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# Reactivity of Intein Thioesters: Appending a Functional Group to a Protein

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*The success of genome sequencing has heightened the demand for new means to manipulate proteins. An especially desirable goal is the ability to modify a target protein at a specific site with a functional group of orthogonal reactivity. Here, we achieve that goal by exploiting the intrinsic electrophilicity of the thioester intermediate formed during intein-mediated protein splicing. Detailed kinetic analyses of the reaction of nitrogen nucleophiles with a chromogenic small-molecule thioester revealed that the α-hydrazino acetyl group was the optimal nucleophile for attacking a thioester at neutral pH to form a stable linkage. A bifunctional reagent bearing an α-hydrazino acetamido and azido group was synthesized in high overall yield. This reagent was used to attack the thioester linkage between a target protein and intein, and thereby append an azido group to the target protein in a single step. The azido protein retained full biological activity. Furthermore, its azido group was available for chemical modification by Huisgen 1,3-dipolar azide–alkyne cycloaddition. Thus, the mechanism of intein-mediated protein splicing provides the means to install a useful functional group at a specific site—the C terminus—of virtually any protein.*

## Introduction

Proteins are nucleophilic. Their side chains contain no electrophiles, other than the disulfide bonds of cystines or functional groups installed by post-translational modification. Accordingly, the chemical reactivity of proteins necessarily entails nucleophilic side chains, such as those of lysine[1] and cysteine.[2,3] The prevalence of these residues obviates control over the regiochemistry of reactions,[4] and produces heterogeneous reaction products—often at the expense of biological function.[3,5]

An intermediate that forms during the intein-mediated splicing of proteins contains an electrophile—a thioester (Scheme 1).[6] The orthogonal reactivity of this functional group can be exploited for the site-specific modification of a protein either by reaction with cysteine derivatives[7] or tandem reaction with a small-molecule thiol and amine.[8] Although thiols are potent nucleophiles for thioesters, the resultant thioesters are inherently unstable to hydrolysis;[9] this makes the simple transthioesterification of an intein-derived thioester unsuitable for the chemical modification of proteins. The powerful methods of native chemical ligation[10] and expressed protein ligation[11] offer an ingenious solution to this problem. After transthioesterification with a cysteine residue, *S*→*N*-acyl transfer regenerates the thiol and forms a stable amide linkage. This approach, which has been used for protein modification and immobilization,[12,13] introduces a residual thiol that can be the focal point for undesirable side reactions. For example, cysteine is by far the most reactive residue toward disulfide bonds, $O_2$(g), and other common electrophiles.[14] In addition, the sulfhydryl group of cysteine can either suffer β elimination to generate dehydroalanine,[15] or disrupt self-assembled monolayers on gold or silver surfaces.[16] These detrimental attributes caused us to search for an alternative means to exploit the intein-derived thioester for the installation of an orthogonal functional group.

In contrast to sulfur nucleophiles, nitrogen nucleophiles can, in theory, react directly with the thioesters formed during intein-mediated protein splicing to form inert linkages. This reaction has been neither explored nor exploited previously. Moreover, we reasoned that an appropriate bifunctional nitrogen nucleophile could both attack an intein-derived thioester to form a stable linkage, and install an orthogonal (and thus useful) functional group.

The azido group can serve as an orthogonal functional group because it is absent from natural proteins, nucleic acids, and carbohydrates.[17] Moreover, chemical reactions of the azido group, such as the $Cu^I$-catalyzed Huisgen 1,3-dipolar azide–alkyne cycloaddition[18] and Staudinger ligation,[19] can lead to site-specific modification or immobilization. Azido proteins have been produced previously. For example, Schultz and co-workers have developed a method for incorporating azidolysine into proteins.[20] Their approach involves the production of an azidolysine-charged suppressor tRNA that inserts the residue into a protein, as directed by an engineered gene. This method, although site-specific, is labor intensive and low yielding. Tirrell, Bertozzi, and co-workers have incorporated an azido group into a protein by using azidohomoalanine, which replaces methionine in proteins produced in methionine-depleted bacterial cultures.[21] This method is not site specific for proteins that contain more than one methionine residue.

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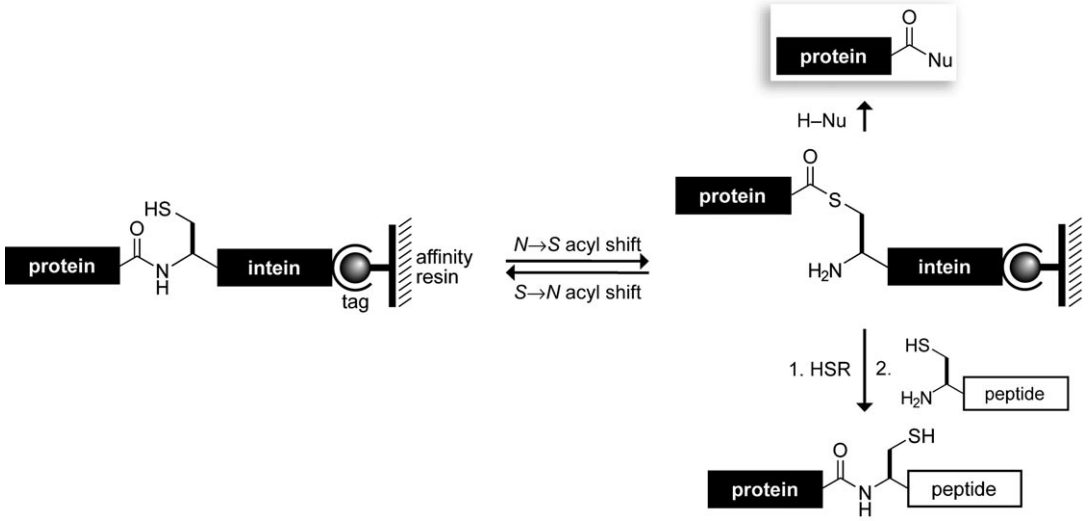


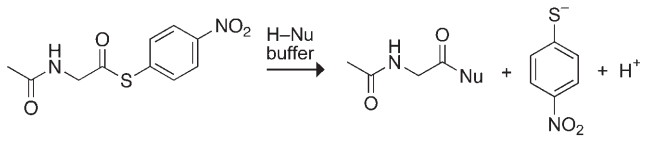

**Scheme 1.** Mechanism of expressed protein ligation[11] and on-resin capture of the thioester intermediate with a small-molecule nucleophile (H–Nu).

