Supporting Information

Mechanistic Studies on the Substrate Tolerant Lanthipeptide Synthetase ProcM

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Contents

Characterization of Small Molecules and Peptides

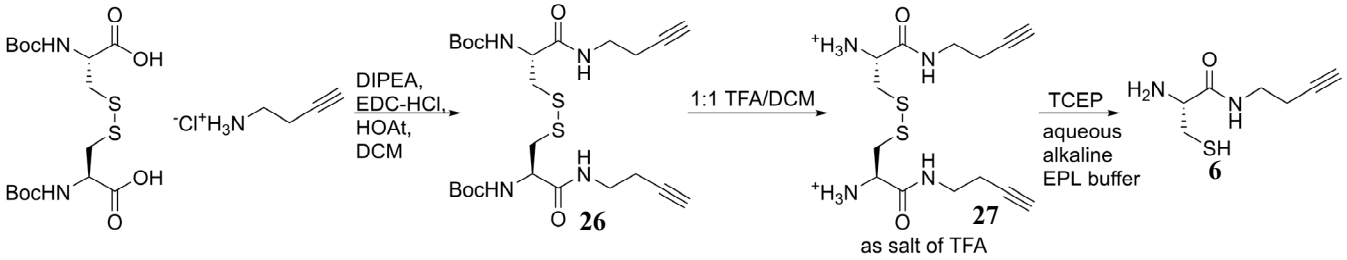
Nuclear magnetic resonance (NMR) spectra were recorded on Varian Unity 400, Unity Inova 500, or Varian VXR 500 spectrometers. Small molecules (MW < 1000 Da) were analyzed by electrospray ionization/time-of-flight (ESI-TOF) mass spectrometry on a Waters Quattro II quadrupole spectrometer. Peptides (MW > 800 Da) were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry on a Bruker UltrafleXtreme spectrometer using a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid in 1:1 H_2O/MeCN with 0.1% TFA. For larger peptides (MW>5000, or for endoproteinase digested mixtures), 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (sinapic acid) dissolved in 1:1 H_2O/MeCN with 0.1% TFA was used as matrix. For smaller peptide fragments (500-1000 Da), MALDI-TOF analysis was performed with 2,5-dihydroxybenzoic acid (DHB) dissolved in 1:1 H_2O/MeCN with 0.1% TFA. ESI-MS on peptides and their tandem MS analysis was carried out with a Synapt Waters G1 system. Samples were separated by liquid chromatography using a Phenomenex Jupitar C_{18} 300a column using an elution gradient of 3% solvent A, 97% solvent B to 40% solvent A, 60% solvent B over 20 min at a flow-rate of 0.2 mL/min (solvent A: 99.9% acetonitrile, 0.1% formic acid, solvent B: 0.1% formic acid in 99.9% water). For analysis of larger framents (MW>4000), an elution gradient of 3% solvent A, 97% solvent B to 70% solvent A, 30% solvent B over 20 min was used. The LC-system was directly connected to the MS chamber, where ESI was used to analyze samples, using Glu-1-Fibrinopeptide B (Glu-Fib) as external calibrant.

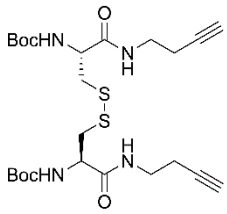
Small Molecule Synthesis

Materials and purifications

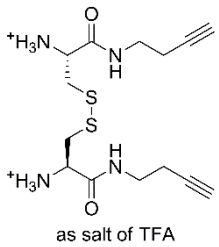
Standard Fmoc-amino acids and resins, Boc-protected Cys and peptide coupling reagents- 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP), *N*,*N'*-diisopropylcarbodiimide (DIC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 1-hydroxy-7-azabenzotriazole (HOAt) and 1-hydroxybenzotriazole (HOBt) were purchased from Chem-Impex. [2,3,3-^{2}H]-L-Ser was purchased from Cambridge Isotope Laboratories, and [2,3-^{2}H]-L-Thr was purchased from CDN-isotopes. Chitin resin was purchased from New England Biolabs. Dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), methanol, and 1,4-dioxane were purchased at reaction grade from Fisher Scientific and dried via a solvent dispensing system prior to use. Other chemical reagents and reaction-grade solvents were purchased from Sigma Aldrich or Alfa Aesar and used without further purification. All reactions and chromatography fractions were monitored by thin layer chromatography (TLC) on silica-gel-coated glass plates with a F254 fluorescent indicator. Visualization was achieved by UV absorption by fluorescence quenching or permanganate stain (1.5 g $KMnO_4$, 10 g K_2CO_3, 1.25 mL 10% NaOH in 200 mL of H_2O), or ninhydrin stain (0.2% ninhydrin in EtOH) for visualizing compounds with free amines. For visualizing compounds with free carboxylic acids, bromocresol green (0.04% in EtOH) was used. Flash chromatography was performed using Silicycle SiliaFlash P60, 230-400 mesh silica gel.

Synthesis of building block 6 used to introduce a *C*-terminal alkyne



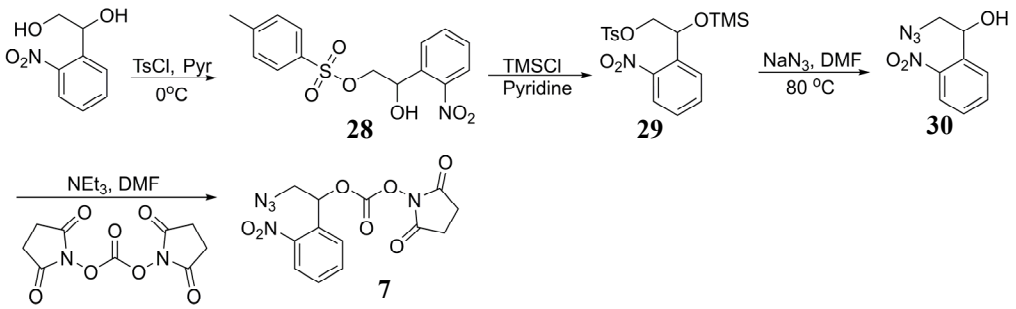


Compound **26.** In a round bottom flask, 3-butyn-1-amine hydrochloride (0.21g, 1.99 mmol) was dissolved in 30 mL of DCM and neutralized with DIPEA (0.38 mL, 2.2 mmol). To the reaction mixture, Boc-L-cystine was added (0.44 g, 1 mmol), followed by EDC-HCl (0.39 g, 2.01 mmol) and HOAt (0.27 g, 2.02 mmol) and the reaction mixture was stirred at room temperature for 30 h. The reaction mixture was washed with sodium bicarbonate (1 x 15 mL), 10% citric acid (1 x 15 mL), and brine (1 x 15 mL), respectively. All aqueous layers were back-extracted with DCM (1 x 10 mL). The organic layers were collected, dried over sodium sulfate and concentrated to yield **26** (0.42 g, 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (t, *J* = 6.2 Hz, 2H), 5.57 (d, *J* = 9.4 Hz, 2H), 4.82 (ddd, J = 14.1, 7.2, 4.0 Hz, 2H), 3.46 (app dq, *J* = 13.3, 6.7 Hz, 2H), 3.34 (app dq, *J* = 13.3, 6.7 Hz, 2H), 3.01-2.97 (m, 2H), 2.92-2.87 (m, 2H), 2.48 (dtd, *J* = 16.5, 6.9, 2.7 Hz, 2H), 2.39 (dtd, *J* = 16.5, 6.9, 2.7 Hz, 2H), 1.96 (t, *J* = 2.5 Hz, 2H), 1.47 (s, 18 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 170.6, 156, 81.4, 80.4, 70, 54.8, 47.4, 38.6, 28.6, 19.5. HRMS (ESI) m/z calc. for $C_{24}H_{39}N_4O_6S_2$ ($M+H^+$) 543.2311, found 543.2310.

