Synthesis of Peptides and Proteins without Cysteine Residues by Native Chemical Ligation Combined with Desulfurization

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Abstract: The highly chemoselective reaction between unprotected peptides bearing an N-terminal Cys residue and a C-terminal thioester enables the total and semi-synthesis of complex polypeptides. Here we extend the utility of this native chemical ligation approach to non-cysteine containing peptides. Since alanine is a common amino acid in proteins, ligation at this residue would be of great utility. To achieve this goal, a specific alanine residue in the parent protein is replaced with cysteine to facilitate synthesis by native chemical ligation. Following ligation, selective desulfurization of the resulting unprotected polypeptide product with H_2/metal reagents converts the cysteine residue to alanine. This approach, which provides a general method to prepare alanyl proteins from their cysteinyl forms, can be used to chemically synthesize a variety of polypeptides, as demonstrated by the total chemical syntheses of the cyclic antibiotic microcin J25, the 56-amino acid streptococcal protein G B1 domain, and a variant of the 110-amino acid ribonuclease, barnase.

Introduction

Chemical protein synthesis from genomic sequence data is gaining significance in the "post-genomic era" of biomedical research by providing direct access to functional proteins. The chemical ligation of unprotected peptides generated by solid-phase peptide synthesis (SPPS) has made considerable progress in recent years.[1−4] Early approaches for protein synthesis using chemoselective reactions of unprotected peptides afforded proteins containing nonnatural peptide bonds at the site of ligation.[2−4] These chemistries continue to be useful for the structure−function analysis of proteins. The development of native chemical ligation using fully unprotected peptide segments has greatly facilitated the synthesis of proteins with all native peptide bonds.[5] This method, which is compatible with chemically synthesized and expressed peptide fragments,[6] has been used to synthesize a variety of protein targets including cyclic proteins.[7−9]

A common required element of native chemical ligation is a cysteine residue at the ligation site.[5] However, not all proteins contain a cysteine residue,or the cysteine residue may not be centrally located for use as a ligation site. In these cases, a noncritical residue may be mutated to cysteine to facilitate the synthesis without loss of protein function. This approach was demonstrated by the synthesis of [^{49}Cys]barnase, in which Lys49 was replaced with Cys.[10] Efforts to extend the applicability of native chemical ligation to non-cysteine ligation sites are ongoing.[11] For example, the requirement for a cysteine residue at the ligation site can be eliminated in the same protein systems by using a combination of C-α-thioester peptides and conformational assistance.[12] Another approach is to use a removable auxiliary to facilitate the peptide ligation.[13,14] Kent and coworkers[13] reported several model studies using X-Gly as the ligation site through an N^{α}-oxyethanethiol ($HSCH_2CH_2O-$) auxiliary. Similarly, an N^{α}-thiobenzyl linker has promise.[14] The challenge of these approaches is to obtain fast ligation while maintaining a cleavable handle. Both of these methods are still under development.

An alternative approach for the synthesis of non-cysteine-containing proteins is to chemically modify the cysteine residue after native chemical ligation. It would significantly extend the applicability of native chemical ligation if X-Ala could be used as the ligation site since alanine is one of the most abundant amino acid residues in proteins. Combining native chemical ligation with desulfurization would yield an X-Ala ligation site (Scheme 1). In principle, the protein is first synthesized by ligation of a peptide containing an N-terminal Cys residue and a second peptide containing a C-terminal thioester. Following ligation, the cysteine thiol is selectively removed by desulfurization to afford a protein with the native sequence. Here we

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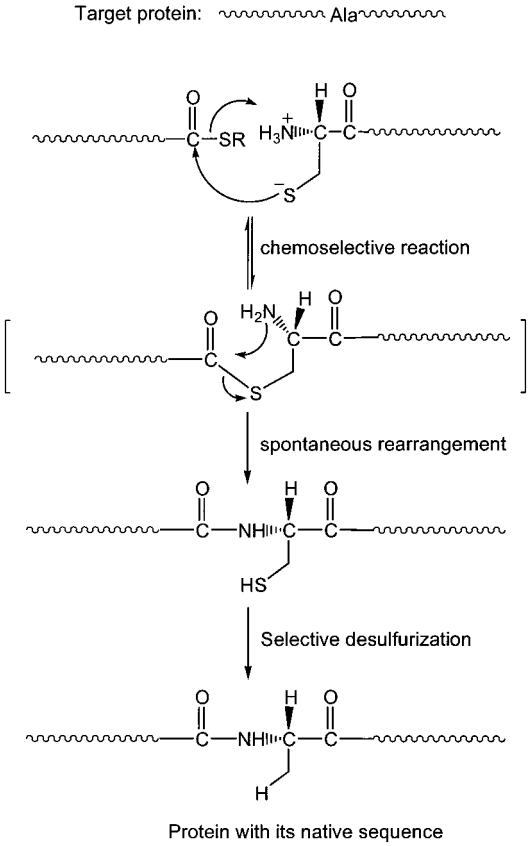
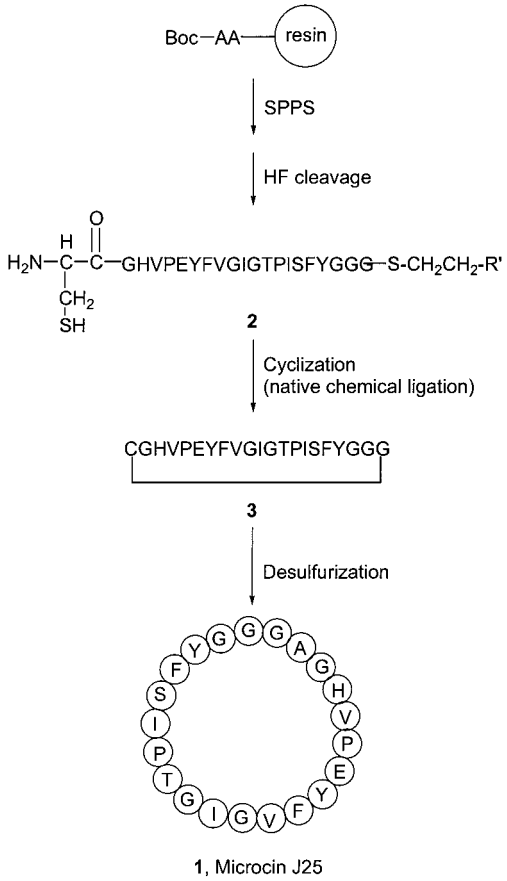
Scheme 1. Strategy to Synthesize Proteins without a Cysteine Residue by Native Chemical Ligation Combined with Desulfurization



Scheme 2. Synthesis of Peptide Antibiotic Microcin J25



report a new and general method for the synthesis of polypeptides without cysteine residue(s) in their native sequences using the native chemical ligation methodology.

