

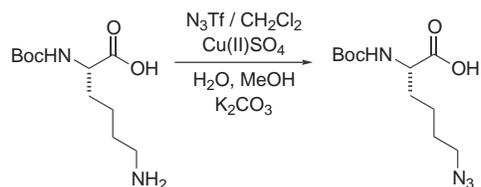
Supporting Information

Site-Specific Protein Immobilization by Staudinger Ligation

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General Experimental. Chemicals and solvents were from Aldrich with the exception of Fmoc-protected amino acids (Novabiochem). Amine-derivatized slides were from CEL Associates (ArrayIt.com). Anhydrous THF, DMF, and CH_2Cl_2 were from a CYCLE-TAINER[®] solvent delivery system (Baker). Other anhydrous solvents were obtained in septum-sealed bottles. Reaction progress was monitored by thin-layer chromatography and visualized by illuminating with UV light or staining with I_2 . Flash chromatography was performed with silica gel 60, 230–400 mesh (Silicycle). Peptide synthesis was performed using standard Fmoc-protection strategies with HATU activation on an Applied Biosystems Pioneer automated synthesizer. Preparative HPLC was performed with a Varian Dynamax C-18 reversed-phase column. Analytical HPLC was performed with a Vydac C-18 reversed-phase column using a linear gradient of H_2O and CH_3CN , both containing TFA (0.1% v/v). NMR spectra were obtained using Bruker AC-300 or Varian UNITY-500 spectrometers. Mass spectra were obtained using electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) techniques.

Synthesis of Boc-Lys(N_3)OH

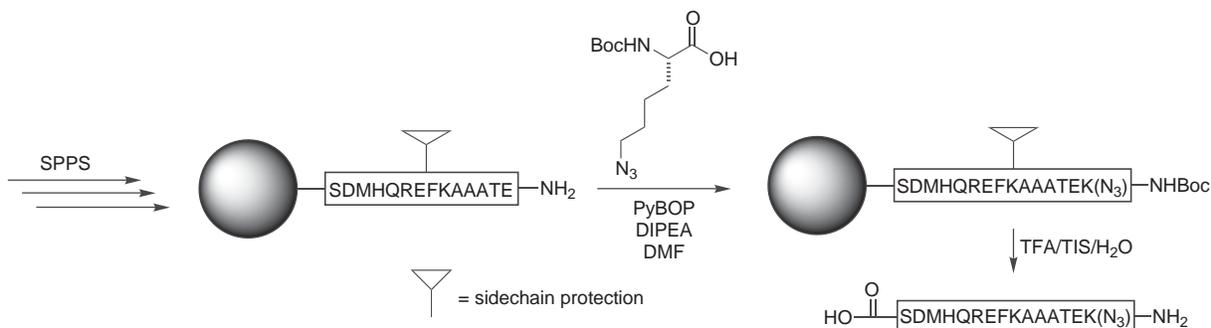


Boc-Lys(N_3)OH was synthesized from Boc-LysOH by using procedures for Cu(II)-catalyzed diazo-transfer to amines.^{S1} Boc-LysOH (4.38 g, 17.8 mmol), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (44.7 mg, 178.2 μmol) and K_2CO_3 (3.69 g, 26.8 mmol) were dissolved in H_2O (57 mL) and MeOH (114 mL). N_3Tf ^{S2} (17.8 mmol in 150 mL CH_2Cl_2) was added, and the reaction mixture was stirred overnight. MeOH was removed under reduced pressure. The resulting aqueous solution (50 mL) was diluted with H_2O (0.10 L), and the pH was adjusted to 6.0 with HCl. Sodium phosphate buffer (150 mL, 0.25 M, pH 6.2) was added, and the solution was washed with EtOAc (3 \times) to remove the triflic amine byproduct. The pH of the washed aqueous layer was adjusted to 2.0 with HCl, and the resulting solution was washed with EtOAc (4 \times) to extract the desired product. The organic layer was then dried over $\text{MgSO}_4(\text{s})$ and filtered, and the solvent was removed under reduced pressure to yield Boc-Lys(N_3)OH as a clear oil (50%). **Spectral data.** ^1H NMR (500 MHz, CDCl_3) δ : 10.93 (broad s, 1H), 5.11 (d, $J = 8.3$ Hz, 1H), 4.34 (m, 1H), 3.30 (d, $J = 7.0$ Hz, 2H), 1.80 (m, 2H), 1.65 (m, 2H), 1.49 (m, 2H), 1.46 (s, 9H) ppm; ^{13}C NMR

