

**Protein Assembly by Orthogonal Chemical Ligation Methods**

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**General Experimental.** Chemicals and solvents were from Aldrich<sup>®</sup> with the exception of Fmoc-protected amino acids and alkanesulfonamide safety-catch resins (Novabiochem<sup>®</sup>). Reactions were monitored by thin-layer chromatography and visualized by UV light or staining with I<sub>2</sub>. Peptide synthesis was performed by using standard Fmoc-protection strategies on an Applied Biosystems Pioneer automated synthesizer with HATU activation. HPLC was performed on a C18 reverse-phase column. NMR spectra were obtained with a Bruker AC-300, Bruker DMX-400, Varian UNITY-500, or Varian Inova-600 spectrometer. Phosphorus-31 NMR spectra were proton-decoupled and referenced against an external standard of deuterated phosphoric acid. Mass spectra were obtained using electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI) techniques.

**NMR spectral assignments of FmocProOH *cis/trans* isomers.** The *cis/trans* assignments for the <sup>1</sup>H<sup>α</sup> resonances of FmocProOH (Figure 1, lower spectrum) were made by correlating the resonances of the α-proton to those of the β- and γ-carbons using a 2D HMBC experiment. The β- and γ-carbons of proline amides each have two distinct <sup>13</sup>C resonances due to *cis/trans* amide bond isomerization. The <sup>13</sup>C chemical shifts for the *cis* and *trans* isomers are constant among different proline derivatives.<sup>1</sup> The <sup>13</sup>C shifts in FmocProOH were correlated to the two <sup>1</sup>H<sup>α</sup> resonances of FmocProOH, and the peak at 4.18 ppm was thereby assigned to arise from the *trans* isomer and the peak at 4.08 ppm to arise from the *cis* isomer.

**Synthesis of FmocCys(Trt)Glu(OtBu)SCH<sub>2</sub>PPh<sub>2</sub>.** FmocGlu(OtBu)OH was loaded onto 4-sulfamylbutyryl resin according to the method of Backes and Ellman.<sup>2</sup> The protocol was repeated twice to give optimal loading. Fmoc-deprotection was performed using 30% (v/v) piperidine in DMF. Coupling was performed by premixing the protected amino acid (3 eq), PyBOP (3 eq), HOBt (3 eq), and DIPEA (6 eq) in DMF, and adding the mixture to the pre-swollen resin for 90 min. The resin containing the synthetic peptide was activated with iodoacetonitrile according to the method of Backes and Ellman.<sup>1</sup> The final phosphinothioester was liberated by incubating the resin (1.0 g, 1.12 mmol peptide loading) with Ph<sub>2</sub>PCH<sub>2</sub>SH (2.1 g, 9.0 mmol) in DMF (15 mL) for 18 h. The resin was then filtered and washed (5 × 10 mL DMF, 5 × 10 mL CH<sub>2</sub>Cl<sub>2</sub>), and solvents were removed under reduced pressure. The residue was purified by chromatography (silica gel, 30% EtOAc in hexanes) to yield 706 mg (0.72 mmol, 64% yield based on a 1.12-mmol resin loading) of FmocCys(Trt)Glu(OtBu)SCH<sub>2</sub>PPh<sub>2</sub>. **Spectral data.** <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.75–7.70 (m, 2H), 7.57–7.55 (m, 2H), 7.42–7.14 (m, 29H), 6.68 (d, *J* = 6.6 Hz, 1H), 5.13 (d, *J* = 8.1 Hz, 1H), 4.56–4.50 (m, 1H), 4.36–4.34 (m, 2H), 4.19–4.17 (m, 1H), 3.81–3.80 (m, 1H), 3.44–3.38 (m, 2H), 2.78–2.68 (m, 1H), 2.61–2.57 (m, 1H), 2.27–2.23 (m, 2H), 2.11–1.95 (m, 1H), 1.83–1.70 (m, 1H), 1.37 (s, 9H) ppm; <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 198.14, 171.89, 170.19, 155.81, 144.17, 143.59, 143.46, 141.10, 136.49 (d, *J* = 14.0 Hz), 132.69 (d, *J* = 4.2 Hz), 132.44 (d, *J* = 4.1 Hz), 129.41, 128.98, 128.38 (d, *J* = 6.6 Hz), 127.93, 127.59, 126.94, 126.74, 124.93, 119.80, 80.74, 67.21, 67.00, 58.50, 53.83, 46.89, 31.00, 27.85, 27.23, 25.45 (d, *J* = 24.8 Hz) ppm; <sup>31</sup>P NMR (121 MHz, CDCl<sub>3</sub>) δ -14.51 ppm; MS (ESI) *m/z* 1007.3340 (MNa<sup>+</sup> [C<sub>59</sub>H<sub>57</sub>N<sub>2</sub>O<sub>6</sub>PS<sub>2</sub>Na] = 1007.3371).

