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

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Barnase, site-specifically labelled with two $^{13}C$ atoms, was synthesized using partially protected peptide thioesters as building blocks. Four partially protected peptide segments (Boc–[Lys(Boc)$^{19,27}$]–barnase(1—34)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$, *i*Noc–[Lys(Boc)$^{39,49}$]–barnase(35—52)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$, *i*Noc–[Lys(Boc)$^{62,66}$]–barnase(53—81)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$, [Lys(Boc)$^{98,108}$]–barnase(82—110)) were successively condensed in the presence of silver ions and *N*-hydroxysuccinimide (HONSu). Finally, barnase with full ribonuclease activity was obtained in a yield of 11% based on the carboxyl terminal peptide segment.

Most protein production is not performed by a chemical method, but by recombinant DNA technology. This is because neither the solid-phase nor solution methods of peptide synthesis are appropriate for the rapid preparation of highly pure proteins. In order to overcome the problems involved in both methods, we developed a procedure in which partially protected peptide thioesters prepared via a solid-phase method are used as building blocks for protein synthesis in a solution. This procedure[1] was applied to the syntheses of *c*-Myb Protein (142—193) amide,[2] HU-type DNA-binding protein (HBs) of 90 amino acids[3] and its stable-isotope-labelled derivative.[4]

To demonstrate the usefulness of this method for enzyme synthesis, we synthesized barnase, a protein comprising 110 amino acids with RNase activity (Fig. 1). In this study, barnase site-specifically labelled with two $^{13}C$ atoms was synthesized for a structural study of barnase in the future. This paper describes the results of the synthesis and the use of this method for protein synthesis.

## Results and Discussion

**Preparation of Peptide Segments:** For synthetic purposes, the barnase sequence was divided into four peptide segments, as shown in Fig. 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 a previous paper.[4] To a 4-methylbenzhydrylamine resin (MBHA resin or $NH_2$-resin), *t*-butoxycarbonyl-L-norleucine (Boc–Nle) and Boc–Gly–SC$(CH_3)_2CH_2COOH$ were successively introduced using dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt) to obtain Boc–Gly–SC$(CH_3)_2CH_2CO$–Nle–NH–resin.[4] On this resin, Boc–amino acids were successively condensed. After peptide chain assembly, the terminal amino group was protected with a 4-pyridylmethoxycarbonyl (*i*Noc) group. The protected peptide resin was treated with anhydrous hydrogen fluoride[5] to give a crude *i*Noc-peptide thioester, which was purified by reversed-phase HPLC (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 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 a previous synthesis.[4]

**Synthesis of Barnase by Segment Coupling:** Segment condensation was performed according to the scheme shown in Fig. 2. The typical coupling conditions were as follows: Peptides **3** (120 mg, 17 μmol) and **4** (100 mg, 13 μmol) were dissolved in dimethyl sulfoxide (DMSO) (2.3 ml). HONSu (30 mg, 260 μmol), $AgNO_3$ (13 mg, 77 μmol), and 4-methylmorpholine (NMM) (9 μl, 82 μmol) were then added in succession. The solution was stirred overnight at room temperature in the dark. The peptide was 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 **5** (170 mg). According to a similar procedure, peptides **2** and **5**, then peptides **1** and **6**, were successively condensed. The condensation reactions were monitored by HPLC using a C4 column. The HPLC elution profiles of the reaction mixtures are shown in Fig. 3. The segment coupling of peptide **5** and **2** and that of peptide **6** and **1** were almost complete within 6 h. During peptide chain elongation by segment coupling no HPLC purification was performed.

