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Modulation of Reactivity in Native Chemical Ligation through the Use of Thiol Additives

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Abstract: In native chemical ligation, an unprotected peptide α-carboxy thioester is reacted with a second peptide containing an N-terminal cysteine residue. It was anticipated that addition of thiophenol to a native chemical ligation reaction would keep cysteine side chains reduced, catalyze the reversal of unproductive thioester formation, and generate a more reactive phenyl thioester through thiol exchange. Several model peptide−α-thioesters were treated with an excess of a competing thiol to investigate their susceptibility to thiol exchange: a highly activated 3-nitro-4-carboxybenzyl α-thioester was smoothly converted to the *less* activated benzyl α-thioester through the addition of an excess of benzyl mercaptan; similarly, a peptide containing the benzyl α-thioester group was converted to a *more* reactive phenyl α-thioester by addition of thiophenol. Even a weakly activated peptide−α-thioester was converted to a substantially more reactive species, as demonstrated by the conversion of peptide−$^{\alpha}$COS-CH_2COOH to peptide−$^{\alpha}$COS-phenyl. The utility of *in situ* transthioesterification in native chemical ligation reactions was demonstrated by model syntheses of the 110-residue barnase polypeptide chain. The use of thiophenol as an additive in the ligation gave clean, rapid reaction to form the desired amide-linked product in high yield. The *in situ* transthioesterification process is broadly applicable to the total chemical synthesis of proteins by native chemical ligation.

Introduction

Chemical ligation, the chemoselective reaction of unprotected peptide segments in solution, has facilitated the total synthesis of long polypeptide chains and has thus opened the world of proteins to the tools of organic chemistry. Use of chemoselective reactions which form a non-amide bond at the ligation site, such as the reaction of a peptide−$^{\alpha}$COSH with an a bromoacetyl peptide to form a thioester-linked product,[1] has enabled the chemical synthesis of a variety of proteins in a straightforward manner.[2−8] Recently, the chemical ligation approach was adapted to allow the formation of a native peptide bond at the ligation site.[9] Using this native chemical ligation approach, the 72-amino acid chemokine protein [Ala^{33}]IL-8 was prepared from two unprotected peptide segments.[9] Native chemical ligation takes advantage of our ability to synthesize peptides up to ∼70 amino acids in length by solid phase peptide synthesis,[10] purify and characterize the unprotected polypeptide products, and then ligate them to form native polypeptide chains up to ∼140 amino acids in length. Denaturing aqueous buffers

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(1) Schnölzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221−225.
(2) Milton, R. C.; Milton, S. C. F.; Schnölzer, M.; Kent, S. B. H. In *Techniques in Protein Chemistry IV*; Angeletti, R. H., Ed.; Academic Press: San Diego, CA, 1993; pp 257−267.
(3) Williams, M. J.; Muir, T. W.; Ginsberg, M. H.; Kent, S. B. H. *J. Am. Chem. Soc.* **1994**, *116*, 10797−10798.
(4) Rose, K. *J. Am. Chem. Soc.* **1994**, *116*, 30−33.
(5) Canne, L. E.; Ferre-D'Amare, A. R.; Burley, S. K.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 2998−3007.
(6) Baca, M.; Muir, T. W.; Schnolzer, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 1881−1887.
(7) Englebretsen, D. R.; Garnham, B. G.; Bergman, D. A.; Alewood, P. F. *Tetrahedron Lett.* **1995**, *38*, 8871−8874.
(8) Liu, C.-F.; Rao, C.; Tam, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 307−312.
(9) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(10) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180−193.

