelled resin, protected peptide resin corresponding to the sequence of HBs(40-60) was obtained. 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. The Troc group was incorporated as described regarding the synthesis of peptide **III-2**. An aliquot of the obtained resin (1.1 g out of 2.1 g) was treated with HF (10 ml) containing *p*-cresol (1 ml) at 0 °C for 1.5 h. A crude peptide obtained (690 mg) was purified on RPHPLC to give 310 mg (87 μmol, 41% based on Gly in the starting resin) of Troc-HBs(40-60)-SC$(CH_3)_2CH_2$CO-Nle-NH_2. Found: m/z 2795.4 $(M+H)^+$. Calcd: m/z 2795.3 $(M+H)^+$. Amino acid composition: $Asp_{1.93}Glu_{2.97}$ $Gly_{3.00}Ala_2Val_{1.90}Ile_{0.93}Leu_{0.96}Nle_{0.94}Phe_{2.02}Lys_{1.91}Arg_{2.95}$.

Troc-HBs(40-60)-SC$(CH_3)_2CH_2$CO-Nle-NH_2 (310 mg, 87 μmol) and Boc-ONSu (94 mg, 440 μmol) was dissolved in DMSO (1.6 ml). The solution was stirred for 5 h after adding TEA (61 μl, 440 μmol) to give peptide **III-3** (330 mg, 87 μmol, 41% based on Gly in the starting resin) after the same posttreatment as described in the preparation of peptide **III-2**. Found: m/z 2995.5 $(M+H)^+$. Calcd: m/z 2995.5 $(M+H)^+$. Amino acid analysis of peptide **III-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) (III-4).

Starting from Boc-Lys(Cl-Z)-OCH$_2$-PAM-resin, protected peptide resin corresponding to the sequence of HBs(61-90) was prepared. 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*-^{2}H$_3$)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. After the completion of peptide chain assembly, the Troc group was introduced to the terminal amino group as described regarding the synthesis of peptide **III-2**. An aliquot of the obtained resin (1.2 g out of 2.7 g) was treated under low-HF conditions (HF-dimethyl sulfide-*p*-cresol, 25:65:10 (v/v), 10 ml) at 0 ^{o}C for 2 h, followed by high-HF conditions (HF-*p*-cresol, 9:1 (v/v), 10 ml) at 0 ^{o}C for 1 h;[3] a crude peptide (880 mg) was obtained by the procedure described regarding peptide **III-2**. This peptide was applied on a Pharmacia HiLoad S-Sepharose HP (16X100 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 40 mM, over 15 min at a flow rate of 2.5 ml min^{-1}. The purified peptide was desalted by RPHPLC to give Troc-[(*guanidino*-$N^{2,3}$-^{15}N$_2$)Arg^{61}, (*methyl*-^{2}H$_3$)Met^{69}, (ε-^{15}N)Lys^{80}]-HBs(61-90) (300 mg, 56 μmol, 27 % based on Lys in the starting resin). Found: m/z 3417.4 $(M+H)^+$. Calcd: m/z 3416.7 $(M+H)^+$. Amino acid composition: Asp$_{2.08}$Thr$_{1.02}$Ser$_{0.98}$Glu$_{4.15}$Pro$_{4.16}$Gly$_{2.03}$Ala$_4$ Val$_{2.34}$Met$_{1.00}$Ile$_{0.99}$Leu$_{1.08}$Phe$_{1.10}$Lys$_{5.51}$Arg$_{0.63}$.

Troc-[(*guanidino*-$N^{2,3}$-^{15}N$_2$)Arg^{61}, (*methyl*-^{2}H$_3$)Met^{69}, (ε-^{15}N)Lys^{80}]-HBs (61-90) (300 mg, 56 μmol) and Boc-ONSu (200 mg, 930 μmol) were dissolved in DMSO (1.5 ml). The solution was stirred for 4 h after adding TEA (131 μl, 940 μmol) to give 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) (340 mg, 56 μmol) Amino acid composition: Asp$_{2.08}$Thr$_{0.98}$ Ser$_{0.92}$Glu$_{4.00}$Pro$_{3.88}$Gly$_{2.09}$Ala$_4$Val$_{1.94}$Met$_{0.75}$Ile$_{0.98}$Leu$_{0.99}$Phe$_{1.07}$Lys$_{4.74}$ Arg$_{0.70}$.

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) (340 mg, 56 μmol) was sonicated with zinc dust (230 mg) in 67% aqueous acetic acid (4.5 ml) under nitrogen atmosphere for 15 min. Zinc dust was removed by centrifugation. The solution was packed in a Spectrapor 6 membrane (M.W. cut off 1000), dialyzed against distilled water (1 lX3) and freeze-dried to give peptide **III-4** (270 mg, 43 μmol). Yield of peptide **III-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 **III-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*-$^{2}H_3$)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*-$^{2}H_3$) 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*-$^{2}H_3$)Met^{69}, (ε-^{15}N)Lys^{80}]-HBs(1-90) (III-7).

Peptides **III-3** (100 mg, 27 μmol), **III-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 (v/v, 5 ml) and acetonitrile (1 ml) under nitrogen for 10 min at room temperature. A mixture (160 mg) containing peptide **III-5** was obtained after dialysis, followed by freeze-drying. Following

the same procedure, peptide **III-2** (110 mg, 27 μmol) was coupled with crude peptide **III-5** to obtain a mixture containing peptide **III-6**, which was further condensed with peptide **III-1** (120 mg, 33 μmol) to obtain a crude product **III-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 **III-7** (56 mg, 2.9 μmol) after freeze-drying. This was further purified by ion-exchange chromatography on Pharmacia Mono-S HR 5/5 (5X50 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 **III-7** (2.3 μmol, 8.5% based upon peptide **III-4**). Amino acid analysis of peptide **III-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 ^{o}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).

References

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

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

3) J. P. Tam, W. F. Heath, and R. B. Merrifield, *J. Am. Chem. Soc.*, **105**, 6442 (1983).

Chapter IV

Synthesis of Barnase Site-Specifically Labelled with Two ^{13}C Atoms Using Partially Protected Peptide Thioester Building Blocks

IV-1 Introduction

To estimate the usefulness of the thioester method in the synthesis of an enzyme, the method was applied to the synthesis of barnase, a protein comprising 110 amino acids with RNase activity (Fig. IV-1).[1] In this study, barnase site-specifically labelled with two ^{13}C atoms was synthesized for future structural studies.

This chapter describes the results of the synthesis as a successful example of the thioester method to protein synthesis with a full enzymatic activity.

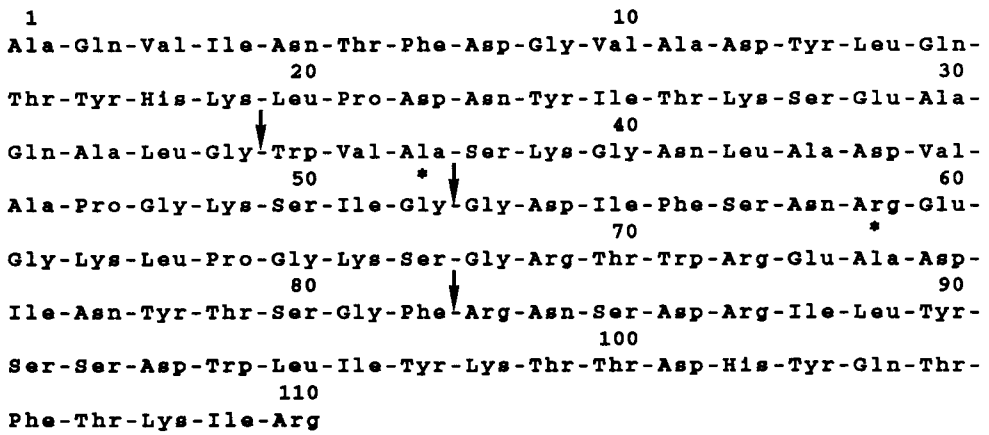


Fig. IV-1. The amino acid sequence of barnase. The arrows indicate the sites of segment coupling; The asterisks indicate amino acids labelled with ^{13}C; (2-^{13}C)Gly^{52} and (1-^{13}C)Ala^{74}.

IV-2 Results and Discussion

Preparation of Peptide Segments.

For synthetic purposes, the barnase sequence was divided into four peptide segments, as shown in Fig. IV-1. Gly^{52} and Ala^{74} were labelled with ^{13}C as (2-^{13}C)Gly and (1-^{13}C)Ala, respectively.

A partially protected peptide thioester was prepared according to the procedure described in chapter III. To an MBHA resin, Boc-Nle and Boc-Gly-$SC(CH_3)_2CH_2COOH$ were successively introduced using DCC in the presence of HOBt to obtain Boc-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH-resin. On this resin, Boc-amino acids were successively condensed. After peptide chain assembly, the terminal amino group was protected with an *i*Noc group. The protected peptide resin was treated with anhydrous HF to give a crude *i*Noc-peptide thioester, which was purified by RPHPLC. Boc groups were introduced to the side-chain amino groups of an HPLC-purified peptide thioester in order to realize selective removal of the amino protecting groups after segment condensation. The partially protected peptide segments were prepared in good yields without any problems, and were used for the barnase synthesis.

All of the partially protected peptide segments used for barnase synthesis are listed in Table IV-1. The yields of the peptide segments were calculated based upon the amino groups in the MBHA resin. The linker containing Nle and *S*-*t*-alkyl thioester moieties, where *t*-alkyl means 1,1-disubstituted alkyl, gave satisfactory yields in the preparation of peptide segments as in chapter III.

Synthesis of Barnase by Segment Coupling.