After segment condensation of peptides **1** and **6**, dis-

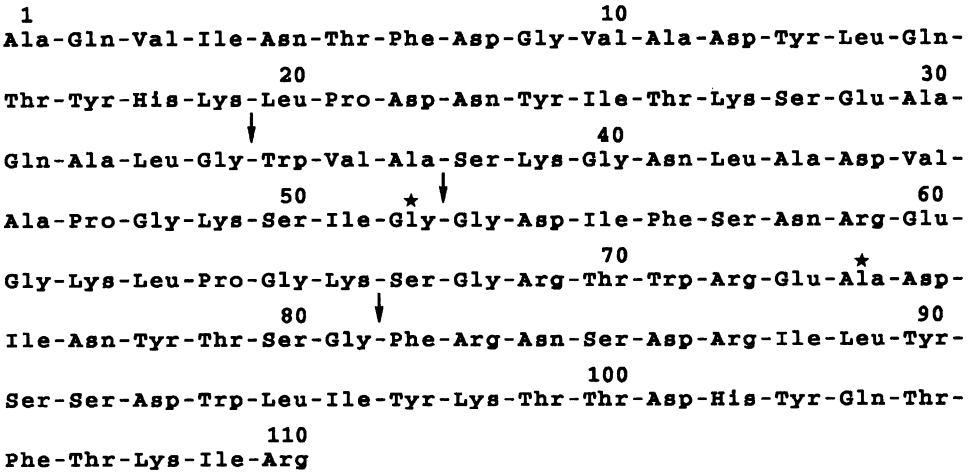


Fig. 1. Amino acid sequence of barnase. The arrows indicate the sites of segment coupling; * indicates amino acids labelled with $^{13}C$; (2-$^{13}C$)Gly$^{52}$ and (1-$^{13}C$)Ala$^{74}$.

Table 1. Partially Protected Peptide Segments Prepared for Segment Coupling

| Peptide segments | Yield/% |
|---|---|
| Boc–[Lys(Boc)$^{19,27}$]–Barnase(1—34)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$<br>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–$NH_2$ | (**1**) 12 |
| *i*Noc–[Lys(Boc)$^{39,49}$]–Barnase(35—52)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$<br>*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–$NH_2$ | (**2**) 32 |
| *i*Noc–[Lys(Boc)$^{62,66}$]–Barnase(53—81)–SC$(CH_3)_2CH_2CO$–Nle–$NH_2$<br>*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–$NH_2$ | (**3**) 12 |
| [Lys(Boc)$^{98,108}$]–Barnase(82—110)<br>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 | (**4**) 12 |

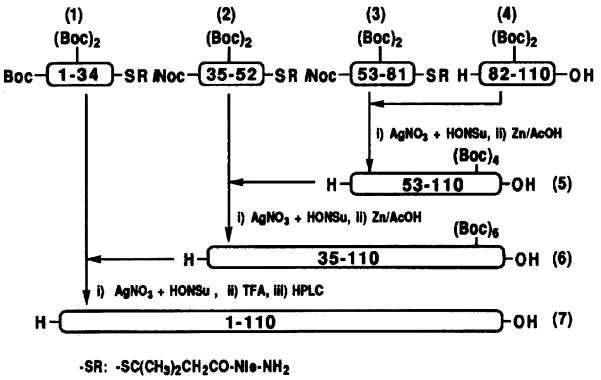


Fig. 2. Synthetic route of barnase (1—110).

tilled water was added to the reaction mixture. The formed precipitate was washed with water and freeze-dried to give a powder which was treated with trifluoroacetic acid (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. 4) to give the final product, barnase (1—110) (**7**) in 11% yield, based upon peptide **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.[6] The synthetic and native barnases had similar activity, as shown in Table 2.

**Application of the Method:** In the barnase synthesis, all of the segments were condensed almost completely within 6 h, even that between peptides **1** and **6** of 34 and 76 amino acid residues, respectively. This fact suggests that the peptide segment with minimum

Table 2. Enzymatic Activity of Barnase

| | Specific activity | Relative activity |
|---|---|---|
| | $\times 10^{-6}$ units/$A_{280}$ | % |
| Native barnase | 2.1±0.2 | 100 |
| Synthetic barnase | 2.4±0.3 | 114 |