Here, we report a general strategy for producing proteins that are site-specifically labeled with an azido group. We produced these azido proteins by semisynthesis by using a variation of expressed protein ligation (Scheme 1).[11] Our strategy involves production of the protein of interest as a fusion protein with an intein molecule and a tag for affinity chromatography. On-resin cleavage of the intein-derived thioester was induced with a bifunctional reagent that carried a nucleophile for thioesters and an azido group. Kinetic analyses with a chromogenic thioester were used to identify the optimal nucleophile for this purpose, and the desired bifunctional reagent was synthesized on a multigram scale. This strategy was used to produce an azido protein that maintained full biological activity and displayed an azido group at its C terminus that is available for chemoselective modification. Thus, exploiting the intrinsic and orthogonal reactivity of the thioester produced during intein-mediated protein splicing enabled site-specific chemical modification of a protein.

## Results and Discussion

### Identification of the optimal nucleophile for thioesters

To identify the optimal nitrogen nucleophile for a thioester, kinetic studies were performed on a model chromogenic thioester, AcGlySC$_6$H$_4$-*p*-NO$_2$ (Scheme 2). The rate of release of the




**Scheme 2.** Cleavage reaction of a model chromogenic thioester.

thiophenolate anion was monitored by measuring the change in absorbance at 410 nm. Nitrogen nucleophiles with conjugate-acid p$K_a$ values ranging from 4.6 to 10.6 (Table 1) were used in the experiments. The logarithmic values of the second-order rate constants ($k_2$) of the unprotonated primary amines

**Table 1.** Nucleophiles used in this study and the p$K_a$ values of their conjugate acids.

| Nucleophiles | p$K_a$ | Nucleophiles | p$K_a$ |
|---|---|---|---|
| $CH_3ONH_2$ | 4.60[22] | $F_3CCH_2NH_2$ | 5.40[23] |
| $C_2H_5O(O)CCH_2NHNH_2$ | 6.45[24] | $CH_3O(O)CCH_2NH_2$ | 7.75[22] |
| $CH_3NHNH_2$ | 7.87[25] | $FCH_2CH_2NH_2$ | 9.19[23] |
| $HOCH_2CH_2NH_2$ | 9.50[22] | $CH_3CH_2NH_2$ | 10.63[22] |

were plotted against the p$K_a$ values of their conjugate acids to yield the Brønsted plot shown in Figure 1 A. The data were fitted to the equation:[26]

$$\log k_2 = \log(\mathrm{AB}) + (\beta + \beta')\,\mathrm{p}K_a - \log(A10^{\beta \mathrm{p}K_a} + B10^{\beta' \mathrm{p}K_a}) \qquad (1)$$

In Equation (1), A and B are constants, $\beta'$ is the slope of the former part of the Brønsted plot, and $\beta$ is the slope of the latter part.

The Brønsted plot in Figure 1 A is biphasic. The slope changes from 0.81 with nucleophiles of low p$K_a$ to 0.42 with nucleophiles of high p$K_a$. This change is due to the known alteration in the rate-determining step from the formation of a tetrahedral-zwitterionic intermediate to the decomposition of the intermediate into products.[26–28] The value of the slope obtained ($\beta' = 0.81$) is in agreement with that for the aminolysis of oxygen esters.[29] As expected,[30] "$\alpha$-effect" nucleophiles (alkoxy amines, $\alpha$-hydrazino acetyl, and alkyl hydrazine) exhibited much greater nucleophilicity than that predicted from their p$K_a$ values. In water, the $\alpha$ effect could arise from the nucleophile being less solvated and hence more reactive because of the inductive withdrawal of electrons by the adjacent heteroatom.[31]

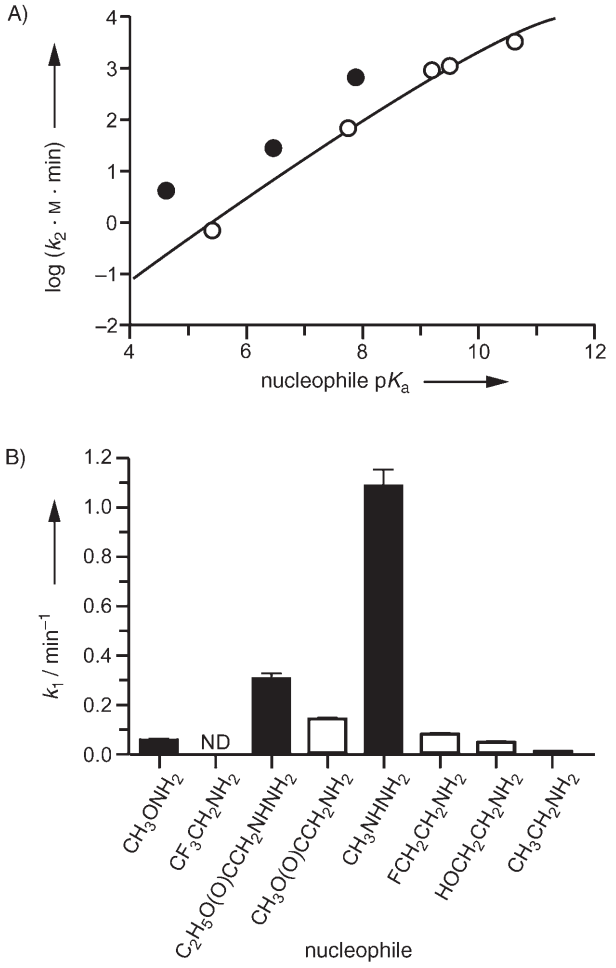



**Figure 1.** Rate constants for the attack of nitrogen nucleophiles on a thioester (Scheme 2). A) Brønsted plot for the reaction of simple amines (○) and α-effect nucleophiles (●) with $AcGlySC_6H_4$-*p*-$NO_2$ at 25 °C. Relevant p$K_a$ values are listed in Table 1. Data were fitted to Equation (1) with $A = 0.21$, $B = 4.0 \times 10^{-5}$, $\beta = 0.42$, and $\beta' = 0.81$. B) Values of the first-order rate constant ($k_1$) at pH 7.0 and 25 °C; ND: not determined.