Segment condensation was performed according to the scheme shown in Fig. IV-2. The typical coupling conditions were as follows: peptides **IV-3** (120 mg, 17 μmol) and **IV-4** (100 mg, 13 μmol) were dissolved in DMSO (2.3 ml). HONSu (30 mg, 260 μmol), $AgNO_3$ (13 mg, 77 μmol), and NMM (9 μl, 82 μmol) were then added in succession. The solution was stirred overnight at room temperature in the dark. The peptide was

Table IV-1. Partially Protected Peptide Segments Prepared for Segment Coupling

Peptide segment		Yield/%[a]
Boc-[Lys(Boc)^{19,27}]-Barnase(1-34)-SC$(CH_3)_2CH_2CO$-Nle-NH_2	**(IV-1)**	12
Boc-Ala-Gln-Val-Ile-Asn-Thr-Phe-Asp-Gly-Val-Ala-Asp-Tyr-Leu-Gln-Thr-Tyr-His-Lys(Boc)-Leu-Pro-Asp-Asn-Tyr-Ile-Thr-Lys(Boc)-Ser-Glu-Ala-Gln-Ala-Leu-Gly-SC$(CH_3)_2CH_2CO$-Nle-NH_2		
*i*Noc-[Lys(Boc)^{39,49}, (2-^{13}C)Gly^{52}]-Barnase(35-52)-SC$(CH_3)_2CH_2CO$-Nle-NH_2	**(IV-2)**	32
*i*Noc-Trp-Val-Ala-Ser-Lys(Boc)-Gly-Asn-Leu-Ala-Asp-Val-Ala-Pro-Gly-Lys(Boc)-Ser-Ile-Gly*-SC$(CH_3)_2CH_2CO$-Nle-NH_2		
*i*Noc-[Lys(Boc)^{62,66}, (1-^{13}C)Ala^{74}]-Barnase(53-81)-SC$(CH_3)_2CH_2CO$-Nle-NH_2	**(IV-3)**	12
*i*Noc-Gly-Asp-Ile-Phe-Ser-Asn-Arg-Glu-Gly-Lys(Boc)-Leu-Pro-Gly-Lys(Boc)-Ser-Gly-Arg-Thr-Trp-Arg-Glu-Ala*-Asp-Ile-Asn-Tyr-Thr-Ser-Gly-SC$(CH_3)_2CH_2CO$-Nle-NH_2		
[Lys(Boc)^{98,108}]-Barnase(82-110)	**(IV-4)**	12
Phe-Arg-Asn-Ser-Asp-Arg-Ile-Leu-Tyr-Ser-Ser-Asp-Trp-Leu-Ile-Tyr-Lys(Boc)-Thr-Thr-Asp-His-Tyr-Gln-Thr-Phe-Thr-Lys(Boc)-Ile-Arg		

a) The yield was calculated based on the amino groups in the MBHA resin.

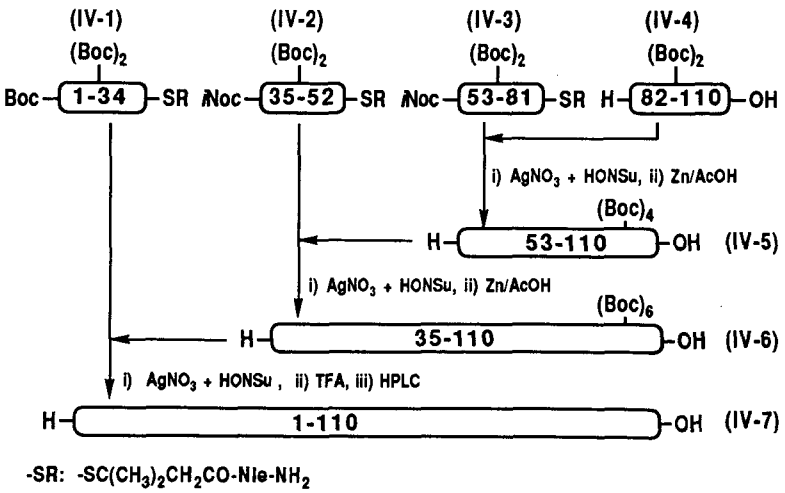


Fig. IV-2. Synthetic route of barnase(1-110).

precipitated with distilled water and washed twice. After the precipitate was dissolved in 70% aqueous acetic acid, 800 mg of zinc dust was added. The solution was sonicated for 7 h under a nitrogen stream. After removing the zinc dust by centrifugation, the supernatant was dialyzed against distilled water using a Spectrapor membrane 6 and freeze-dried to give a mixture containing peptide **IV-5** (170 mg). According to a similar procedure, peptides **IV-2** and **IV-5**, then peptides **IV-1** and **IV-6**, were successively condensed. The condensation reactions were monitored by RPHPLC using a C_4 column. The HPLC elution profiles of the reaction mixtures are shown in Fig. IV-3. The segment coupling of peptide **IV-5** and **IV-2** and that of peptide **IV-6** and **IV-1** were almost complete within 6 h. During peptide chain elongation by segment coupling, no RPHPLC purification was performed.

After segment condensation of peptides **IV-1** and **IV-6**, distilled water was added to the reaction mixture. The precipitate formed was washed with water and freeze-dried to give a powder which was treated with TFA containing 5% 1,4-butanedithiol (v/v) for 15 min. After removing the TFA under a nitrogen stream, the product was washed twice with ether. The crude product was purified on PROTEIN-RP, followed by Pharmacia HiLoad S-Sepharose HP (Fig. IV-4) to give the final product, barnase(1-110) (**IV-7**) in 11% yield, based upon peptide **IV-4**. Its amino acid composition agreed well with that predicted. The RNase activity of the synthetic barnase was determined by measuring the hydrolytic activity toward yeast RNA according to the method described by Rushizky et al. [2] The synthetic and native barnases had practically same activities, as shown in Table IV-2.

Application of the Method.

In the barnase synthesis, all of the segment condensation reactions were almost completed within 6 h, even those between peptides **IV-1** and **IV-6** of 34 and 76 amino

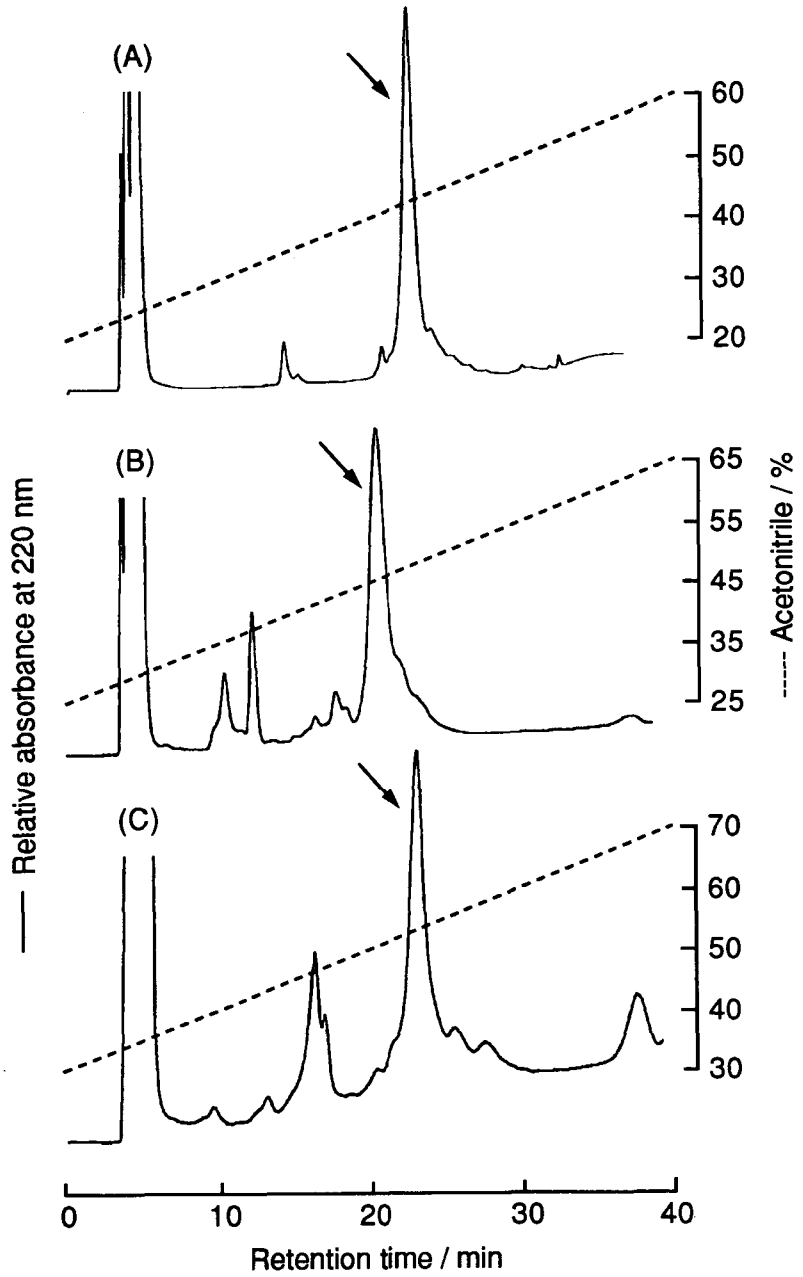


Fig. IV-3. RPHPLC elution profiles of the reaction mixtures of segment couplings after an overnight reaction. Arrows in panel A,B,C indicate *i*Noc-[Lys(Boc)^{62,66,98,108}]-barnase (53-110), *i*Noc-[Lys(Boc)^{39,49,62,66,98,108}]-barnase(35-110) and Boc-[Lys(Boc)^{19,27,39,49,62,66,98,108}]-barnase(1-110), respectively. Column: YMC-Pack C_4 (4.6X250 mm) at a flow rate of 1 ml min^{-1}. Eluent: aqueous acetonitrile containing 0.1% TFA.

acid residues, respectively. This fact suggests that the peptide segment with minimum protecting groups was well solvated by DMSO, kept good flexibility around the reaction sites and, hence, retained high reactivity. Thus, the thioester method, which uses a minimal protection strategy, is suitable for protein synthesis, not only because of the ease of segment preparation, but also because of the high reactivity during segment condensation. The points which must be overcome in this method are to find an easily-removable protecting group instead of Troc or *i*Noc for the terminal amino group, and to establish a strategy with which cysteine-containing proteins can be synthesized.