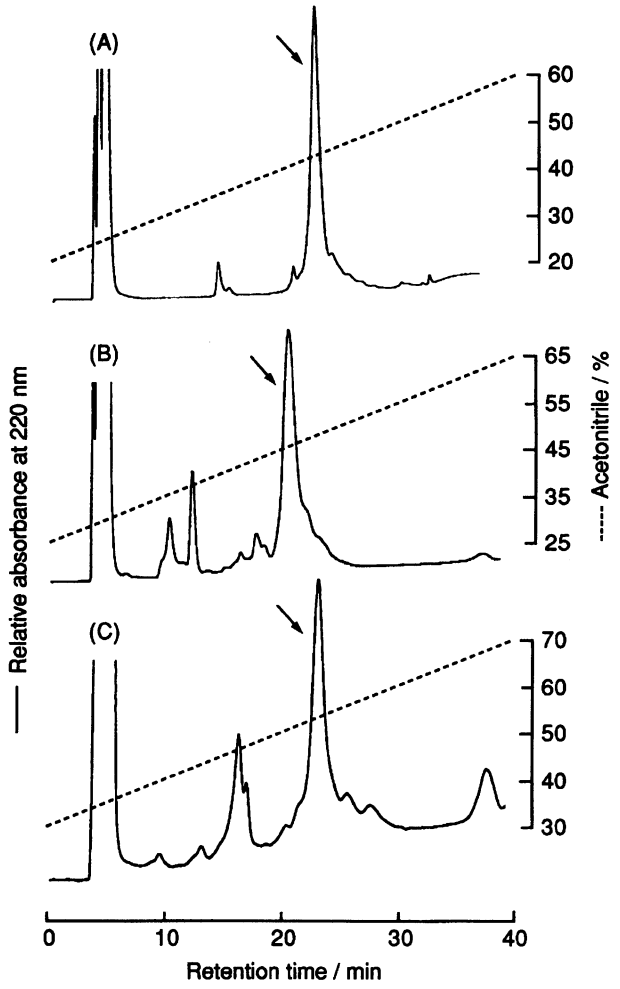


Fig. 3. HPLC elution profiles of the reaction mixtures of segment couplings after an overnight reaction. Arrows in panel A, B, C indicate *i*Noc-[Lys(Boc)$^{62,66,98,108}$]-barnase(53—110), *i*Noc-[Lys(Boc)$^{39,49,62,66,98,108}$]-barnase(35—110), and Boc-[Lys(Boc)$^{19,27,39,49,62,66,98,108}$]-barnase(1—110), respectively. Column: YMC-Pack C4 (4.6×250 mm). The broken line indicates the acetonitrile concentration in 0.1% TFA.

protecting groups was well solvated by DMSO, kept good flexibility around the reaction sites and, hence, retained high reactivity. Thus, the thioester building block 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 2,2,2-trichloroethoxycarbonyl (Troc) or *i*Noc for the terminal amino group, and to establish a strategy with which to synthesize cysteine-containing proteins.[7]

## Materials and Methods

Boc-amino acid derivatives and MBHA resin were purchased from the Peptide Institute Inc. (Osaka). Boc-Arg(Tos)-$OCH_2$-$C_6H_4CH_2CONHCH_2$-$C_6H_4$-resin (Boc-Arg(Tos)-$OCH_2$-PAM-resin) was purchased from Applied Biosystems Inc. (Foster City, CA.). (1-$^{13}$C)Ala and (2-$^{13}$C)Gly were purchased from Isotec Inc. (Miamisburg, OH.). The solvents and reagents used for solid-phase peptide synthesis were purchased from Watanabe Chemical Ind. Ltd. (Hiroshima). Analytical RPHPLC was performed on YMC-Pack ODS-AM or C4 (4.6×250 mm) and preparative RPHPLC was on YMC-Pack ODS-AM or PROTEIN-RP (20×250 mm) (YMC, Kyoto). The amino acids were analyzed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo) after hydrolysis with 4 M# methanesulfonic acid at 110 °C for 24 h in an evacuated sealed tube. The peptide mass number was determined by fast atom bombardment mass spectrometry using a JMS-HX100 (JEOL Ltd., Tokyo) equipped with a JMA-3100 mass data system. Although the peptide weight was an observed value, the yield was calculated based upon the amino acid analysis data. Sonication was performed using a Branson Model B-220. Dialysis was carried out using a Spectrapor 6 membrane (M. W. cut off 1000). Native barnase was a gift from Dr. H. Yanagawa of Mistubishi-Kasei Institute of Life Sciences. Yeast RNA was purchased from Kohjin Co., Ltd. (Tokyo).