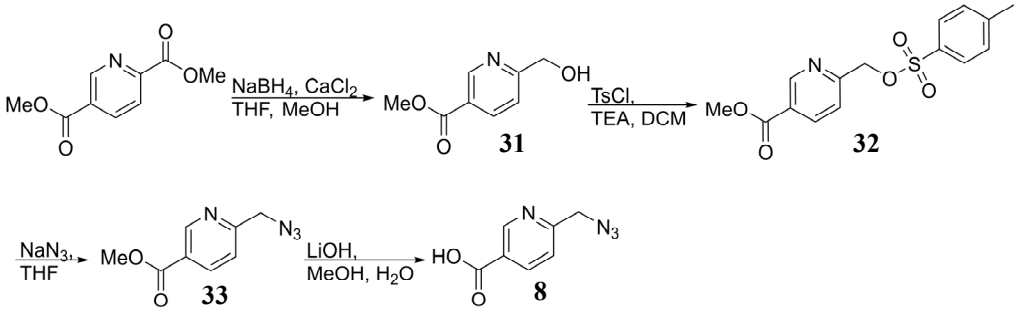


Compound **27.** Compound **26** (0.42 g, 0.79 mmol) was suspended in 6 mL of dry DCM to form a milky suspension. To this suspension, 6 mL of trifluoroacetic acid (TFA) was added when a clear yellowish solution formed, which was stirred at room temperature for 1 h. TFA was then evaporated using a stream of N_2. The residue was dissolved in water and lyophilized to yield **27** as white fluffy solid (0.34 g, 95%). ^{1}H NMR (500 MHz, D_2O) δ 4.31 (t, *J* = 6.5 Hz, 2H), 3.49-3.43 (m, 2H), 3.37-3.33 (m, 2H), 3.33-3.28 (m, 2H), 3.23-3.18 (m, 2H), 2.47-2.42 (m, 2H), 2.36-2.35 (t, *J* = 2.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm = 168, 82.4, 70.8, 52.1, 38.5, 37.7, 18.4. HRMS (ESI) m/z calc. for $C_{14}H_{23}N_4O_2S_2$ 343.1262, found 343.1256. During EPL, compound **27** was reduced in situ to **6**.

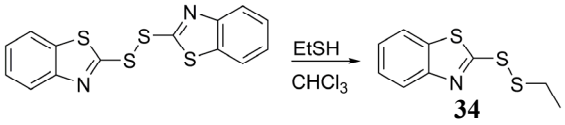
Scheme for synthesis of azide building block 7[1]



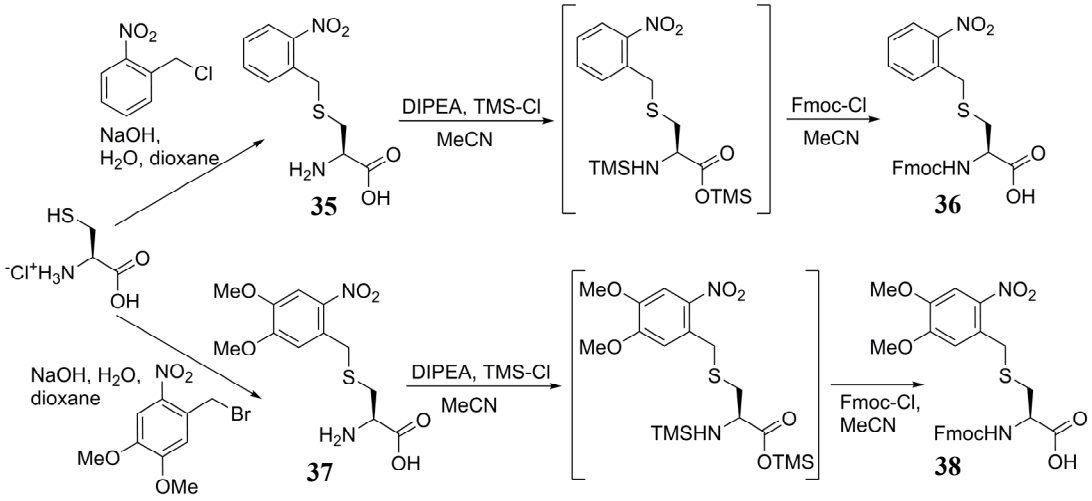
Scheme for synthesis of azide building block 8[2]



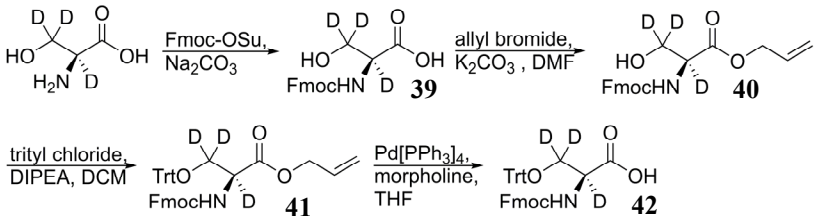
Synthesis of benzothiazolyl-ethyl disulfide 34[3,4]



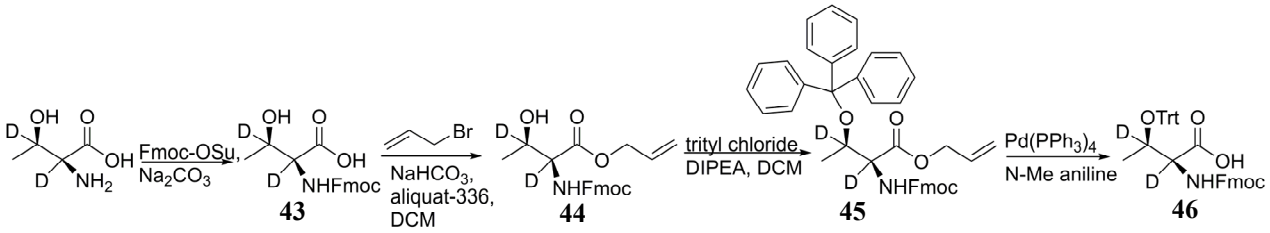
Scheme for synthesis of Cys(o-NO_2Bn) building blocks 36[5,6] and 38[7] for SPPS

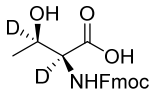


Scheme for synthesis of [2,3,3-^{2}H]-Ser building block for SPPS[8]

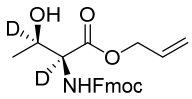


Scheme for synthesis of protected [2,3-^{2}H]-Thr building block for SPPS

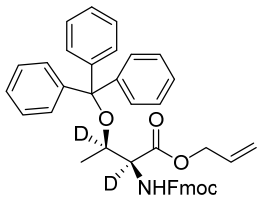




Compound **43**. L-[2,3-^{2}H]-Thr-OH (0.25 g, 2.06 mmol) and Na_2CO_3 (0.25 g, 2.36 mmol) was dissolved in 3 mL of H_2O. Fmoc-OSu (0.75 g, 2.22 mmol) dissolved in 4 mL of dry dioxane was stirred in an ice-bath, and the amino acid solution was added slowly to the dioxane solution. The ice-bath was removed and the reaction was stirred for 20 h to form a milky suspension. The solvent was then removed on a rotary evaporator, and the residue was dissolved in H_2O (20 mL) to generate a colorless solution. 10% citric acid was used to acidify the solution to pH 4.0, when a thick white solution formed. The aqueous layer was extracted with EtOAc (10 x 25 mL) and the organic layer was washed with brine (1 x 50 mL) and concentrated on a rotary evaporator followed by drying using a vacuum pump to generate a white solid, which was carried over to the next step without purification. Yield (crude): 0.97 g (calculated 0.71 g)