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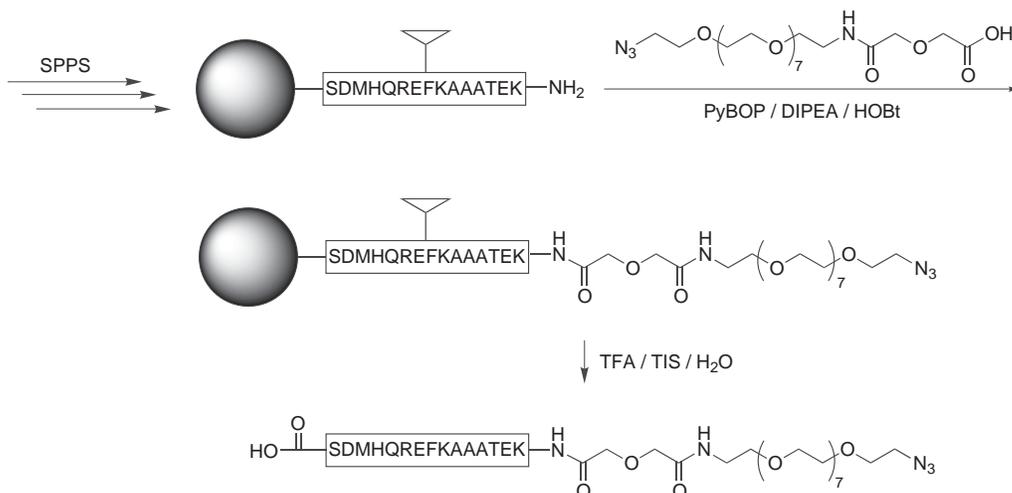
(125 MHz, CDCl₃) δ : 179.96, 156.30, 97.44, 55.79, 53.79, 34.66, 31.07, 30.96, 25.20 ppm; MS (ESI) m/z 295.1383 (MNa⁺ [C₁₁H₂₀N₄O₄Na] = 295.1382).

Synthesis of (N₃)Lys1 S15



S-Peptide residues 2–15 was synthesized on a 0.2-mmol scale using pre-loaded Fmoc-Ser-PEG-PS (Applied Biosystems) as the solid support. Boc-Lys(N₃)OH (218 mg, 0.8 mmol) was dissolved in DMF (15 mL), and PyBOP (416 mg, 0.8 mmol) was added to the resulting solution. The solution was placed under Ar(g), and DIPEA (206.8 mg, 1.6 mmol) was added. The resulting mixture was added to a peptide synthesis vessel containing S-peptide residues 2–15 on a solid support. The coupling reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The resin was then filtered and washed with DMF followed by CH₂Cl₂. Cleavage cocktail (9.5 mL TFA, 0.5 mL TIS, and 0.5 mL H₂O) was then added to the resin. The cleavage reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The filtrate was then added dropwise to ice-cold diethyl ether (30 mL), and the deprotected peptide was collected by centrifugation. The pellet was washed (2×) in ice-cold diethyl ether and dissolved in H₂O/CH₃CN (90:10). The peptide was purified by reversed-phase HPLC on a C-18 column. **Spectral data.** MS (MALDI) m/z 1775.0 (MH⁺ = 1774.8).