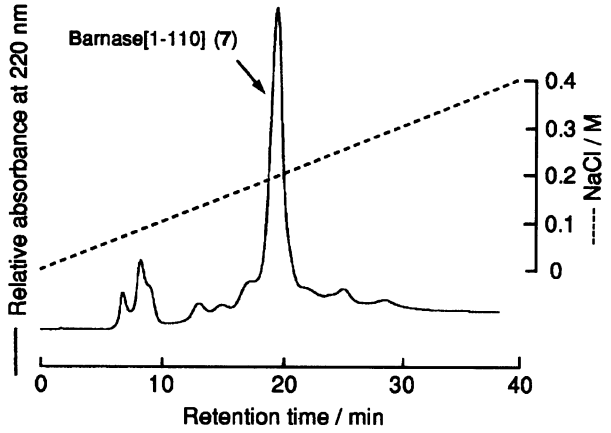


Fig. 4. Ion-exchange chromatography of the HPLC-purified barnase(1—110) (**7**) by Pharmacia HiLoad S-Sepharose HP (16×100 mm). The broken line indicates the NaCl concentration in 0.05 M sodium phosphate buffer (pH 6.0).

## Synthesis

**Peptide Chain Elongation on a Solid Support.** Solid-phase syntheses of peptide segments were performed on a peptide synthesizer 430A (Applied Biosystems Inc.) using the 0.5 mmol scale, double-coupling protocol of the benzotriazole-active ester method of the system software version 1.40 NMP/HOBt *t*-Boc. Unreacted amino groups were capped by acetic anhydride after each amino acid introduction. The side-chain-protecting groups of Boc-amino acids were *o*-chlorobenzyloxycarbonyl (Cl-Z) for the $N^{\varepsilon}$ of Lys, benzyl (Bzl) for the alcoholic OH of Thr and Ser, cyclohexyl ester (O*c*Hex) for the $\beta$-carboxyl group of Asp, benzyl ester for the $\gamma$-carboxyl group of Glu, tosyl (Tos) for the $N^{g}$ of Arg, benzyloxymethyl for the $N^{\pi}$ of His, 2-bromobenzyl-

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

oxycarbonyl for the phenolic OH of Tyr and formyl (For) for the $N^{i}$ of Trp. Boc–Gly–$SC(CH_3)_2CH_2COOH$ was prepared according to the method described in a preceding paper.[4]

**iNoc–[Lys(Boc)$^{39,49}$, (2-$^{13}$C)Gly$^{52}$]–barnase(35—52)–$SC(CH_3)_2CH_2CO$–Nle–$NH_2$ (2).** 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 30 min, this solution was mixed with neutralized MBHA-resin (850 mg, 0.54 mequiv of $NH_2$ group) and shaken for 4 h. After the Boc group was removed with 55% TFA in dichloromethane (DCM) for 5 and 15 min followed by neutralization with 5% *N,N*-diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF) for 5 min (×2), Boc–(2-$^{13}$C)Gly–$SC(CH_3)_2CH_2COOH$ (220 mg, 0.75 mmol), prepared as the same procedure described previously,[4] 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–(2-$^{13}$C)Gly–$SC(CH_3)_2CH_2CO$–Nle–NH–resin. Using this resin, 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(OcHex)–Val–Ala–Pro–Gly–Lys(Cl-Z)–Ser(Bzl)–Ile–(2-$^{13}$C)Gly–$SC(CH_3)_2CH_2CO$–Nle–NH–resin was prepared on a synthesizer by means of double coupling. After this resin was treated with 55% TFA in DCM for 5 and 15 min, followed by neutralization with 5% DIEA in DMF for 5 min (×2), 4-pyridylmethyl *p*-nitrophenyl carbonate (*i*Noc–ONp) (410 mg, 1.5 mmol) was allowed to react with the terminal amino group in 80% DMSO–NMP overnight, to give a protected peptide resin (1.9 g). An aliquot of the resin (510 mg) was treated with HF (8 ml), *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)–$SC(CH_3)_2CH_2CO$–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 for $(M+H)^+$: 2134.1. Amino acid composition: $Asp_{2.16}Ser_{2.03}Pro_{1.03}Gly_3\,Ala_{3.27}Val_{2.04}\,Ile_{0.98}Leu_{1.06}Nle_{0.99}\,Lys_{2.08}Trp_{0.54}$.