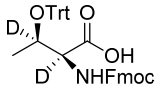


Compound **44**. Compound **43** (0.97 g) was suspended in a solution of $NaHCO_3$ (0.18 g, 2.1 mmol) in 6 mL of H_2O. The suspension was stirred under N_2 and to it, 13 mL of dry DCM was added, while stirring vigorously. Aliquat-336 (0.96 mL, 2.1 mmol) was added followed by allyl bromide (1.3 mL, 15 mmol) and then the reaction was continued to be stirred vigorously for 22 h. The reaction mixture was then diluted with 15 mL of H_2O and extracted with DCM (4 x 20 mL). The organic layers were collected, washed with brine (1 x 20 mL), dried over Na_2SO_4 and concentrated on a rotary evaporator to generate a pale-yellow oil. The crude mass was purified by flash chromatography (SiO_2, 30% EtOAc in hexanes) to generate **44** as a white solid upon concentrating on a rotary evaporator. R_f 0.21 (30% EtOAc in hexanes). Yield: 0.59 g (75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.7 (d, *J* = 7.5 Hz, 2H), 7.61 (t, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 6.10-5.89 (m, 1H), 5.60 (s, 1H), 5.39-5.33 (m, 1H), 5.26 (d, *J* = 10.5 Hz, 1H), 4.68 (d, *J* = 5.5 Hz, 2H), 4.46-4.39 (m, 2H), 4.25 (t, *J* = 7.0 Hz, 1H), 2.70 (s, 1H), 1.26 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.5, 156.9, 143.9, 143.7, 141.3, 131.5, 131, 127.8, 127.1, 125.2, 122.7, 120, 119, 67.3, 66.3, 47.2, 25.5, 19.9. HRMS (ESI) calculated for $C_{22}H_{22}D_2NO_5$ ($M+H^+$) 384.1777, observed 384.1780.



Compound **45**. Compound **44** (0.59 g, 1.54 mmol) was dissolved in 13 mL of dry DCM and DIPEA (0.83 mL, 4.8 mmol) was added to the solution. Trityl chloride (1.09 g, 3.9 mmol) dissolved in 5 mL of dry DCM was added dropwise and the reaction was stirred for 21 h. The reaction turned from colorless to light pink to dark pink over time. The reaction was concentrated on a rotary evaporator and the

product purified twice by SiO_2 gel flash chromatography. Elution was performed by a stepwise gradient from 10% EtOAc in Hex to 20% to 30% EtOAc in Hex. Product **45** eluted with an R_f of 0.57 (3:1 Hex:EtOAc). Yield: 0.38 g (39%). Unreacted starting material **44** (0.27 g, 45%) was also recovered (R_f 0.15, 3:1 Hex:EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (dd, *J* = 7.6, 2.9 Hz, 2H), 7.70 (dd, *J* = 19.5, 7.5 Hz, 2H), 7.43-7.41 (m, 4H), 7.32-7.27 (m, 15H), 5.77-5.69 (m, 1H), 5.23-5.17 (m, 2H), 4.54-4.53 (dd, *J* = 13.0, 6.1 Hz, 1H), 4.32-4.27 (m, 2H), 0.91 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.8, 147.0, 144.6, 144.2, 144.0, 141.5, 131.7, 129.0, 128.0, 127.9, 127.8, 127.4, 127.2, 125.3, 120.1, 119.0, 82.2, 67.4, 66.4, 47.4, 18.8. HRMS (ESI) calculated for $C_{41}H_{35}D_2NO_5Na$ ($M+Na^+$) 648.2695, observed 648.2695.



Compound **46**. Compound **45** (0.38 g, 0.61 mmol) was dissolved in 4 mL of dry THF and the round bottom flask was covered in Al-foil. $Pd[PPh_3]S_4$ (0.05 g, 0.04 mmol) was added followed by N-methyl aniline (0.07 mL, 0.65 mmol) as a solution in 3 mL of dry THF and the reaction was stirred for 2.5 h. The reaction mixture was diluted with 100 mL of EtOAc and washed with brine (2 x 30 mL). The aqueous layer was extracted with EtOAc (5 x 25 mL) and concentrated on a rotary evaporator and dried using a vacuum pump. The product was used without further purification. HRMS (ESI) calculated for $C_{38}H_{31}D_2NO_5Na$ ($M+Na^+$) 608.2380, observed 608.2378.

Solid Phase Peptide Synthesis (SPPS)

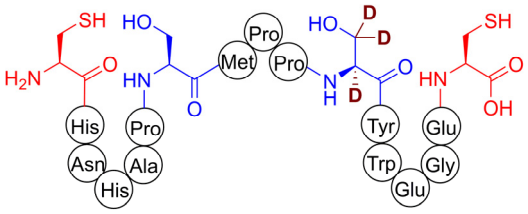
General methodology

The automated peptide coupling was performed on a CEM Liberty microwave peptide synthesizer using standard Fmoc protected amino acids, 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) as activator, 2 M *N*,*N*-diisopropylethylamine (DIPEA) in *N*-methyl-2-pyrrolidone (NMP) as the activator base, 20% piperidine in dimethylformamide (DMF) as the deprotection agent and 90/8/2 DMF/acetic anhydride/DIPEA as the capping agent. Coupling of each amino acid in the microwave synthesizer occurred at 75 °C except for Cys and His residues for which coupling was performed at 50 °C to prevent racemization. Typically each residue was double coupled followed by a capping step, unless otherwise noted. Coupling of any synthesized non-proteinogenic amino acid residues and the azide linker at the *N*-terminus was performed manually using either diisopropyl carbodiimide (DIC) as the coupling agent with 1-hydroxy-7-azabenzotriazole (HOAt) as the racemization suppressant, or HCTU as activator. Unless otherwise stated, manual couplings were performed as follows. Fmoc deprotection was performed using 20% piperidine in DMF while sparging the resin with N_2 for agitation (2 x 5 min). After draining the reaction vessel, the resin was washed with DMF (6 x 30 s). The appropriate moiety to be coupled (4 equiv.) was dissolved in DMF (5-10 mL) and pre-activated with DIC/HOAt (4 equiv.) for 5 min, then added to the resin and the reaction was agitated by sparging with N_2 for 1 h. After draining the reaction vessel, the resin was washed as before. Kaiser test was performed to monitor the completion of coupling and double coupling was performed as needed. The coupling of [2,3,3-^{2}H]-Ser and [2,3-^{2}H]-Thr building blocks

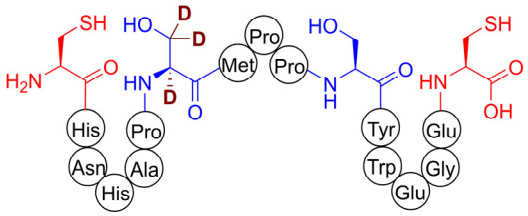
were performed under optimized conditions using (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) as activator and HOAt as racemization suppressant using 2,4,6-collidine as base to prevent partial loss of the α-deuterium during coupling. After the completion of SPPS, the resin was washed with ca. 5 mL of DMF (3 x 30 s) and then ca. 5 mL of DCM (3 x 30 s) and drained under vacuum to dry the resin. The cleavage of the synthesized peptide from the resin was performed in a cleavage cocktail of 95/2.5/2.5 TFA/triisopropyl silane/H_2O for 1 h and then the solution was filtered though a fritted funnel and the filtrate was evaporated under a stream of N_2 to remove most of the TFA. Peptide was precipitated by adding 10-15 mL of cold diethyl ether to the solution. The mixture was centrifuged and the supernatant was discarded. The precipitate was dissolved in 50% MeCN, 0.05% TFA in H_2O, flash frozen in liquid N_2 and lyophilized to generate white to pale yellow amorphous powder, which was stored at – 20 °C.