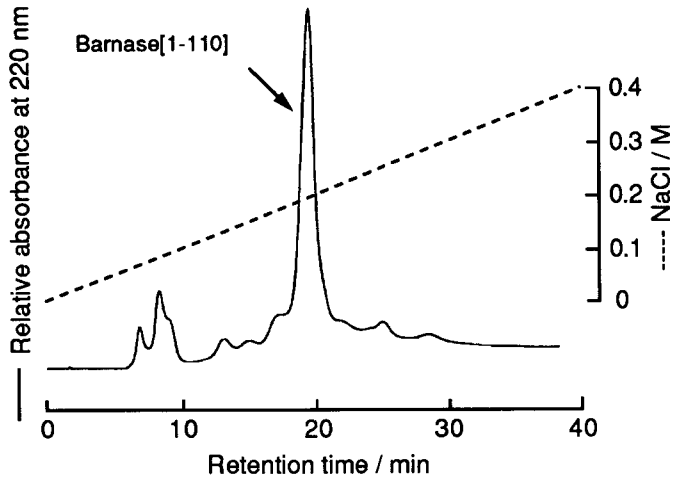


Fig. IV-4. Ion-exchange chromatography of the RPHPLC-purified barnase(1-110) (**IV-7**) by Pharmacia HiLoad S-Sepharose HP (16x100 mm) at a flow rate of 2.5 ml min^{-1}. The broken line indicates the NaCl concentration in 0.05 M sodium phosphate buffer (pH 6.0).

Table IV-2. Enzymatic Activity of Barnase

	Specific Activity x 10^{-6} units /A_{280}	Relative Activity %
Native Barnase	2.1 ± 0.2	100
Synthetic Barnase	2.4 ± 0.3	114

IV-3 Materials and Methods

Boc-Arg(Tos)-OCH_2-PAM-resin was purchased from Applied Biosystems Inc. (1-^{13}C)Ala and (2-^{13}C)Gly were purchased from Isotec Inc. Analytical RPHPLC was performed on YMC-Pack ODS-AM or C_4 (4.6X250 mm) and preparative RPHPLC was on YMC-Pack ODS-AM or PROTEIN-RP (20X250 mm) (YMC, Kyoto). Native barnase was a gift from Dr. H. Yanagawa of Mitsubishi-Kasei Institute of Life Sciences. Yeast RNA was purchased from Kohjin Co. Ltd. (Tokyo).

Peptide Chain Elongation on a Solid Support.

Solid-phase synthesis of a peptide segment was carried out according to the procedure described in the preceding chapter.

Boc-[Lys(Boc)^{19,27}]-barnase(1-34)-SC(CH_3)$_2$$CH_2$CO-Nle-$NH_2$ (IV-1).

Boc-Nle (170 mg, 0.75 mmol) was mixed with 1 M HOBt in NMP (0.75 ml) and 1 M DCC in NMP (0.75 ml). After 30 min, this solution was mixed with neutralized MBHA-resin (810 mg, NH_2: 0.51 meq) and shaken for 4 h. After the Boc group was removed with 55% TFA in DCM (v/v) for 5 and 15 min followed by neutralization with 5% DIEA in DMF (v/v) for 5 min (X2), Boc-Gly-SC(CH_3)$_2$$CH_2$COOH (220 mg, 0.75 mmol), prepared as the same procedure described in the previous chapter, was introduced to the Nle-NH-resin using 1 M HOBt in NMP (0.75 ml) and 1 M DCC in NMP (0.75 ml) in a similar manner, to give Boc-Gly-SC(CH_3)$_2$$CH_2$CO-Nle-NH-resin. Using this resin, a protected peptide resin corresponding to the sequence of barnase(1-34), Boc-Ala-Gln-Val-Ile-Asn-Thr(Bzl)-Phe-Asp(O*c*Hex)-Gly-Val-Ala-Asp(O*c*Hex)-Tyr(Br-Z)-Leu-Gln-Thr(Bzl)-Tyr(Br-Z)-His(Bom)-Lys(Cl-Z)-Leu-Pro-Asp(O*c*Hex)-Asn-Tyr(Br-Z)-Ile-Thr(Bzl)-Lys(Cl-Z)-Ser(Bzl)-Glu(OBzl)-Ala-Gln-Ala-Leu-Gly-SC(CH_3)$_2$$CH_2$CO-Nle-NH-resin was prepared on a synthesizer by means of double coupling. After *N*-terminal Boc group

was removed by treatment with 55% TFA in DCM (v/v) for 5 and 15 min followed by neutralization by 5% DIEA in DMF (v/v) for 5 min (X2), 2.8 g of protected peptide resin was obtained. An aliquot of the resin (1.4 g) was treated with anhydrous HF (30 ml) containing *p*-cresol (3 ml) at 0 ^{o}C for 90 min to give 740 mg of crude product. This peptide was purified on RPHPLC to obtain 180 mg (39 μmol, 15% based on the amino groups in the MBHA resin) of barnase(1-34)-SC$(CH_3)_2CH_2$CO-Nle-NH_2. Found: m/z 4012.4 $(M+H)^+$. Calcd: m/z 4012.0 $(M+H)^+$. Amino acid composition: $Asp_{5.02}$ $Thr_{2.76}Ser_{0.93}Glu_{4.16}Pro_{1.02}Gly_{2.03}Ala_4Val_{1.61}Ile_{1.60}Leu_{3.00}(Tyr+Nle)_{4.06}$ $Phe_{0.93}Lys_{2.02}His_{1.07}$.

Barnase(1-34)-SC$(CH_3)_2CH_2$CO-Nle-NH_2,(180 mg, 39 μmol) dissolved in DMSO (1.1 ml) was reacted with Boc-ONSu (57 mg, 270 μmol) in the presence of TEA (37 μl, 270 μmol). The resulting solution was stirred for 8 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 200 mg (30 μmol, 12% based on the amino groups in the MBHA resin) of peptide **IV-1**. Amino acid analysis of peptide **IV-1**: $Asp_{5.06}Thr_{2.82}Ser_{0.89}Glu_{4.08}Pro_{1.02}Gly_{2.01}Ala_4Val_{1.69}$ $Ile_{1.61}Leu_{2.99}(Tyr+Nle)_{4.02}Phe_{0.97}Lys_{2.00}His_{1.00}$.

iNoc-[Lys(Boc)^{39,49}, (2-^{13}C)Gly^{52}]-barnase(35-52)-SC$(CH_3)_2CH_2$CO-Nle-NH_2 (IV-2).

Starting from MBHA resin (850 mg, NH_2: 0.54 meq), a protected peptide resin corresponding to the sequence of barnase(35-52), Boc-Trp(For)-Val-Ala-Ser(Bzl)-Lys(Cl-Z)-Gly-Asn-Leu-Ala-Asp(O*c*Hex)-Val-Ala-Pro-Gly-Lys(Cl-Z)-Ser(Bzl)-Ile-(2-^{13}C)Gly-SC$(CH_3)_2CH_2$CO-Nle-NH-resin was prepared. Boc-(2-^{13}C)Gly-SC$(CH_3)_2CH_2$COOH was prepared according to the procedure described in the previous chapter. After removing the *N*-terminal Boc group as regarding the synthesis of peptide **IV-1**, the peptide resin was treated with *i*Noc-ONp (410 mg, 1.5 mmol) in 80% DMSO-NMP (v/v) overnight to give a

protected peptide resin (1.9 g). An aliquot of the resin (510 mg) was treated with HF (10 ml) containing *p*-cresol (0.5 ml) and 1,4-butanedithiol (1.5 ml) at 0 °C for 90 min to give 250 mg of a crude product. This product was purified on RPHPLC to obtain *i*Noc-[(2-^{13}C)Gly^{52}]-barnase(35-52)-SC$(CH_3)_2CH_2$CO-Nle-NH_2 (150 mg, 50 μmol, 35 % based on the amino groups in the MBHA resin). Found: m/z 2134.5 $(M+H)^+$. Calcd: m/z 2134.1 $(M+H)^+$. Amino acid composition: $Asp_{2.16}Ser_{2.03}Pro_{1.03}Gly_3Ala_{3.27}Val_{2.04}Ile_{0.98}Leu_{1.06}Nle_{0.99}Lys_{2.08}Trp_{0.54}$.

To the *i*Noc-[(2-^{13}C)Gly^{52}]-barnase(35-52)-SC$(CH_3)_2CH_2$CO-Nle-NH_2 (150 mg, 50 μmol) dissolved in DMSO (2.6 ml), Boc-ONSu (56 mg, 260 μmol) and TEA (36 μl, 260 μmol) were added; the resulting solution was 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 180 mg of peptide **IV-2** (46 μmol, 32% based on the amino groups in the MBHA resin). Found: m/z 2335.0 $(M+H)^+$. Calcd: m/z 2334.2 $(M+H)^+$. Amino acid analysis of peptide **IV-2**: $Asp_{2.08}Ser_{1.88}Pro_{1.07}Gly_{3.05}Ala_3Val_{1.93}Ile_{0.98}Leu_{1.02}Nle_{0.99}Lys_{2.02}Trp_{0.50}$.

*i*Noc-[Lys(Boc)^{62,66}, (1-^{13}C)Ala^{74}]-barnase(53-81)-SC$(CH_3)_2CH_2$CO-Nle-NH_2 (IV-3).

Starting from MBHA resin (820 mg, NH_2: 520 meq), *i*Noc-Gly-Asp(O*c*Hex)-Ile-Phe-Ser(Bzl)-Asn-Arg(Tos)-Glu(OBzl)-Gly-Lys(Cl-Z)-Leu-Pro-Gly-Lys(Cl-Z)-Ser(Bzl)-Gly-Arg(Tos)-Thr(Bzl)-Trp(For)-Arg(Tos)-Glu(OBzl)-Ala-Asp(O*c*Hex)-Ile-Asn-Tyr(Br-Z)-Thr(Bzl)-Ser(Bzl)-Gly-SC$(CH_3)_2CH_2$CO-Nle-NH-resin (2.5 g) was prepared regarding the synthesis of peptide **IV-2**. Ala^{74} was incorporated manually, by mixing with Boc-(1-^{13}C)Ala (0.75 mmol), 1 M HOBt in NMP (0.75 ml) and 1 M DCC in NMP (0.75 ml) for 4 h. An aliquot of the resin 1.2 g was treated with HF (20 ml) containing *p*-cresol (1.0 ml) and 1.4-butanedithiol (3.0 ml) at 0 °C for 90 min to give 650 mg of a crude product. This peptide was purified on RPHPLC to obtain 150 mg (36 μmol, 15% based on

the amino groups in the MBHA resin) of *i*Noc-[(1-^{13}C)Ala^{74}]-barnase(53-81)-$SC(CH_3)_2CH_2CO$-Nle-NH_2. Found: m/z 3576.1 $(M+H)^+$. Calcd: m/z 3575.8 $(M+H)^+$. Amino acid composition: $Asp_{4.26}Thr_{2.06}Ser_{2.96}Glu_{2.15}Pro_{0.98}Gly_5Ala_{1.04}Ile_{1.88}$ $Leu_{0.99}(Tyr+Nle)_{2.03}Phe_{0.87}Lys_{2.03}Trp_{0.78}Arg_{3.02}$.