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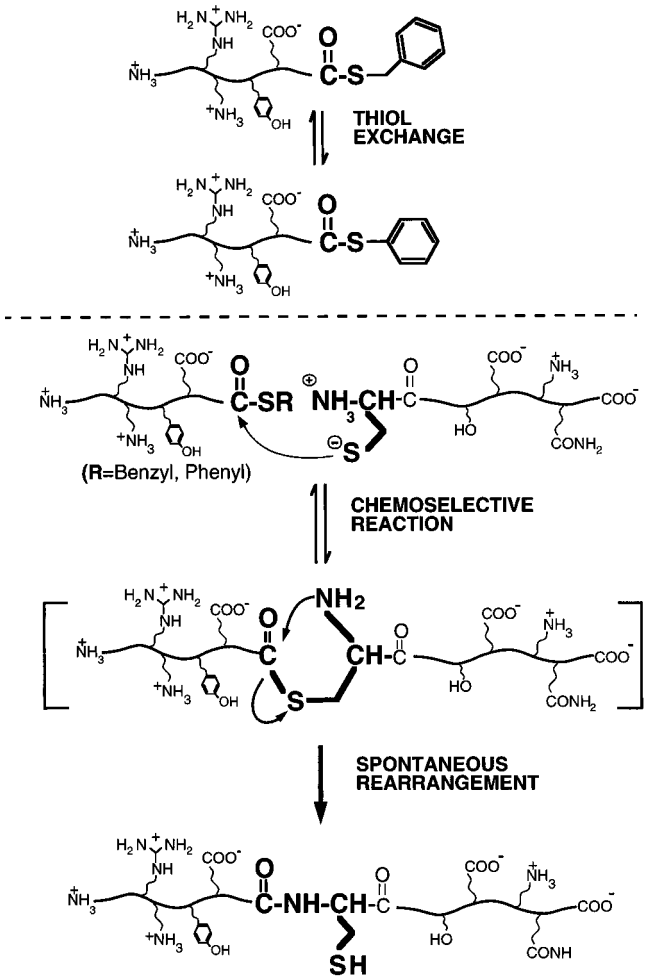
Scheme 1



such as 6 M Gn·HCl allow high peptide concentrations (typically >1 mM) to be employed so that ligations proceed cleanly and in high yield. Native chemical ligation is a robust synthetic procedure and has been used to synthesize a series of model peptides,[11] the serine proteinase inhibitor protein OMTKY3,[12] and a variety of other proteins.[13]

In native chemical ligation, an unprotected peptide−α-thioester is reacted with a second unprotected peptide containing an N-terminal cysteine residue. Exchange of the thioester with the thiol moiety of the N-terminal cysteine side chain gives a thioester-linked intermediate as the initial covalent product. Without change in the reaction conditions, a rapid intramolecular reaction occurs, forming the thermodynamically favored amide bond at the ligation site.[9,14] Our conception of the reaction mechanism is shown in Scheme 1. The first (thioester-forming) step is reversible, whereas the rearrangement to the amide is irreversible under the reaction conditions used, leading to the formation of the ligation product in high yield. Following ligation, the polypeptide product is obtained in final form with no further chemical manipulation.

The rate of ligation is dependent on the nature of the thiol leaving group of the peptide−α-thioester. Several thioester groups have been successfully employed in native chemical ligation,[9] one of the most synthetically convenient being the benzyl thioester. Reaction of a crude thioacid peptide with benzyl bromide in aqueous buffers at pH 4.0 generates the desired α-carboxy benzyl thioester. In previous work on interleukin 8, the ligation reaction using a benzyl thioester peptide proceeded to ∼60% completion over 3 days.[9] These ligation conditions made use of a large excess of benzyl mercaptan to keep cysteine side chains reduced as well as to restore any newly-formed unproductive thioesters (formed from other Cys residues in either peptide segment) back to the benzyl thioester. It was anticipated that addition of thiophenol to the reaction instead of benzyl mercaptan would still keep the cysteine side chains reduced, reverse the formation of unproductive thioesters, and also convert the benzyl thioester into a more reactive phenyl thioester through thiol exchange (Scheme 1). This was expected to speed up the ligation reaction and increase yields of the desired product. Here we report model studies designed to explore thiol exhange in peptide−α-thioesters and the application of thiol-modified native chemical ligation to the synthesis of a 110-residue polypeptide chain.

Results and Discussion

Thioester Exchange in Model Peptides. Amino acid α-thioesters are known to undergo transthioesterification reactions when exposed to thiol-containing compounds.[15] To investigate the susceptibility of peptide−α-thioesters to thiol exchange, several model thioester peptides were treated with an excess of a competing thiol. For example, a peptide containing the highly reactive 2-nitrobenzoic acid α-thioester group[16] was converted to the *less* activated benzyl α-thioester through the addition of an excess of benzyl mercaptan (2% by volume) in aqueous 100 mM sodium phosphate buffer at pH 6.5. Under these conditions, the transthioesterification of Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSNB to yield Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COS-benzyl proceeded to ∼50% completion in <30 min, without significant hydrolysis. Similarly, a peptide containing the benzyl α-thioester group was converted to a *more* reactive thioester by addition of thiophenol. Thus, the peptide Ala-Glu-Ile-Ala-Ala-$^{\alpha}$COS-benzyl was ∼50% converted to the corresponding phenyl thioester, Ala-Glu-Ile-Ala-Ala-$^{\alpha}$COS-phenyl by reaction under the same conditions with thiophenol (2% by volume) over a time period of 3 h (Figure 1). Even a weakly activated peptide−α-thioester was converted to a substantially more reactive species, as demonstrated by the conversion of Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COS-CH_2COOH to Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COS-phenyl in ∼50% yield in 1 h under identical conditions.