Hydrogenolytic desulfurization[15,16] has had limited use in peptide and protein chemistry. Raney nickel has been used to prepare alanine analogues from the corresponding cysteinyl peptides and proteins.[17−21] It has been long confirmed that desulfurization using Raney nickel does not affect the absolute configuration of the α-carbon of sulfur-containing amino acids.[22−24] Nevertheless, these early studies had limited characterization of the reaction products due to the unavailability of analytical methods, such as electrospray ionization mass spectrometry (ESI-MS) and high performance liquid chromatography (HPLC). Here we report palladium can also be used to desulfurize proteins and compare its use to Raney nickel. The technique developed in this study combines native chemical ligation with hydrogenolytic desulfurization and can be used to synthesize both linear and cyclic polypeptides, as demonstrated by the syntheses of peptide antibiotic microcin J25, streptococcal protein G B1 domain, and an analogue of barnase.

Results and Discussion

Synthetic Strategy for Microcin J25. Native chemical ligation has been utilized in the synthesis of cyclic peptides and proteins. Muir and co-workers reported the successful syntheses of a 15-mer head-to-tail cyclic peptide[7] and the WW domain of Yes kinase-associated protein.[8] Tam and co-workers reported the successful synthesis of a 31-amino acid cyclic peptide through native chemical ligation approach.[9] Similarly, a removable auxiliary of N^{α}-oxyethanethiol was also exploited to synthesize cyclic peptides.[25] These results demonstrate the effectiveness of an intramolecular chemical ligation for the synthesis of cyclic peptides and proteins. As mentioned earlier, all of these peptides and proteins contain at least one cysteine residue that is required for the ligation site.

Microcin J25 **1** is a small antibiotic naturally produced by a strain of *Escherichia coli* originally isolated from human feces.[26] The primary structure of this peptide was elucidated recently[27] as a cyclic peptide containing 21 amino acid residues (Scheme 2). There is no report for the total synthesis of this cyclic peptide. Cyclic microcin J25 contains one alanine residue, but no cysteine residue. A linear analogue of microcin J25 was designed that incorporated a C-terminal thioester and an N-terminal Cys in

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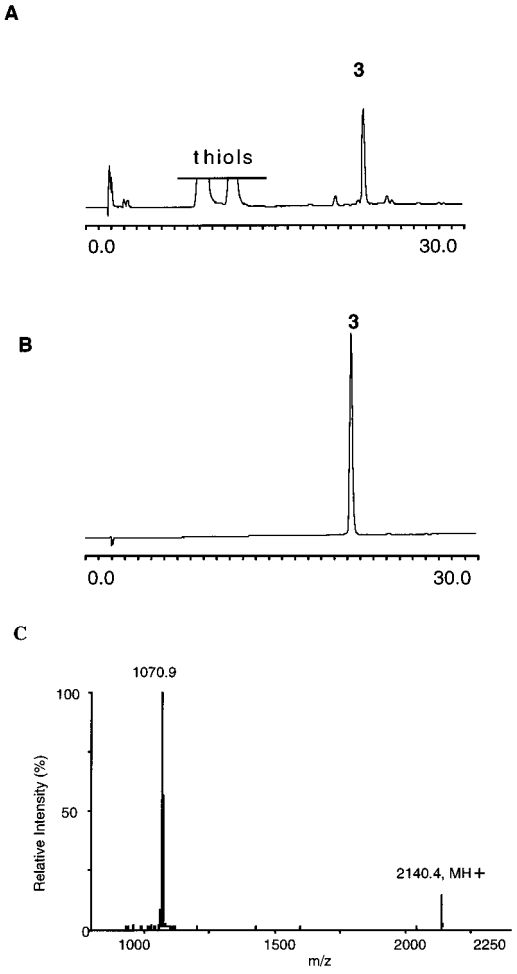

Figure 1. (A) Cyclization of linear peptide **2** to microcin J25-Cys **3**. HPLC conditions: running at 15% of buffer B for 10 min, then linear increased to 67% of buffer B over 20 min on an analytical Vydac C4 column. (B) Chromatogram of the purified cyclic microcin J25-Cys **3**. HPLC conditions: 0−67% of buffer B over 30 min on an analytical Vydac C4 column. (C) ESI-MS of microcin J25-Cys **3**. Observed molecular mass 2139.6 ± 0.3 Da; calculated molecular mass (average isotopic composition): 2139.4.

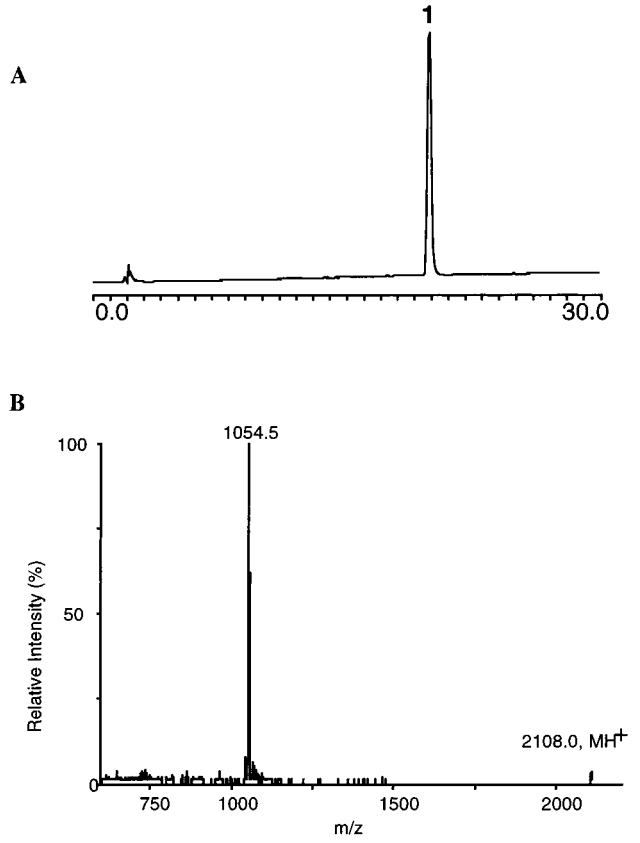

Figure 2. (A) The analytical HPLC chromatograms of the crude and purified microcin J25 **1**, respectively. Desulfurization of **3** was carried out in 20% aqueous acetic acid with 10 times (weight) of Pd/Al_2O_3 (10%) for 2 h under a hydrogen balloon. HPLC conditions: 0−67% of buffer B over 30 min on an analytical Vydac C4 column. (B) ESI-MS of microcin J25 **1**. Observed molecular mass 2108.2 ± 0.4 Da; calculated molecular mass (average isotopic composition): 2107.4.

place of the natural Ala (Scheme 2), which allowed the synthesis of a cyclic analogue of microcin J25 through native chemical ligation. Desulfurization of this cyclic analogue afforded microcin J25 **1**.

