Semisynthesis of Human Ribonuclease–S

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1. General Materials and Methods

Materials. The BL21(DE3) strain of *Escherichia coli* was from Novagen (Madison, WI). Ribonuclease A (RNase A) was product number R6513 from Sigma–Aldrich (St. Louis, MO). A fluorogenic ribonuclease substrate (6-FAM–dArU$(dA)_2$–6-TAMRA) and DNA oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Phosphate-buffered saline (PBS) contained Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), NaCl (137 mM), and KCl (2.7 mM) at pH 7.4. Immobilized TCEP was product 77712 from Thermo Fischer Scientific (Waltham, MA). A Spectra Multicolor Broad Range Protein Ladder from Thermo Fisher Scientific was used as a molecular mass standard during SDS–PAGE. Gels were stained with a solution of Bio-Safe™ Coomassie Brilliant Blue G-250 from Bio-Rad (Hercules, CA). SYPRO Orange was from Sigma–Aldrich. All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Instrumentation. Aqueous solutions were made with water that was generated with a Milli-Q® IQ 7000 water purification system from Millipore Sigma and had resistivity ≥18 $M\Omega \cdot cm^{-1}$ at 25 °C. SDS–PAGE analyses were performed with Any kD™ Mini-PROTEAN® TGX™ Precast Gels in a Mini-PROTEAN Tetra cell. Gels were imaged with an Amersham Imager 600 from GE Life Sciences and images were processed with ImageJ software. FPLC chromatography was performed with an ÄKTA Pure system from GE Healthcare Life Sciences (Piscataway, NJ), and the results were analyzed with the UNICORN Control System. HiTrap SPHP and HiLoad® 26/600 Superdex® 75 pg columns for protein purification were from GE Healthcare Life Sciences. Protein concentrations were determined by using either a bicinchonic acid (BCA) assay kit from Thermo Fisher Scientific and an infinite M1000 microplate reader from Tecan (Zürich, Switzerland) or by absorbance at 280 nm with a DS-11 UV–Vis spectrophotometer from DeNovix (Wilmington, DE). Mass spectrometry of synthetic peptides was performed with a nominal mass 6125B mass spectrometer attached to a 1260 Infinity LC from Agilent Technologies (Santa Clara, CA). The intact molecular masses of RNase–S conjugates were determined by ESI mass spectrometry with a 6530 Accurate-Mass Q-TOF LC/MS equipped with a PLRP-S column (1000-Å pore size, 5-μM particle size, 50-mm length, and 2.1-mm ID) from Agilent Technologies. Synthetic peptides were purified with a 1260 Infinity Preparative LC System from Agilent Technologies equipped with a Nucleosil C18 column (100 Å, 5 μm, 250 × 21 mm) from Macherey–Nagel (Düren, Germany).

Conditions. All procedures were performed in air at ambient temperature (~22 °C) and pressure (1.0 atm) unless indicated otherwise.

1.1. Solid-Phase Peptide Synthesis (SPPS)

1.1.1. Preloading Rink Amide ProTide Resin

Rink Amide ProTide resin was initially washed with CH_2Cl_2 (5×) and DMF (5×), followed by removal of the Fmoc protecting group by treatment with 20% v/v 4-methyl piperidine in DMF (2 × 5 min). The resin was washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×). To a solution of the first residue to be coupled (Fmoc-AA-OH, 4 equiv) in DMF (final concentration 0.1 M) was added PyBOP (4 equiv) and DIPEA (8 equiv). After 2 min of preactivation, the mixture was added to the resin and incubated with agitation for 2 h. The resin was washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×). Any unreacted amino groups remaining on the resin were capped by treatment with a solution of 10% v/v acetic anhydride in pyridine (2 × 3 min) and then washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×). The resin was subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

1.1.2. Preloading Cl-TCP(Cl) ProTide Resin

Cl-TCP(Cl) ProTide resin was swollen in CH_2Cl_2 for 30 min then washed with CH_2Cl_2 (5×) and DMF (5×). A solution of Fmoc-AA-OH (0.2 M) in DMF was added to the resin. To a 0.125M KI solution in DMF was added DIPEA (1.0 M final concentration), and the resulting solution was added to the resin. The resin was agitated overnight. The resin was washed with DMF (5×), CH_2Cl_2 (5×) and treated with a solution of 17:2:1 CH_2Cl_2/MeOH/*i*Pr_2NEt for 30 min to cap any unreacted 2-chlorotrityl chloride resin handles. Finally, the resin was washed with CH_2Cl_2 (5×) and DMF (5×) and subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

1.1.3. Loading Estimation of First Amino Acid

The resin was treated with 20% v/v 4-methyl piperidine in DMF (2 × 3 min). The combined filtrate was diluted to 10 mL with DMF. A 10-μL aliquot of the solution was further diluted 200-fold with DMF, and the UV absorbance of the piperidine–fulvene adduct was measured (λ = 301 nm, ε = 7800 $M^{-1}cm^{-1}$). The amount of amino acid loaded onto the resin could then be estimated.

1.1.4. General Iterative Peptide Assembly (Fmoc-SPPS)

Deprotection. The resin was treated with 20% v/v 4-methyl piperidine in DMF (2 × 3 min) and washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×).

General Amino Acid Coupling. A preactivated solution of Fmoc-AA-OH (4 equiv), HATU (4 equiv) and DIPEA (8 equiv) in DMF (final concentration: 0.10 M) was added to the resin. After 2 h of agitation, the resin was washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×).

Capping. A solution of 10% v/v acetic anhydride in pyridine was added to the resin (2 × 3 min). The resin was washed with DMF (5×), CH_2Cl_2 (5×), and DMF (5×).

Global Deprotection and Resin Cleavage. A solution of 90:5:2.5:2.5 TFA/triisopropylsilane (*i*Pr_3SiH)/thioanisole/H_2O was added to the resin. After 2 h of agitation, the resin was washed with TFA (3 × 2 mL).

Work-up. The cleavage filtrate was concentrated to dryness under a stream of N_2(g). The residue was suspended in diethyl ether and cooled to 0 °C. The precipitate was pelleted by centrifugation at 4000 rpm for 5 min, the supernatant decanted and the pellet dried under a gentle stream of N_2(g). The resulting white powder was stored at −20 °C until it could be purified *via* reversed-phase preparative HPLC.

1.1.5. Automated SPPS

Microwave-assisted peptide synthesis was performed at 90 °C on a Liberty Blue® automated microwave peptide synthesiser from CEM (Matthews, NC). The general coupling method was employed as follows: 2-min coupling (90 °C), 1-min deprotection (90 °C), and 1-min associated washes and liquid handling. A 4-fold excess of Fmoc-AA-OH, HOBt, and DIC was used, as is standard.

1.2. Conjugation Protocol

1.2.1. Disulfide Conjugation

Lyophilized A5C S-peptide (10 equiv) was dissolved in PBS, pH 8.0, containing EDTA (40 mM), to a final concentration of 1 mg/mL. An equivalent volume of immobilized TCEP was drained using a fritted syringe, and then the A5C S-peptide solution was taken up and allowed to reduce for 30 min at 37 °C. Then, the reduced A5C S-peptide solution was ejected directly into a solution of NTB-protected V118C S-protein (0.1 mg/mL in sodium acetate buffer, pH 5.5). The conjugation

reaction was allowed to proceed for 1 h with gentle agitation at 37 °C. The solution gradually turns yellow due to removal of the NTB protecting group on Cys118. Complete conversion to the disulfide-linked RNase–S conjugate was confirmed by MALDI–TOF mass spectrometry. The solution was concentrated to ≤500 µL using a Vivaspin® 20 centrifugal concentrator (5000 MWCO PES) and then purified by passage through a HiTrap® SP cation-exchange column from GE Healthcare Life Sciences with 50 mM sodium acetate buffer, pH 5.0, and a gradient of 0.0–0.8 M NaCl over 80 min. The conjugated product elutes at approximately 0.5 M NaCl.