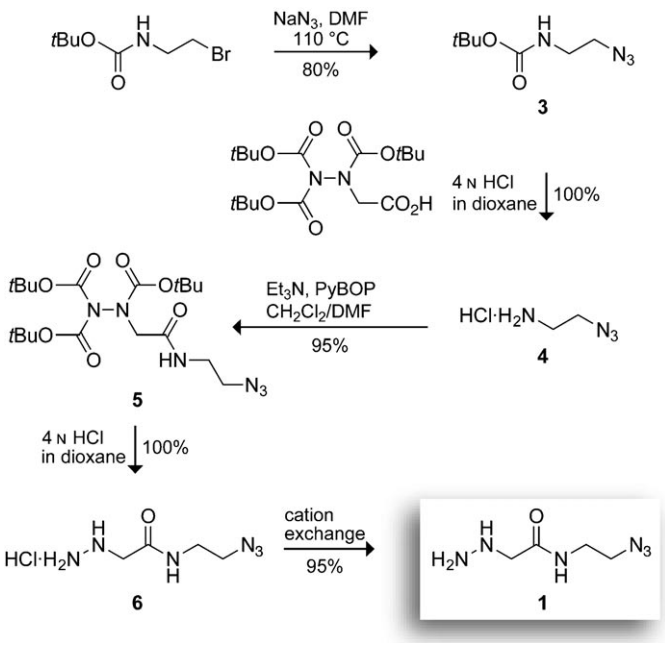
The Brønsted plot reports on the nucleophilicity of unprotonated (that is, neutral) amines. According to Figure 1 A, the best nucleophile for thioesters in an environment in which all the amines are deprotonated, is ethyl amine. Indeed, other simple amines have been used in tandem with a small-molecule thiol to modify an intein-derived thioester.[8] At the high pH necessary to deprotonate an amine, however, thioesters are prone to hydrolysis.[9] Moreover, proteins are subject to unfolding and subsequent aggregation at high pH values.[32] Performing the reaction at pH 7.0 provides an acceptable tradeoff between deprotonation of the nitrogen nucleophile and hydrolysis of the thioester. Data on the first-order rate constant ($k_1$) for the reaction of various nitrogen nucleophiles at pH 7.0 under pseudo-unimolecular reaction conditions are shown in Figure 1 B. At pH 7.0, the α-hydrazino acetyl and alkyl hydrazine functionalities are much more nucleophilic than the simple amines and alkoxy amine (e.g., 30- and 100-fold greater $k_1$ value than ethyl amine, respectively), and are therefore the optimal nucleophiles for reacting with a thioester to form a stable linkage.

Jencks,[29,33] Bruice,[30] Castro,[28,34] and others have reported in detail on the mechanism and kinetics of the nucleophilic attack on esters and, to a lesser extent, thioesters. This previous work was not, however, performed with the intent of making a stable linkage with a bifunctional reagent. For example, hydroxylamine was identified in ca. 1950 as an exceptional nucleophile for a thioester.[35] Indeed, this attribute of hydroxylamine has led to its use in revealing transient thioesters formed during intein-mediated protein splicing.[6] The cleavage of thioesters by hydroxylamine, however, relies on the formation of an *O*-acylated hydroxylamine intermediate[36,37] that is inaccessible during the attack of an alkoxy amine on a thioester. Accordingly, an alkoxy amine is not an especially potent nucleophile for a thioester (Figure 1).

### Synthesis of bifunctional azides

After identification of two optimal nucleophiles, we proceeded to synthesize two bifunctional reagents that carried those nucleophiles on one end and an azido group on the other. Azides **1** and **2** are both amides of 1-azido-2-aminoethane. Azide **1** has an α-hydrazino acetamido group, which is a more stable analogue of the α-hydrazino acetyl group of $C_2H_5O(O)CCH_2NHNH_2$ (Table 1; Figure 1); azide **2** has a γ-hydrazino acetamido group and is effectively an alkyl hydrazine.

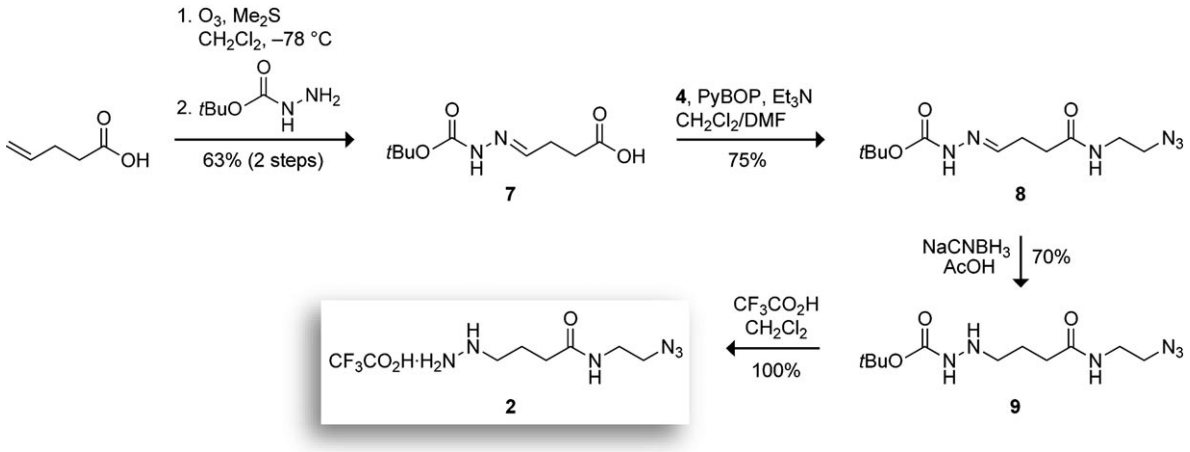
Azide **1** was synthesized by the route shown in Scheme 3. Briefly, Boc-protected 1-azido-2-aminoethane was synthesized




**Scheme 3.** Synthetic route to azide **1**.

from Boc-protected 1-bromo-2-aminoethane. After Boc deprotection, the amine was coupled to tri-Boc-protected α-hydrazino acetic acid. The Boc groups were removed, and azide **1** was isolated as a free base after cation-exchange chromatography with an overall yield of 72%.




**Scheme 4.** Synthetic route to azide **2**.

Azide **2** was synthesized by the route shown in Scheme 4. Briefly, 4-pentenoic acid was subjected to ozonolysis, and the resulting aldehyde was treated, in situ, with Boc-protected hydrazine. The azido group was installed by coupling 1-azido-2-aminoethane (**4**) to the carboxylic acid. The hydrazone was then reduced selectively with $NaCNBH_3$. The Boc group was removed to produce azide **2** as its trifluoroacetic acid (TFA) salt with an overall yield of 26% (which includes the 80% yield for the synthesis of azide **3**). Attempts to produce the free hydrazine base by cation-exchange chromatography resulted in decomposition of the molecule by an (as yet) unknown mechanism. Likewise, the TFA salt was unstable even upon storage under vacuum, and was hence used immediately after its synthesis.