To the solution of *i*Noc-[(1-^{13}C)Ala^{74}]-barnase(53-81)-$SC(CH_3)_2CH_2CO$-Nle-NH_2 (150 mg, 36 μmol) dissolved in DMSO (0.9 ml), Boc-ONSu (36 mg, 170 μmol) and TEA (35 μl, 250 μmol) were added and the resulting solution was stirred for 4 h to give peptide **IV-3** (160 mg, 30 μmol, 12% based on the amino groups in the MBHA resin) as regarding the synthesis of peptide **IV-1**. Found: m/z 3775.5 $(M+H)^+$. Calcd: m/z 3775.9 $(M+H)^+$. Amino acid analysis of peptide **IV-3**: $Asp_{4.29}Thr_{2.03}Ser_{3.00}Glu_{2.16}Pro_{1.03}$ $Gly_{5.05}Ala_1Ile_{1.90}Leu_{1.00}(Tyr+Nle)_{1.98}Phe_{0.97}Lys_{2.01}Trp_{0.63}Arg_{2.98}$.

[Lys(Boc)^{98,108}]-barnase(82-110) (IV-4).

Starting from Boc-Arg(Tos)-OCH_2-PAM-resin (0.83 g, 0.5 mmol), *i*Noc-Phe-Arg(Tos)-Asn-Ser(Bzl)-Asp(O*c*Hex)-Arg(Tos)-Ile-Leu-Tyr(Br-Z)-Ser(Bzl)-Ser(Bzl)-Asp(O*c*Hex)-Trp(For)-Leu-Ile-Tyr(Br-Z)-Lys(Cl-Z)-Thr(Bzl)-Thr(Bzl)-Asp(O*c*Hex)-His(Bom)-Tyr(Br-Z)-Gln-Thr(Bzl)-Phe-Thr(Bzl)-Lys(Cl-Z)-Ile-Arg(Tos)-OCH_2-PAM-resin (3.0 g) was prepared. An aliquot of the resin (1.3 g) was treated with HF (15 ml) containing *p*-cresol (0.75 ml) and 1,4-butanedithiol (2.3 ml) at 0 °C for 90 min to give 690 mg of crude peptide. This peptide was purified on RPHPLC to give 190 mg (34 μmol, 15% based on Arg in the starting resin) of *i*Noc-barnase(82-110). Amino acid composition: $Asp_{3.91}Thr_{3.90}Ser_{2.66}Glu_{1.10}Ile_{2.93}Leu_2Tyr_{3.04}Phe_{1.88}Lys_{2.04}His_{1.04}Trp_{0.54}Arg$ 2.96.

*i*Noc-barnase(82-110) (190 mg, 34 μmol) and Boc-ONSu (39 mg, 180 μmol) were dissolved in DMSO (1.6 ml). The solution was stirred for 5 h after adding TEA (25 μl, 180 μmol) to give *i*Noc-[Lys(Boc)^{98,108}]-barnase(82-110) (200 mg, 28 μmol) as the

procedure described for peptide **IV-1**. Amino acid composition: $Asp_{4.21}Thr_{3.99}Ser_{2.96}Glu_{1.09}Ile_{2.67}Leu_2Tyr_{3.20}Phe_{1.70}Lys_{2.19}His_{1.20}Trp_{0.60}Arg_{2.89}$.

*i*Noc-[Lys(Boc)^{98,108}]-barnase(82-110) (200 mg, 28 μmol) was sonicated with zinc dust (320 mg) in 75% aqueous acetic acid (6.4 ml) under nitrogen for 2 h. After removing zinc dust, the solution was dialyzed against distilled water (1 l X3) and freeze-dried to give peptide **IV-4** (130 mg, 27 μmol, 12% based on Arg in the starting resin). Found: m/z 3868.3 $(M+H)^+$. Calcd: m/z 3868.0 $(M+H)^+$. Amino acid analysis of peptide **IV-4**: $Asp_{4.19}Thr_{4.13}Ser_{3.02}Glu_{1.11}Ile_{2.73}Leu_2Tyr_{3.05}Phe_{1.79}Lys_{2.10}His_{1.03}Trp_{0.40}Arg_{2.77}$.

Synthesis of [(2-^{13}C)Gly^{52}, (1-^{13}C)Ala^{74}]-barnase(1-110) (IV-7).

Peptides **IV-3** (120 mg, 17 μmol), **IV-4** (100 mg, 13 μmol) and HONSu (30 mg, 260 μmol) were dissolved in DMSO (2.3 ml) containing NMM (9.0 μl, 82 μmol). $AgNO_3$ (13 mg, 77 μmol) was then added and the mixture was stirred for 5 h at room temperature in the dark. The solution was stirred overnight after adding more NMM (4.0 μl, 36 μmol). A precipitate obtained by adding distilled water to the solution, was freeze-dried to give a powder (220 mg). This peptide was sonicated with zinc dust (800 mg) in 70% acetic acid (25 ml) under nitrogen for 7 h at room temperature. The solution was dialyzed against distilled water (1 l X3) and freeze-dried to give a mixture (170 mg) containing peptide **IV-5**. Following the same procedure, peptides **IV-2** (81 mg, 20 μmol) and **IV-1** (93 mg, 14 μmol) were successively condensed. The crude peptide obtained (320 mg) was treated with TFA (2.6 ml) containing 5% 1,4-butanedithiol (v/v) at room temperature for 15 min. TFA was removed under a nitrogen stream and the peptide was precipitated with ether. This peptide was purified on RPHPLC using PROTEIN-RP to give powdered peptide **IV-7** (81 mg, 3.7 μmol) after freeze-drying. This peptide was further purified by ion-exchange chromatography using Pharmacia HiLoad S-Sepharose HP (16X100 mm), which was

equilibrated with 0.05 M sodium phosphate (pH 6.0) and eluted with a 0 to 0.3 M NaCl gradient in the buffer over 30 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 desalted by RPHPLC to give [(2-^{13}C)Gly^{52}, (1-^{13}C)Ala^{74}]-barnase(1-110) (22 mg, 1.4 μmol, 11% based on peptide **IV-4**). Amino acid analysis of peptide **IV-7**: $Asp_{14.94}Thr_{8.50}Ser_{8.39}Glu_{7.25}Pro_{2.96}Gly_{10.21}Ala_{8}Val_{3.54}Ile_{6.87}Leu_{6.77}Tyr_{6.80}Phe_{3.81}Lys_{8.13}His_{2.12}Trp_{1.79}Arg_{5.82}$.

Measurement of RNase Activity.

Yeast RNA (1.6 mg) was dissolved in 0.125 M Tris-HCl pH 8.5 (0.8 ml) and 0.2 ml of appropriately diluted enzyme was added. The mixture was incubated at 37 ^{o}C for 15 min. The reaction was stopped by adding a solution containing 6% $HClO_4$ and 1% lanthanum acetate (1 ml). The mixture was kept at 0 ^{o}C for 15 min and the precipitate was removed by centrifugation. The supernatant (0.5 ml) was diluted with 4.5 ml of water, and the absorbance at 260 nm (A_{260}) was measured. An increase in A_{260} of 1.0 under these conditions was defined as 100 units of enzyme activity.

References

1) C. J. Paddon and R. W. Hartley, *Gene*, **40**, 231 (1986).

2) G. W. Rushizky, A. E. Greco, R. W. Hartley, and H. A. Sober, *Biochemistry*, **2**, 787 (1963).

Chapter V

Preparation of Partially Protected Peptide Thioesters Containing Cysteine Residues and Their Segment Condensation

V-1 Introduction

The thioester method was successfully applied to protein synthesis. However, cysteine-containing proteins could not be prepared using this method, because there is no known route to an adequately protected cysteine-containing peptide segment. To expand this method to a general procedure for the preparation of proteins, methods for the preparation of partially protected cysteine-containing peptide thioesters should be developed.

Concurrently, it is also necessary to find a new candidate for an α-amino protecting group. The zinc-dust treatment of a peptide having Troc or *i*Noc groups at the terminal amino group is accompanied by a side-reaction and zinc ions have to be removed by dialysis. This post treatment is rather tedious.

This chapter describes the procedures for the preparation of partially-protected peptide thioesters containing a cysteine residue(s) and the use of the segments with 9-fluorenylmethoxycarbonyl (Fmoc) group at the terminal amino group for the synthesis of the barnase-like domain (RPSc(299-410)) in DNA-directed RNA polymerase II of *Saccharomyces cerevisiae* .[1)]

V-2 Results and Discussion

Preparation of Cysteine-Containing Partially Protected Peptide Segment.

The 4-methylbenzyl (MeBzl)[2)] and 2,4,6-trimethylbenzyl (Tmb)[3)] groups were chosen to protect the thiol group of a cysteine residue, because both groups are stable

toward silver ions under segment coupling conditions. Three methods of preparing a peptide segment bearing MeBzl or Tmb group on the cysteine residue were examined.

Method A: A protected peptide was prepared on Merrifield resin and treated under low-HF conditions.[4] As a model, *S*-protected human atrial natriuretic peptide (1-9) (hANP(1-9)) (**V-8**) was prepared in a yield of 19% based on the alanine content in a starting resin as shown in Fig. V-1. However, low-HF treatment of protected hANP(10-28) on Merrifield resin, Boc-Gly-Arg(Tos)-Met-Asp(OBzl)-Arg(Tos)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr(Br-Z)-OCH_2-resin, gave a partially deprotected peptide mixture and the yield of a desired product, Gly-Arg(Tos)-Met-Asp-Arg(Tos)-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg(Tos)-Tyr, was as low as 5%.