***In Situ* Transthioesterification in the Synthesis of Barnase Analogues.** We tested the utility of *in situ* transthioesterification in ligation reactions by the synthesis of two analogues of the enzyme barnase. Barnase is a small microbial ribonuclease that has been intensively studied as a model for protein folding[17]

(11) Tam, J. P.; Lu, Y. A.; Liu, C. F.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485−12489.

(12) Lu, W; Qasim, M. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996,** *118*, 8518−8523.

(13) Kent, S. B. H.; Bark, S. J.; Canne, L. E.; Dawson, P. E.; Fitzgerald, M. C.; Hackeng, T.; Lu, W.; Muir, T. W. *Proceedings of the 24th European Peptide Symposium*, 1996 (in press).

(14) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Liebigs Ann. Chem.* **1953**, *583*, 129−149.

(15) Wieland, V. T.; Lamber, R.; Lang, H. L.; Günther Schramm, M. *Liebigs Ann. Chem.* **1956**, *591*, 181−195.

(16) The peptide−2-nitrobenzoic acid α-thioester (peptide−α-COS-NB) can be synthesized through reaction of the peptide $^{\alpha}$COSH with an excess of Ellman's reagent in 6 M Gn·HCl, 100 mM sodium phosphate (pH 6.5) for 15 min.[9] Although the product of this reaction has been described as a perthioester [(COSSNB) Liu, C. F.; Rao, C.; Tam, J. P. *Tetrahedron Lett.* **1996**, *37*, 933−936], in our hands, the peptide−α-thioester is the final reaction product under these conditions, as characterized by electrospray mass spectrometry (manuscript in preparation). It is important to note that the majority of peptide−α-thioesters used in this work have been synthesized by a different synthetic procedure, alkylation of peptide−α-thioacids, and therefore can be unambiguously characterized as thioesters.

(17) Sali, D.; Bycroft, M.; Fersht, A. R. *J. Mol. Biol.* **1991**, *220*, 779−188.

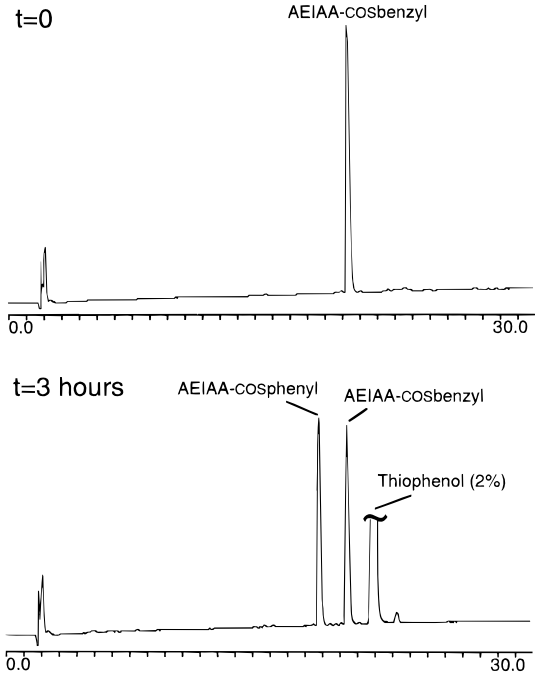


Figure 1. Model study of transthioesterification of α-carboxy thiobenzyl ester peptides. The peptide Ala-Glu-Ile-Ala-Ala-COS-benzyl (0.2 mg, 0.3 μmol) was dissolved in 100 μL of 100 mM sodium phosphate (pH 6.5) and 2% thiophenol. (A) Analytical reverse phase HPLC of Ala-Glu-Ile-Ala-Ala-COS-benzyl before treatment with thiophenol. (B) Analytical reverse phase HPLC after 3 h at room temperature in the presence of 2% thiophenol. Approximately equal amounts of the Ala-Glu-Ile-Ala-Ala-COS-benzyl (observed mass, 579.0 ± 0.5 Da; calculated average isotope composition for $C_{27}H_{41}N_5O_7S_1$, 579.6 Da) and Ala-Glu-Ile-Ala-Ala-COS-phenyl (observed mass: 566.0 ± 0.5 Da; calculated average isotope composition for $C_{26}H_{39}N_5O_7S_1$, 565.6 Da) were present. HPLC gradient 0%−60% acetonitrile and 0.1% TFA in H_2O over 30 min.

and has been previously prepared by classical chemical synthesis using several partially protected peptide segments.[18] We wanted to establish straightforward synthetic access to barnase in order to be able to use nongenetic variation of the protein structure.