Often, the free thiols were protected as ethylthio disulfide to increase the yield of CuAAC. Such protection was carried out in solution phase. HPLC purified peptide was dissolved in H_2O and about 10 equiv. of **34** (2-(ethyldisulfanyl)benzo[*d*]thiazole) dissolved in 20-50 µL of EtOH was added and the reaction was stirred at room temperature for 7 h. The reaction mixture was centrifuged and the supernatant was further purified by HPLC using methods described later.

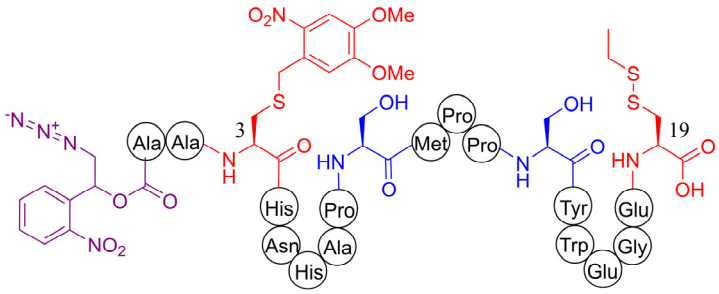
Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1260 Infinity system with a Phenomenex Luna C_{18}, a Waters Vydac C_{18}, or a Waters Vydac C_4 column with a flow rate of 1 mL/min and a solvent gradient of 2-100% solvent A over 45 min. Preparatory RP-HPLC was performed on a Waters 600 system with a Phenomenex Luna C_{18} or C_5 semi-preparative column using a gradient of 2% solvent A to 67% solvent A in 30 min. Any change from these standard HPLC conditions is noted in the procedures. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 µm nylon membrane filter prior to use. HPLC solvent compositions: solvent A was 80% acetonitrile in water with 0.086% trifluoroacetic acid (TFA), solvent B was 0.1% TFA in water.



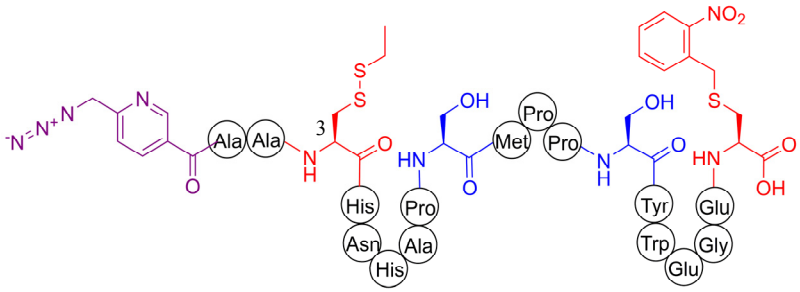
Peptide 2. H-Cys(Trt)-2-chlorotrityl resin (0.05 mmol) was used. All amino acids (0.25 mmol) were manually coupled using HCTU (0.25 mmol) as activator and DIPEA (0.5 mmol) as base, except for the deuterated Ser residues. All residues were double coupled (2 x 1 h coupling time) followed by capping procedure. Coupling of [2,3,3-^{2}H]-Ser was performed using PyBOP (0.25 mmol) as activator, HOAt (0.25 mmol) as racemization suppressant, and 2,4,6-collidine as base, with a 3 h coupling time. Post cleavage from the resin, 54 mg of crude peptide was obtained. Of the crude peptide, 25 mg was purified in 7 injections by preparative RP-HPLC using the standard gradient to elute **2** with a retention time (R_t) of 15.0 – 15.7 min (34% to 36% solvent A). Yield: 7.5 mg. MALDI-TOF (LR-MS) m/z calculated for $C_{83}H_{111}D_3N_{23}O_{26}S_3$ 1947.76 ($M+H^+$), observed 1947.69.



Peptide 3. Using similar chemistry as for peptide **2**, 53 mg of crude peptide was synthesized on a 0.05 mmol scale. Of the crude peptide, 22 mg was purified in 8 injections by RP-HPLC using the standard gradient to elute **3** with an R_t of 15.2 – 16.1 min (35% to 37% solvent A). Yield: 8 mg. MALDI-TOF (LR-MS) m/z calculated for $C_{83}H_{111}D_3N_{23}O_{26}S_3$ 1947.76 ($M+H^+$), observed 1947.69.

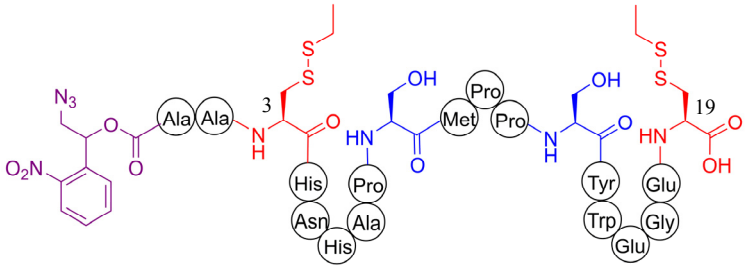


Peptide 47. The first residue Glu was coupled manually to H-Cys(Trt)-2-chlorotrityl resin using DIC/HOAt chemistry in the absence of base. Compound **38** was used to manually install Cys3 with 4,5-dimethoxy-2-nitrobenzyl protection using DIC/HOAt chemistry. All other residues were coupled using a CEM microwave synthesizer. The final coupling of the *N*-terminal azide-containing moiety was performed as follows. The peptide-bearing 2-chlorotrityl resin (0.05 mmol) was placed in a 5 mL round bottom flask. To the resin, a solution of compound **7** (45 mg, 0.13 mmol, 2.6 equiv.) in 2 mL of dry DMF was added, followed by 50 µL of DIPEA (0.22 mmol, 4.5 equiv) and the reaction was stirred overnight for 15 h. The solution was drained over a fritted funnel and the resin was washed with DMF (3 x 30 s) and DCM (3 x 30 s). Standard cleavage conditions yielded 5.5 mg of crude product, which was purified using a Phenomenex Luna C_{18} column using the standard gradient and flow-rate conditions as previously described to yield pure precursor peptide to **47** (1.1 mg) with a free Cys19. R_t 22.7 min, eluting at 52% of solvent A. The peptide was reacted with **34** and further purified by RP-HPLC using the standard conditions to generate **47** eluting with a R_t of 23.5 min to 25 min (53% solvent A). Yield: 0.25 mg. LR-MS (MALDI-TOF) calculated for $C_{109}H_{143}N_{30}O_{36}S_4$ 2575.9 ($M+H^+$), observed 2576.3.