Boc-Ser(Bzl)-Leu-Arg(Tos)-Arg(Tos)-Ser(Bzl)-Ser(Bzl)-Cys(MeBzl)-Phe-Gly-$SC(CH_3)_2CH_2CO$-Ala-OCH_2-Merrifield resin,

↓ 1) Low-HF treatment, 2) RPHPLC

Ser-Leu-Arg(Tos)-Arg(Tos)-Ser-Ser-Cys(MeBzl)-Phe-Gly-$SC(CH_3)_2CH_2CO$-Ala (**V-8**)

Fig. V-1. Preparation of hANP(1-9)-$SC(CH_3)_2CH_2CO$-Ala (**V-8**) by low-HF treatment.

Method B: As shown in Fig. V-2, a protected peptide thioester prepared on MBHA resin was treated under high-HF conditions.[4] After purifying the crude peptide by RPHPLC, a MeBzl group was introduced to the thiol group of a cysteine residue using MeBzl-Cl. This method was successfully applied to several peptides, which contain tryptophan, histidine or methionine residues in addition to cysteine.

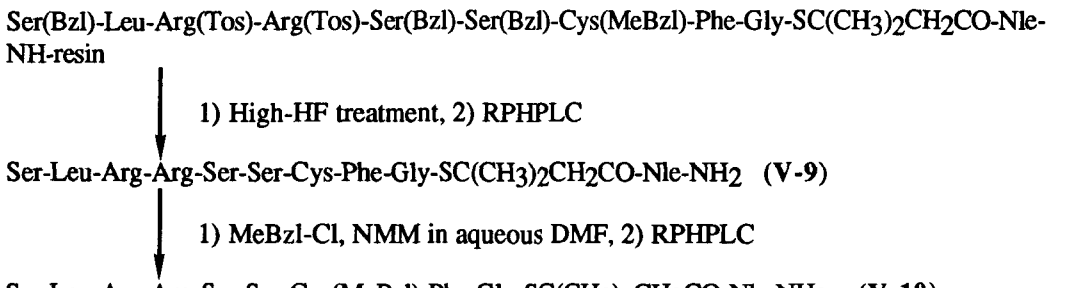


Fig. V-2. Preparation of hANP(1-9)-$SC(CH_3)_2CH_2CO$-Nle-NH_2 (**V-10**) by high-HF treatment.

Method C: As shown in Fig. V-3, a protected peptide resin corresponding to the sequence of RPSc(299-321) was prepared on an acid labile SAL resin[5] using Npys amino acids.[6] The resin was treated with reagent K[7] and a peptide bearing Tmb groups on cysteine residues was obtained. This peptide was easily purified on RPHPLC and

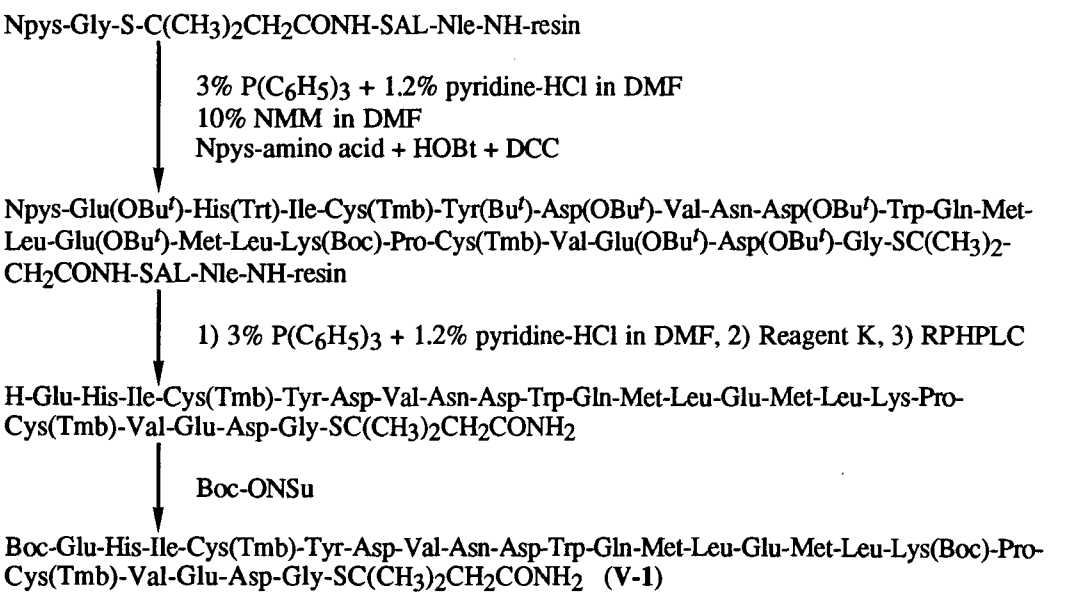


Fig. V-3. Preparation of Boc-[$Cys(Tmb)^{302,317}$, $Lys(Boc)^{315}$]-RPSc(299-321)-$SC(CH_3)_2CH_2CONH_2$ (**V-1**) using Npys-amino acids.

[Cys(Tmb)^{302,317}]-RPSc(299-321)-SC$(CH_3)_2CH_2CONH_2$ was obtained in a 14% yield based upon Nle in the starting resin. As the desired peptide was directly obtained without re-introducing the Tmb group, this method avoids the problem involved in method B. In addition, the side chain protecting groups, (except Tmb), can be completely removed by deprotection. Thus, this method is widely applicable to the preparation of *S*-protected peptide thioesters regardless of the number of Cys and Arg residues in the peptide.

Preparation of a *C*-terminal peptide segment containing a cysteine residue(s): In the case of *C*-terminal segment, which does not contain thioester group, an *S*-protected peptide can be conveniently synthesized using Fmoc-amino acids[8] as shown in Fig. V-4. The protected peptide resin corresponding to the sequence of RPSc(380-410) was synthesized on Wang resin[9] by *FastMoc* chemistry. The resin was treated with reagent K[7] and crude peptide was obtained. The peptide was purified on RPHPLC to give Fmoc-[Cys(MeBzl)^{388}]-RPSc(380-410) in a yield of 20%.

Fmoc-Gly-$OCH_2C_6H_4OCH_2C_6H_4$-resin (Fmoc-Gly-Wang-resin)

↓ ABI 430A peptide synthesizer, *FastMoc* Chemistry

Fmoc-Tyr(Bu^t)-Met-Ile-Asn-Arg(Pmc)-Leu-Leu-Leu-Cys(MeBzl)-Ala-Leu-Asp(OBu^t)-Arg(Pmc)-Lys(Boc)-Asp(OBu^t)-Gln-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-Wang-resin

↓ 1) Reagent K, 2) RPHPLC

Fmoc-Tyr-Met-Ile-Asn-Arg-Leu-Leu-Leu-Cys(MeBzl)-Ala-Leu-Asp-Arg-Lys-Asp-Gln-Asp-Asp-Arg-Asp-His-Phe-Gly-Lys-Lys-Arg-Leu-Asp-Leu-Ala-Gly

↓ 1) Boc-ONSu, 2) 5% piperidine-DMSO

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 **(V-4)**

Fig. V-4. Preparation of [Cys(MeBzl)^{388}, Lys(Boc)^{393,403,404}]-RPSc(380-410) **(V-4)** using Fmoc-amino acids.

Selection of the Terminal Amino Protecting Group.

In previous syntheses, Troc or *i*Noc groups provided terminal amino protection. These protecting groups are stable under the conditions of HF treatment and are orthogonally deprotected against Boc groups on the side chain amino groups by zinc-dust treatment in aqueous acetic acid. However, the removal of Troc group is usually accompanied by formation of a by-product. The cleavage of the *i*Noc group is not accompanied by a distinct by-product under restricted conditions, but it takes several hours to remove the *i*Noc group from a long chain peptide with zinc-dust. The prolonged treatment tends to cause gradual random decomposition of the peptide. Furthermore, zinc ions have to be removed by dialysis afterwards.

In order to overcome these problems, several protecting groups were examined in place of Troc or *i*Noc group. The Fmoc group,[8] which is cleaved by piperidine, proved to be used for this purpose.

Synthesis of RPSc(299-410).

To demonstrate the usefulness of a partially protected cysteine-containing peptide thioester obtained by a solid-phase method using Npys-amino acids, and the applicability of the Fmoc group to the method, the barnase-like domain (RPSc(299-410)) in the second largest subunit of RNA polymerase II of *Saccharomyces cerevisiae* (Fig. V-5)[1] was synthesized. This domain shows sequence similarity with the bacterial RNase, barnase. All of the catalytic sites of barnase are conserved in the domain. Thus this domain is expected to have an RNase activity. This is the second reason to choose this domain as the target of synthesis.

Preparation of Peptide Segments.

RPSc(299-410) was divided into four segments. The synthetic procedures for

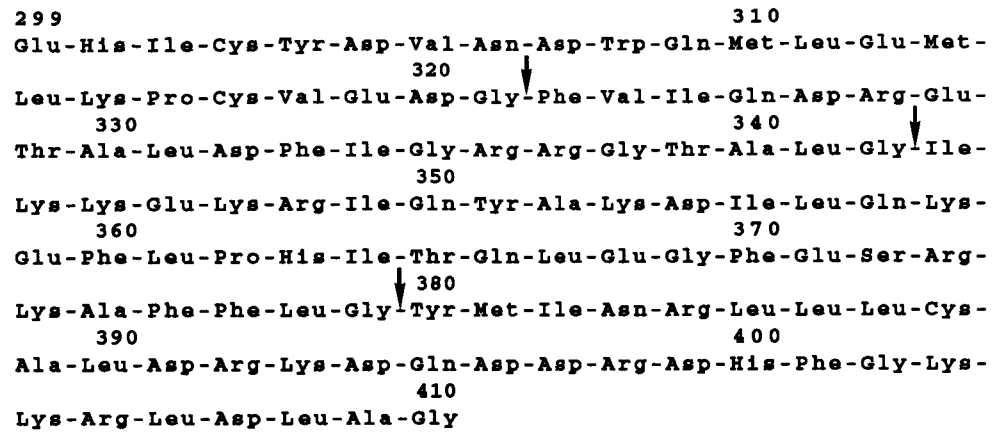


Fig. V-5. The amino acid sequence of RPSc(299-410). The arrows indicate the sites of segment coupling.

peptides **V-1** and **V-4** are shown in Fig. V-3, V-4. Peptides **V-2** and **V-3** were prepared by the same procedure described in Chapter IV. Starting from Boc-Gly-$SC(CH_3)_2CH_2$-CO-Nle-NH-resin, Boc-amino acids were successively condensed. After the completion of the chain assembly, an 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 purified by RPHPLC to give a crude Fmoc-peptide thioester. The Fmoc group was quite stable during HF treatment. The side-chain amino groups of the peptide were protected with Boc groups by Boc-ONSu in the presence of DIEA to give partially protected peptide thioesters of **V-2** and **V-3**. Under the conditions for the introduction of Boc groups, the Fmoc group was not removed. Peptide segments prepared for the synthesis of RPSc(299-410) were listed in Table V-1. The yields of the peptide segments were calculated based upon the amino acids in the starting resins.