2. Semisynthetic Enzyme Production

2.1. RNase 1 Variant

2.1.1. Plasmid Preparation

Synthetic cDNA encoding the H12A/S20_S21insDDDDK/V118C RNase 1 variant flanked by regions of homology near the T7 promoter and terminator of the pET22b vector were obtained from Integrated DNA Technologies. Linear pET22b was prepared by PCR using primers that complement the DNA encoding RNase 1:

```
5′-ACTGAGCTCGTGGTGGTGG-3′
3′-CCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG-5′
```

Each gene and plasmid fragment were combined with Gibson assembly for expression in *E. coli*. The corresponding amino acid sequence for the RNase 1 variant is as follows (changes in **red**). Note that the variant is missing the inconsequential C-terminal threonine residue (Thr128), which was absent from both the initial report of the amino acid sequence[1] and the crystal structure.[2]

```
         10          20          30          40          50          60          70
KESRAKKFQR QAMDSDSSPS DDDDKSSSTY CNQMMRRRNM TQGRCKPVNT FVHEPLVDVQ NVCFQEKVTC
         80          90         100         110         120         130
KNGQGNCYKS NSSMHITDCR LTNGSRYPNC AYRTSPKERH IIVACEGSPY VPCHFDASVE DS_
```

2.1.2. Expression and Purification

The RNase 1 variant was produced and purified essentially as described previously.[3] Briefly, transformed BL21(DE3) cells were grown at 37 °C with shaking in Terrific Broth containing ampicillin (400 µg/mL) until OD = 1.8–2.2 at 600 nm. Gene expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3–4 h, cells were harvested by centrifugation and lysed at 19.0 kpsi with a benchtop cell disruptor from Constant Systems (Kennesaw, GA). After centrifugation at 10,500 rpm for 45 min, the resulting inclusion bodies were dissolved in 20 mM Tris–HCl buffer, pH 8.0, containing guanidine–HCl (7 M), EDTA (10 mM), and DTT (100 mM). This solution was diluted 10-fold by the slow addition of degassed 20 mM acetic acid, then subjected to centrifugation at 10,500 rpm for 45 min. The resulting supernatant was then dialyzed using 3.5-kDa MWCO tubing from Spectrum Labs (Rancho Dominguez, CA) against 16 L of 20 mM acetic acid overnight. After centrifugation at 10,500 rpm for 40 min, the retentate was added dropwise to re-folding solution, which was 100 mM Tris–HCl buffer, pH 7.8, containing NaCl (100 mM), reduced glutathione (1.0 mM), and oxidized glutathione (0.2 mM), then allowed to re-fold at 4 °C for 5 days. The pH of the solution was adjusted to 5.0, and the resulting solution was concentrated to 10 mL with an Amicon® Stirred Cell concentrator from EMD Millipore (Billerica, MA) with Hydrostart® 10-kDa filters from Sartorius.

The resulting protein solution was purified by chromatography on a HiLoad® 26/600 Superdex® 75 pg gel-filtration column from GE Healthcare Life Sciences with 50 mM sodium acetate buffer, pH 5.0, containing NaCl (100 mM) and sodium azide (0.05% w/v).

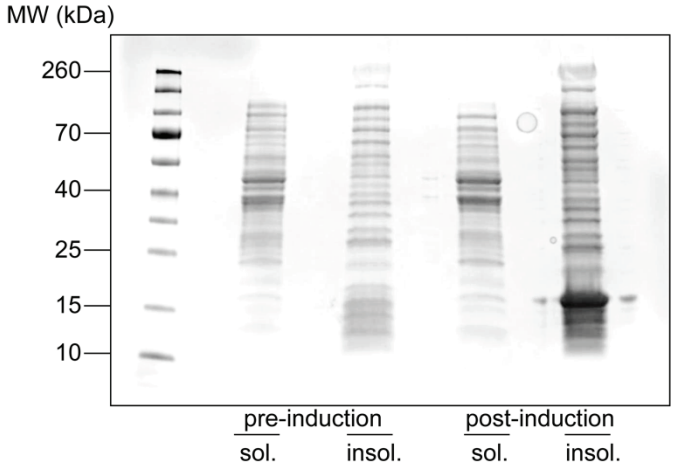


Figure S1. I SDS–PAGE analysis of the production of the RNase 1 variant, stained with Coomassie. Expression was induced by the addition of IPTG.

2.1.3. Protection of Ribonuclease Variant with NTB

After gel-filtration chromatography, the fractions containing ribonuclease were combined, and the pH of the resulting solution was increased by adding 2.0 M Tris–HCl buffer, pH 8.3, containing EDTA (10 mM). To this solution was added 50 mM Tris–HCl buffer, pH 8.0, containing DTNB (5 mM) and EDTA (50 mM) such that the DTNB was in a 4-fold molar excess to the protein. After incubation for 10 min at 4 °C, the pH of the solution was adjusted to 5.0 by the addition of 3.0 M sodium acetate, and the resulting solution was incubated at 4 °C overnight. The protected protein was purified further by passage through a HiTrap® SP cation-exchange column from GE Healthcare Life Sciences with 50 mM sodium acetate buffer, pH 5.0, and a gradient of 0.0–1.0 M NaCl over 10 min.

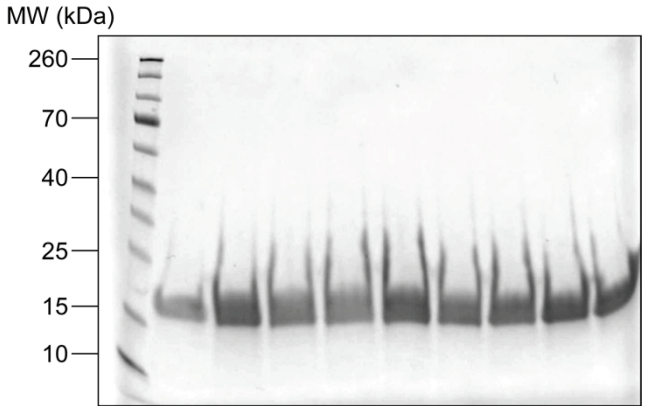


Figure S2. SDS–PAGE analysis of fractions collected after cation-exchange chromatography of the RNase 1 variant, stained with Coomassie.

2.2. V118C(NTB) S-Protein

2.2.1. Enterokinase Digestion

$CaCl_2$ (2 mM final concentration) was added to the RNase 1 variant (10 mL of a 1 mg/mL solution) in 20 mM Tris–HCl buffer, pH 7.4, containing NaCl (50 mM). Enterokinase (light chain) was product number P8070S from New England Biolabs (Ipswich, MA). Enterokinase (1 U per 0.1 mg

of protein) was added, and the reaction mixture was incubated with gentle agitation. Aliquots were removed over 24 h and subjected to SDS–PAGE analysis.

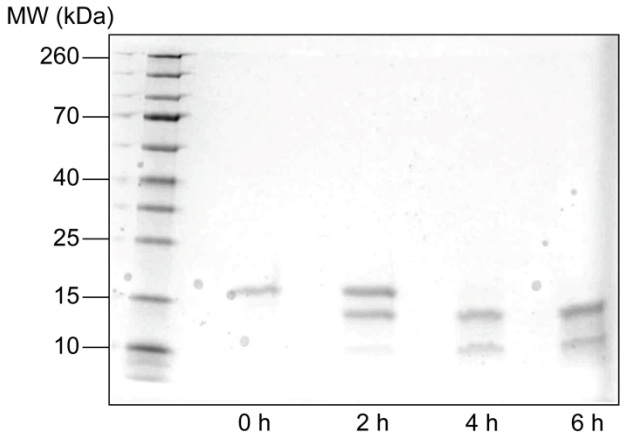


Figure S3. SDS–PAGE analysis of the enterokinase digestion of the RNase 1 variant, stained with Coomassie.