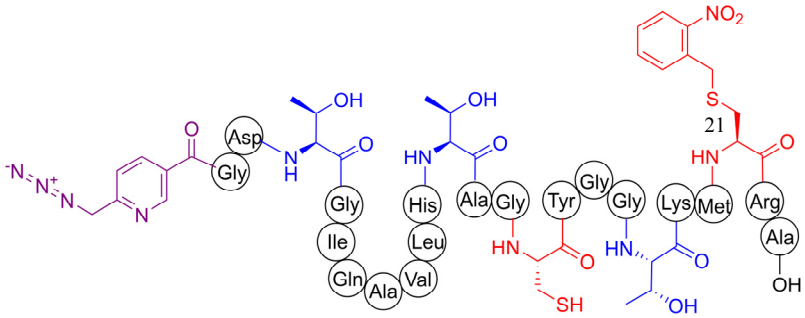


Peptide 48. Compound **36** (0.16 g, 0.33 mmol) was dissolved in 5 mL of dry DCM and 1 mL of dry DMF and this solution was added to 500 mg of 2-chlorotrityl choride resin (0.65 meq g^{-1}) pre-swelled in DMF. To this mixture, DIPEA (0.23 mL, 1.3 mmol) was added and the reaction was stirred for 14 h under N_2. The solution was drained through a fritted funnel and the resin was washed successively with 5 mL of DCM, MeOH and again DCM. Subsequent iterative Fmoc deprotection and amino acid coupling (DIC/HOBt chemistry) was carried out on 0.1 mmol of resin. Standard cleavage generated the precursor to **48** with a free thiol at Cys3. About 11 mg of crude peptide was purified by RP-HPLC using the standard conditions to generate pure precursor to **48** (3.6 mg), R_t: 20.2 min, eluted at 46% solvent A.

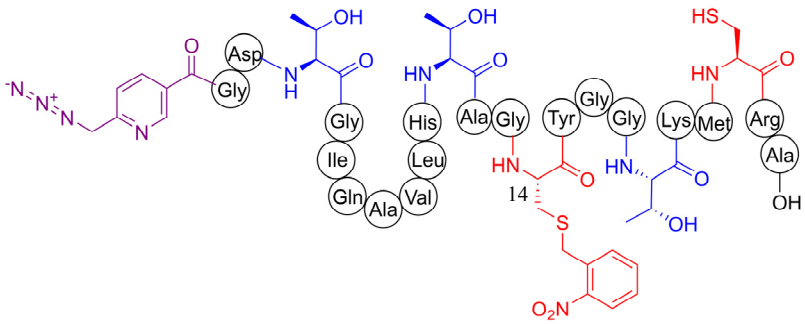
The free thiol at the Cys3 was protected by reaction with **34** and the obtained peptide was purified by HPLC using a Phenomenex Luna C_{18} column using the standard conditions to obtain pure **48** (2.2 mg), R_t: 23.5 min, eluting at 54% solvent A. LR-MS (MALDI-TOF) calculated for $C_{105}H_{137}N_{30}O_{31}S_4$ 2441.88, ($M+H^+$), observed 2441.94.



Peptide 49. H-Cys(Trt)-2-chlorotrityl resin was used. The first residue Glu was coupled manually to H-Cys(Trt)-2-chlorotrityl resin using DIC/HOAt chemistry in the absence of base. All residues except Cys3 were coupled on the microwave synthesizer using the standard conditions. Cys3 was coupled manually using diisopropylcarbodiimide (DIC) as activator and HOAt as racemization suppressant. Standard cleavage generated the precursor to **49** with a free thiol at Cys3 and Cys19. Crude peptide (30.6 mg) was purified by RP-HPLC using the standard conditions to elute precursor to **49** (7.4 mg, R_t 20.2 min, 46% solvent A). The precursor to **49** (4.2 mg) was reacted with **34** and the product was purified by RP-HPLC using the standard conditions to yield pure **49** (2.2 mg), R_t 23.8 min (54% solvent A). LR-MS (MALDI-TOF) calculated for $C_{102}H_{138}N_{29}O_{32}S_5$ 2440.88, ($M+H^+$), observed 2441.11.

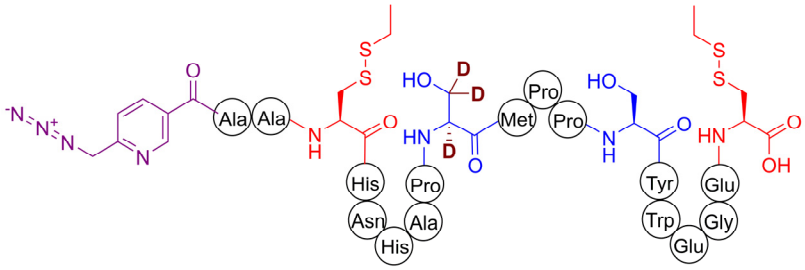


Peptide 50. Fmoc-Ala-Wang resin (0.1 mmol) was used. Each residue was triple coupled followed by capping on a microwave synthesizer except for the following. Cys21 with an o-nitrobenzyl protected thiol was installed by coupling **36** manually using HCTU as activator and DIPEA as base. Final coupling of 6-azidomethyl nicotinic acid (compound **8**) was performed manually using the same chemistry. After cleavage of the peptide from the resin, the yield of crude peptide was 103 mg. Of the crude peptide, 59 mg was loaded onto a Phenomenex C_{18} column in 5 injections and purified using the standard gradient to elude **50** with a R_t of 21.1 min (48% solvent A). Yield: 2.2 mg. MALDI-TOF (LR-MS) for $C_{109}H_{166}N_{35}O_{34}S_3$ calculated 2605.1 ($M+H^+$), observed 2605.1.

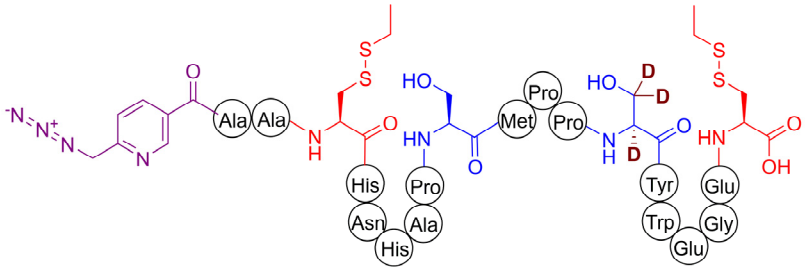


Peptide 51. Fmoc-Ala-Wang resin (0.05 mmol) was used. Each residue was triple coupled followed by capping on a microwave synthesizer except for the following. Coupling of **36** (0.2 mmol) was performed manually on 0.05 mmol of resin using 1-[bis(dimethylamino)methylene]-1*H*-

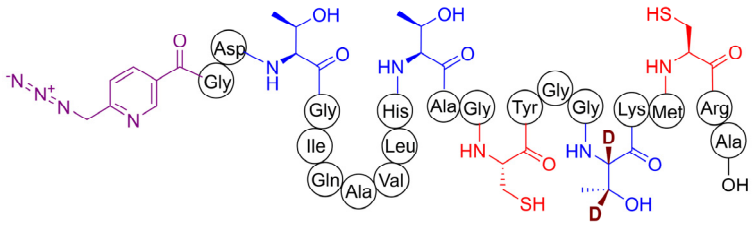
1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU, 0.2 mmol) as activator and DIPEA (0.4 mmol) as base, with 5 h coupling time. Final coupling of **8** (0.15 mmol) was performed manually using HATU (0.15 mmol) and DIPEA (0.3 mmol) for 4 h. After cleavage from the resin, 43 mg of crude peptide was obtained. The standard gradient was used to purify the crude peptide, with **51** eluting with a R_t of 21.5 min. Yield: 0.9 mg. MALDI-TOF MS for $C_{109}H_{166}N_{35}O_{34}S_3$ calculated 2605.1 $(M+H^+)$, observed 2604.7.