Design of Barnase Ligation Site. The 110-residue polypeptide chain contains no cysteine residues.[19] To take advantage of native chemical ligation, Lys[49] was replaced by Cys[49], creating a Gly[48]-Cys[49] sequence at the ligation site.[20] Substitution of the solvent-exposed Lys[49], which is remote from the enzyme's active site, with a cysteine residue was expected to have little or no effect on the folding and activity of barnase.[21] The Cys residue has the further advantage of being centrally located in the polypeptide chain, so that peptides corresponding to barnase(1−48)$^{\alpha}$COS-benzyl (48 residues, C-terminal thioester) and [Cys[49]]barnase (49−110) (62 residues) were readily generated by solid phase synthesis.[10]

Comparison of Benzyl Mercaptan and Thiophenol as Thiol Additives. In pilot studies to explore the effects of different thiol additives, synthetic peptides corresponding to barnase(1−48)-$^{\alpha}$COS-benzyl and the analogue sequence [Cys[49],His[80],Ala[102]]barnase(49−110) were ligated in the presence of either benzyl mercaptan or thiophenol. After just 30 min, both ligated product and (1−48)-$^{\alpha}$COS-phenyl were observed in the reaction containing 4% thiophenol (Figure 2A). After 7 h of reaction in the presence of thiophenol, formation of the amide-linked product had proceeded essentially to completion (Figure 2B). Over the same time period, reaction in the presence of 4% benzyl mercaptan was less than 25% complete (Figure 2C). These experiments demonstrate that the use of a thiol additive corresponding to a more potent leaving group can, through transthioesterification, significantly increase the rate of native ligation to form the 110-residue polypeptide chain.

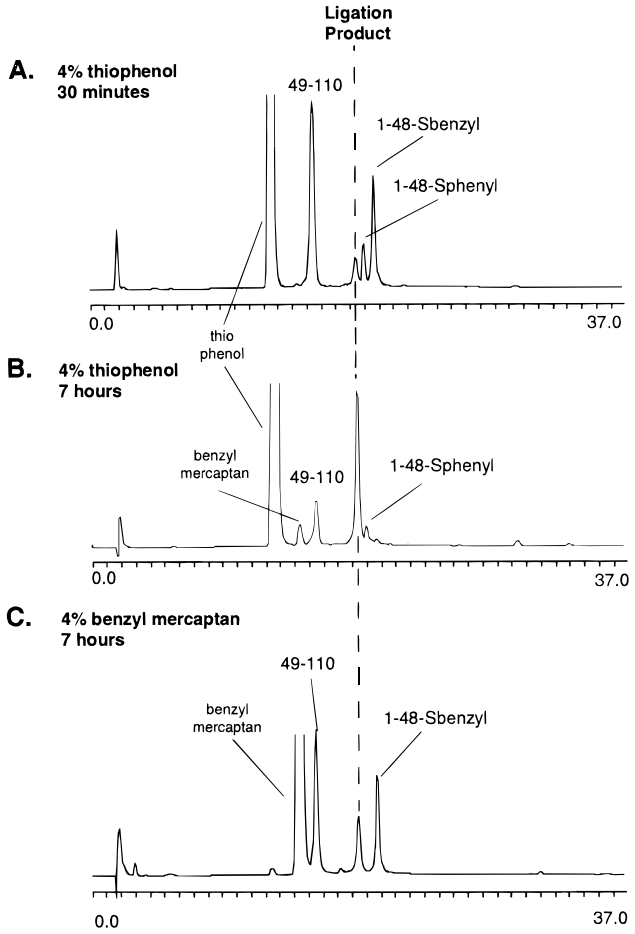


Figure 2. Effect of thiol additive on the native chemical ligation reaction between a 48-residue peptide α-carboxy thiobenzyl ester and a 62-residue N-terminal cysteine peptide. The peptides corresponding to barnase(1−48)$^{\alpha}$COS-benzyl and [Cys[49],His[80],Ala[102]]barnase(49−110) were dissolved in 6 M GnHCl and 100 mM sodium phosphate (pH 7.5) to give a final concentration of 1 mM (∼5 mg/mL) for each peptide. Analytical reverse phase HPLC traces are shown of a 27%−54% acetonitrile vs 0.1% TFA in H_2O gradient over 30 min. Labeled components were identified by electrospray mass spectrometry.[24] (A) Analytical HPLC of ligation reaction after 30 min in the presence of 4% thiophenol. (B) Analytical HPLC of ligation reaction after 7 h in the presence of 4% thiophenol. (C) Analytical HPLC of ligation reaction after 7 h in the presence of 4% benzyl mercaptan.