2.2.2. Purification of V118C(NTB) S-Protein

The V118C(NTB) S-protein fragment was isolated by semi-preparative reversed-phase HPLC with a C-4 column and two-step linear gradient (Step 1: 20–50% v/v B over 3 min. Step 2: 50–100% v/v B over 25 min. A: 50 mM sodium phosphate buffer, pH 2.7. B: 40% v/v A + 60% v/v acetonitrile). Fractions containing V118C(NTB) S-protein were pooled and concentrated under a stream of N_2(g) to remove acetonitrile. The concentration of V118C(NTB) S-protein was determined by absorbance at 280 nm using ε = 7950 $M^{-1}cm^{-1}$. The identity of each peak on the HPLC trace was confirmed by Q-TOF mass spectrometry.

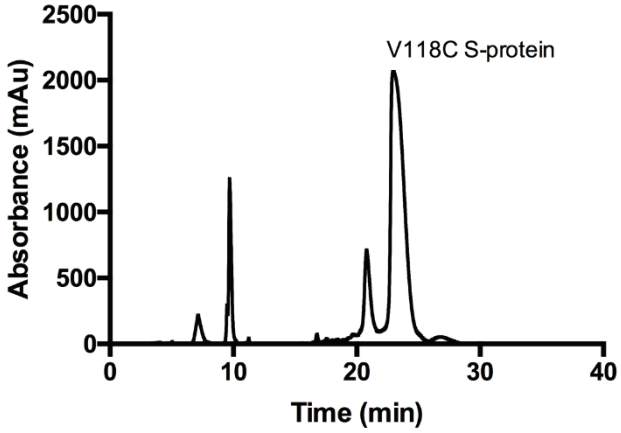


Figure S4. Preparative HPLC trace of crude digestion reaction (20–50% v/v B over 3 min, then 50–100% v/v B over 25 min). V118C(NTB) S-protein RT, 22.97 min.

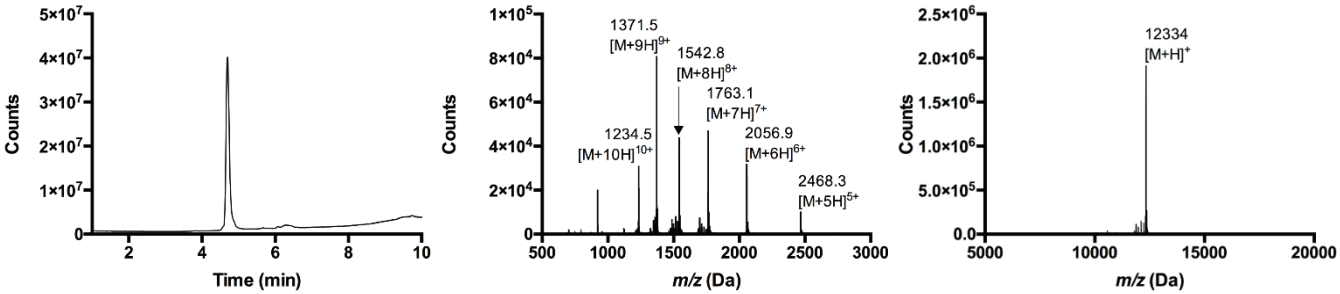


Figure S5. Q-TOF LC/MS analysis of purified V118C(NTB) S-protein; RT, 4.70 min (5–95% v/v B over 10 min). Left, TIC trace. Middle, *m*/*z* expected $[M + 5H]^{5+}$, 2468.0; $[M + 6H]^{6+}$, 2056.8; $[M + 7H]^{7+}$, 1763.1; $[M + 8H]^{8+}$, 1542.9; $[M + 9H]^{9+}$, 1371.5; $[M + 10H]^{10+}$, 1234.5. *m*/*z* found (ESI^+) $[M + 5H]^{5+}$, 2468.3; $[M + 6H]^{6+}$, 2056.9; $[M + 7H]^{7+}$, 1763.1; $[M + 8H]^{8+}$, 1542.8; $[M + 9H]^{9+}$, 1371.5; $[M + 10H]^{10+}$, 1234.5. Right, deconvoluted mass spectrum. *m*/*z* expected $[M + H]^+$, 12334.9. *m*/*z* found (ESI^+) $[M + H]^+$, 12334.0.

2.3. Disulfide Crosslink Design

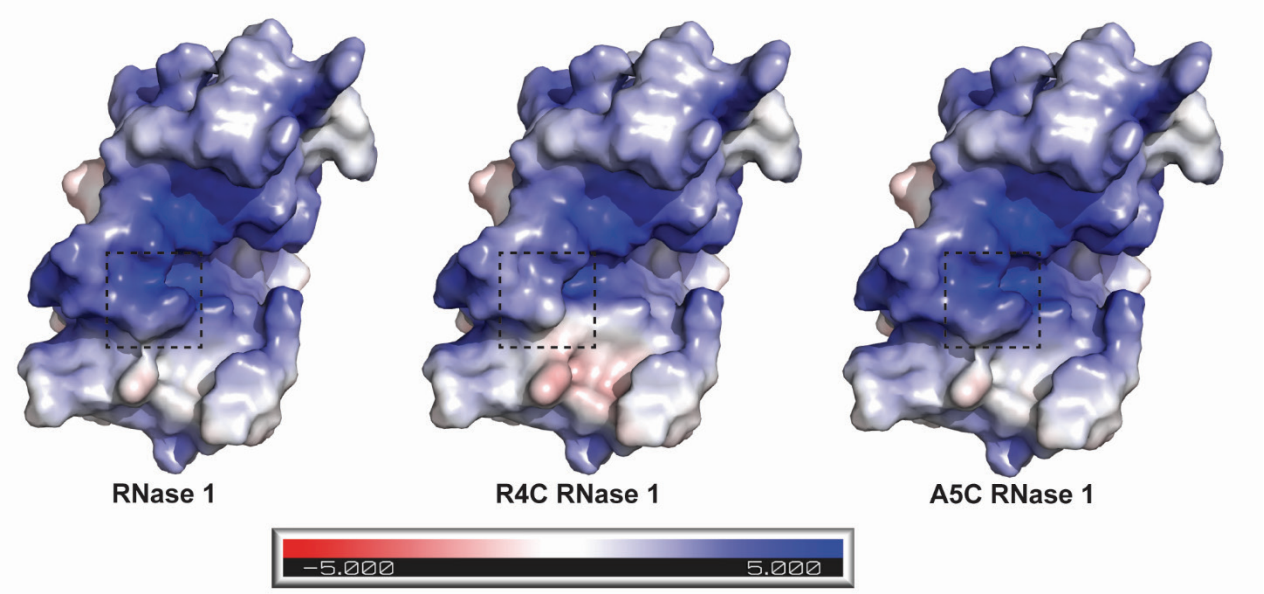


Figure S6. Electrostatic potential molecular surfaces of RNase 1 (PDB entry 1z7x) calculated with the Adaptive Poisson–Boltzmann Solver of PyMOL software from Schrödinger (New York, NY). Left, Unmodified RNase 1 sequence. Middle, R4C RNase 1 variant. Right, A5C RNase 1 variant. The positive electrostatic potential in the region highlighted by the dashed box is reduced substantially by the R4C substitution but less so by the A5C substitution.