### Kinetics of thioester cleavage

Kinetic studies were performed by treating azides **1** and **2** with a model chromogenic thioester (Scheme 2). The rate constants ($k_2$ and $k_1$) for azide **1** were found to be indistinguishable from those of the α-hydrazino acetyl group. The rate constants for azide **2** were, surprisingly, much lower than those of methylhydrazine. This result is contrary to our finding that methyl hydrazine is a somewhat better nucleophile than the α-hydrazino acetyl functional group (Figure 1). The intrinsic instability of azide **2** is likely to be responsible for this apparent decrease in reactivity.

### Production of an azido protein

Next, we sought to use our bifunctional reagents to install an azido group at the C terminus of a model protein. As our protein, we chose bovine pancreatic ribonuclease (RNase A), which has been the object of much seminal work in protein chemistry,[38] and has been manipulated previously with expressed protein ligation.[39–41] RNase A has valine as its C-terminal residue. A valine residue at the C terminus of a target protein is known to have a debilitating effect on the cleavage efficiency of protein–intein thioesters.[12,42] In order to avert this problem, we inserted a glycine residue between the C terminus of RNase A and the intein. The resulting Met(−1)RNase A–Gly–*mxe* intein–chitin-binding domain fusion protein ($M_W$ ~ 36 kDa) was produced in *E. coli*, and the cell lysate was loaded onto chitin resin. Azides **1** and **2** were used to induce the on-resin cleavage of the fusion protein. As expected from the kinetic studies, azide **1** was found to be much more effective than azide **2** in cleaving the Met(−1)RNase A–Gly–*mxe* intein thioester. Its shorter and higher yielding synthesis, superior stability, and higher cleavage efficiency make azide **1** the optimal bifunctional reagent for the semisynthesis of proteins labeled with the azido group. The purity of the azido-RNase A (even upon elution from the chitin column) was apparent from SDS–PAGE analysis (Figure 2A); the integrity of the azido-RNase A was verified by using MALDI-TOF mass spectrometry (Figure 2B). This procedure produced an overall yield of ~1 mg of purified azido-RNase A per liter of *E. coli* culture.

Incubating a protein with a potent nucleophile, such as the α-hydrazino acetamido group of azide **1**, could compromise the structure of the protein. For example, the target protein in this study has 142 amide bonds in its main chain and side chains that could be attacked by the α-hydrazino acetamido group, but it has only one thioester bond. In addition, the target protein has eleven amino groups that could serve as intramolecular nucleophiles for that thioester bond. Enzymatic catalysis provides an extremely sensitive measure of native-protein structure.[43] This measure is especially useful for detecting the inadvertent modification of RNase A, as one of its eleven amino groups is both especially reactive and critical for enzymatic activity.[38,44] Purified azido-RNase A had $k_{cat}/K_M = (3.2 \pm 1.0) \times 10^7\ M^{-1} s^{-1}$ for the cleavage of RNA. This value was in gratifying agreement with that of the wild-type enzyme, which had $k_{cat}/K_M = (5.2 \pm 0.4) \times 10^7\ M^{-1} s^{-1}$.[45]

The α-hydrazino acetamido group was found to be the optimal nitrogen nucleophile for producing azido proteins. Still, we had to use a 450 mM solution of azide **1** to produce the desired hydrazide product. Lower concentrations led to the hydrolyzed protein (that is, the protein with a C-terminal carboxyl group) to be a dominant product. In contrast, a 50 mM solu-

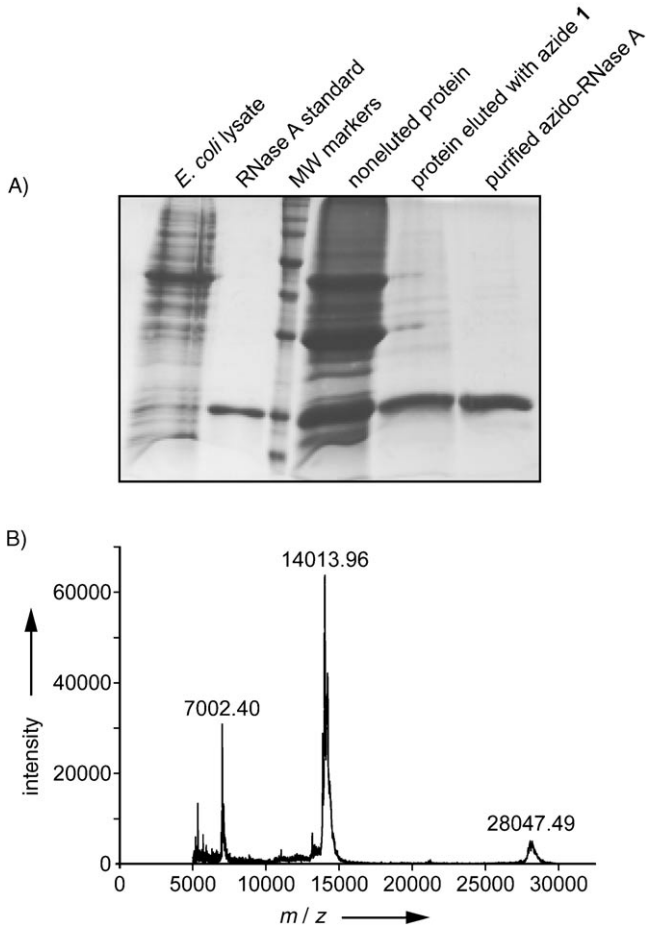
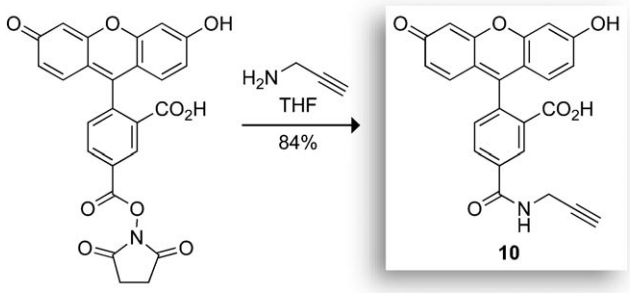



**Figure 2.** A) SDS–PAGE analysis of the preparation of azido-RNase A. B) MALDI-TOF mass spectrum of azido-RNase A (expected for Met(−1)-RNase A–Gly–$NHNHCH_2C(O)NHCH_2CH_2N_3$ [$C_{586}H_{92a}N_{179}O_{195}S_{13}$] = 14011).

tion of thiol typically suffices for transthioesterification during expressed protein ligation.[11] The resulting thioester must then, however, react with a peptide (present in vast excess) that contains an N-terminal cysteine residue.[12,13] The ability to obtain an azido protein in a single step by on-resin cleavage and the absence of the residual sulfhydryl group installed during expressed protein ligation are noteworthy advantages of our strategy (Scheme 1). These attributes are of particular importance for high-throughput procedures, such as the fabrication of protein microarrays.[46]