Peptide 52. H-Cys(Trt)-2-chlorotrityl resin (0.05 mmol) was used. Each residue was double coupled followed by capping on a CEM microwave synthesizer except for the following. Compound **42** (0.2 mmol) was coupled with PyAOP (0.2 mmol) as activator, HOAt (0.2 mmol) as racemization suppressant, and 2,4,6-collidine (0.4 mmol) as base in 5 mL of DMF with 3 h coupling time. Final coupling of **8** (0.15 mmol) was performed manually using HCTU (0.15 mmol) and DIPEA (0.3 mmol) for 3 h. Post cleavage from the resin, 3.2 mg of crude peptide was obtained. RP-HPLC using the standard gradient was used to purify the crude peptide, with the precursor to **52** with free thiols eluting with a R_t of 17.4 min (40% solvent A). Yield: 0.7 mg. This peptide (0.7 mg, 0.31 µmol) was dissolved in 0.4 mL of H_2O and stirred. Compound **34** (0.92 mg, 4.05 µmol) dissolved in 20 µL of EtOH was added to the peptide solution and the reaction mixture was stirred for 4 h. The reaction mixture was diluted with 3 mL of starting eluent for RP-HPLC (2% solvent A, 98% D) and then centrifuged (15000 x g, 5 min) and the supernatant was purified by HPLC in two injections using the standard conditions to elute **52** with a R_t of 21.1 min. Yield: 0.18 mg. MALDI-TOF MS calculated for $C_{100}H_{133}D_3N_{29}O_{29}S_5$ $(M+H)^+$ 2369.9, observed 2370.1.

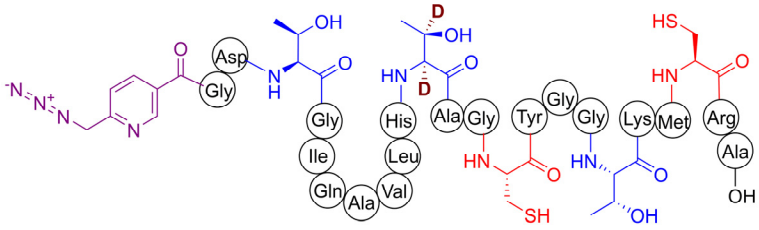


Peptide 53. Using similar chemistry as described for peptide **52**, this peptide was synthesized from 0.05 mmol resin to yield 9.8 mg of crude peptide, which was purified using the standard conditions to yield 1.4 mg of pure peptide with unprotected Cys3 and Cys19 (R_t 17.5 min, 40% solvent A). Compound **34** (2.22 mg, 9.8 µmol) dissolved in 40 µL of EtOH was added to the 5 mL of peptide solution (1.37 mg) and the reaction mixture was stirred for 12 h at room temperature. The reaction mixture was diluted with 3 mL of starting eluent for RP-HPLC (2% solvent A, 98% solvent D) and then centrifuged (15000 x g, 5 min) and the supernatant was purified by HPLC in two injections using the standard conditions to elute **53** with a R_t of 21.1 min. Yield: 0.17 mg. MALDI-TOF MS calculated for $C_{100}H_{133}D_3N_{29}O_{29}S_5$ $(M+H)^+$ 2369.9, observed 2370.2.



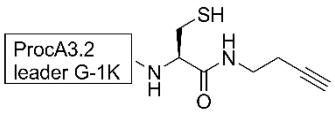
Peptide 54. Each residue was triple coupled followed by capping on a microwave synthesizer except for coupling [2,3-^{2}H]-Thr. Coupling of **46** was performed manually using PyBOP as

activator, HOAt as racemization suppressant and 2,4,6-collidine as base. Final coupling of **8** was performed using HCTU/DIPEA chemistry. Post cleavage from the resin, 41 mg of crude peptide was obtained, which was purified using a C_{18} Phenomenex column using the standard conditions to elute target peptide with a R_t of 19.4 min (44% solvent A). Yield: 1.7 mg. MALDI-TOF (LR-MS), m/z calculated for $C_{102}H_{159}D_2N_{34}O_{32}S_3$ 2472.12, observed 2471.97.



Peptide 55. Using similar chemistry as for the synthesis of peptide **54**, peptide **55** was prepared. Post cleavage from the resin, 37.7 mg of crude peptide was obtained, which was purified using a C_{18} Phenomenex column using the standard conditions in four injections, with **55** eluting at a R_t of 19.4 min (44% solvent A). Yield: 0.61 mg. MALDI-TOF (LR-MS), m/z calculated for $C_{102}H_{159}D_2N_{34}O_{32}S_3$ ($M+H^+$) 2472.12, observed 2472.06.

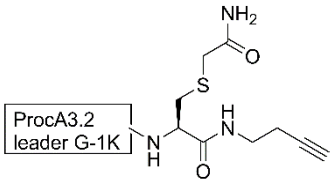
Generation of the Alkyne Modified ProcA3.2 Leader Peptide



Peptide 56. An aliquot of BL21 (DE3) *E. coli* cells was transformed with pET15b plasmid encoding His_6-ProcA3.2-leader-intein-CBD (CBD: chitin binding domain) and then the transformed cells were spread on a LB + ampicillin (100 µg/mL) agar plate and incubated overnight at 37 °C. Cells from a single colony were used to innoculate two 25 mL LB + Amp (100 µg/mL) cultures, which were incubated overnight. The starter cultures were used to innoculate 2 x 1.5 L cultures in LB + Amp (100 µg/mL) and the cells were grown until the $OD_{600\ nm}$ for each set was ca. 0.6 when IPTG (final concentration 0.5 mM) was added and the cultures were incubated in a 18 °C shaker overnight (final $OD_{600\ nm}$ 2.5-3.0). The cells were harvested by centrifugation (10,500 x g, 30 min). The cell pellets were suspended in 40 mL of lysis buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 0.1 % Triton-X, PMSF (dissolved in 20 mL of isopropanol, final concentration ca. 1 mM), TCEP-HCl (1 mM, pH- 7.5) and the cells were lysed by sonication (4.4 s pulse, 9.9 s interval, 35% amplitude). The cell lysate was centrifuged (22800 x g, 30 min) and the supernatant was filtered through 0.45 µm amicon filters. The chitin resin (NEB) was loaded into a column and equilibrated with column buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA). To the resin, the cell lysate was applied and the column was shaken in the cold room (4 °C) on a rocker for 4 h to allow proper mixing. The cell lysate was allowed to drain from the resin at 2 mL/min. The resin was washed further with column buffer (ca. 400 mL at 2 mL/min). Cleavage buffer (40 mL) consisted of column buffer containing MESNa (50 mM), TCEP-HCl (5 mM), compound **27** (60 mg, ca. 2 mM), and the pH was adjusted to 7.5. The cleavage buffer was added to the resin and the column was rocked for 16 h, after which the buffer was eluted. The resin was washed with 20 mL of column buffer and the washing along with the eluted cleavage buffer was concentrated on 3000 Da MWCO Amicon filter (4000 x g, 45 min) and the concentrated peptide was desalted on a C_4 SPE column and lyophilized. Yield: 45 mg. LR-MS (MALDI-TOF) calculated for $C_{384}H_{610}N_{121}O_{128}S_2$ 9028.4 ($M+H^+$), observed 9031. His-tag-ProcA3.2 G–1K sequence:

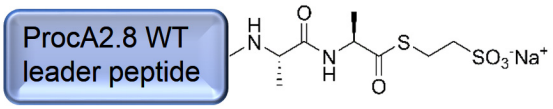
GSSHHHHHHSSGLVPRGSHMSEEQLKAFIAKVQADASLQEQLRTEGADVVAIAKAAGFSITTED
LNSHRQNLSDDELEGVAGK

Iodoacetamide capping of the free Cys generated during EPL



Peptide 57. Peptide **56** (41.8 mg, 4.6 μmol) was dissolved in 2 mL of 100 mM NH_4HCO_3 buffer, pH 8.1. TCEP-HCl (7.2 mg, 25.3 μmol, 5.4 equiv.) was dissolved in 1 mL of 100 mM NH_4HCO_3 buffer and was added to the peptide solution. Iodoacetamide (7.5 mg, 40.5 μmol, 8.7 equiv.) dissolved in 1 mL of H_2O was added to the reaction mixture and the solution was stirred at room temperature for 16 h. The 4 mL of reaction mixture was diluted in 14 mL of 2% solvent A (80% MeCN in 0.086% TFA) and 98% solvent D (0.1% aqueous TFA) and purified using a Phenomenex Luna C_5 column in Waters Delta 600 HPLC purification system using a flow rate of 7 mL/min. A gradient of 2% A to 67% solvent A in 30 min was employed and **57** eluted with a R_t between 21.4 min – 23.6 min. Yield: 28 mg. LR-MS (MALDI-TOF) calculated for $C_{386}H_{613}N_{122}O_{129}S_2$ 9085.45 ($M+H^+$), observed 9089.

Generation of ProcA2.8 WT Leader-AlaAla-MESNa Thioester



Peptide 1. *E.coli* BL21(DE3) cells were transformed with pTXB1 plasmid encoding His_6-tag-ProcA2.8 leader-AlaAla-intein-chitin binding domain, and the cells were spread on a LB + Ampicillin (100 μg/mL) agar plate and incubated overnight at 37 °C. Cells from a single colony were used to innoculate two 25 mL LB + Amp (100 μg/mL) starter cultures, which were incubated overnight. The starter culture was used to inoculate 5 L (2 x 1.5 L, and 2 x 1 L) LB + Amp (100 μg/mL) medium. The cultures were shaken at 37 °C to an OD_{600nm} of ca. 0.6. After cooling the cultures to room temperature, IPTG (final concentration: 0.5 mM) was added to induce the cells and the cells were shaken at 18 °C for 17 h. The cells were harvested by centrifugation (10,500 x g, 20 min) and frozen at – 80 °C until further use. The cell pellet (from 2.5 L culture) was thawed on ice and 30 mL of lysis buffer [NaP_i (50 mM, pH 7.0), NaCl (500 mM), and TCEP (1 mM)] was added and the cells were homogenized. Cells were then lysed by sonication (4.4 sec pulse, 9.9 sec interval, 35% amplitude) on ice. The cells were centrifuged (22,800 x g, 30 min) and the supernatant was filtered through a 0.45 μm filter. The filtered supernatant was loaded onto a column packed with chitin resin (25 mL) equilibrated with column buffer [NaP_i (50 mM, pH 7.0), NaCl (500 mM)]. The column was shaken on a rocker in a cold room (4 °C) for 2 h. The slurry was drained (1 mL/min) and the column was then washed with 250 mL (~10 CV) of column buffer. To the resin, 30 mL of cleavage buffer (column buffer + 50 mM MESNa) was applied and the column was shaken overnight on a rocker at 4 °C. The eluent was collected (~35 mL) and acidified to 0.1 % TFA to lower the pH (~4), desalted on a C_4 SPE column and eluted with 80% MeCN, 0.1% TFA in H_2O and lyophilized. The lyophilized peptide was dissolved in 15 mL of starting eluent: 2% solvent A (80% MeCN, 0.086% TFA in H_2O), 98% solvent D (0.1%

TFA in H_2O) and then purified in 5 injections using a C_5-Phenomenex semi-prep column using a flow-rate of 7 mL/min. Elution gradient: 2% A to 67% solvent A over 30 min. Product eluted with a R_t of 21.9 min - 22.9 min (50% - 52% solvent A). Yield: 9 mg (from 2.5 L culture). MALDI-TOF MS calculated 9117 ($M+H^+$), observed 9119.

Peptide sequence of His-tag-ProcA2.8-AlaAla:

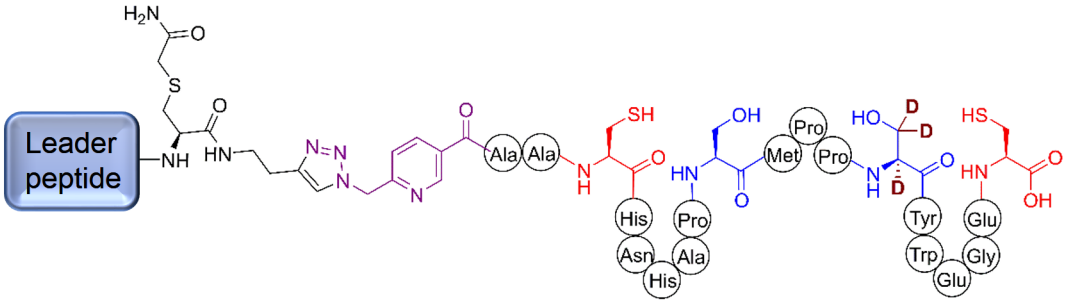
GSSHHHHHHSSGLVPRGSHMSEEQLKAFLTKVQADTSLQEQLKIEGADVVAIAKAAGFSITTED LNSHRQNLSDDELEGVAGGAA

Copper Catalyzed Azide–Alkyne Cycloaddition (CuAAC) - 'Click Chemistry'

Optimized methodology

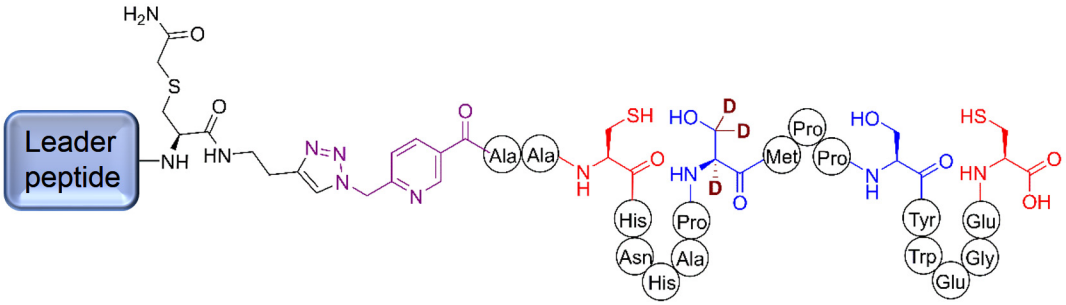
Equivalent amounts of the two reacting peptide partners were dissolved in H_2O or 50% aqueous MeCN (if not completely soluble in H_2O), frozen with liquid N_2 and lyophilized. The lyophilized mixture of two peptides was dissolved in 30 mM aqueous degassed phosphate buffer, pH 7.8 to a concentration of about 1 to 2 mM for each peptide. Activated catalyst solution was prepared by mixing 5 µL of 100 mM $CuSO_4$ in degassed H_2O, 25 µL of 20 mM TBTA in MeOH, 10 µL of 200 mM sodium ascorbate in degassed H_2O and stirred under N_2 for 5 min. The activated catalyst solution (40 µL) was added to the peptide solution (60 µL) and the reaction was stirred for 1 h. The final concentrations in the reaction mixture were 5 mM $CuSO_4$, 5 mM TBTA, and 20 mM sodium ascorbate. Generally completion of the reaction was achieved in 1 h, as observed by MS (MALDI-TOF). If after 1 h, a significant fraction of starting peptide remained, further addition of activated catalyst followed by stirring at room temperature under N_2 was performed. The crude reaction mixture was desalted using a C_4 solid phase extraction (SPE) column and lyophilized.