**Solid-Phase Staudinger Ligation.** Asn(Trt)ProTyr(*t*Bu)ValProValHis(Trt)PheAsp(OtBu)AlaSer(*t*Bu)Val was synthesized on a polystyrene resin with PEG linkers by using standard

methods.  $\alpha$ -Azido glycine<sup>3</sup> was coupled using PyBOP/HOBt/DIPEA preactivation of the azido acid in DMF prior to its addition to the resin. The coupling reaction was allowed to proceed for 2 h at room temperature.

Four equivalents of FmocCys(Trt)Glu(O $t$ Bu)SCH<sub>2</sub>PPh<sub>2</sub> were added to the resin-linked peptide in DMF/H<sub>2</sub>O (10:1). Approximately 1.5 mL of solvent were used per 180 mg of resin (25- $\mu$ mol loading). The mixture was agitated gently for 12 h, after which the solvent was removed by filtration and the resin was rinsed with DMF (5  $\times$  10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (5  $\times$  10 mL). The resin was dried under vacuum and then treated with a cleavage cocktail (95% TFA, 2.5% H<sub>2</sub>O, 2.5% ethanedithiol) for 2 h. The cleavage cocktail was separated from the resin by filtration and added to ice-cold diethyl ether to precipitate the deprotected peptide product. The peptide product was purified by reverse-phase HPLC and analyzed by MALDI mass spectrometry. The results are listed in Table S1.

**Table S1.** Monoisotopic Mass of RNase A(110–124) Produced by Solid-Phase Staudinger Ligation

Phosphinothioester peptide <sup>a</sup>	Azido peptide <sup>a</sup>	Product peptide <sup>b</sup>	MALDI (MH <sup>+</sup> )	expected (MH <sup>+</sup> )
Fmoc–RNase A(110–111)–SCH <sub>2</sub> PPh <sub>2</sub>	N <sub>3</sub> -RNase A(112–124)–resin	RNase A(110–124)	1633.4	1633.7
Fmoc–RNase A(110–111)–SCH <sub>2</sub> PPh <sub>2</sub>	[ <sup>13</sup> C, <sup>15</sup> N] <sub>3</sub> -RNase A(112–124)–resin <sup>c</sup>	[ <sup>13</sup> C, <sup>15</sup> N]RNase A(110–124) <sup>c</sup>	1637.6	1637.7

<sup>a</sup> Side chains are protected. <sup>b</sup> Side chains are not protected. <sup>c</sup> [<sup>13</sup>C,<sup>13</sup>C $\alpha$ ,<sup>15</sup>N]ProOH was used at position 114, and [<sup>13</sup>C $\alpha$ ]ProOH was used at position 117. Note: In the present study, no NMR experiments were performed using the [<sup>13</sup>C $\alpha$ ]Pro residue at position 117, which has a *trans* peptide bond in native RNase A.

**Expressed protein ligation.** Expressed protein ligation of RNase A(1–109) thioester, which also has an N-terminal methionine residue [Met(–1)], to RNase A(110–124) was performed as described previously.<sup>4</sup> MALDI *m/z* 13,832; expected, 13,819.

**References**

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