### Huisgen 1,3-dipolar cycloaddition to an azido protein

For our strategy to be useful, the azido group in azido-RNase A must be available for further reaction. We used a chemoselective reaction, $Cu^I$-catalyzed Huisgen 1,3-dipolar azide–alkyne cycloaddition,[18,47,48] to probe for the availability of the azide functionality. To effect this "functional-group test", alkynyl fluorescein **10** was synthesized by the route shown in Scheme 5. Azido-RNase A was treated with **10** in the presence of the $Cu^I$ catalyst and its polytriazole ligand.[48] The resulting protein had a molecular mass of *m/z* 14449, which agreed well with that expected for the conjugate ($C_{610}H_{936}N_{180}O_{201}S_{13}$ = 14424). The




**Scheme 5.** Synthetic route to alkynyl fluorescein **10**.

protein was also subjected to SDS–PAGE and visualized by staining with Coomassie blue and fluorescence imaging. The azido-RNase A was found to be fluorescent as a result of the cycloaddition, whereas wild-type RNase A treated in the same manner was not fluorescent (Figure 3). Neither the mass spectrum nor the SDS–PAGE gel showed evidence of cleavage products, as have been observed in an azido protein exposed to reducing agents.[49] Thus, an azido group was not only installed into a specific site on RNase A, but was also available for reaction. In ongoing work, the Staudinger ligation[19] is being used for the site-specific immobilization of proteins produced by the novel route shown in Scheme 1.

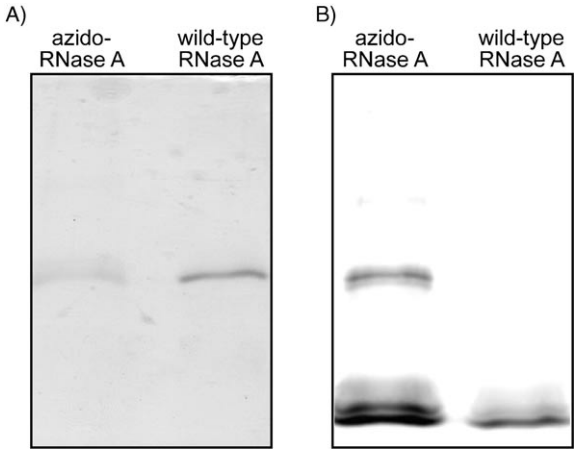



**Figure 3.** SDS–PAGE analysis of the reaction of azido-RNase A and wild-type RNase A with alkynyl fluorescein **10**. A) Visualization with Coomassie-blue staining. B) Visualization with fluorescence imaging.

## Conclusion

We have exploited the mechanism of intein-mediated protein splicing to develop a general strategy for installing a functional group at the C terminus of a protein. The strategy is based on the capture of an intein-derived thioester with a nucleophile that was selected by using Brønsted analysis. We used this strategy to append an azido group to a model protein. The azido group did not affect the function of the protein and was

available for Huisgen 1,3-dipolar azide–alkyne cycloaddition of a fluorophore. We anticipate that such a C-terminal azido group could also be used for site-specific protein immobilization, which can be preferable to random immobilization,[3,5] and for modification by Staudinger ligation.[19] Finally, we note that our strategy can be used to append other functional groups with orthogonal reactivity (such as an alkene, alkyne, or nitrile) to a target protein.

## Experimental Section

The chromogenic thioester $AcGlySC_6H_4$-*p*-$NO_2$ (a generous gift from B. L. Nilsson[40]) was purified by recrystallization from methylene chloride and stored in a tightly sealed vial in a desiccator to prevent hydrolysis by moisture present in air. Fluorescein–NHS ester was a generous gift from L. D. Lavis. All other chemicals were commercial reagent grade or better, and were used as received except for benzyl azide, which was purified by flash chromatography before use. Anhydrous THF, DMF, and $CH_2Cl_2$ were obtained from a CYCLE-TAINER® solvent delivery system (J. T. Baker, Phillipsburg, NJ, USA). Other anhydrous solvents were obtained in septum-sealed bottles. Synthetic reactions were monitored by thin-layer chromatography (TLC) and visualized by using UV-light or stained with vanillin, ninhydrin, or $I_2$. In all reactions involving anhydrous solvents, glassware was flame-dried. Flash chromatography was performed with columns of silica gel 60, 230–400 mesh (Silicycle, Quebec City, Quebec, Canada).

**Instrumentation**: A Cary Model 3 UV/VIS spectrophotometer (Varian, Palo Alto, CA, USA) was used to perform kinetic assays and measure ultraviolet absorbance. NMR spectra were acquired with a Bruker AC+ 300 spectrometer ($^1$H: 300 MHz; $^{13}$C: 75 MHz) at the Magnetic Resonance Facility in the Department of Chemistry, or (as indicated) on a Bruker DMX-400 Avance spectrometer ($^1$H: 400 MHz; $^{13}$C: 100 MHz) at the National Magnetic Resonance Facility at Madison (NMRFAM); $^{13}$C spectra were proton-decoupled. Mass spectra on small organic molecules were obtained with ESI techniques. Mass spectra of proteins were obtained with MALDI-TOF mass spectrometry by using a Voyager-DE-PRO Biospectrometry workstation (Applied Biosystems, Foster City, CA, USA) and a 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix (Aldrich). Fluorescence measurements were made with a QuantaMaster 1 photon counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ, USA). A Typhoon 9410 variable mode fluorimager (Amersham Biosciences) was used to visualize fluorescein-labeled protein after SDS–PAGE.