Synthesis of Microcin J25 1. The linear thioester peptide **2**, in which the alanine residue was replaced by cysteine, was synthesized by stepwise solid-phase peptide synthesis on a MBHA resin and a thioester MAPAL linkage.[11] Native chemical ligation of the linear peptide thioester **2** in 0.1 M Tris-HCl containing 6.0 M guanidine afforded the cysteine analogue of microcin J25 **3** in an excellent yield (90% by HPLC and 50% isolated) with no apparent polymerization (Figure 1A). The cyclization product was purified by HPLC (Figure 1B) and ESI-MS showed the desired mass (Figure 1C). The subsequent treatment of **3** in 20% aqueous acetic acid with palladium/Al_2O_3 (10%, Sigma-Aldrich) under hydrogen yielded the native form of microcin J25 **1**. The reaction was fast and clean (Figure 2A) with an isolated yield of 52%. The mass spectrum the purified product **3** is shown in Figure 2B.

Structural Characterization of Microcin J25 and its Cysteine Analogue. The identity of peptides **1** and **3** was confirmed by HPLC, mass spectrometry, and amino acid analysis. They were homogeneous on reversed phase HPLC and their ESI-MS showed the expected mass. Standard amino acid analysis revealed that peptide **3** had no alanine residue, while peptide **1** (microcin J25) contained one alanine residue.

Functional Studies of Microcin J25. In recent years, antimicrobial peptides have become the focus of an increasing amount of fundamental and applied research due to the importance of developing new types of antimicrobial agents to counter the increase in antibiotic-resistant bacterial strains.[28] Antimicrobial peptides or peptide antibiotics exist widely in nature, being produced by virtually all living systems.[29,30] Preliminary experiments indicated that the synthetic microcin J25 **1** was highly active toward the tested *E. coli* strain. It was also found that the cysteine analogue **3** was much less potent than the native form microcin J25 **1**. The synthesis of various structural analogues of microcin J25 and the structure−activity relationship of this antibiotic will be reported separately elsewhere.

Alternative Conditions for Microcin J25 Desulfurization with Pd/Al_2O_3. Initially, desulfurization was performed in 0.1 M phosphate buffer containing 6 M guanidine at pH 7.5 (Table 1, entry 1). Under these conditions, a small amount of a dimer of **2** linked by an intermolecular disulfide bond was isolated. Interestingly, the oxidized form was stable to the reduction conditions, a property that may be of utility in systems where

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Table 1. Studies on the Desulfurization Conditions for Microcin J25

entry	metal reagent	reaction medium	yield (%)	advantage or disadvantage
1	Pd/Al_2O_3 (10%)	0.1 M phosphate, 6 M guanidine, pH 7.5	90	disulfide formation
2	Pd/Al_2O_3 (10%)	0.1 M acetate, 6 M guanidine, pH 4.5	>99	desalting required
3	Pd/Al_2O_3 (10%)	20% aqueous AcOH	>99	directly lyophilizable
4	Pd/Carbon (10%)	20% aqueous AcOH	>99	directly lyophilizable
5	$Pd/BaSO_4$ (10%)	20% aqueous AcOH	>90	directly lyophilizable
6	PdO	20% aqueous AcOH	<30	reaction incomplete
7	Raney nickel	20% aqueous AcOH	>99	directly lyophilizable

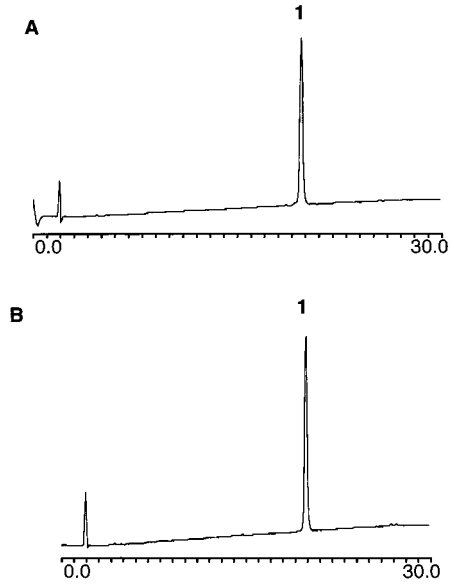

Figure 3. (A) The analytical HPLC chromatogram of microcin J25 **1** prepared by using Raney nickel. Desulfurization of **3** was carried out in 20% aqueous acetic acid with approximately 10 times (weight) of Raney nickel. (B) The analytical HPLC chromatogram of a mixture (1:1) of microcin J25 **1** prepared by using Raney nickel or Pd/Al_2O_3. HPLC conditions for both A and B: 0−67% of buffer B over 30 min on an analytical Vydac C4 column.

selective desulfurization is desired. The undesired disulfide product could be eliminated by performing desulfurization at a low pH. No dimerization product was found when the reaction was carried out at pH 4.5 in 0.1 M sodium acetate containing 6.0 M guanidine (entry 2). Desulfurization was also carried out in aqueous acetic acid, allowing the reaction to be monitored directly by mass spectrometry and recovered by lyophilization following removal of the metal reagent by filtration. The reaction was complete in 2 h with an isolated yield of 50% (entry 3).

Metal Reagents Used for Desulfurization. Besides Pd/Al_2O_3, several other metal reagents, such as Pd/carbon (10%, Table 1, entry 4) and $Pd/BaSO_4$ (10%, entry 5), were also able to desulfurize the peptide **3** to give the target product **1** within 2 h. The reaction with PdO was slow and incomplete (entry 6). Raney nickel (entry 7) could effectively desulfurize microcin J25-Cys **3** to give the target product **1** with a similar efficiency and speed (see Figure 3A). Desulfurization with palladium reagent or Raney nickel afforded identical products as demonstrated by the same mass and HPLC retention time. When a mixture of the two products was loaded onto the reversed-phase HPLC, it showed a single peak (Figure 3B), which further confirmed their identity.[31]

Synthesis of Protein G B1 Domain 4. The synthesis of PGB1 **4** by native chemical ligation and desulfurization demonstrates that this this methodology can be applied to the synthesis of linear proteins with a diverse set of the natural amino acid functional side chains. PGB1 is a 56 amino acid immunoglobulin binding domain from *Streptococcus*[32] and is composed of four β-strands and one α-helix (PDB: 1PGA).[33] PGB1 contains six alanine residues, but no cysteine residues.