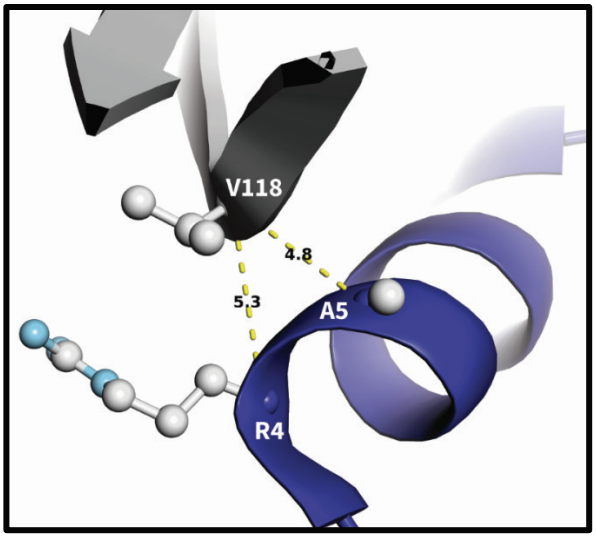


Figure S7. Image of C^{α}–C^{α} distances from Val118 to Arg4 (5.3 Å) and Ala5 (4.8 Å) as measured in PDB entry 1z7x with PyMOL software.

2.4. Chemical Synthesis

2.4.1. A5C S-Peptide

A5C S-Peptide was prepared on Rink Amide ProTide resin (116 mg, 0.43 mmol/g, 50 μmol) *via* automated Fmoc-SPPS as described in Section 1.1.5. Following complete elongation of the sequence, the peptide was subjected to global deprotection/resin cleavage and precipitated from cold Et_2O (Section 1.1.4) to produce a white powder. The crude material was purified *via* reversed-phase preparative HPLC (10–40% v/v B over 20 min, 0.1% v/v TFA) to afford A5C S-peptide as a white solid after lyophilization (19 mg, 7.98 μmol, 12% yield).

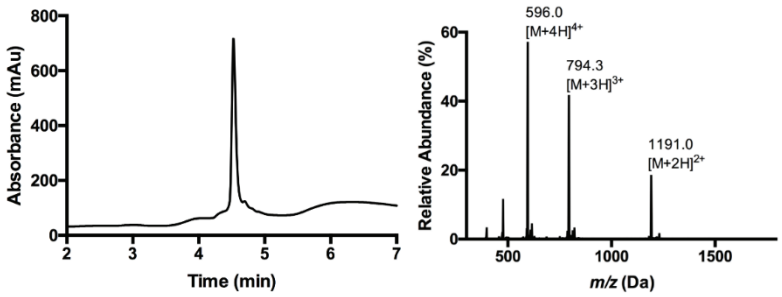


Figure S8. LC/MS analysis of purified A5C S-peptide; RT, 4.53 min (0–10% v/v B over 7 min, λ = 210 nm); *m*/*z* expected $[M + 2H]^{2+}$: 1191.3, $[M + 3H]^{3+}$: 794.5, $[M + 4H]^{4+}$: 596.2. *m*/*z* found (ESI^{+}) $[M + 2H]^{2+}$: 1191.0, $[M + 3H]^{3+}$: 794.3, $[M + 4H]^{4+}$: 596.0.

2.4.2. Alkyne–A5C S-Peptide

Alkyne-tagged A5C S-peptide was prepared on Rink Amide ProTide resin (77 mg, 0.43 mmol/g, 33 μmol). The sequence was elongated to Lys1 *via* automated Fmoc-SPPS as described in Section 1.1.5. Fmoc-propargyl-Gly-OH (22 mg, 66 μmol, 2 equiv) was coupled to the N terminus according to the general amino acid coupling procedure described in Section 1.1.4. Following deprotection of the N-terminal Fmoc protecting group, the crude peptide was deprotected, cleaved from the resin and precipitated from cold Et_2O (section 1.1.4). Finally, the crude material was purified *via* reversed-phase preparative HPLC (10–40% v/v B over 20 min, 0.1% v/v TFA) to afford alkyne–A5C S-peptide as a white solid after lyophilization (8 mg, 3.22 μmol, 10% yield).

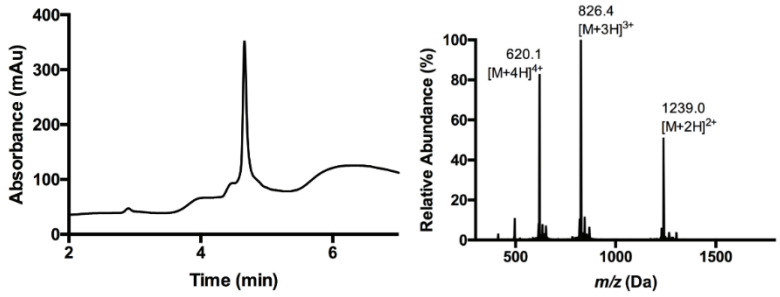
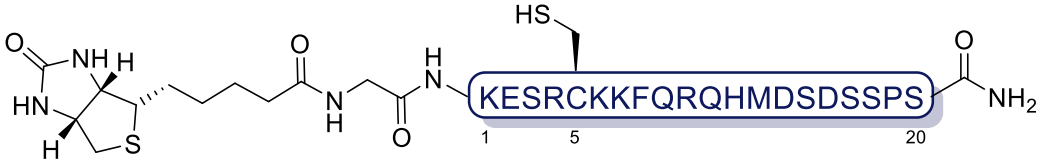


Figure S9. LC/MS analysis of purified Alkyne–A5C S-peptide; RT, 4.66 min (0–10% v/v B over 7 min, λ = 210 nm); *m*/*z* expected (Met13 oxidized) $[M + 2H]^{2+}$, 1239.6; $[M + 3H]^{3+}$, 826.7; $[M + 4H]^{4+}$, 620.3; *m*/*z* found (ESI^+) $[M + 2H]^{2+}$, 1239.0; $[M + 3H]^{3+}$, 826.4; $[M + 4H]^{4+}$, 620.1.

2.4.3. Biotin–A5C S-Peptide



Biotin-tagged A5C S-peptide was prepared on Rink Amide ProTide resin (77 mg, 0.43 mmol/g, 33 μmol). The sequence was elongated *via* automated Fmoc-SPPS as described in Section 1.1.5, with an additional Gly residue added to the N terminus. Following Fmoc-deprotection, (+)-biotin *N*-hydroxysuccinimide ester (23 mg, 66 μmol, 2 equiv) was coupled as a solution in DMF with DIPEA (12 μL, 66 μmol, 2 equiv) for 2 h. The crude peptide was deprotected, cleaved from the resin, and precipitated from cold Et_2O (Section 1.1.4) before purification *via* reversed-phase preparative HPLC (10–40% v/v B over 20 min, 0.1% v/v TFA) to afford biotin–A5C S-peptide as a white solid after lyophilization (11 mg, 4.13 μmol, 12% yield).

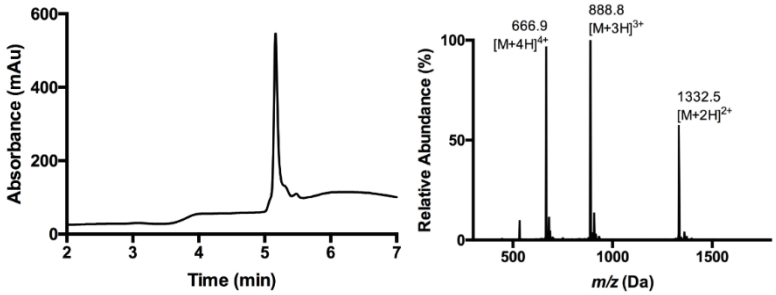
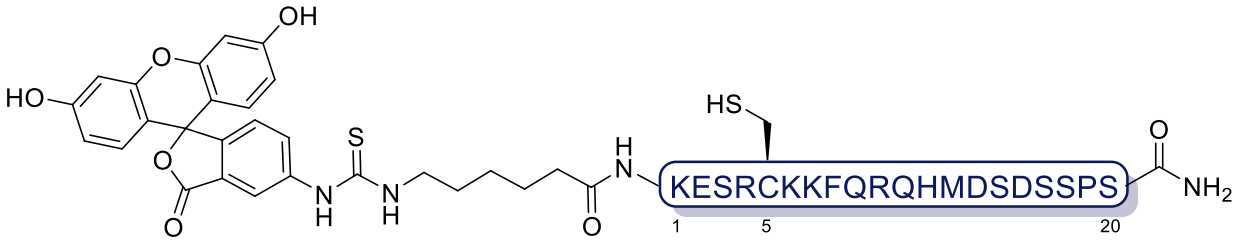


Figure S10. LC/MS analysis of purified biotin–A5C S-peptide; RT, 5.16 min (0–10% v/v B over 7 min, λ = 210 nm); *m*/*z* expected $[M + 2H]^{2+}$: 1333.0, $[M + 3H]^{3+}$: 889.0, $[M + 4H]^{4+}$: 667.0.