**Kinetics of thioester cleavage**: $AcGlySC_6H_4$-*p*-$NO_2$ was dissolved in anhydrous acetonitrile to a concentration of 0.45 mM and used immediately. Amine solutions (except $CF_3CH_2NH_2$·HCl) were prepared by dissolving the amine hydrochloride salts in sodium phosphate buffer (0.10 M) at pH 7.0 to a concentration of 0.15 M. Reaction mixtures were prepared (final volume 1.1 mL) and equilibrated at 25 °C. All reactions contained $\leq$3% (*v*/*v*) acetonitrile. The ionic strength of each reaction mixture was $I$ = 0.22–0.25. All reactions were carried out with a large excess of amine and followed pseudo-first-order kinetics. Amine solution (0.15 M, 100 μL) was added to a cuvette containing sodium phosphate buffer (0.10 M) at pH 7.0 (967 μL), and the absorbance at 410 nm was adjusted to zero. The thioester (33 μL of a 0.45 mM solution in acetonitrile) was then added to the cuvette, and the absorbance was monitored at 410 nm over time ($\varepsilon$ = 11,230 $M^{-1}cm^{-1}$ for *p*-nitrothiophenolate anion[40]). The p$K_a$ of $HSC_6H_4$-*p*-$NO_2$ is 4.77 in 40% (*v*/*v*) ethanol in water.[50] This value is likely to be lower in an aqueous buffer, such that the ionization of the *p*-nitrothiophenol product is virtually complete in all our assays. The final concentration of the thioester in the reaction was 13.64 μM and the final concentration of the amines (except $CF_3CH_2NH_2$·HCl) was 13.64 mM. Each reaction was performed in triplicate. The reactions were allowed to go to completion, and $t_{1/2}$ was determined from the kinetic trace. The $t_{1/2}$ was corrected by subtracting $t_{1/2}$ for hydrolysis from the observed value. Pseudo-first-order rate constants were calculated by using the equation $k_1 = 0.693/t_{1/2}$. Second-order rate constants were obtained by dividing each observed first-order rate constant by the concentration of free amine. Logarithmic values of second-order rate constants were plotted against the p$K_a$ values of the conjugate acids of the respective amines to yield a Brønsted plot.

**Kinetics of thioester cleavage by $CF_3CH_2NH_2$**: The procedure for the kinetics of thioester cleavage (vide supra) was found to be problematic for $CF_3CH_2NH_2$ because this amine is a poor nucleophile at pH 7.0. Indeed, the rate of hydrolysis was found to be greater than the rate of aminolysis by $CF_3CH_2NH_2$ under standard reaction conditions. A higher concentration of the hydrochloride salt (40.92 mM) resulted in the alteration of the final reaction pH to 5.8. Using a higher buffer concentration was not ideal, as that would increase the ionic strength to a value much larger than that in other reaction mixtures. To overcome these problems, the reaction with $CF_3CH_2NH_2$ was carried out by using 40.92 mM $CF_3CH_2NH_2$·HCl and allowing the pH of the reaction mixture to decrease to 5.8. The observed $t_{1/2}$ was corrected by subtracting the $t_{1/2}$ for hydrolysis at pH 5.8. The second-order rate constant was calculated by accounting for the concentration of free amine at pH 5.8, and the resulting value was used in the Brønsted plot. The first-order rate constant thus obtained was not compared to those from other nucleophiles.

**Synthesis of $BocNHCH_2CH_2N_3$ (3)**: $BocNHCH_2CH_2Br$ (10.00 g, 44.62 mmol) was dissolved in DMF (200 mL). $NaN_3$ (14.48 g, 223.1 mmol) was added, and the mixture was stirred at 110 °C for 12 h. The solvent was removed under reduced pressure, and the residue was dissolved in water (200 mL). The resulting aqueous solution was extracted with ethyl acetate (2×200 mL). The organic layers were combined and dried over anhydrous $MgSO_4$(s). After filtration, the organic layer was concentrated under reduced pressure and the residue was dissolved in methylene chloride (10–20 mL) and purified by flash chromatography (silica gel, methylene chloride). $BocNHCH_2CH_2N_3$ (6.60 g, 80%) was isolated as a colorless oil. HRMS (ESI) $[M+Na]^+$ calcd for $C_7H_{14}N_4O_2Na$, 209.1014; found, 209.1010; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ = 4.88 (br s, 1H), 3.42 (t, $J$ = 5.3 Hz, 2H), 3.34–3.26 (m, 2H), 1.45 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ = 155.8, 79.8, 51.4, 40.2, 28.5.

**Synthesis of $(Boc)_2NN(Boc)CH_2CONHCH_2CH_2N_3$ (5)**: $HCl{\cdot}H_2NCH_2CH_2N_3$ (**4**) was synthesized by dissolving azide **3** (2.11 g, 11.33 mmol) in HCl (4 N) in dioxane (100 mL). The mixture was then stirred at room temperature for 1 h. The solvent was removed under reduced pressure to give a dirty-white powder. $(Boc)_2NN(Boc)CH_2CO_2H$ (4.42 g, 11.33 mmol) was then added, and the mixture was dissolved in methylene chloride (70 mL) and DMF (45 mL). The mixture was cooled to 0 °C, and PyBOP (5.9 g, 11.33 mmol) and $Et_3N$ (3.2 mL, 22.66 mmol) were added. The reaction mixture was allowed to warm to room temperature and stirred under Ar(g) for 21 h. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 50% (*v*/*v*) ethyl acetate in hexanes) to give $(Boc)_2NN(Boc)CH_2CONHCH_2CH_2N_3$ as a colorless viscous oil (4.9 g, 95%). HRMS (ESI) $[M+Na]^+$ calcd for $C_{19}H_{34}N_6O_7Na$, 481.2387; found,

481.2389; $^1$H NMR (400 MHz, $CDCl_3$, 2 rotamers) $\delta = 8.51$ and 8.27 (br s, 1H), 4.06 and 3.99 (s, 2H), 3.49–3.40 (m, 4H), 1.56–1.44 (m, 27H); $^{13}$C NMR (100 MHz, $CDCl_3$, 2 rotamers) $\delta = 169.5$, 169.2, 154.0, 153.5, 151.4, 151.2, 85.2, 85.1, 83.5, 82.8, 56.5, 54.8, 50.6, 38.9, 34.1, 29.9, 28.2, 28.1.

**Synthesis of $H_2NNHCH_2CONHCH_2CH_2N_3$ (1)**: Azide **5** (4.66 g, 10.17 mmol) was dissolved in HCl (4 N) in dioxane (200 mL), and the solution was stirred at room temperature for 5 h. Solvent was removed under reduced pressure, the residue was dissolved in water (15 mL), and purified by cation-exchange chromatography (Dowex 50WX8-200 ion-exchange resin, 1 M $NH_4OH$) to give $H_2NNHCH_2CONHCH_2CH_2N_3$ as a yellow oil (1.52 g, 95%). HRMS (ESI) $[M+Na]^+$ calcd for $C_4H_{10}N_6ONa$, 181.0814; found, 181.0805; $^1$H NMR (300 MHz, [D$_6$]DMSO) $\delta = 8.03$ (app br s, 1H), 3.80–3.40 (br s, 3H), 3.40–3.34 (m, 2H), 3.32–3.24 (m, 2H), 3.16 (s, 2H); $^{13}$C NMR (75 MHz, [D$_6$]DMSO) $\delta = 171.2$, 57.1, 50.0, 37.8.