Segment Condensation.

Segment condensation proceeded according to the scheme shown in Fig. V-6.

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

Peptide segments		Yield/%[a)]
Boc-[Cys(Tmb)^{302,317}, Lys(Boc)^{315}]-RPSc(299-321)-SC$(CH_3)_2CH_2CONH_2$	(V-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	(V-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	(V-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)	(V-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		

a) The yield was calculated based upon the amino acid in the starting resin.

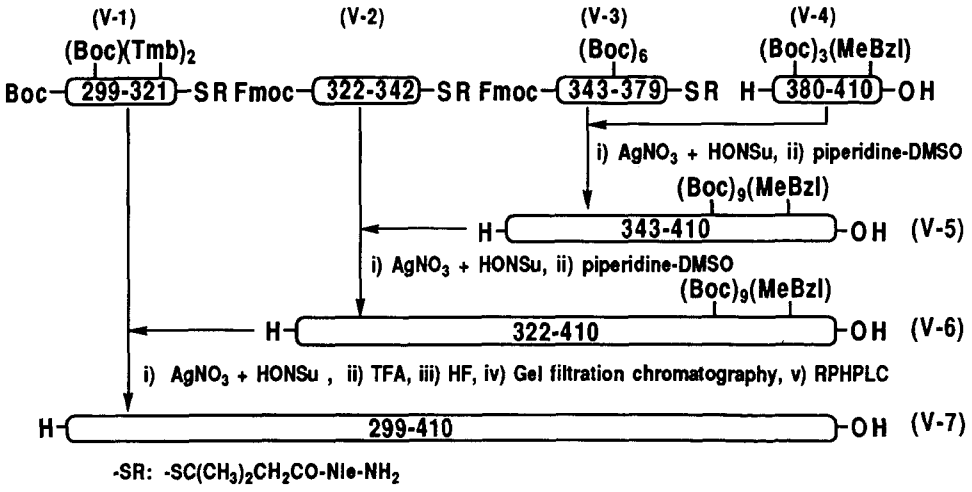


Fig. V-6. Synthetic route of RPSc(299-410).

Typical conditions are described below. Peptides **V-3** (7.7 mg, 1.1 μmol), **V-4** (5.7 mg, 0.9 μmol) and HONSu (1.6 mg, 14 μmol) were dissolved in DMSO (150 μl) containing DIEA (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. DIEA (0.1 μl, 0.6 μmol) was added and the solution was stirred for another 3 d. During this period, about 5% of the Fmoc group was removed from the product. This minor component was combined with the main product after its Fmoc group was removed with 5% (v/v) piperidine in DMSO. Under these conditions the Fmoc group was cleaved cleanly 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 **V-5** (13 mg) was obtained after lyophilization from distilled water. Following the same procedure, peptides **V-2** and **V-1** were successively condensed. The MeBzl and Tmb groups were stable during the segment condensation. The crude peptide was treated with TFA containing 5% 1,4-butanedithiol (v/v) at room temperature for 10 min. TFA was removed under a nitrogen stream and the peptide was precipitated with ether. This peptide was treated with HF containing 7.5% (v/v) 1,4-butanedithiol and 7.5% (v/v) anisole at 0 ^{o}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. V-7, followed by RPHPLC purification on YMC-Pack ODS-AM to give RPSc(299-410) at 12% yield based upon peptide **V-4**. The RPHPLC profile of the purified RPSc(299-410) (**V-7**) was shown in Fig. V-8. The structure of the product was confirmed by acid hydrolysis and electrospray ionization (ESI) mass spectroscopy.

Contrary to our expectation, however, enzymatic activity of RPSc(299-410) was not detected under the 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. The RNase activity of

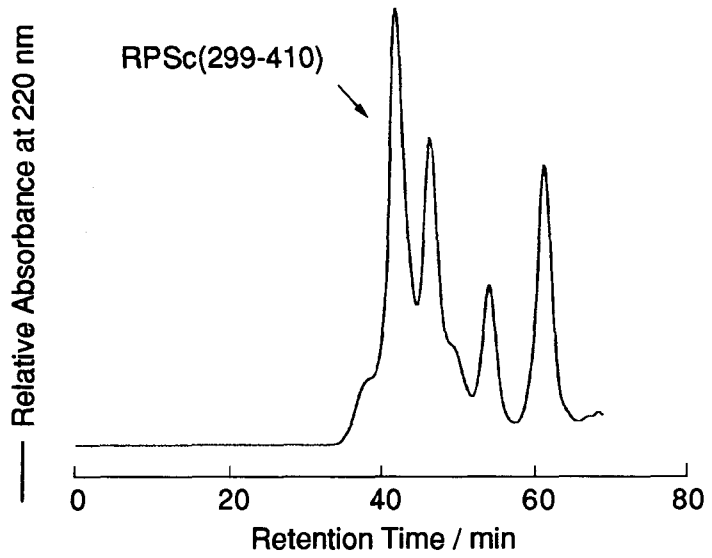


Fig. V-7. Gel filtration chromatogram of crude RPSc(299-410). Column: TSKgel G3000SW (7.5X600 mm) at a flow rate of 0.3 ml min^{-1}. Eluent: 50% aqueous acetonitrile containing 0.1% TFA.

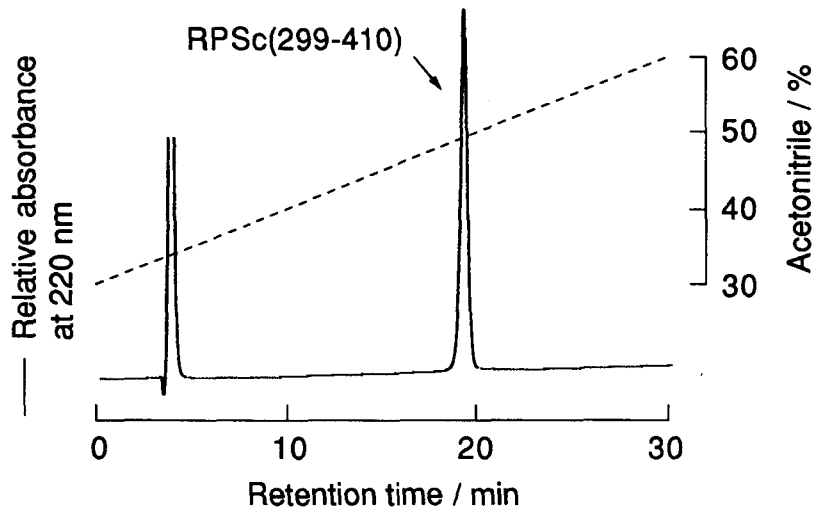


Fig. V-8. RPHPLC profile of RPSc(299-410) (V-7) after gel filtration and RPHPLC purification. Column: YMC-Pack ODS-AM (4.6X250 mm) at a flow rate of 1 ml min^{-1} at 40 °C. Eluent: 50% aqueous acetonitrile containing 0.1% TFA.

RPSc(299-410) will be measured in the presence of the amino and/or carboxyl terminal flanking peptide segments, which should interact with the hydrophobic part of the RPSc(299-410). The data will be published elsewhere.

Evaluation of the Method.

Partially protected cysteine-containing peptide thioesters were prepared by a solid-phase method using Npys-amino acids. 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 proven useful even for the preparation of cysteine-containing proteins.

As a terminal amino protecting group, Fmoc was superior to Troc or *i*Noc groups in its ease of removal. In addition, the product was obtained as a precipitate only by adding ether to the reaction mixture.

V-3 Materials and Methods

Fmoc-amino acids and *p*-[(R,S)-α-{1-(9H-Fluoren-9-yl)-methoxyformamido}-2,4-dimethoxybenzyl]phenoxyacetic acid (Fmoc-NH-SAL resin linker) was 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.[7] ESI mass spectrum of peptides **V-7** and **V-12** were measured by JMS-SX102 mass spectrometer (JEOL Ltd., Tokyo) equipped with an ESI ion source (Analytica of Branford, Conn.).

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 **V-2**, **V-3**, **V-8**, **V-10**, and **V-11** 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 **V-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*. Peptide **V-1** was synthesized manually by a solid-phase method using a Npys-amino acids.[6)]

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, 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 $C_{11}H_{10}O_6N_4S_1$: C, 40.30; H, 3.10; N, 17.23%.

Npys-Gly-SC(CH$_3$)$_2$CH$_2$COOH.

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). DIEA (580 mg, 4.5 mmol) was added and the solution was stirred for 3 h at room temperature. More DIEA (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 (X5) 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$_3$)$_2$CH$_2$COOH (190 mg, 550 μmol, 20%), m.p.

107.0-107.5 °C. Found: C, 41.66; H, 4.36; N, 12.16%. Calcd for $C_{12}H_{15}O_5N_3S_2$: 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% DIEA in DMF (v/v) for 5 min (X2). 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).

[Arg(Tos)^{3,4}, Cys(MeBzl)^7]-hANP(1-9)-SC$(CH_3)_2CH_2$CO-Ala (V-8).