2.4.4. Fluorescein–A5C S-Peptide



Fluorescein-tagged A5C S-peptide was prepared on Rink Amide ProTide resin (116 mg, 0.43 mmol/g, 50 μmol). The sequence was elongated *via* automated Fmoc-SPPS as described in Section 1.1.5, with an additional 6-aminohexanoic acid residue added to the N terminus. Inclusion of this non-α amino acid spacer was necessary to avoid acid-catalyzed thiohydantoin formation via the Edman degradation pathway.[4] Following Fmoc-deprotection, fluorescein isothiocyanate (98 mg, 250 μmol, 5 equiv) was coupled as a solution in DMF with DIPEA (45 μL, 250 μmol, 5 equiv) for 2 h. The crude peptide was deprotected, cleaved from the resin, and precipitated from cold Et_2O (Section 1.1.4) before purification *via* reversed-phase preparative HPLC (10–40% v/v B over 20 min, 0.1% v/v TFA) to afford fluorescein–A5C S-peptide as a white solid after lyophilization (13 mg, 4.50 μmol, 9% yield).

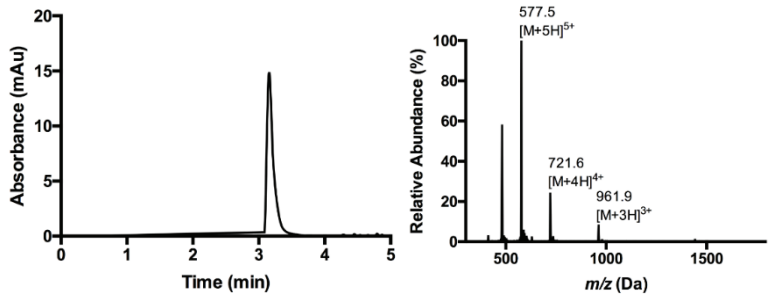


Figure S11. LC/MS analysis of purified fluorescein–A5C S-peptide; RT, 3.16 min (5–95% v/v B over 7 min, λ = 280 nm); *m*/*z* expected $[M + 3H]^{3+}$, 962.1; $[M + 4H]^{4+}$, 721.8; $[M + 5H]^{5+}$, 577.6. *m*/*z* found (ESI^+) $[M + 3H]^{3+}$, 961.9; $[M + 4H]^{4+}$, 721.6; $[M + 5H]^{5+}$, 577.5.

2.4.5. A5Dha S-Peptide

To a solution of A5C S-peptide (20 mg, 8.40 μmol) in 50 mM sodium phosphate buffer, pH 8.0, containing EDTA (20 mM), was added 2,5-dibromohexanediamide (20 mg, 66.2 μmol, 7.9 equiv).[5] The reaction mixture was agitated overnight at 37 °C. Purification was performed *via* reversed-phase preparative HPLC (10–30% v/v B over 40 min, 0.1% v/v TFA) to afford A5Dha S-peptide as a white solid after lyophilization (7.05 mg, 3.00 μmol, 35% yield).

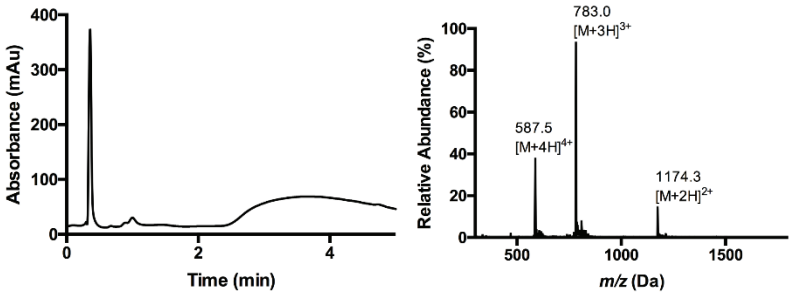


Figure S12. LC/MS analysis of purified A5Dha S-peptide; RT, 0.36 min (5–95% v/v B over 7 min, λ = 210 nm); *m*/*z* expected $[M + 2H]^{2+}$, 1174.3; $[M + 3H]^{3+}$, 783.2; $[M + 4H]^{4+}$, 587.6. *m*/*z* found (ESI^+) $[M + 2H]^{2+}$, 1174.3; $[M + 3H]^{3+}$, 783.0; $[M + 4H]^{4+}$, 587.5.

2.5. Conjugation

2.5.1. RNase–S

Conjugation of V118C(NTB) S-protein and A5C S-peptide was performed as described in Section 1.2.1 to generate disulfide-linked RNase–S.

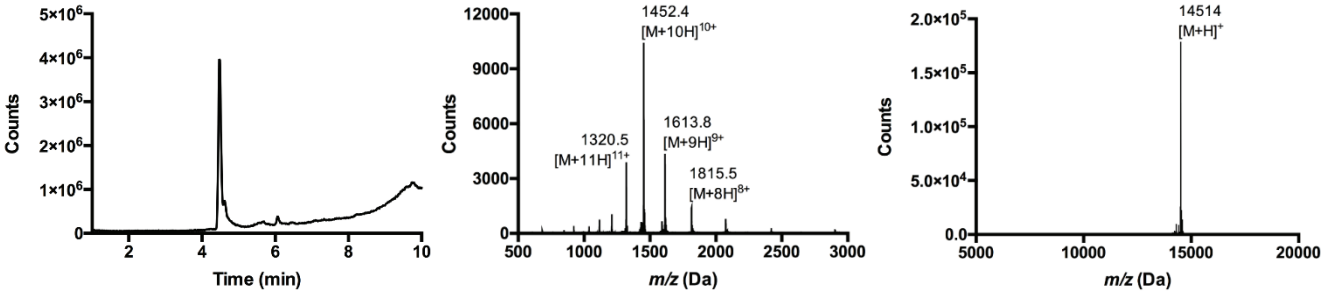


Figure S13. Q-TOF LC/MS analysis of purified RNase–S conjugate; RT, 4.47 min (5–95% v/v B over 10 min). Left, TIC trace. Middle, *m*/*z* expected $[M + 8H]^{8+}$, 1815.5; $[M + 9H]^{9+}$, 1613.9; $[M + 10H]^{10+}$, 1452.6; $[M + 11H]^{11+}$, 1320.7. *m*/*z* found (ESI^+) $[M + 8H]^{8+}$, 1815.5; $[M + 9H]^{9+}$, 1613.8; $[M + 10H]^{10+}$, 1452.4; $[M + 11H]^{11+}$, 1320.5. Right, deconvoluted mass spectrum. *m*/*z* expected $[M + H]^+$, 14516.3. *m*/*z* found (ESI^+) $[M + H]^+$, 14514.0.

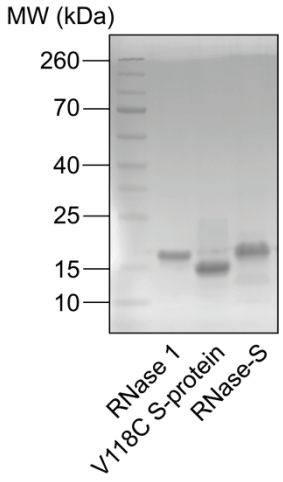


Figure S14. SDS–PAGE analysis of RNase–S conjugate under non-reducing conditions with Coomassie staining. Lane 1: Wild-type RNase 1. Lane 2: V118C(NTB) S-protein. Lane 3: RNase–S conjugate.