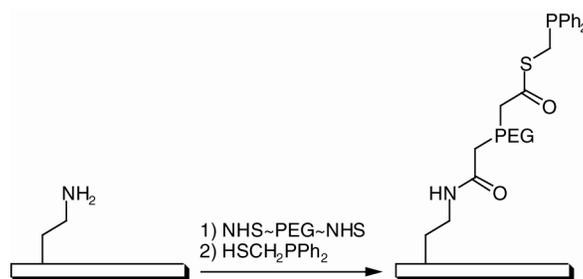
Synthesis of N₃-PEG-S15



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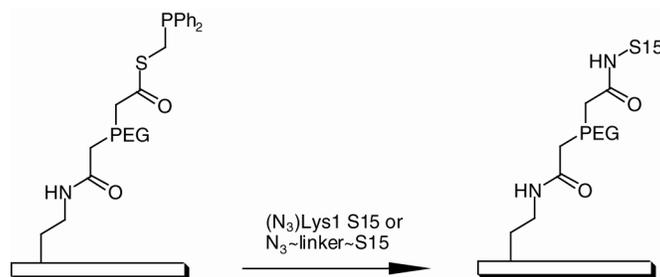
S15 was synthesized on the 0.2-mmol scale using pre-loaded Fmoc-Ser-PEG-PS (Applied Biosystems) as the solid support. N_3 -PEG-CO₂H (444 mg, 0.8 mmol, Novabiochem) was dissolved in 15 mL DMF and PyBOP (416 mg, 0.8 mmol) was added to the resulting solution. The reaction was placed under Ar(g) and DIPEA (206.8 mg, 1.6 mmol) was added. The resulting mixture was added to a peptide synthesis vessel containing S15 on a solid support. The coupling reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The resin was then filtered and washed with DMF followed by CH₂Cl₂. Cleavage cocktail (9.5 mL TFA, 0.5 mL TIS, and 0.5 mL H₂O, premixed) was then added to the resin. The cleavage reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The filtrate was then added dropwise to ice-cold diethyl ether (30 mL) and centrifuged to collect the deprotected peptide. The pellet was washed twice in ice-cold diethyl ether and dissolved in H₂O/CH₃CN (90:10). The peptide was purified by reversed-phase HPLC on a C-18 column. **Spectral data.** MS (MALDI) m/z , 2285.8 (MH⁺ = 2285.4).

Preparation of Phosphinothioester-Derivatized Surface



PEG (M_r 3400) disuccinimidyl propionate (NHS-C(O)-PEG-C(O)-NHS, Shearwater Polymers, 0.1 M in anhydrous DMF) was added to a glass microscope slide derivatized with 3-aminopropyltriethoxysilane. The reaction was allowed to proceed for 2 h under Ar(g). The reaction mixture was washed extensively with anhydrous DMF. To generate a surface-bound phosphinothioester, phosphinomethanethiol^{S3} (0.10 M in anhydrous DMF) was added to the slide and allowed to react for 2 h under Ar(g). The slide was then washed with anhydrous DMF (20 × 10 mL) and allowed to dry under a stream of Ar(g).

Staudinger Ligation on Phosphinothioester-Derivatized Surface

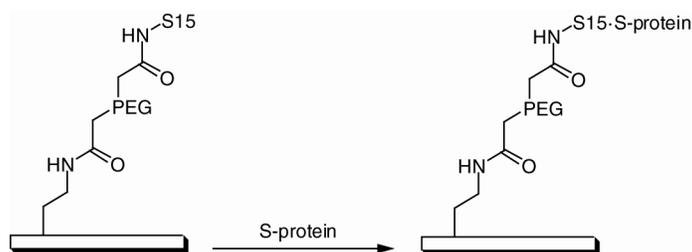


An azido-peptide ((N₃)Lys1 S15 or N₃-PEG-S15) in DMF/H₂O (50:1) was added to a phosphinothioester-derivatized slide at a desired concentration. (Peptide concentrations were spotted with a micropipettor in 0.8-mm spots. Peptide concentrations ranged from ~5 μM to 50 pM, see text.) The Staudinger ligation was allowed to proceed for 8 h in an enclosed chamber

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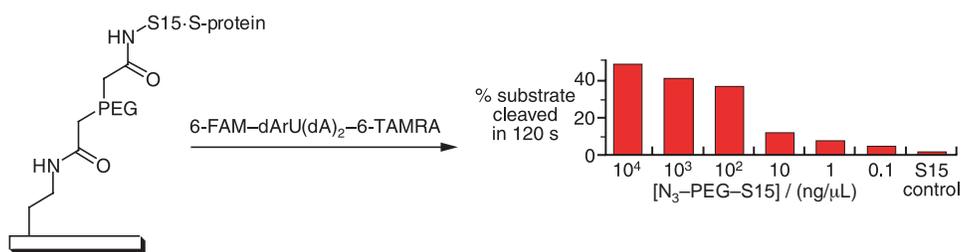
saturated with DMF. The slide was then washed with DMF (20 × 10 mL) and 0.10 M phosphate buffer (pH 7.2, 20 × 10 mL).

Generation of Immobilized Ribonuclease S'



RNase A and RNase S were removed from commercial S-protein (Sigma Chemical) by reversed-phase HPLC on a Vydac C-4 column. Purified S-protein (10 mg/mL in 0.10 M sodium phosphate buffer, pH 7.2) was incubated for 8 h on a slide presenting immobilized S15 (see above). The slide was then washed with buffer (30 × 10 mL). Slides were stored in 0.10 M sodium phosphate buffer, pH 7.2.