To the *i*Noc–[(2-$^{13}$C)Gly$^{52}$]–barnase (35—52)–$SC(CH_3)_2CH_2CO$–Nle–$NH_2$ (150 mg, 50 μmol) dissolved in DMSO (2.6 ml), *N*-(*t*-butoxycarbonyloxy)succinimide (Boc–ONSu) (56 mg, 260 μmol) and triethylamine (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 **2** (46 μmol, 32% based on the amino groups in the MBHA resin). Found: *m/z* 2335.0 $(M+H)^+$, Calcd for $(M+H)^+$: 2334.2. Amino acid analysis of peptide **2**: $Asp_{2.08}Ser_{1.88}Pro_{1.07}Gly_{3.05}Ala_3Val_{1.93}Ile_{0.98}Leu_{1.02}\,Nle_{0.99}Lys_{2.02}Trp_{0.50}$.

**Boc–[Lys(Boc)$^{19,27}$]–barnase(1—34)–$SC(CH_3)_2CH_2CO$–Nle–$NH_2$ (1).** This peptide was prepared following the procedure described for the synthesis of peptide **2**. Yield of peptide **1**: 12% based on the amino groups in the MBHA resin. Amino acid analysis of peptide **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)$^{62,66}$, (1-$^{13}$C)Ala$^{74}$]–barnase(53—81)–$SC(CH_3)_2CH_2CO$–Nle–$NH_2$ (3).** This peptide was prepared by the procedure described for the synthesis of peptide **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. Yield of peptide **3**: 12% based on the amino groups in the MBHA resin. Found: *m/z* 3775.5. Calcd for $(M+H)^+$: 3775.9. Amino acid analysis of peptide **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) (4).** Starting from Boc–Arg(Tos)–$OCH_2$–PAM–resin (0.83 g, 0.5 mmol), *i*Noc–[Lys(Boc)$^{98,108}$]–barnase(82—110) (440 mg, 62 μmol) was prepared by the procedure described for peptide **2**. This peptide (130 mg, 18 μmol) was sonicated with zinc dust (200 mg) in 75% aqueous acetic acid (4 ml) under nitrogen for 2 h. After removing zinc dust, the solution was dialyzed against distilled water (1 $dm^3$×3) and freeze-dried to give peptide **4** (120 mg, 17 μmol, 12% based on Arg in the starting resin). Found: *m/z* 3868.3. Calcd for $(M+H)^+$: 3868.0. Amino acid analysis of peptide **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) (7).** Peptides **3** (120 mg, 17 μmol), **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 $dm^3$×3) and freeze-dried to give a mixture (170 mg) containing peptide **5**. Following the same procedure, peptides **2** (81 mg, 20 μmol) and **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 **7** (81 mg, 3.7 μmol) after freeze-drying. This peptide was further purified by ion-exchange chromatography using Pharmacia HiLoad S-Sepharose HP (16×100 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 **4**). Amino acid analysis of peptide **7**: $Asp_{14.94}Thr_{8.50}Ser_{8.39}Glu_{7.25}Pro_{2.96}Gly_{10.21}Ala_8Val_{3.54}\,Ile_{6.87}Leu_{6.77}Tyr_{6.80}Phe_{3.81}Lys_{8.13}His_{2.12}Trp_{1.79}Arg_{5.82}$.

**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 °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 °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.

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## References

1) In this method, segment condensation proceeds according to the process shown below:
$R\text{–}CO\text{–}SR' + Ag^+ + HONSu \xrightarrow{DIEA} [R\text{–}CO\text{–}ONSu + Ag - SR'] \xrightarrow{NH_2\text{–}R''} R\text{–}CO\text{–}NH\text{–}R''$, where R–CO–SR′ is a partially protected peptide thioester, –SR′ is $-SC(CH_3)_2CH_2CO\text{–Nle–}NH_2$ and $NH_2$-R″ is an amino component peptide.

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