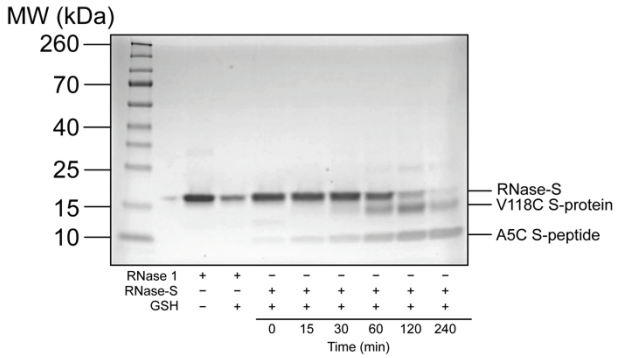


Figure S15. SDS–PAGE analysis of RNase–S conjugate incubated at 37 °C with glutathione (1 mM, 10:1 GSH/GSSG). Lane 1, wild-type RNase 1. Lane 2: RNase 1 plus glutathione.

2.5.2. Alkyne–RNase–S

Conjugation of V118C(NTB) S-protein and alkyne–A5C S-peptide was performed as described in Section 1.2.1 to generate alkyne-tagged RNase–S.

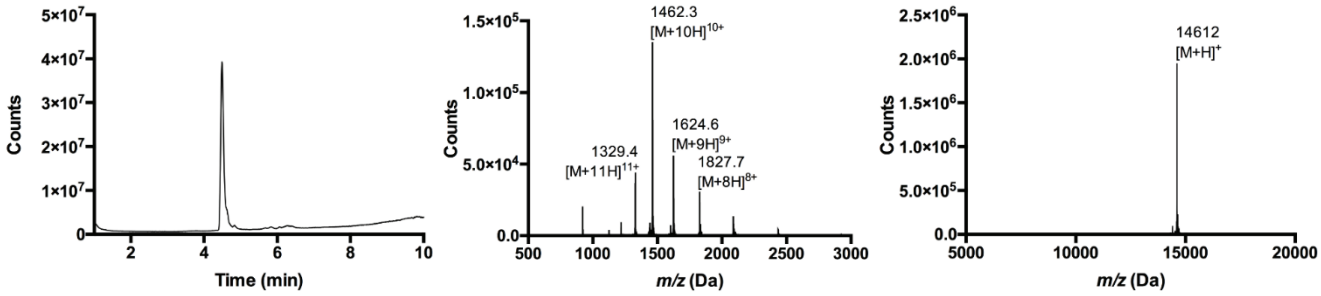


Figure S16. Q-TOF LC/MS analysis of purified alkyne–RNase–S conjugate; RT, 4.49 min (5–95% v/v B over 10 min). Left, TIC trace. Middle, *m*/*z* expected $[M + 8H]^{8+}$: 1827.6, $[M + 9H]^{9+}$, 1624.7; $[M + 10H]^{10+}$, 1462.3; $[M + 11H]^{11+}$, 1329.4. *m*/*z* found (ESI^+) $[M + 8H]^{8+}$, 1827.7; $[M + 9H]^{9+}$, 1624.6; $[M + 10H]^{10+}$, 1462.3; $[M + 11H]^{11+}$, 1329.4. Right, deconvoluted mass spectrum. *m*/*z* expected $[M + H]^+$, 14612.9. *m*/*z* found (ESI^+) $[M + H]^+$, 14612.0.

Alkyne–RNase–S (1 mg/mL in PBS) was reacted with 5-carboxytetramethylrhodamine-azide (Azide-Fluor 545; 5-TAMRA–azide; Sigma–Aldrich product number 760757) in a CuAAC reaction. The reaction was performed by the addition of $CuSO_4$ (to 1 mM from a 100× stock solution in water), Tris((1-benzyl-4-triazolyl)methyl)amine (to 0.1 mM from a 100× stock solution in DMSO), Azide-fluor 545 (to 0.1 mM from a 100× stock solution in DMSO), and sodium ascorbate (to 2 mM from a 200× stock solution in water). The reaction mixture was agitated for 1 h before analysis by SDS–PAGE.

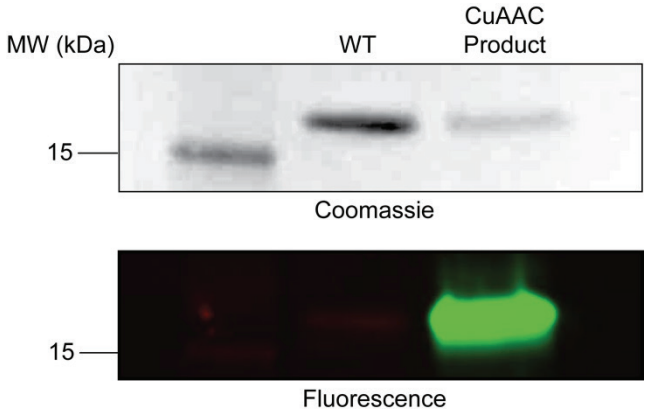


Figure S17. Analysis of CuAAC reaction of alkyne–RNase–S and Azide-Fluor 545 by SDS–PAGE with Coomassie staining and fluorescence imaging. Lane 1: wild-type RNase 1. Lane 2: Product of the CuAAC reaction.

2.5.3. Biotin–RNase–S

Conjugation of V118C(NTB) S-protein and biotin–A5C S-peptide was performed as described in Section 1.2.1 to generate biotin-tagged RNase–S.

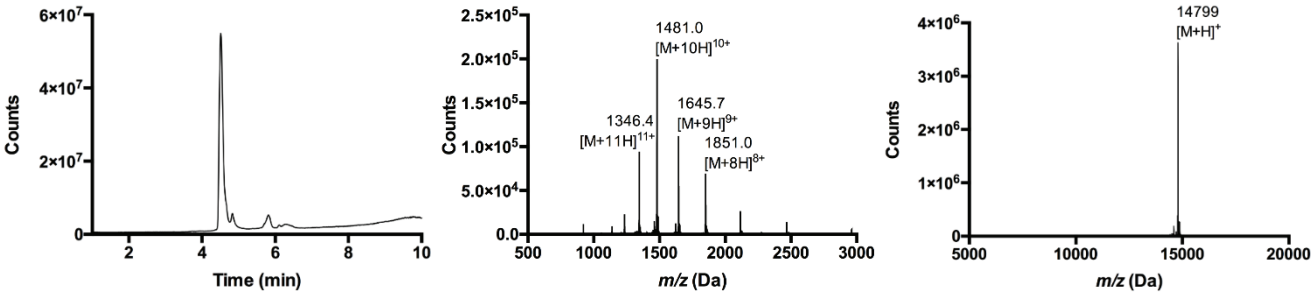


Figure S18. Q-TOF LC/MS analysis of purified biotin–RNase–S conjugate; RT, 4.52 min (5–95% v/v B over 10 min). Left, TIC trace. Middle, *m*/*z* expected $[M + 8H]^{8+}$, 1851.0; $[M + 9H]^{9+}$, 1645.4; $[M + 10H]^{10+}$, 1481.0; $[M + 11H]^{11+}$, 1346.4. *m*/*z* found (ESI^+) $[M + 8H]^{8+}$, 1851.0; $[M + 9H]^{9+}$, 1645.7; $[M + 10H]^{10+}$, 1481.0; $[M + 11H]^{11+}$, 1346.4. Right, deconvoluted mass spectrum; *m*/*z* expected $[M + H]^+$, 14799.7. *m*/*z* found (ESI^+) $[M + H]^+$, 14799.0.