N^{α}-Ac-[^{24}Cys]PGB1 **5** was synthesized by ligation of the two segments prepared by SPPS. The N-terminal thioester segment contains residues 1−23, and the C-terminal segment contains residues 24−56, in which Ala24 was replaced with Cys to facilitate the ligation. The identity of the ligated product N^{α}-Ac-[^{24}Cys]PGB1 was confirmed by HPLC and mass spectrometry (Figure 4A). The ligated N^{α}-Ac-[^{24}Cys]PGB1 **5** was subject to desulfurization with palladium/Al_2O_3 in 0.1 M phosphate buffer containing 6 M guanidine, which afforded N^{α}-Ac-PGB1 **6** with a yield of 80%. The ESI-MS of the purified product is shown in Figure 4B.

Comparative Studies on Pd/Al_2O_3 and Raney Nickel in the Preparation of PGB1. Pd/Al_2O_3 selectively removed the cysteine thiol of PGB1 and left the methionine thiol untouched (Table 2, entry 1). However, when Pd/Al_2O_3 was utilized to desulfurize [Cys^{24}]PGB1 **7**, the small, unidentified fractions (up to 20% in total) observed on HPLC were presumably due to multiple low-abundance side reactions (Figure 5). In contrast, Raney nickel (Table 2, entry 2) yielded a highly clean reaction product when the reaction time is controlled (Figure 6). Prolonged treatement with Raney nickel resulted in demethylthiolation of Met residues. As shown in Figure 6, more than 80% of cysteine thiol is removed from N^{α}-Ac-[Cys^{24}]PGB1 **5** during the first 30 min (Figure 6A) to give target product N^{α}-Ac-PGB1 **6**. The demethylthiolated product N^{α}-Ac-[Abu^{1}]PGB1 **8** (Abu: α-amino butyric acid) began to appear in 60 min, as shown by the small peak in front of the alanyl product **6** (Figure 6B). After 4 h, more than half of the methionine residue was converted to Abu (Figure 6C). Both the thiol of the cysteine residue and the methylthiol of the methionine residue were removed in 14 h by Raney nickel, converting Cys and Met were converted to Ala and Abu, respectively (Figure 6D). N^{α}-Ac−PGB1 **6** prepared by using either Pd/Al_2O_3 or Raney nickel was identical by ESMS and coeluted on HPLC. The differences between Pd/Al_2O_3 and Raney nickel have also been studied using model peptides (see the Supporting Information).

Synthesis of [^{49}Ala]barnase 9. [^{49}Cys]Barnase **10** was synthesized several years ago by ligation of two fragments: barnase(1−48) benzyl thioester and [^{49}Cys]barnase(49−110).[10] The two fragments were ligated and purified as previously

(31) It has been shown that pretreatment of Raney nickel with H_2S gas or H_2O_2 abolished its desulfurization activity (Ohmori, S.; Takahashi, K.; Ikeada, M.; Ubuka, T. *Z. Naturforsch.* **1981**, *36B*, 370−374), suggesting that the sulfurs on the peptide are removed by poisoning the metal reagent. While hydrogen gas was not necessary for Raney nickel to desulfurize the peptide, it was required for palladium reagents. Freshly prepared Raney nickel contains a large amount of absorbed hydrogen, which is the hydrogen source during hydrogenolytic desulfurization (Petitt, G. R. In *Organic Reaction*; John Wiley: New York, 1962; Vol. 12, pp 356−447; Otieno, S. *Biochemistry* **1978**, *17*, 5468−5474).

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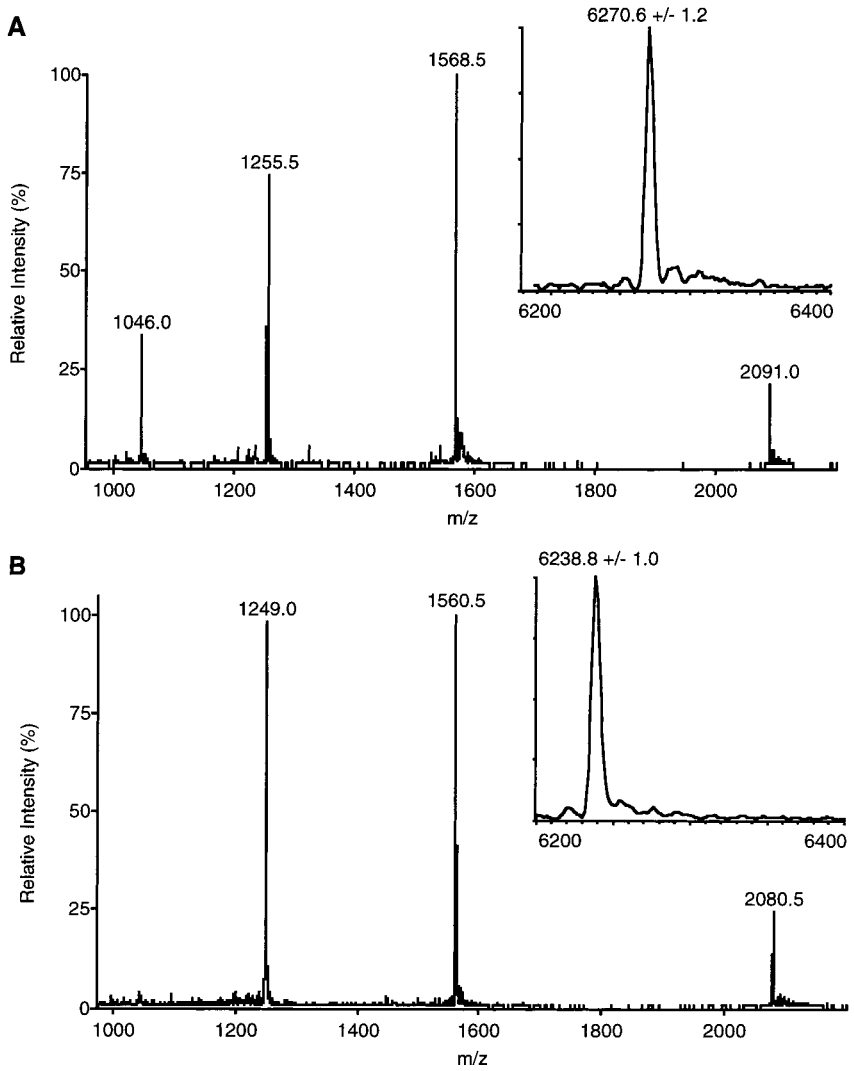

Figure 4. (A) ESI-MS of $^{\alpha}$N-Ac-[^{24}Cys]PGB1. Observed molecular mass 6270.6 ± 1.2 Da; calculated molecular mass (average isotopic composition): 6269.8. (B) ESI-MS of $^{\alpha}$N-Ac-[^{24}Ala]PGB1. Observed molecular mass 6238.8 ± 1.0 Da; calculated molecular mass (average isotopic composition): 6237.8.