Synthesis of [Cys[49]]Barnase. A second protein analogue, [Cys[49]]barnase, was then synthesized utilizing thiophenol to achieve rapid ligation. Peptides corresponding to barnase(1−48)-$^{\alpha}$COS-benzyl and [Cys[49]]barnase(49−110) were synthesized by highly optimized solid phase peptide synthesis,[10] purified by reverse phase HPLC, and characterized by electrospray mass spectrometry. Approximately equimolar quantities of these two peptides were dissolved at a concentration of 1 mM each in 6

(18) Aimoto, S.; Kwon, Y.; Hojo, H. In *Peptide Chemistry 1992: Proceedings of the 2nd Japan Symposium on Peptide Chemistry*; Yanaihara, N., Ed.; ESCOM: Leiden, The Netherlands, 1992; pp 54−56.

(19) The barnase wild-type sequence is as follows: AQVINTFDGV. ADYLQTYHKL. PNDYITKSEA. QALGWVASKG. NLADVAPGKS. IGGDIFSNRE. GKLPGKSGRT. WREADINYTS. GFRNSDRILY. SSDWLIYKTT. DHYQTFTKIR. Hartley, R. W. *J. Mol. Biol.* **1988**, *202*, 913−915.

(20) Although an N-terminal Cys is necessary for native ligation, a more general approach has been developed which allows a wider selection of amino acids at this position. Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891−5896.

(21) Buckle, A. M.; Fersht, A. R. *Biochemistry* **1994**, *33*, 1644−1653.

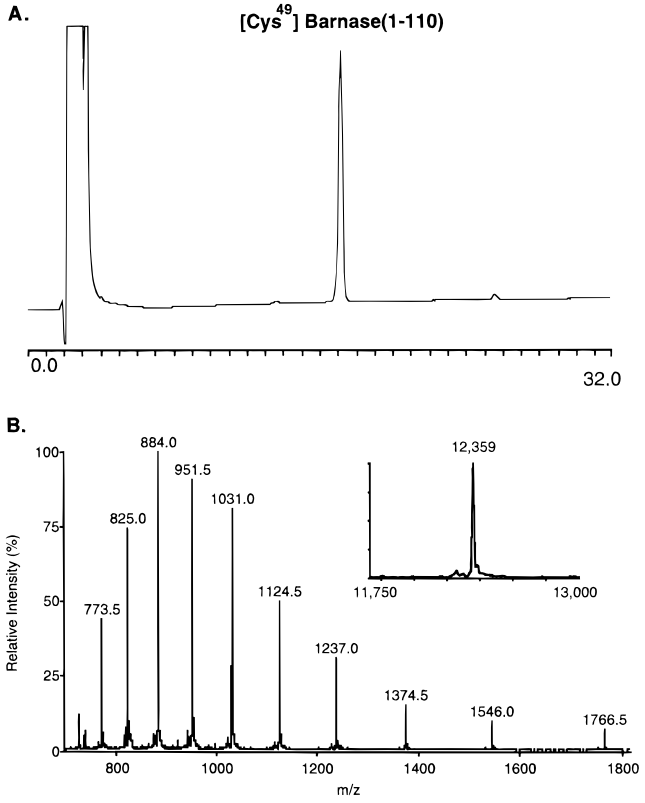


Figure 3. Characterization of the purified [Cys[49]]barnase(1−110) polypeptide product prepared by native ligation. (A) Analytical reverse phase HPLC. Conditions: 27%−54% acetonitrile and 0.1%TFA in water over 30 min. (B) Electrospray mass spectrum of the lyophilized product dissolved in 40% acetonitrile and 0.1% TFA in water. ESMS: observed mass, 12359 ± 2 Da; calculated average isotope composition for $C_{552}H_{840}N_{152}O_{170}S_1$, 12357.8 Da.

M Gn·HCl and 100 mM phosphate (pH 7.5) containing 2% thiophenol. As in the pilot study using thiophenol, native chemical ligation to form the [Cys[49]]barnase polypeptide was very efficient; the reaction was essentially complete after 4.5 h. The product was purified by reverse phase HPLC and characterized by analytical HPLC and electrospray mass spectrometry (Figure 3). The purified polypeptide chain was then folded by dilution from 6 M Gn·HCl and assayed for enzymatic activity. Synthetic [Cys[49]]barnase had enzymatic activity comparable to recombinant barnase of wild-type sequence,[22] as monitored by cleavage of the fluorogenic substrate polyethenoadenosine phosphate.[23]