The biotin–RNase–S conjugate was subjected to SDS–PAGE under non-reducing conditions and then transferred onto a PVDF membrane using an iBlot® Gel Transfer Device. After protein transfer, the membrane was washed with Tris-buffered saline supplemented with Tween® 20 (0.1% v/v) (TBST, 5 × 5 min). The membrane was blocked with TBST containing non-fat dry milk (5% w/v) with gentle agitation at 4 °C overnight and then washed with TBST (5 × 5 min). The membrane was incubated with anti-biotin HRP-linked antibody, which was product number 7075S from Cell Signaling Technologies (Danvers, MA) and was diluted 1:1500 in TBST containing bovine serum albumin (3% w/v), with gentle agitation for 1 h. After incubation, the membrane was washed with TBST (5 × 5 min) and the blot was then developed using SuperSignal West Pico PLUS Chemiluminescent Substrate according to the manufacturer's protocol (Thermo Scientific).

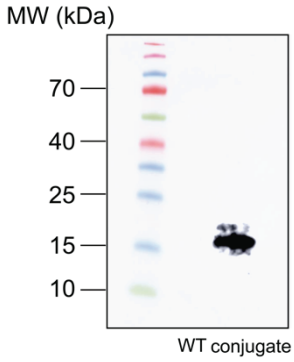


Figure S19. Immunoblot of biotin–RNase–S conjugate. Lane 1, wild-type RNase 1; Lane 2, Biotin–RNase–S conjugate.

2.5.4. Fluorescein–RNase–S

Conjugation of V118C(NTB) S-protein and fluorescein–A5C S-peptide was performed as described in Section 1.2.1 to generate fluorescein-tagged RNase–S.

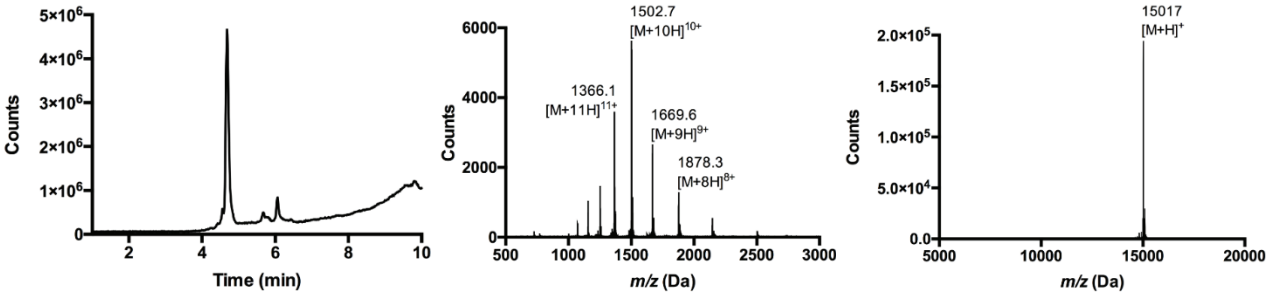


Figure S20. Q-TOF LC/MS analysis of purified fluorescein–RNase–S conjugate; RT, 4.68 min (5–95% v/v B over 10 min). Left, TIC trace. Middle, *m*/*z* expected $[M + 8H]^{8+}$, 1878.4; $[M + 9H]^{9+}$, 1669.8; $[M + 10H]^{10+}$, 1502.9, $[M + 11H]^{11+}$, 1366.4. *m*/*z* found (ESI^+) $[M + 8H]^{8+}$: 1878.3, $[M + 9H]^{9+}$, 1669.6; $[M + 10H]^{10+}$, 1502.7; $[M + 11H]^{11+}$, 1366.1. Right, deconvoluted mass spectrum. *m*/*z* expected $[M + H]^+$, 15019.0. *m*/*z* found (ESI^+) $[M + H]^+$, 15017.0.

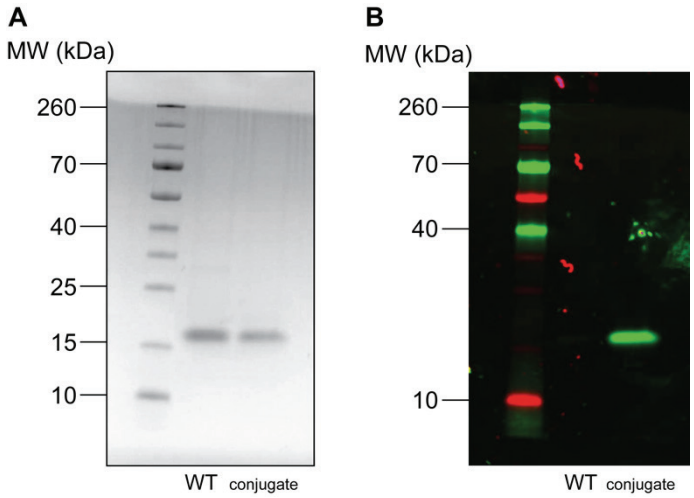


Figure S21. Analysis of fluorescein–RNase–S conjugate by SDS–PAGE with Coomassie staining (A) and fluorescence imaging (B). Lane 1: wild-type RNase 1. Lane 2: Fluorescein RNase–S conjugate.

2.6. Thioether-Linked RNase–S

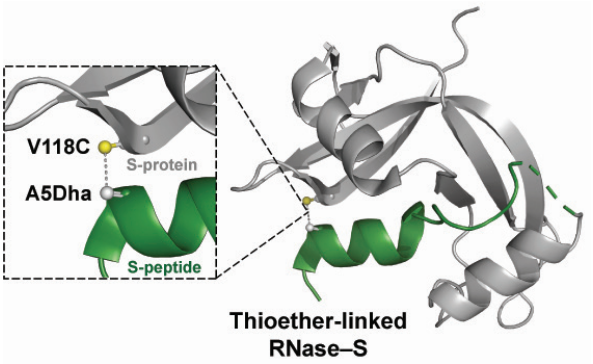


Figure S22. Image of the envisioned thioether crosslink between V118C S-protein and A5Dha S-peptide as generated with PyMOL software and adapted from PDB entry 1rnu.

NTB-protected V118C S-protein (0.3 mg/mL in 50 mM sodium phosphate buffer, pH 2.7) was deprotected for 1 h at 37 °C with immobilized TCEP with gentle agitation. After this time, the yellow solution was desalted and exchanged into 50 mM Tris–HCl buffer, pH 7.0, containing NaCl (50 mM) and EDTA (10 mM) using a PD MiniTrap G-25 column (spin protocol) from GE Healthcare Life Sciences. The solution of deprotected V118C S-protein was immediately combined with A5Dha S-peptide (12 equiv, 0.4 mg/mL) in 50 mM Tris–HCl buffer, pH 7.0, containing NaCl (50 mM) and EDTA (10 mM). The conjugation reaction was allowed to proceed overnight with gentle agitation. The solution was concentrated to ≤500 μL using a Vivaspin® 20 centrifugal concentrator (5000 MWCO PES) and then purified by passage through a HiTrap® SP cation-exchange column from GE Healthcare Life Sciences with 50 mM sodium acetate buffer, pH 5.0, and a gradient of 0.0–0.8 M NaCl over 80 min. The thioether-linked RNase–S product elutes at approximately 0.5 M NaCl.

It should be noted that a substantial amount of S-protein dimerization was observed under the conditions required for thioether conjugation. We were, however, able to isolate a small amount of the desired thioether-linked RNase–S conjugate. To confirm that the thioether-crosslink enhanced the stability of the conjugate in a reducing environment, we incubated thioether-linked RNase–S with glutathione and monitored the conjugate stability over 4 h at 37 °C (Figure S23).