Table 2. Desulfurization Conditions for PGB1

entry	metal reagent	reaction medium	yield (%)	potential problem
1	Pd/Al_2O_3	0.1 M phosphate, pH 5.8, 6 M guanidine	80	hydrogenation of tryptophan
2	Raney nickel	0.1 M phosphate, pH 5.8, 6 M guanidine	82	demethylthiolization of methionine

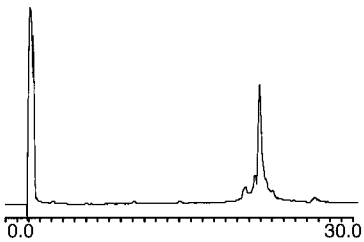

Figure 5. Desulfurization of PGB1 using Pd/Al_2O_3. HPLC conditions: 0−67% of buffer B over 30 min on an analytical Vydac C4 column.

described.[10] The product [^{49}Cys]barnase showed the expected mass by ESI-MS. [^{49}Cys]Barnase was successfully converted to [^{49}Ala]barnase by Pd/Al_2O_3 in 0.1 M phosphate buffer containing 6 M guanidine at pH 5.8. It was homogeneous on HPLC and ESI-MS revealed the desired mass.

Compatibility of Desulfurization Conditions with Fully Unprotected Proteins. The results from microcin J25, PGB1, and barnase demonstrate that the desulfurization conditions are compatible with all of the functional side chains of the natural amino acids. Desulfurization of linear proteins is preferentially performed in unfolding buffers containing 6 M guanidine, even though various solvents can be used for the preparation of microcin J25. This difference could be attributed to residual protein structure in 20% acetic acid. It has been reported that some thiol groups of folded lysozyme are inaccessible to desulfurization reaction.[17]

Raney nickel is a highly effective desulfurization agent[17,18] and should be the primary choice for most proteins. However, care should be exercised to prevent demethylthiolation if the cysteinyl proteins contain methionine residue(s). Newly developed desulfurization methods could potentially eliminate these limitations.[34] In synthetic proteins, Met → Nle is a highly conservative modification that is often used to avoid Met(O) formation that occurs in both expressed and synthetic proteins

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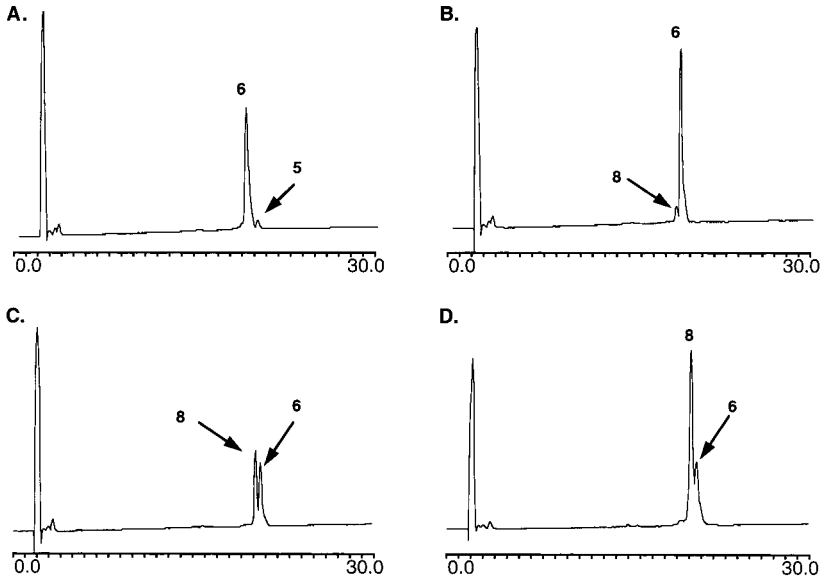

Figure 6. Desulfurization of N^{α}-Ac-[Cys^{24}]PGB1 **5** with Raney nickel. (A) 30 min, more than 80% of cysteine thiol was removed to give the target product **6**; (B) 60 min, the demethylthiolated product **8** (α-amino butyric acid, Abu) began to appear as shown by the little peak in front of the alanyl product **6**; (C) 4 h, more than half of methionine residue in the protein was converted to Abu; (D) 14 h. Most of the cysteine thiol and the methylthiol of methionine residue were removed and compound **8** was obtained as the major product. HPLC conditions: 0−67% of buffer B over 30 min on an analytical Vydac C4 column.

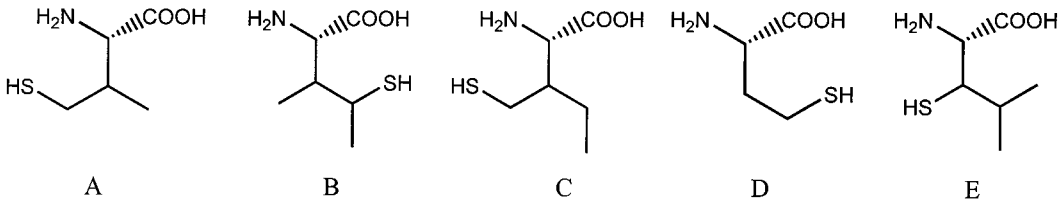

Figure 7. Unnatural amino acids that can potentially be used at the ligation site in protein synthesis.

upon storage. This modification would eliminate concerns about demethylthiolation.

Model Study of Using α-Amino Butyric Acid (Abu) as the Ligation Site in Protein Synthesis. Tam and co-workers reported an alternative to native chemical ligation by using homocysteine at the ligation site. After ligation, the homocysteine was then converted to methionine by methylation.[35] The utility of this approach was limited by the low selectivity in the methylation of the fully unprotected peptides. Using our current method, we investigated the possibility of converting homocysteine to Abu after ligation. A model peptide of Homocys-Ala-Tyr-Gly-Gly-Phe-Leu-NH_2 **11** was ligated to the N-terminal thioester fragment of PGB1. The ligation reaction was completed in 36 h with an excellent yield (> 80%). The resulted product (Ac-MTYKLILNGKTLKGETTT EAVDA-HomoCys-AYGGFL-NH_2) **12** was purified and lyophilized to afford the target product with a recovered yield of 45%.