Starting from Boc-Ala-OCH_2-Merrifield resin (1.4 g, Ala: 0.5 mmol), a protected peptide resin corresponding to the sequence of hANP(1-9), Boc-Ser(Bzl)-Leu-Arg(Tos)-Arg(Tos)-Ser(Bzl)-Ser(Bzl)-Cys(MeBzl)-Phe-Gly-SC$(CH_3)_2CH_2$CO-Ala-OCH_2-Merrifield resin (2.2 g) was prepared using the single coupling protocol. An aliquot of the resin (300 mg) was treated under low-HF conditions (HF-dimethylsulfide-*p*-cresol, 25:65:10 (v/v), 5 ml) at 0 °C for 2 h.[4] After complete evaporation of HF under a high vacuum, the residual solid was washed with ether (X2) and ethyl acetate (X2). The peptide was extracted with 30% aqueous acetonitrile containing 0.1% TFA and lyophilized to give a crude peptide (170 mg), which was purified on RPHPLC to give peptide **V-8** (37mg, 13 μmol, 19% based on Ala in the starting resin). Found: m/z 1611.7 $(M+H)^+$. Calcd: m/z 1611.6 $(M+H)^+$. Amino acid analysis of peptide **V-8**: $Ser_{2.63}Gly_1Ala_{1.07}Leu_{0.93}Phe_{1.00}(Arg+Cys(MeBzl))_{2.77}$.

[Cys(MeBzl)^7]-hANP(1-9)-SC(CH_3)$_2$$CH_2$CO-Nle-$NH_2$ (V-10).

Starting from Boc-Gly-SC(CH_3)$_2$$CH_2$CO-Nle-NH-resin (1.2 g, Gly: 0.21 mmol), the protected peptide resin corresponding to the sequence of hANP(1-9), Boc-Ser(Bzl)-Leu-Arg(Tos)-Arg(Tos)-Ser(Bzl)-Ser(Bzl)-Cys(MeBzl)-Phe-Gly-SC(CH_3)$_2$-CH_2CO-Nle-NH-resin (1.4 g) was obtained using the single coupling protocol. An aliquot of the resin (540 mg) was treated under high-HF conditions (HF:anisole, 85:15 (v/v), 10 ml) at 0 °C for 90 min and 98 mg of the crude peptide was obtained. This peptide was purified on RPHPLC to give 60 mg (39 μmol, 49% based on Gly in the starting resin) of Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-SC(CH_3)$_2$$CH_2$CO-Nle-$NH_2$ (peptide **V-9**). This peptide, 30 mg (19 μmol), was dissolved in 90% aqueous DMF (2.0 ml) and 70 mg (0.5 mmol) of MeBzl-Cl was added. The reaction mixture was stirred at room temperature for 90 min after the addition of NMM (120 μl). The product was purified on RPHPLC to give 17 mg (9.7 μmol, 51% based on peptide **V-9**) of peptide **V-10**. Found: m/z 1344.5 $(M+H)^+$. Calcd: m/z 1344.7 $(M+H)^+$. Amino acid analysis of peptide **V-10**: $Ser_{2.43}Gly_1$ $Leu_{0.89}Nle_{0.92}Phe_{1.03}(Arg+Cys(MeBzl))_{2.44}$.

[Arg(Tos)^{11,14,27}, Cys(MeBzl)^{23}]-hANP(10-28) (V-11).

Starting from Boc-Tyr(Br-Z)-OCH_2-Merrifield resin (1.2 g, Tyr: 0.5 mmol), a protected peptide resin corresponding to the sequence of hANP(10-28), Boc-Gly-Arg(Tos)-Met-Asp(OBzl)-Arg(Tos)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser(Bzl)-Phe-Arg(Tos)-Tyr(Br-Z)-OCH_2-Merrifield resin (2.7 g) was obtained using the double coupling protocol. An aliquot of the resin (500 mg) was treated under low-HF conditions (HF-dimethylsulfide-*p*-cresol, 25:65:10 (v/v), 7 ml) at 0 °C for 2 h. Gly-Arg(Tos)-Met-Asp-Arg(Tos)-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(MeBzl)-Asn-Ser-Phe-Arg(Tos)-Tyr (15 mg, 4.6 mmol, 5% based on Tyr in the starting resin) was obtained by the procedure described for the synthesis of peptide **V-8**. Found: m/z 2654.1 $(M+H)^+$.

Calcd: m/z 2654.1 $(M+H)^+$. Amino acid analysis of peptide **V-11**: $Asp_{2.06}Ser_{1.73}Glu_{1.07}Gly_4Ala_{1.02}Met_{0.76}Ile_{1.05}Leu_{1.07}Tyr_{1.03}Phe_{1.19}(Arg+Cys(MeBzl))_{4.20}$.

Boc-[Cys(Tmb)^{302,317}, Lys(Boc)^{315}]-RPSc(299-321)-SC$(CH_3)_2CH_2CO$-NH_2 (V-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 (X6). 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 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 minX3); (2) DMF (1 minX3); (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 minX3). An aliquot of the resin (220 mg) was treated with reagent K (5 ml) at room temperature for 120 min. 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: m/z 3146.4

$(M+H)^+$. 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)-$SC(CH_3)_2CH_2CONH_2$ (26 mg, 6.9 μmol) dissolved in DMSO (150 μl) was reacted with Boc-ONSu (6.9 mg, 32 μmol) in the presence of DIEA (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 **V-1**. Found: m/z 3346.7 $(M+H)^+$. Calcd: m/z 3346.5 $(M+H)^+$. Amino acid analysis of peptide **V-1**: $Asp_{3.89}Glu_{4.16}Pro_{0.96}$ $Gly_{1.32}$ 1/2Cystine$_{0.64}$ $Val_{1.98}Met_{1.91}Ile_{0.65}Leu_2Tyr_{0.94}Lys_{1.19}His_{1.17}Trp_{0.50}$.

Fmoc-RPSc(322-342)-$SC(CH_3)_2CH_2CO$-Nle-NH_2 (V-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(O*c*Hex)-Arg(Tos)-Glu(OBzl)-Thr(Bzl)-Ala-Leu-Asp(O*c*Hex)-Phe-Ile-Gly-Arg(Tos)-Arg(Tos)-Gly-Thr(Bzl)-Ala-Leu-Gly-$SC(CH_3)_2CH_2CO$-Nle-NH-resin was prepared by the synthesizer using double coupling protocol. The *N*-terminal Boc group was removed by 55% TFA in DCM (v/v) for 5 and 15 min, followed by neutralization with 5% DIEA in DMF (v/v) for 5 min (X2). Fmoc-ONSu (670 mg, 2 mmol) was then allowed to react with the terminal amino group in DMF overnight, to give a protected peptide resin (1.8 g). An aliquot of the resin (740 mg) was treated with HF (10 ml) containing anisole (1 ml) at 0 ^oC 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: m/z 2785.5 $(M+H)^+$. Amino acid analysis of peptide **V-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 (V-3).

Starting from Boc-Gly-SC$(CH_3)_2CH_2$CO-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-Tyr(Br-Z)-Ala-Lys(Cl-Z)-Asp(O*c*Hex)-Ile-Leu-Gln-Lys(Cl-Z)-Glu(OBzl)-Phe-Leu-Pro-His(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$(CH_3)_2CH_2$CO-Nle-NH-resin (3.3 g) was prepared by the method described for the synthesis of peptide **V-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$(CH_3)_2CH_2$CO-Nle-NH_2. (peptide **V-12**). Found: 4901.5 daltons. Calcd: (average) 4901.9 daltons. Amino acid analysis of peptide **V-12**: $Asp_{0.95}Thr_{0.94}Ser_{0.97}Glu_{6.79}Pro_{0.70}Gly_2Ala_{1.77}Ile_{3.29}Leu_{3.91}$ $(Tyr+Nle)_{2.03}Phe_{4.02}Lys_{5.48}His_{1.01}Arg_{1.84}$.

To the solution of peptide **V-12** (41 mg, 6.6 μmol) dissolved in DMSO (200 μl), Boc-ONSu (22 mg, 100 μmol) and DIEA (18 μl, 100 μmol) were added and the solution was stirred for 2.5 h to give peptide **V-3** (47 mg, 6.6 μmol, 6.4% based on Gly in the starting resin) as described for the synthesis of peptide **V-1**. Amino acid analysis of peptide **V-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) (V-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(OBu^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, precipitated with ether was treated with reagent K in the same manner. The peptide (370 mg), precipitated with ether was purified on RPHPLC 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: m/z 4015.1 $(M+H)^+$. Amino acid composition: $Asp_{7.15}Glu_{1.46}Gly_{2.22}Ala_{1.94}Met_{0.56}Ile_{0.90}Leu_{5.63}Tyr_{0.72}Phe_1Lys_{3.13}His_{1.08}(Arg+Cys(MeBzl))_{4.57}$.

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 DIEA (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 **V-1**. Amino acid composition: $Asp_{6.98}Glu_{1.01}Gly_{1.97}Ala_{1.93}Met_{0.67}Ile_{0.86}Leu_{5.71}Tyr_{0.68}Phe_1Lys_{2.96}His_{1.00}(Arg+Cys(MeBzl))_{4.72}$.

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 was 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 **V-4** (110 mg, 17 μmol, 17% based on Gly in the starting resin). Amino acid analysis of peptide **V-4**: $Asp_{7.15}Glu_{1.10}Gly_{1.97}Ala_{1.93}Met_{0.83}Ile_{0.95}Leu_{5.94}Tyr_{0.97}Phe_1Lys_{2.67}His_{1.02}(Arg+Cys(MeBzl))_{4.93}$.

Synthesis of RPSc(299-410) (V-7).