Conclusion

As demonstrated by these model syntheses of a 110-residue polypeptide chain, the use of appropriate thiol additives in native chemical ligation gives clean, rapid reaction to form the desired amide-linked ligation product in high yield. The straightforward modulation of the thioester reactivity made possible by the transthioesterification reaction allows great flexibility in the synthesis and use of peptide−α-thioesters. Weakly activated thioesters are less susceptible to hydrolysis and are more easily handled than their highly reactive counterparts. These thioesters can be simply generated through reaction of a peptide−α-thiocarboxylate with an alkyl halide such as bromoacetic acid or benzyl bromide. Thiol reducing agents such as benzyl mercaptan or thiophenol can then be added to a ligation mixture to regulate the reactivity of the peptide−α-thioester.

We have applied this transthioesterification approach to the total chemical synthesis of a number of small proteins: the serine proteinase inhibitors turkey ovomucoid third domain (51 residues)[12] and eglin C (63 residues), the b/HLH domains of the transcription factor Deadpan (61 residues), $\beta_2$microglobulin (99 residues), phospholipase A2 (124 residues), and the HIV-1 protease (2 × 99 residues).[13] The *in situ* transthioesterification process, modulated by the addition of suitable thiols, appears to be broadly applicable to the total chemical synthesis of proteins by native chemical ligation.

Experimental Methods

General Materials and Methods. Boc-amino acids and HBTU were obtained from Novabiochem (San Diego, CA). Boc-Arg-OCH_2-Pam-resin and DIEA were purchased from Applied Biosystems (Foster City, CA). *N*,*N*-Dimethylformamide (Photrex grade) was obtained from Baker, and HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ). Trifluoroacetic acid was purchased from Halocarbon (River Edge, NJ).

Reverse Phase HPLC. Analytical and semipreparative gradient HPLC were performed on a Rainin dual-pump high-pressure mixing system with 214 nm detection. Semipreparative HPLC was run on a Vydac C18 column (10 μm, 10 × 250 mm) at a flow rate of 3 mL/min. Analytical HPLC was performed on a Vydac C18 column (5 μm, 4.6 × 150 mm) at a flow rate of 1 mL/min. Preparative HPLC was performed on a Waters Prep 4000 system fitted with a Waters 486 tunable absorbance detector using a Vydac C18 column (15−20 μm, 50 × 250 mm) at a flow rate of 30 mL/min. All runs used linear gradients of 0.1% TFA in water vs 90% acetonitrile/10% water plus 0.1% TFA.

Mass Spectrometry. Electrospray mass spectrometric analysis was routinely applied to all synthetic peptides and components of reaction mixtures. ESMS was performed on a Sciex API-III triple-quadrupole electrospray mass spectrometer as previously described.[24] Calculated masses were obtained using the program MacProMass (Sunil Vemuri and Terry Lee, City of Hope, Duarte, CA).

Solid-Phase Peptide Synthesis. All peptides were synthesized according to the *in situ* neutralization/HBTU activation protocol for stepwise Boc solid-phase peptide synthesis as previously described.[10] Short model peptides were synthesized by manual methods, while assembly of the longer barnase fragments was achieved by established machine-assisted synthesis on a custom-modified Applied Biosystems 430A peptide synthesizer.[10] Coupling yields were monitored by the quantitative ninhydrin determination of residual free amine.[25] Peptides with C-terminal α-thiocarboxylates were constructed on the corresponding amino acid−thioester support.[26] Peptide α-carboxylates were constructed on Boc-Arg-OCH_2-Pam-resin. Side-chain protection was as previously described,[10] except for peptides assembled on thioacid resins where it was necessary to use Boc-His(Bom)-OH and unprotected Boc-Trp-OH to avoid reaction of the thioester resin under nucleophilic deprotection conditions. In all cases, side-chain protecting groups were removed and the peptides cleaved from the resin by treatment with liquid HF containing 4% *p*-cresol, for 1 h at 0 °C. Crude peptide products were triturated with diethyl ether before being dissolved in 40% acetonitrile in water plus 0.1% TFA and lyophilized.

Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSNB. The crude, lyophilized C-terminal $^{\alpha}$thiocarboxylate peptide was dissolved in 6 M Gn·HCl and 0.1 M sodium phosphate at pH 6.5 (10 mg/mL) followed by addition of 10 mol equiv of a solution of 10 mM Ellman's reagent in 0.1 M sodium phosphate. After stirring for 45 min, the product was purified by semipreparative HPLC and lyophilized. The resulting α-carboxythioester peptide was characterized by ESMS (observed, 759.6 ± 0.2 Da; calculated average isotope composition for $C_{33}H_{45}N_9O_{10}S_1$, 759.7 Da), which was consistent with the formation of a thioester rather

(22) Recombinant barnase was expressed from vector strain PMT416 in *E. coli* strain J107, obtained from R. W. Hartley.