The purified model peptide was subjected to desulfurization. Raney nickel removed the thiol moiety of the homocysteine residue in 30 min, and the Abu analogue of (Ac-MTYKLIL-NGKTLKGETTTEAVDA-Abu-AYGGFL-NH_2) **13** was prepared in 82% yield. Prolonged exposure of the peptide to Raney nickel resulted in further demethylthiolation of the methionine residue. However, the demethylthiolated product showed a shorter retention time on reversed phase HPLC and was easily separated. On the other hand, the reaction could be controlled to give only desulfurized product by limiting the reaction time. The above experiment indicated that X-Abu could be successfully used as the ligation site through homocysteine.

Combining native chemical ligation and selective desulfurization, a logical extension of the above result would be to use a variety of other amino acids, such as X-Val, X-Leu, X-Ile, at the ligation site through their corresponding β- or γ-thiol amino acids. Several examples of these kinds of amino acids are shown in Figure 7. These unnatural amino acids or any other β- and γ-thiol amino acids could be incorporated in peptide chain by SPPS just like the common amino acids as demonstrated by homocysteine. Selective removal of the thiol group after ligation will convert compounds A, B, C, D, and E (Figure 7) to Val, Ile, Ile, Abu, and Leu, respectively.

Conclusions

Native chemical ligation combined with selective desulfurization is an effective method for the synthesis of proteins that do not contain cysteine residues. This technique, which includes a process of Ala → Cys → Ala, can be used to synthesize both linear and cyclic proteins, as demonstrated by the syntheses of microcin J25, streptococcal protein G B1 domain, and an analogue of barnase. The sequence of X-Ala appears more often than X-Cys due to its high abundance in proteins.[36] For example, the residues of Ala/Cys in lysozyme (chicken), cytochrome *c* (human), and α-chain of hemoglobin (human) are 12/8, 6/2, and 21/0, respectively. Hence, design of protein synthesis through native chemical ligation will permit more choices for the ligation site. One limitation is that unprotected Cys or Acm-protected Cys residues are not compatible since all cysteine thiols are removed with no selectivity.[37]

(35) Tam, J. P.; Yu. Q. *Biopolymers* **1998**, *46*, 319−327.

(36) McCaldon, P.; Argos, P. *Proteins* **1988**, *4*, 99−122.

The current study will provide an important alternative method to prepare alanyl proteins from their cysteinyl forms, which can be either chemically synthesized or of biological origin. Preliminary results have indicated (data not shown) that all of the eight cysteine residues in unfolded ribonuclease A could be effectively converted to alanine using Pd/Al_2O_3. As a result, the present method could also significantly broaden the application of protein semitotal synthesis to synthesize proteins without cysteine residues.[6]

Experimental Methods

Materials. Boc-amino acids were obtained from Midwest Biotech (Fishers, IN), except Boc-Arg (*p*-toluenesulfonyl)-OH and Boc-Asn-(xanthyl)-OH, which were obtained from Bachem Bioscience (King of Prussia, PA). *S*-trityl-mercaptopropionic acid was obtained from Peptides International (Louisville, KY). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Boc-Glu-OCH_2-Pam-resin and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Applied Biosystems (Foster City, CA). MBHA resin was obtained from Peninsula Laboratories (Belmont, CA). All solvents (HPLC-grade *N*,*N*-dimethylformamide (DMF), dichloromethane, acetonitrile) of high purity were purchased from Fisher. Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (River Edge, NJ). HF was purchased form Matheson Gas (Cucamonga, CA). Palladium on alumina (10%), palladium on activated carbon (10%), palladium on barium sulfate (10%), palladium oxide, nickel acetate, sodium boronhydride, benzyl mercaptan, thiophenol, *p*-cresol were all purchased from Sigma-Aldrich (St. Louis, MO).

HPLC. Analytical reversed-phase HPLC was performed on a Hewlett-Packard HPLC 1050 system with 214 nm UV detection, using Vydac C-18 or C-4 columns (5 μm, 0.46 $\times$ 15 cm, flow rate 1 mL/min). Semipreparative reversed-phase HPLC was performed on a Rainin HPLC system by using a Vydac C-18 or C4 column (10 μm, 1.0 $\times$ 25 cm, flow rate 5 mL/min). Preparative reversed-phase HPLC was performed on a WatersDelta Prep-4000 HPLC system using Vydac C-18 column (5 $\times$ 25 cm, flow rate 30 mL/min). Linear gradients (2%/min analytical, 0.3%/min preparative) of acetonitrile in water with 0.1% TFA were used for all systems to elute bound peptides. Buffer A is MilliQ water containing 0.1% TFA; Buffer B is acetonitrile with 10% water and 0.09% TFA. Fractions that contained the desired peptide were detected by electrospray ionization mass spectrometry (ESI-MS).

Mass Spectrometry. ESI-MS was performed on an API-III triple quadrupole mass spectrometer (PE-Sciex, Thornhill, ON, Canada). Peptide masses were calculated from the experimental mass to charge (*m*/*z*) ratios from all the observed protonation states of a peptide using MacSpec software (Sciex). Theoretical masses of peptides and proteins were calculated using MacProMass software (Beckman Research Institute, Duarte, CA). Matrix-assisted laser desorption/ionization-Fourier transform mass spectrometry (MALDI-FTMS) experiments are performed on an IonSpec FTMS mass spectrometer. Samples are irradiated with a nitrogen laser (Laser Science Inc.) operated at 337 nm, and the laser beam is attenuated by a variable attenuator and focused on the sample target. The ions are then differentiated according to their *m*/*z* using an ion cyclotron resonance mass analyzer.

Amino Acid Analysis. Amino acid analysis was carried out at the Protein and Nucleic Acids Core Facility in The Scripps Research Institute. Peptide samples were fully hydrolyzed using a vapor-phase hydrolysis in 6 M HCl containing 2% saturated phenol/water at 105 °C for 24 h. Hydrolyzed amino acids were quantitatively analyzed by Waters AccQ*Tag chemistry using a Waters Alliance HPLC system equipped with a fluorescence detector.