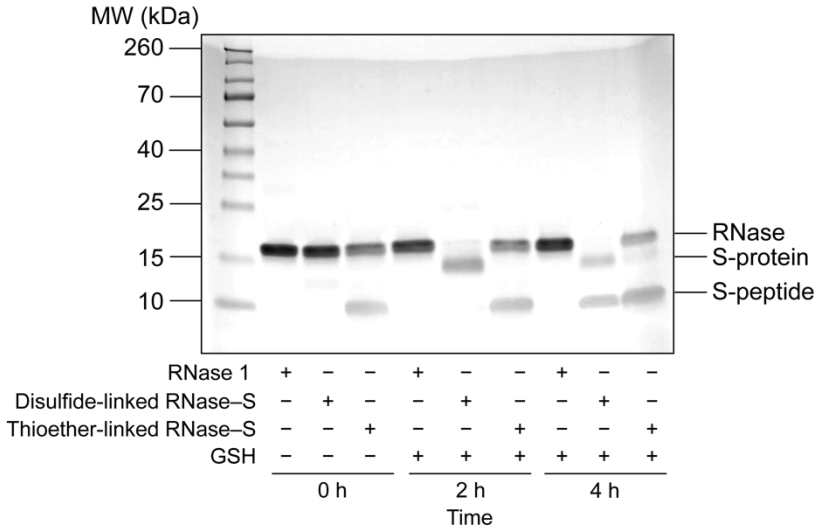


Figure S23. SDS–PAGE analysis of RNase 1, disulfide-linked RNase–S and thioether-linked RNase–S incubated at 37 °C with glutathione (1 mM, 10:1 GSH/GSSG).

3. Assays of Ribonucleolytic Activity

Assays were carried out in 0.10 M DEPC-treated[6] OVS-free[7] MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M), 6-FAM–dArUdAdA–6-TAMRA (200 nM), and a ribonuclease (5 nM).[8] Fluorescence intensity was measured with a M1000 microplate reader from Tecan (Männedorf, Switzerland) by monitoring emission at 515 nm, upon excitation at 493 nm. Assays were performed in triplicate in a flat, black 96-well plate from Corning (Corning, NY). Kinetic parameters were determined by using the equation:

$$I = I_{\mathrm{o}} + (I_{\mathrm{max}} - I_{\mathrm{o}})(k_{\mathrm{cat}}/K_{\mathrm{M}})[\mathrm{ribonuclease}]t \quad (1)$$

The background intensity of the substrate (I_{o}) was determined by measuring fluorescence intensity prior to the addition of enzyme. Product intensity (I_{max}) was determined by measuring fluorescence

intensity after the addition of enzyme to a final concentration of 5 μM, which was deemed to be sufficient to cleave all of the substrate. Values of k_{cat}/K_M were determined by linear least-squares regression analysis of the initial velocity with eq 1, which assumes that assays were performed at a substrate concentration below the value of K_M, which has been estimated to be 22 μM for RNase A.[8]

Table S1. Values of k_{cat}/K_M for Catalysis by Ribonucleases

Ribonuclease	k_{cat}/K_M ($M^{-1}s^{-1}$)
RNase 1	$(9.47 \pm 0.33) \times 10^6$
RNase–S	$(3.63 \pm 0.15) \times 10^5$
Alkyne–RNase–S	$(2.23 \pm 0.05) \times 10^4$
Biotin–RNase–S	$(2.08 \pm 0.21) \times 10^4$
Fluorescein–RNase–S	$(2.74 \pm 0.10) \times 10^4$

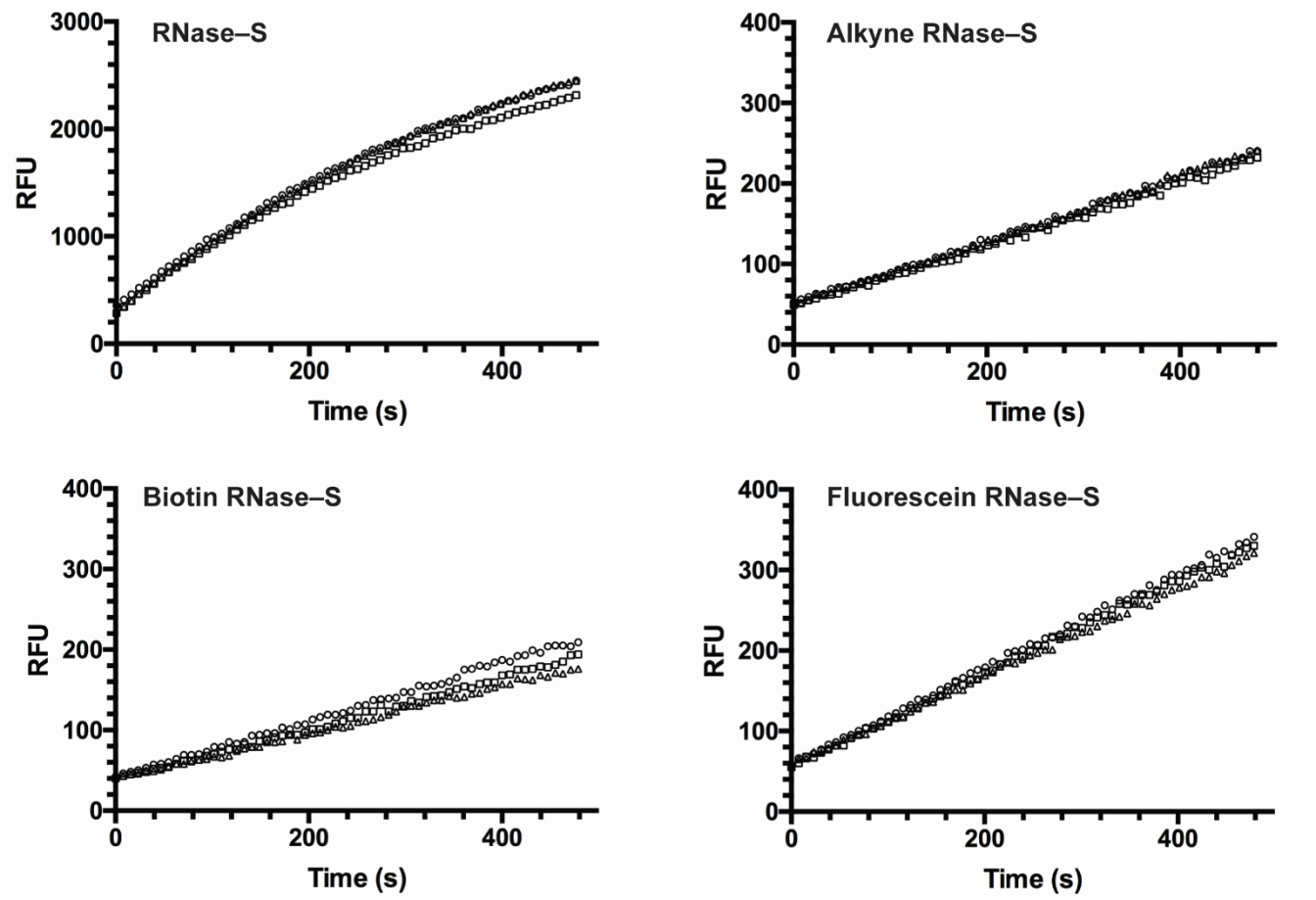


Figure S24. Graph showing the ribonucleolytic activity of RNase–S and its conjugates (5 nM) with a fluorogenic substrate, 6-FAM–dArUdAdA–6-TAMRA (200 nM), in 0.10 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M); excitation: 493 nm, emission: 515 nm. Assays were performed in triplicate. Data for RNase–S are also shown in Figure 3.

4. Assays of Thermostability

The thermostability of RNase 1 and RNase–S was determined by differential scanning fluorimetry using a QuantStudio 7 Flex Real-Time PCR instrument from Applied Biosystems (Foster City, CA) essentially as described previously.[9,10] Briefly, samples of protein (20 μg) in 20 μL of PBS, pH 7.3, containing SYPRO Orange (1% v/v) were heated from 15–95 °C at a continuous rate of 1 °C/min. As the solution was heated, its fluorescence emission was monitored at (586 ± 10) nm after excitation at (470 ± 15) nm. Values of T_m were determined with Protein Thermal Shift software from Applied Biosystems using the Boltzmann model and are reported as the mean ± SE of quadruplicate measurements.

5. References

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