**Synthesis of BocNHN=$CHCH_2CH_2CO_2H$ (7)**: 4-Pentenoic acid (10.00 g, 99.88 mmol) was dissolved in methylene chloride (150 mL), and the mixture was cooled to −78 °C under $N_2$(g). Ozone was bubbled through the reaction mixture, and the course of the reaction was monitored by TLC. When TLC showed the disappearance of the starting material (ca. 2.5 h), methyl sulfide (15.0 mL, 205.23 mmol) was added, and the reaction mixture was allowed to warm to room temperature. Subsequently, solvent was removed under reduced pressure, and the residue was dissolved in THF (200 mL). $BocNHNH_2$ (13.2 g, 99.88 mmol) was then added, and the reaction mixture was refluxed overnight under Ar(g). Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 6% (*v*/*v*) methanol in methylene chloride) to give BocNHN=$CHCH_2CH_2CO_2H$ as a white solid (13.60 g, 63%). HRMS (ESI) $[M-H]^-$ calcd for $C_9H_{15}N_2O_4$, 215.1032; found, 215.1035; $^1$H NMR (300 MHz, [D$_6$]DMSO) $\delta = 12.16$ (s, 1H), 10.45 (s, 1H), 7.30 (app br s, 1H), 2.45–2.30 (m, 4H), 1.41 (s, 9H); $^{13}$C NMR (100 MHz, [D$_6$]DMSO) $\delta = 173.5$, 152.4, 145.6, 78.9, 30.4, 28.1, 27.2.

**Synthesis of BocNHN=$CHCH_2CH_2CONHCH_2CH_2N_3$ (8)**: $HCl{\cdot}H_2NCH_2CH_2N_3$ (**4**) was synthesized as described earlier. Compound **7** (4 g, 18.5 mmol) and $HCl{\cdot}H_2NCH_2CH_2N_3$ (2.27 g, 18.5 mmol) were dissolved in methylene chloride (180 mL) and DMF (70 mL). The mixture was cooled to 0 °C, and PyBOP (9.63 g, 18.5 mmol) and $Et_3N$ (20 mL, 142.8 mmol) were added. The reaction mixture was allowed to warm to room temperature and stirred for 21 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, ethyl acetate). BocNHN=$CHCH_2CH_2CONHCH_2CH_2N_3$ was obtained as a white solid (3.94 g, 75%). HRMS (ESI) $[M+Na]^+$ calcd for $C_{11}H_{20}N_6O_3Na$, 307.1495; found, 307.1494; $^1$H NMR (300 MHz, [D$_6$]DMSO) $\delta = 10.43$ (br s, 1H), 8.13 (t, $J = 5.3$ Hz, 1H), 7.28 (app br s, 1H), 3.33 (t, $J = 5.7$ Hz, 2H), 3.28–3.19 (m, 2H), 2.4–2.3 (m, 2H), 2.29–2.22 (m, 2H), 1.41 (s, 9H); $^{13}$C NMR (100 MHz, [D$_6$]DMSO) $\delta =$ 171.6, 152.5, 146.1, 79.0, 50.0, 38.3, 32.1, 28.2, 27.8.

**Synthesis of $BocNHNHCH_2CH_2CH_2CONHCH_2CH_2N_3$ (9)**: Compound **8** (1.12 g, 3.9 mmol) was dissolved in acetonitrile (27 mL) and acetic acid (3.7 mL). $NaCNBH_3$ (1.11 g, 16.75 mmol) was added to the resulting solution. The reaction mixture was stirred at room temperature for 3 h. Solvent was removed under low pressure, and the residue was dissolved in water (45 mL). The pH of the solution was increased to 13.0 by adding NaOH (10 N), and the aqueous solution was extracted with ether (3×45 mL) and then methylene chloride (50 mL). The organic layers were combined and dried over anhydrous $MgSO_4$(s). After filtration, the organic layer was concentrated under reduced pressure, and the residue was purified by flash chromatography (silica gel, 2% (*v*/*v*) methanol in methylene chloride) to give $BocNHNH(CH_2)_3CONHCH_2CH_2N_3$ as a colorless oil (0.78 g, 70%). HRMS (ESI) $[M+Na]^+$ calcd for $C_{11}H_{22}N_6O_3Na$, 309.1651; found, 309.1641; $^1$H NMR (300 MHz, [D$_6$]DMSO, 2 rotamers) $\delta = 8.15$ (br s, 1H), 8.09–7.90 (m, 1H), 4.34 (br s, 1H), 3.32 (t, $J =$ 5.7 Hz, 2H), 3.26–3.18 (m, 2H), 2.66–2.53 (m, 2H), 2.22–2.07 (m, 2H), 1.64–1.48 (m, 2H), 1.46–1.22 (m, 9H); $^{13}$C NMR (100 MHz, [D$_6$]DMSO, 2 rotamers) $\delta = 172.5$, 156.4, 78.2, 56.1, 54.9, 50.4, 50.0, 38.2, 33.0, 28.2, 23.5, 22.9.

**Synthesis of $CF_3CO_2H{\cdot}H_2NNH(CH_2)_3CONHCH_2CH_2N_3$ (2)**: Compound **9** (197.8 mg, 0.69 mmol) was dissolved in methylene chloride (6.4 mL), and trifluoroacetic acid (6.4 mL) was added to the resulting solution. The reaction mixture was stirred at room temperature for 15 min. Solvent was removed under reduced pressure to afford $CF_3CO_2H{\cdot}H_2NNH(CH_2)_3CONHCH_2CH_2N_3$ as a yellow oil (130 mg, 100%). HRMS (ESI) $[M+H]^+$ calcd for $C_6H_{15}N_6O$, 187.1307; found, 187.1299; $^1$H NMR (400 MHz, [D$_6$]DMSO) $\delta = 8.19$ (t, $J = 4.8$, 1H), 7.9–5.5 (br s, 3H), 3.34 (t, $J = 5.8$, 2H), 3.28–3.20 (m, 2H), 2.89 (t, $J = 7.5$ Hz, 2H), 2.21–2.11 (m, 2H), 1.85–1.68 (m, 2H); $^{13}$C NMR (100 MHz, [D$_6$]DMSO) $\delta = 171.9$, 50.1, 50.0, 38.2, 32.2, 21.0.