Peptide Synthesis. Side-chain protected amino acids used in this study were: Arg(Tos), Asp(OChx), Glu(OChx), Cys(pMeBzl) or Cys-(Acm), His(Dnp), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(2-Br-Z). Other amino acids were used without side chain protection. All peptide segments were synthesized manually in stepwise fashion by established solid-phase peptide synthesis (SPPS) methods typically on a 0.4 mmol scale using in situ neutralization/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation procedure for *tert*-butoxycarbonyl (Boc) chemistry as previously described.[38] The solid supports were either pre-loaded PAM-resin or MBHA resin. The peptide coupling was carried out with a 5-fold excess (2.2 mmol) of activated amino acid for a minimum of 15 min. After each coupling, yields were determined by measuring residual free amine with the quantitative ninhydrin assay.[39] Acylation of the α-amino group was performed as follows: the Boc-protecting group was first removed, and the resin was then allowed to react with acetic anhydride/Et_3N in DMF for 10 min. After chain assembly was complete, the peptide was deprotected and cleaved from the resin by treatment with anhydrous HF for 1 h at 0 °C with 4% *p*-cresol as a scavenger. After cleavage, peptides were precipitated with ice-cold diethyl ether, dissolved in aqueous acetonitrile, and lyophilized. All peptides were purified by reversed phase HPLC and characterized by electrospray ionization mass spectrometry (ESI-MS).

Trityl-Associated Mercaptopropionic Acid-Leucine (TAMPAL) Resin.[11] Boc-Leu-OH (2.2 mmol) was activated with 2.0 mmol of HBTU in the presence of 3.2 mmol DIEA and coupled for 20 min to 0.4 mmol MBHA resin (99.9% coupling yield). *S*-Trityl mercaptoporpionic acid (0.6 mmol) was activated with 0.54 mmol HBTU in the presence of 1.2 mmol DIEA and then coupled for 20 min to Leu-MBHA resin (99.9% coupling yield). The resulting trityl-mercaptopropionic acid-leucine (TAMPAL) resin was used as a starting resin for polypeptide chain assembly. Trityl-protecting group was removed with 2 $\times$ 1 min treatments with a cocktail containing 2.5% triisopropylsilane, 2.5% H_2O, and 95% TFA.

After chain assembly was complete, the final peptide was deprotected and cleaved from the resin by treatment with anhydrous HF as described above. The resulting C-terminal mercaptopropionic acid-leucine (MPAL) thioester peptides are ready for participation in native chemical ligation after HPLC purification. In all cases, the imidazole side-chain 2,4-dinitropheny (Dnp)-protecting group remained on His residues because the removal of Dnp is incompatible with the C-terminal thioester group.[11] However, Dnp is gradually removed by thiols during the ligation reaction, yielding a ligation product with unprotected His.

General Procedures for Desulfurization. Otherwise specified, desulfurization reactions were performed in 0.1 M phosphate buffer containing 6.0 M guanidine at pH 5.8. The buffer was normally degassed by bubbling argon through for 10 min before each use. The catalyst used was 5−10 times of the peptide weight. Catalysts used in this study include Pd/Al_2O_3 (10%), Pd/carbon (10%), $Pd/BaSO_4$ (10%), PdO, and Raney nickel. Unless otherwise specified, Pd/Al_2O_3 was the primary choice for all the desulfurization reactions. The reactions were performed under a hydrogen balloon with magnetic stirring. Peptide concentrations ranged 0.5−2 mg/mL. Desulfurization reaction was monitored by analytical HPLC and mass spectrometry. Raney nickel was prepared as follows:[17] Nickel acetate (3.0 g) was dissolved in 75 mL of H_2O, and then sodium borohydride (0.5 g) was slowly added with stirring. The black amorphous nickel precipitate was filtered and washed with distilled water until the washing became neutral. The nickel was then tranferred into a closed bottle and stored in H_2O at 4 °C. Preparations more than 1 week old were not used.

Preparation of Microcin J25 1. The linear peptide **2** (ESI-MS: 2523.5 $\pm$ 0.5; calculated average isotope composition for **2**: 2523.5) was prepared using MBHA resin with a C-terminal MPAL thioester. Cyclization (native chemical ligation) of linear peptide **2** was performed as follows: 17.6 mg of **2** was dissolved in 8 mL of ligation buffer (0.1 M Tris-HCl, 6.0 M guanidine, pH 8.5), and then 2% benzyl mercaptan and 2% thiophenol were added. The reaction was more than 90% complete within 6 h, as demonstrated by HPLC. However, the reaction was allowed to proceed overnight at room temperature with occasional mixing. The cyclized product was purified on a semipreparative HPLC with an isolated yield of 50% (7.0 mg). As shown by the mass spectra, the Dnp-protecting group on His was successfully removed by the thiols during cyclization. ESI-MS of **3**: 2139.6 $\pm$ 0.3; calculated average

(37) In principle, it would be possible to oxidatively fold a protein with an unpaired Cys, which could be selectively desulfurized.

(38) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180−193.

(39) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.

isotope composition for $C_{101}H_{139}N_{23}O_{27}S$: 2139.4. High-resolution MALDI-FTMS: 2138.9939 (MNa^+: 2160.964); calculated average isotope composition: 2139.0004; MNa^+: 2160.982.

Desulfurization of peptide **3** was carried out in 0.1 M phosphate or acetate buffer containing 6.0 M guanidine at pH 4.5−7.5, or in 20% aqueous acetic acid. The reaction was monitored by analytical HPLC and mass spectrometry. Pd/Al_2O_3, Pd/carbon, and Raney nickel all quantitatively converted **3** to **1** in 2 h. When the reaction was carried out at pH 7.5, a small amount of the dimer of **3** was isolated, which had a much longer retention time on reversed-phase HPLC and was easily separated. The desulfurization reaction with PdO was slow and incomplete; the starting material was recovered with a small amount of product. ESI-MS of **1**: 2107.2 ± 0.4; calculated average isotope composition for $C_{101}H_{139}N_{23}O_{27}$: 2107.4. High-resolution MALDI-FTMS: 2107.0284; MNa^+: 2129.010; calculated average isotope composition: 2107.0284; MNa^+: 2129.015.

Standard amino acid analysis revealed that peptide **3** contained the following amino acid residues: Ser (1.0), Glx (1.1), Gly (6.0), His (1.0), Thr (1.0), Ala (0.2), Pro (2.1), Tyr (2.1), Val (1.9), Ile (1.9), Phe (2.0). On the other hand, the amino acid component of peptide **1** (microcin J25) was as follows: Ser (1.1), Glx (1.1), Gly (6.2), His (0.9), Thr (1.0), Ala (1.3), Pro (2.1), Tyr (1.9), Val (1.8), Ile (1.9), Phe (2.0).