(23) Fitzgerald, P. C.; Hartley, W. *Anal. Biochem.* **1993**, *214*, 544−547.

(24) Schnölzer, M.; Jones, A.; Alewood, P. F. and Kent, S. B. H. *Anal. Biochem.* **1992**, *204*, 335−343.

(25) Sarin, V. K.; Kent, S. B. H.; Tam, J. P. and Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−153.

(26) Canne, L. E.; Walker, S. M.; Kent, S. B. H. *Tetrahedron Lett.* **1995**, *36*, 3795−3798.

than a perthioester (calculated average isotope composition for $C_{33}H_{45}N_9O_{10}S_2$: 791.8 Da).[16]

Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSCH$_2$COOH. The crude, lyophilized C-terminal α-thiocarboxylate peptide was dissolved in 6 M Gn·HCl and 0.1 M sodium acetate at pH 3.8. Bromoacetic acid (2 equiv) was added, and the peptide was reacted at room temperature for 30 min and then purified by semipreparative HPLC. The resulting α-carboxythioester peptide was characterized by ESMS (observed, 652.5 ± 0.5 Da; calculated average isotope composition for $C_{28}H_{44}N_8O_8S_1$, 652.7 Da).

Ala-Glu-Ile-Ala-Ala-$^{\alpha}$COS-benzyl. The crude, lyophilized C-terminal α-thiocarboxylate peptide obtained from HF cleavage was taken up in 6 M guanidine·HCl and 0.1 M sodium phosphate at pH 6.5 at a concentration of 10 mg/mL. Benzyl bromide (10 equiv) was added, and the solution was briefly vortexed and then stirred at room temperature for 30 min. The product was purified by semipreparative HPLC, lyophilized, and characterized by ESMS (observed, 579.0 ± 0.5 Da; calculated average isotope composition for $C_{27}H_{41}N_5O_7S$, 579.6 Da).

Model Studies on Thio Acid and Thioester Groups. Purified model peptides were reacted under the conditions described, and in each case, the reaction was followed by analytical reverse phase HPLC. Peaks were collected on the basis of their UV absorbance (214 nm) and analyzed by ESMS.

Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSNB + Benzyl Mercaptan. Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSNB (0.2 mg, 0.3 μmol) was dissolved in 0.1 mL of 6 M Gn·HCl and 0.1 M sodium phosphate at pH 6.5 (peptide concentration 2 mg/mL, 3 mM) containing benzyl mercaptan (2 μL, 20 μmol). After stirring for 1 h at room temperature, the peptide solution was analyzed by HPLC. In addtion to the starting peptide, Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSNB, a later eluting peak of approximately equal area was collected and analyzed by ESMS. The mass of this peptide was consistent with converstion to the benzyl thioester peptide, Lys-Tyr-Arg-Ala-Gly-$^{\alpha}$COS-benzyl (observed: 684.8 ± 0.3 Da; calculated average isotope composition, 684.7 Da).

Ala-Glu-Ile-Ala-Ala-$^{\alpha}$COS-benzyl + Thiophenol. The benzyl thioester peptide (0.2 mg, 0.3 μmol) was dissolved in 0.1 mL of 6 M Gn·HCl and 0.1 M sodium phosphate at pH 6.5 (2 mg/mL, 3 mM) containing thiophenol (2 μL, 20 μmol). After stirring for 3 h at room temperature, the peptide solution was analyzed by HPLC. In addition to the starting benzyl thioester peptide, an early eluting peptide was collected and analyzed by ESMS. The mass of this peptide was consistent with thioester exchange to the phenyl thioester peptide, Ala-Glu-Ile-Ala-Ala-$^{\alpha}$COS-phenyl (observed, 566.0 ± 0.5 Da; calculated average isotope composition for $C_{26}H_{39}N_5O_7S$, 565.5 Da).

Leu-Tyr-Arg-Ala-Gly-$^{\alpha}$COSCH$_2$COOH + Thiophenol. The peptide thioester acetate (0.2 mg, 0.3 μmol) was dissolved in 0.1 mL of 6 M Gn·HCl and 0.1 M sodium phosphate at pH 6.5 (2 mg/mL, 3 mM) containing thiophenol (2 μL, 20 μmol). After stirring for 1 h at room temperature, the peptide solution was analyzed by HPLC. In addition to the starting peptide−α-thioester, a second, later eluting peptide was collected and analyzed by ESMS. The mass of this peptide was consistent with thioester exchange to the peptide−$^{\alpha}$thiophenyl ester (observed, 670.5 ± 0.5 Da; calculated average isotope composition for $C_{32}H_{46}N_8O_6S_1$, 670.7 Da).