**Synthesis of alkynyl fluorescein (10)**: Fluorescein–NHS ester (100 mg, 0.21 mmol) was dissolved in THF (10 mL) and propargylamine (23.27 mg, 0.42 mmol) was added. The reaction mixture was stirred at room temperature for 4 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 35% (*v*/*v*) hexanes in ethyl acetate containing 1% (*v*/*v*) AcOH) to give the desired product as a red solid (72.80 mg, 84%). HRMS (ESI) $[M+Na]^+$ calcd for $C_{24}H_{15}NO_6Na$, 436.0797; found, 436.0777; $^1$H NMR (400 MHz, [D$_6$]DMSO) $\delta = 10.20$ (br s, 2H), 9.31 (t, $J = 5.1$ Hz, 1H), 8.47 (s, 1H), 8.25 (dd, $J = 7.9$, 1.4 Hz, 1H), 7.39 (d, 8.3 Hz, 1H), 6.68 (d, $J = 2.3$ Hz, 2H), 6.59 (d, $J =$ 8.6 Hz, 2H), 6.54 (dd, $J = 8.7$, 2.3 Hz, 2H), 4.11 (dd, $J = 5.3$, 2.2 Hz, 2H), 3.17 (t, $J = 2.5$ Hz, 1H); $^{13}$C NMR (100 MHz, [D$_6$]DMSO) $\delta =$ 168.1, 164.4, 159.7, 154.8, 151.9, 135.5, 134.7, 129.2, 126.7, 124.4, 123.5, 112.8, 109.1, 102.3, 83.8, 81.0, 73.1, 28.8.

**Synthesis of polytriazole ligand**: A polytriazole ligand for the $Cu^I$ catalyst was synthesized essentially as described.[48] Tripropargyl amine (0.56 g, 4.28 mmol) was dissolved in acetonitrile (5.7 mL), and benzyl azide (2 g, 15.02 mmol) and 2,6-lutidine (0.46 g, 4.28 mmol) were added to the resulting solution. The reaction mixture was cooled to 0 °C, and $Cu(CH_3CN)_4PF_6$ (81.25 mg, 0.22 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred under Ar(g) for 2.5 days. The reaction mixture was filtered, and the white precipitate obtained was dried under high vacuum to yield the polytriazole ligand (0.34 g, 15%). HRMS (ESI) $[M+Na]^+$ calcd for $C_{30}H_{30}N_{10}Na$, 553.2553; found, 553.2570; $^1$H NMR (400 MHz, $CDCl_3$) $\delta = 7.66$ (s, 3H), 7.40–7.20 (m, 15H), 5.50 (s, 6H), 3.70 (s, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta = 134.8$, 129.2, 128.8, 128.1, 123.9, 54.2, 47.2.

**Production of RNase A–intein–chitin-binding domain fusion protein.** The plasmid that directs the expression of an RNase A–*mxe* intein–chitin-binding domain fusion protein was a generous gift from U. Arnold. A glycine codon was introduced between RNase A and the *mxe* intein genes by using the QuickChange site-directed mutagenesis kit from Stratagene (LaJolla, CA, USA). The resulting plasmid, pJK01, was transformed into *E. coli* BL21(DE3) cells, and the production of Met(−1)RNase A–Gly–intein-chitin binding domain fusion protein was induced as described previously.[41]

**Production of azido–RNase A**: Cells were resuspended in 3-(*N*-morpholino)propane sulfonic acid (MOPS; 20 mM)–NaOH buffer at

pH 6.8 containing NaCl (0.50 M), ethylenediaminetetraacetic acid (EDTA; 0.10 mM), and Triton X-100 (0.1%, *v/v*). Cells were lyzed with a French pressure cell, and the cell lysate was subjected to centrifugation at 15,000 *g* for 30 min. The supernatant was diluted to a final volume of 25 mL (per g of cells) and applied (flow rate: 0.75 mL $min^{-1}$) to a chitin column that had been equilibrated with the same buffer. The loaded resin was washed with two column volumes of buffer and then with four column volumes of MOPS–NaOH (0.5 M) buffer at pH 7.0 containing NaCl (0.50 M) and EDTA (0.10 mM). Azide **1** was dissolved in the latter buffer to a final concentration of 450 mM, and three column volumes of this solution were loaded onto the resin, out of which, two column volumes were allowed to flow through and one column volume was allowed to sit on top of the resin. This incubation was carried out for three days at room temperature in order to enable the reaction to proceed to completion. The hydrazide product was eluted with three column volumes of an aqueous solution of NaCl (2 M). Azido-RNase A was precipitated out of the eluate by adding an aqueous solution of sodium deoxycholate (NaDOC; to 0.72 mM) and trichloroacetic acid (TCA; to 260 mM). This precipitate was washed with acetone and dissolved in an aqueous solution of guanidine-HCl (4 M).

The solution of protein was added, with gentle stirring, in 20 μL aliquots into a refolding solution (50 mL) consisting of Tris–HCl (100 mM) buffer at pH 8.0 containing NaCl (100 mM), reduced glutathione (1 mM), and oxidized glutathione (0.2 mM). The final concentration of guanidine-HCl was 0.05 M. The refolding solution was incubated at room temperature for 24 h.

The refolding solution was dialyzed for 12 h against sodium acetate (50 mM) buffer at pH 5.0. The azido protein was purified by cation-exchange chromatography as described previously.[51]

**Ribonucleolytic activity**: Values of $k_{cat}/K_M$ for the enzymatic cleavage of a fluorogenic substrate, 6-carboxyfluorescein-dArU$(dA)_2$-6-carboxytetramethylrhodamine, were determined as described previously.[52]

**Huisgen 1,3-dipolar cycloaddition**: Alkynyl fluorescein **10** (1.1 μL, 2.23 mM suspension in 20% (*v/v*) ethanol in water), tris(2-carboxyethyl)phosphine hydrochloride (1.0 μL, 50 mM), $CuSO_4 \cdot 5H_2O$ (1.0 μL, 50 mM), and polytriazole ligand (5.0 μL, 20 mM suspension in 80% (*v/v*) *tert*-butanol in water) were added to a solution of azido-RNase A (9.6 μM) in potassium phosphate buffer (0.10 M) at pH 8.0 (41.9 μL). The reaction mixture was agitated gently, and incubated at room temperature for 1 h and then at 4 °C for 16 h. The same procedure was followed for the control reaction with unmodified wild-type RNase A (Sigma Chemical). Protein precipitation was observed in both reaction mixtures, as is common (but not well appreciated) during $Cu^I$-catalyzed Huisgen 1,3-dipolar azide–alkyne cycloaddition to a protein,[47] obviating the calculation of a yield for this reaction. The reaction mixtures were subjected to centrifugation at 5900 *g* for 4 min, and the supernatant was discarded. The pellet was resuspended in 2× denaturing buffer (20 μL) and subjected to SDS–PAGE. The resulting gel was visualized with a fluorescence imager and was then stained with Coomassie blue.

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