Antimicrobial assay was performed according to the literature method.[26] Both **1** and **3** showed antimicrobial activity toward the tested *E. coli* strain. However, native form of microcin J25 **1** showed a more specific potency.

Preparation of Protein G B1 Domain (PGB1) and $^{\alpha}$N-Ac-PGB1. Ligation of the two fragments of PGB1 was performed in 0.1 Tris-HCl buffer, pH 8.5 containing 6.0 M guanidine. Typically, 3.8 mg (1.0 μmol) of C-terminal fragment and 2.3 mg (1.1 μmol) of N-terminal fragment (thioester) were dissolved in 1 mL of above buffer together with 2% thiophenol and 2% benzyl mercaptan. The ligation was performed at room temperature and was vortexed periodically to equilibrate the thiol additives. The reaction was monitored by analytical HPLC and electrospray ionization mass spectrometry until completion. The ligation product [^{24}Cys]PGB1 **7** was purified on a semipreparative HPLC using a Vydac C-4 column with an isolated yield of 20% (1.2 mg, not optimized). Preparation of $^{\alpha}$N-Ac-[^{24}Cys]PGB1 **5** was carried out in the same way using the acylated N-terminal fragment and similar yield was obtained. ESI-MS of [^{24}Cys]PGB1 **7**: 6228.8 ± 1.1; calculated average isotope composition for $C_{273}H_{424}N_{67}O_{95}S_2$: 6227.8. ESI-MS of $^{\alpha}$N-Ac-[Cys^{24}]PGB1 **5**: 6270.6 ± 1.2; calculated average isotope composition for $C_{275}H_{426}N_{67}O_{96}S_2$: 6269.8.

Desulfurization of [Cys^{24}]PGB1 or $^{\alpha}$N-Ac-[Cys^{24}]PGB1 was performed in 0.1 M phosphate buffer, pH 5.8 containing 6.0 M guanidine. The reaction was monitored by analytical HPLC and ESI-MS. Typically the reaction was complete in 2 h with a yield of 30%. Interestingly, for the $^{\alpha}$N-Ac-PGB1, the yield was tremendously increased to 80% or more according to HPLC peak integration. ESI-MS of PGB1 **4**: 6194.3 ± 0.8; calculated average isotope composition for $C_{273}H_{424}N_{67}O_{95}S$: 6195.8. ESI-MS of $^{\alpha}$N-Ac-PGB1 **6**: 6238.8 ± 1.0; calculated average isotope composition for $C_{275}H_{426}N_{67}O_{96}S$: 6237.8.

When Pd/Al_2O_3 was used as the catalyst, the side chain of the methionine residue was stable even with a prolonged reaction time. However, the demethylthiolated product of the methionine residue was found when the protein was exposed to Raney nickel longer than 2 h. After 14 h, both thiol of the cysteine residue and methylthiol of the methionine residue were completely removed. ESI-MS of $^{\alpha}$N-Ac-[^{1}-Abu]PGB1 **8**: 6192.1 ± 0.3; calculated average isotope composition for $C_{274}H_{424}N_{67}O_{96}$: 6191.8.

Preparation of [^{49}Ala]barnase 9. The two fragments of [^{49}Cys]-barnase: barnase(1−48) benzyl thioester and [^{49}Cys]barnase(49−110) were prepared by this laboratory several years ago.[10] The ligation of the two fragments was carried out under the same conditions as those previously described.[10] The ligated product **10** was purified to homogeneity on HPLC using a semipreparative reversed-phase Vydac C-4 column. ESI-MS **10**: 12359.3 ± 1.8; calculated average isotope composition for $C_{552}H_{840}N_{152}O_{170}S$: 12357.8.

After the ligation, [^{49}Cys]barnase **10** was converted to [^{49}Ala]barnase **9** by Pd/Al_2O_3 desulfurization in 0.1 M phosphate buffer containing 6.0 M guanidine at pH 5.8. The yield is about 60% according to HPLC integration. ESI-MS **9**: 12326.6 ± 1.8; calculated average isotope composition for $C_{552}H_{840}N_{152}O_{170}$: 12325.8.

Model Study of Using α-Amino Butyric Acid (Abu) as the Ligation Site in Protein Synthesis. A model peptide of Homocys-Ala-Tyr-Gly-Gly-Phe-Leu-NH_2 **11** (ESI-MS 742.6; calculated average isotope composition for $C_{35}H_{51}N_8O_8S_1$: 742.9) was ligated to the N-terminal thioester fragment of PGB1. Specifically, 5.5 mg (2.0 μmol) of the C-terminal fragment of PGB1 and 1.5 mg (2.0 μmol) Homocys-Ala-Tyr-Gly-Gly-Phe-Leu-NH_2 were dissolved in 1.2 mL of 0.1 M phosphate buffer containing 6.0 M guanidine at pH 8.2. The mixture of 2% thiophenol and of 2% benzylmercaptan was then added and was vortexed periodically to equilibrate the thiol additives. The ligation was performed at 37 °C and was monitored by analytical HPLC and ESI-MS. After 36 h the reaction was completed. The product **12** was purified on a semipreparative HPLC using a Vydac C-4 column and lyophilized to give an isolated yield of 45% (2.9 mg, 0.9 μmol). ESI-MS **12**: 3265.2 ± 0.4; calculated average isotope composition for $C_{146}H_{237}N_{35}O_{45}S_2$: 3264.9.

The purified peptide was subjected to desulfurization using either Pd/Al_2O_3 or Raney nickel in 0.1 M sodium acetate buffer containing 6.0 M guanidine at pH 5.5. The buffer was briefly degassed by bubbling argon for 10 min to avoid oxidation. The removal of the thiol moiety of the homocysteine residue was monitored by analytical HPLC. Desulfurization using Pd/Al_2O_3 was completed in 2 h and afforded the target product **13** in 44% yield. A yield of 82% was obtained when Raney nickel was exploited. Hydrogen gas was not necessary for the desulfurization when Raney nickel was used, and the reaction was completed in 30 min. Prolonged exposure of Raney nickel to the peptide resulted in demethylthiolation of the methionine residue. However, the demethylthiolated product showed a shorter retention time on reversed phase HPLC and was easily separated. ESI-MS of the target product **13**: 3232.2 ± 0.4; calculated average isotope composition for $C_{146}H_{237}N_{35}O_{45}S_1$: 3232.8. ESI-MS of the demethylthiolated product: 3187.7 ± 0.4; calculated average isotope composition for $C_{145}H_{235}N_{35}O_{45}$: 3186.7.

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Supporting Information Available: Model peptide studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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