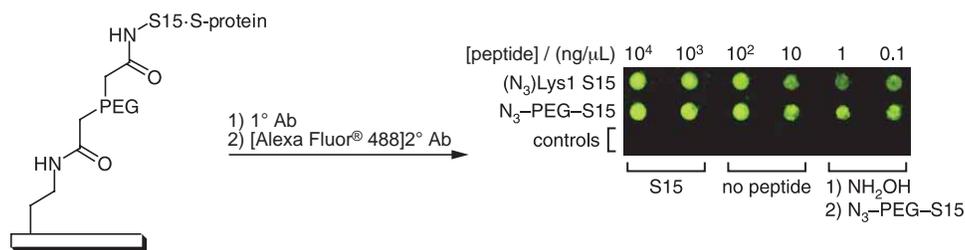
Activity Assay of Immobilized Ribonuclease S'



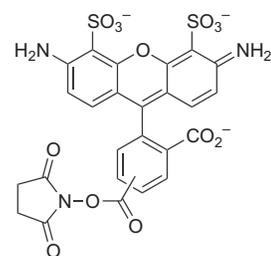
The ribonucleolytic activity of each spot was determined using the fluorogenic substrate, 6-FAM-(dA)rU(dA)₂-6-TAMRA.^{S4} Cleavage of this substrate results in a *ca.* 200-fold increase in fluorescence intensity (excitation at 494 nm; emission at 515 nm). Assays were performed by adding 0.10 M MES-NaOH buffer (pH 6.0) containing 0.10 M NaCl and 6 μM 6-FAM-(dA)rU(dA)₂-6-TAMRA to each spot. At various times, an aliquot was removed from the plate and its fluorescence was recorded. The concentration of RNase S' in each spot was determined by using the equation $[RNase\ S'] = (\Delta I / \Delta t) / [(I_f - I_0)(k_{cat} / K_M)]$, where $\Delta I / \Delta t$ is the initial velocity of the reaction, I_0 is the fluorescence intensity prior to exposure to the plate, I_f is the fluorescence intensity after complete hydrolysis of the substrate with excess RNase A, and $k_{cat} / K_M = 3.6 \times 10^7\ M^{-1}s^{-1}$.^{S5}

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Immunoassay of Immobilized Ribonuclease S'



RNase S' attached to the plate was visualized by immunostaining. Rabbit IgG raised against RNase A (Bioscience International) was used at a concentration of 2 μg/mL. Each spot was incubated with primary antibody for 30 min at 23 °C. After exposure to primary antibody, the entire slide was washed with PBS (4 × 20 mL). Secondary antibody conjugated to Alexa Fluor® 488 (Molecular Probes) was diluted in PBS to 1 μg/mL and incubated as described for primary antibody. The slide was then washed with PBS (4 × 20 mL). RNase S' was detected by using a FluorImager SI fluorescence scanner (Molecular Dynamics). The quantity of immobilized protein was determined with IMAGEQUANT densitometry software (Molecular Dynamics) and a standard curve generated by spotting various concentrations of Alexa Fluor® 488-conjugated secondary antibody.



Alexa Fluor® 488

References

- (S1) Alper, P. B.; Hung, S. -C.; Wong, C. -H. *Tetrahedron Lett.* **1996**, *37*, 6029–6032.
- (S2) Triflyl azide was prepared as follows. A solution of sodium azide (11.42 g, 175.0 mmol) was dissolved in distilled H₂O (27 mL) with CH₂Cl₂ (54 mL) and cooled in an ice bath. Triflyl anhydride (10.0 g, 35.4 mmol) was added slowly over 5 min while stirring continued for 2 h on ice. The mixture was placed in a separatory funnel, and the organic phase was removed. The aqueous portion was extracted with CH₂Cl₂ (2 × 37.5 mL). The organic fractions, which contain the triflyl azide, were pooled, washed once with saturated Na₂CO₃(aq), and used without further purification.
- (S3) (a) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2001**, *3*, 9–12. (b) Soellner, M. B.; Nilsson, B. L.; Raines, R. T. *J. Org. Chem.* **2002**, *67*, 4993–4996. (c) Nilsson, B. L.; Hondal, R. J.; Soellner, M. B.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 5268–5269.
- (S4) Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. *Nucleic Acids Res.* **1999**, *27*, 3696–3701.
- (S5) This value of k_{cat}/K_M is for the cleavage of 6-FAM-(dA)rU(dA)₂-6-TAMRA by RNase A (ref. S4). The catalytic activity of RNase S' is reported to be identical to that of RNase A (Potts, J. T. Jr.; Young, D. M.; Anfinsen, C. B. *J. Biol. Chem.* **1963**, *238*, 2593–2594). If it were lower, then the actual yield of active enzyme immobilized on the slide would be higher than reported herein.