Peptides **V-3** (7.7 mg, 1.1 μmol), **V-4** (5.7 mg, 0.9 μmol) and HONSu (1.6 mg, 14 μmol) were dissolved in DMSO (150 μl) containing DIEA (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. DIEA (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, which was washed with distilled water containing 0.1% TFA (X2). A mixture containing peptide **V-5** (13 mg) was obtained after lyophilization. Following the same procedure, peptides **V-2** (7.8 mg, 2.3 μmol) and **V-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 ^{o}C for 90 min. This peptide was purified by gel filtration on G3000SW (7.5X600 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) (**V-7**) (2.0 mg, 110 nmol, 12% based on peptide **V-4**). Found: 13193.9 daltons. Calcd: (average) 13191.2 daltons. Amino acid analysis of peptide **V-7**: $Asp_{14.40}$ $Thr_{2.74}Ser_{0.95}Glu_{13.15}Pro_{1.95}Gly_{8}Ala_{6.04}$ 1/2$Cys_{0.86}Val_{2.28}Met_{2.65}Ile_{5.28}$ $Leu_{12.86}Tyr_{2.81}Phe_{6.05}Lys_{8.76}His_{2.49}Trp_{0.64}Arg_{8.24}$.

Measurement of RNase Activity.

RNase activity of RPSc(299-410) was measured according to the procedure described in the previous chapter for barnase.

References

1) T. Shirai and M. Go, *Proc. Natl. Acad. Sci. USA*, **88**, 9056 (1991).

2) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973).

3) F. Brtnik, M. Krojidlo, T. Barth, and K. Jost, *Coll. Czech. Chem. Commun.*, **46**, 286 (1981).

4) J. P. Tam, W. F. Heath, and R. B. Merrifield, *J. Am. Chem. Soc.*, **105**, 6442 (1983).

5) M. S. Bernatowicz, S. B. Daniels, and H. Köster, *Tetrahedron Lett.*, **30**, 4645 (1989).

6) R. Matsueda and R. Walter, *Int. J. Peptide Protein Res.*, **16**, 392 (1980).

7) D. S. King, C. G. Fields, and G. B. Fields, *Int. J. Peptide Protein Res.*, **36**, 255 (1990).

8) L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, **92**, 5748 (1970).

9) S. S. Wang, *J. Am. Chem. Soc.*, **95**, 1328 (1973).

Summary

A novel strategy of protein synthesis, which has overcome the problems involved in the solution and solid-phase methods, was developed. Partially protected peptide thioesters are used as building blocks. The application of this method to the synthesis of several proteins led to the following conclusions.

1) Partially protected peptide thioesters are easily prepared by means of the solid-phase method.
2) The thioester moiety is rapidly converted to an active ester in the presence of silver ions and HONSu and segment condensation can be accomplished using partially protected peptide segments.
3) Consecutive condensation of the partially protected peptide segments is realized by the selective removal of the terminal amino protecting group after segment condensation.
4) Peptide segments retain high reactivity even in the condensation between high molecular weight peptide segments. This fact suggests that peptides with the minimum number of protecting groups are well solvated by DMSO and hence the peptide can maintain good flexibility around the reaction sites.
5) Large peptide segments can be used. Thus, all the products obtained by the thioester method were separated from a reaction mixture as distinct peaks by RPHPLC, even when there is no purification process during successive segment condensation. This fact suggests that a protein without any specific features such as enzymatic or biological activities, can be isolated solely by its chromatographic profile.
6) The thioester method will provide a new basis for protein studies.

7) The problem of racemization during segment condensation remains to be solved. Thus, condensation has to proceed using a peptide thioester with a glycine residue at the *C*-terminal at the moment.

List of Publications

1) Development of a Facile Method for Polypeptide Synthesis. Synthesis of Bovine Pancreatic Trypsin Inhibitor (BPTI)

S. Aimoto, N. Mizoguchi, H. Hojo, and S. Yoshimura

Bull. Chem. Soc. Jpn., **62**, 524-531 (1989).

2) Mast Cell Degranulating Peptide Forms Voltage Gated and Cation-Selective Channels in Lipid Bilayers

T. Ide, T. Taguchi, T. Morita, M. Sato, K. Ikenaka, S. Aimoto, T. Kondo, H. Hojo, M. Kasai, and K. Mikoshiba

Biochem. Biophys. Res. Commun., **163**, 155-160 (1989).

3) Application of Protected-Peptide *S*-Alkyl Thioester Method to the Synthesis of HU-Type DNA-Binding Protein (HBs)

H. Hojo, C. Maegawa, S. Yoshimura, and S. Aimoto

"Peptide Chemistry 1988", ed. by M. Ueki, Protein Research Foundation, Osaka (1989), 97-102.

4) Synthesis of DNA-Binding Protein II (HBs) by the Use of Protected-Peptide *S*-Alkyl Thioesters

S. Aimoto, C. Maegawa, S. Yoshimura, and H. Hojo

"Peptides 1988", ed. by G. Jung and E. Bayer, Walter de Gruyter, Berlin (1989), 25-27.

5) Long-Term Enhancement of Synaptic Transmission by Synthetic Mast Cell Degranulating Peptide and Its Localization of Binding Sites in Hippocampus

T. Kondo, K. Ikenaka, H. Kato, K. Ito, S. Aimoto, H. Hojo, and K. Mikoshiba

Neuroscience Research, **8**, 147-157 (1990).

6) Studies on the Disulfide Bridges of Sarafotoxins. Chemical Synthesis of Sarafotoxin S6b and Its Homologue with Different Disulfide Bridges

S. Aimoto, H. Hojo, and C. Takasaki

Biochemistry International, **21**, 1051-1057 (1990).

7) Re-Synthesis of DNA-Binding Protein II (HBs) Using Protected-Peptide *S*-Alkyl Thioester Method

H. Hojo, C. Maegawa, S. Yoshimura, and S. Aimoto

"Peptide Chemistry 1989", ed. by N. Yanaihara, Protein Research Foundation, Osaka (1990), 297-302.

8) Polypeptide Synthesis Using the *S*-Alkyl Thioester of a Partially Protected Peptide Segment. Synthesis of the DNA-Binding Domain of *c*-Myb Protein(142-193)-NH_2

H. Hojo and S. Aimoto

Bull. Chem. Soc. Jpn., **64**, 111-117 (1991).

9) Pro-Peptide as an Intermolecular Chaperone: Renaturation of Denatured Subtilisin E with a Synthetic Pro-Peptide

Y. Ohta, H. Hojo, S. Aimoto, T. Kobayashi, X. Zhu, F. Jordan, and M. Inouye

Molecular Microbiology, **5**, 1507-1510 (1991).

10) A New α-Helical Motif in Membrane Active Peptides

Y. Kobayashi, A. Sato, H. Takashima, H. Tamaoki, S. Nishimura, Y. Kyogoku, K. Ikenaka, T. Kondo, K. Mikoshiba, H. Hojo, S. Aimoto, and L. Moroder

Neurochem. Int., **18**, 525-534 (1991).

11) Synthesis of DNA-Binding Protein Using Partially Protected Peptide Thioesters

H. Hojo, S. Yoshimura, C. Maegawa, and S. Aimoto

"Peptide Chemistry 1990", ed. by Y. Shimonishi, Protein Research Foundation, Osaka (1991), 23-28.

12) Rotational Resonance NMR Studies of an Undecapeptide of Alamethicin: Comparison of Crystal and Membrane Structure

O. Peersen, S. Yoshimura, H. Hojo, S. Aimoto, and S. Smith

"Peptide Chemistry 1990", ed. by Y. Shimonishi, Protein Research Foundation, Osaka (1991), 399-404.

13) Total Synthesis of DNA-Binding Protein Using Partially Protected Peptide Thioesters as Building Blocks

S. Aimoto and H. Hojo

"Peptides 1990", ed. by E. Giralt and D. Andreu, ESCOM, Leiden (1991), 113-114.

14) Rotational Resonance NMR Measurement of Internuclear Distances in an α-Helical Peptide

O. B. Peersen, S. Yoshimura, H. Hojo, S. Aimoto, and S. O. Smith

J. Am. Chem. Soc., **114**, 4332-4335 (1992).

15) Solution Structure of a DNA-Binding Unit of Myb: a Helix-Turn-Helix-Related Motif with Conserved Tryptophans Forming a Hydrophobic Core

K. Ogata, H. Hojo, S. Aimoto, T. Nakai, H. Nakamura, A. Sarai, S. Ishii, and Y. Nishimura

Proc. Natl. Acad. Sci. USA, **89**, 6428-6432 (1992).

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

H. Hojo and S. Aimoto

Bull. Chem. Soc. Jpn., **65**, 3055-3063 (1992).

17) Assembly Regulatory Domain of Glial Fibrillary Acidic Protein

Y. Nakamura, M. Takeda, S. Aimoto, H. Hojo, T. Takao, Y. Shimonishi, S. Hariguchi, and T. Nishimura

J. Biol. Chem., **267**, 23269-23274 (1992).

18) Improvement of the Method for the Preparation of Partially Protected Peptide Thioester

H. Hojo, Y. D. Kwon, and S. Aimoto

"Peptide Chemistry 1991", ed. by A. Suzuki, Protein Research Foundation, Osaka (1992), 115-120.

19) Preparation of Partially Protected Peptide Thioesters Containing a Cysteine Residue and Their Segment Condensation

Y. D. Kwon, R. H. Zhang, M. P. Bemquerer, M. Tominaga, H. Hojo, and S. Aimoto

Chem. Lett., **1993**, 881-884.

20) 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

H. Hojo, Y. D. Kwon, Y. Kakuta, S. Tsuda, I. Tanaka, K. Hikichi, and S. Aimoto

Bull. Chem. Soc. Jpn., **66**, 2700-2706 (1993).

21) Synthesis of Barnase Site-Specifically Labelled with Two ^{13}C Atoms Using Partially Protected Peptide Thioester Building Blocks

H. Hojo and S. Aimoto

Bull. Chem. Soc. Jpn., **66**, 3004-3008 (1993).

22) Protein Synthesis Using Partially Protected Peptide Thioesters as Building Blocks

S. Aimoto, Y. D. Kwon, and H. Hojo

"Peptide Chemistry 1992", ed. by N. Yanaihara, ESCOM, Leiden (1993), 54-57.

23) Preparation of Partially Protected Peptide Thioesters Containing Cysteine Residue and Their Segment Condensation

Y. D. Kwon, R. H. Zhang, H. Hojo, and S. Aimoto

"Peptide Chemistry 1992", ed. by N. Yanaihara, ESCOM, Leiden (1993), 58-60.