Synthesis of Barnase Analogues. Barnase(1−48)-$^{\alpha}$COS-benzyl was synthesized on a glycine thioester resin[26] by highly optimized Boc-chemistry SPPS[10] and cleaved by HF using 5% *p*-cresol as a scavenger. The resulting crude thio acid peptide was lyophilized and then alkylated with benzyl bromide to produce the desired benzyl thioester as follows: barnase(1−48)-$^{\alpha}$COSH (123 mg) was dissolved in 25 mL of 6 M guanidine and 100 mM sodium acetate (pH 4.0) (5 mg/mL, 1 mM). Benzyl bromide (5 equiv) was added and the solution was briefly vortexed. The reaction was checked after 20 min by analytical HPLC and electrospray mass spectrometry and then purified by preparative HPLC to give 10 mg of lyophilized thioester peptide. The product was characterized by ESMS (observed, 5258 ± 1 Da; calculated average isotope composition $C_{239}H_{360}N_{60}O_{72}S_1$, 5257.8 Da).

[^{49}Cys]Barnase(49−110) and [Cys^{49},His^{80},Ala^{102}]Barnase (49−110) were separately synthesized on Boc-Phe-OCH$_2$Pam resin by highly optimized SPPS[10] and individually cleaved by treatment with HF containing 5% 1:1 *p*-cresol:*p*-thiocresol. The resulting crude peptides were lyophilized and purified by semipreparative HPLC. A typical run used 14 mg of crude peptide and yielded 1.8 mg of highly purified peptide. The peptide products were characterized by HPLC and electrospray mass spectrometry. [Cys^{49}]Barnase(49−110): observed, 7224 ± 1 Da; calculated average isotope composition for $C_{320}H_{488}N_{92}O_{98}S_1$, 7225.0 Da. [Cys^{49},His^{80},Ala^{102}]Barnase(1−110): observed, 7209 ± 1 Da; calculated average isotope composition for $C_{320}H_{488}N_{92}O_{97}S_1$, 7208.0 Da.

[Cys^{49},His^{80},Ala^{102}]Barnase(1−110) Model Ligation. The peptides corresponding to barnase(1−48)-$^{\alpha}$COS-benzyl and [Cys^{49},His^{80},Ala^{102}]-barnase(49−110) were dissolved in 6 M Gn·HCl and 100 mM sodium phosphate (pH 7.5) to give a final concentration of 1 mM (∼5 mg/mL) of each peptide. Half of the solution was removed to a separate reaction vessel. Benzyl mercaptan (4%) was added to one ligation reaction, and 4% thiophenol was added to the other, followed by a brief vortex of the solution to dissolve the thiol additive. The ligation reactions were monitored by analytical reverse phase HPLC on a 27%−54% acetonitrile and 0.1% TFA in H_2O over 30 min gradient. Eluted peptides were identified by electrospray mass spectrometry.[24] Two new peptide productss were observed with masses corresponding to barnase-(1−48)-$^{\alpha}$COS-phenyl (observed, 5244 ± 1 Da; calculated average isotope composition for $C_{238}H_{358}N_{60}O_{72}S_1$, 5243.8 Da) and [Cys^{49},His^{80},-Ala^{102}]barnase(1−110) (observed, 12343 ± 2 Da; calculated average isotope composition for $C_{552}H_{840}N_{152}O_{169}S_1$, 12341.8 Da).

[Cys^{49}]Barnase(1−110) Ligation. [Cys^{49}]Barnase(49−110) (3.7 mg, 0.5 μmol) and barnase(1−48)-S-benzyl (2.6 mg, 0.5 μmol) were dissolved in 370 μL of 6 M Gn·HCl and 100 mM sodium phosphate (pH 7.5) (∼1 mM each peptide) by gentle mixing with a pipet. Thiophenol (7 μL, 50 μmol) was added, and the mixture was vigorously mixed by pipet to saturate the ligation buffer with thiophenol. The ligation reaction was allowed to proceded for 12 h. Following ligation, 300 μL of the reaction mixture was purified by analytical HPLC in two runs. Recovered yield after ligation 2.52 mg, 53% from limiting (1−48)-S-benzyl component. ESMS: observed mass, 12359 ± 2 Da; calculated average isotope composition for $C_{552}H_{840}N_{152}O_{170}S_1$, 12357.8 Da).

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