The thioethyl protection group was removed from Cys by dissolving the semi-synthetic precursor in 3 mM TCEP-HCl in 2 mM Tris buffer, pH 7.5, and stirring at room temperature for 3 to 5 h, unless otherwise noted. The reaction mixture was purified using a Phenomenex Luna C_5 column using a gradient of 2% to 67% solvent A in 30 min with a flow rate of 7 mL/min using a Waters HPLC instrument to generate the precursor peptide with one free Cys.

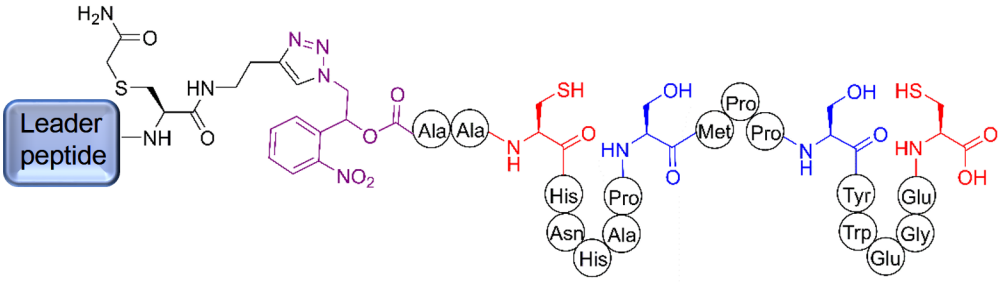


Peptide 9. Peptide **57** (0.63 mg, 0.07 µmol) and peptide **53** (0.17 mg, 0.07 µmol) were mixed and to the reaction was added four batches of 40 µL of freshly prepared catalyst solution with an interval of one hour between each addition. The crude reaction mixture was desalted using a C4 SPE column to yield peptide precursor to **9** with thioethyl protected Cys residues. The peptide was reduced with TCEP and purified

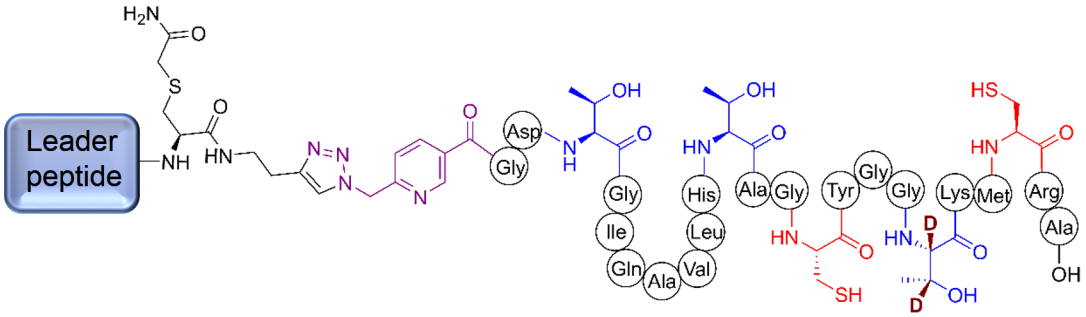
by RP-HPLC using the standard conditions to generate peptide **9** (0.2 mg, 26%). MALDI-TOF MS calculated 11336 ($M+H^+$), observed 11339.



Peptide **10**. Peptide **57** (0.46 mg, 0.05 µmol) and peptide **52** (0.13 mg, 0.05 µmol) were mixed and to the reaction was added three batches of 40 µL of freshly prepared catalyst solution with an interval of 1 h between additions. The product was reduced with TCEP as described in the section on General Methodology and further purified by RP-HPLC using a C_5 Phenomenex column. The gradient was 2% to 100% solvent A over 45 min with a flow rate of 8 mL/min. Yield: 0.15 mg (27%). MALDI-TOF MS calculated 11336 ($M+H^+$), observed 11338.

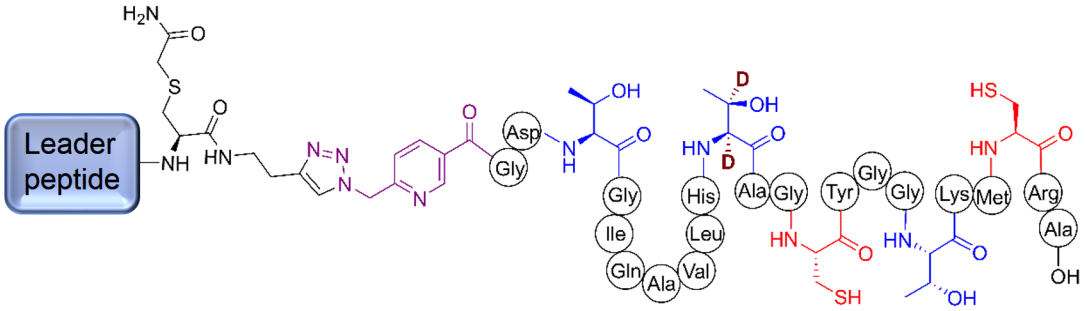


Peptide **11**. Peptide **57** (0.95 mg, 0.1 µmol) and peptide **49** (0.37 mg, 0.15 µmol) were mixed and to the reaction was added two batches of 40 µL of freshly prepared catalyst solution with an interval of one hour. The reaction was stirred for 2 h after the second addition of catalyst solution. The reaction mixture was desalted using a C_4 SPE column to yield peptide precursor with thioethyl protected Cys residues, which was reduced with TCEP and purified by RP-HPLC using a C_5 Phenomenex column, the standard gradient, and a 8 mL/min flow rate. The product **11** eluted with a R_t of 21.4 min. MALDI-TOF MS calculated 11410, observed 11396. Yield: 0.6 mg (50 %).

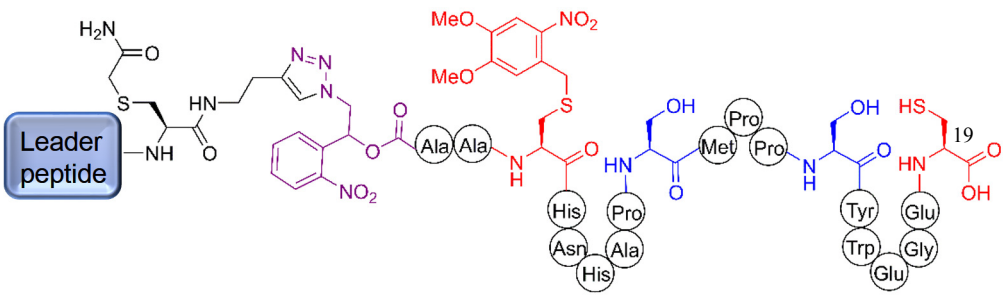


Peptide **12**. Peptide **57** (0.73 mg, 0.08 µmol) and peptide **54** (0.24 mg, 0.1 µmol) were mixed and to the reaction was added three batches of 40 µL of freshly prepared catalyst solution with an interval of 1 h after each

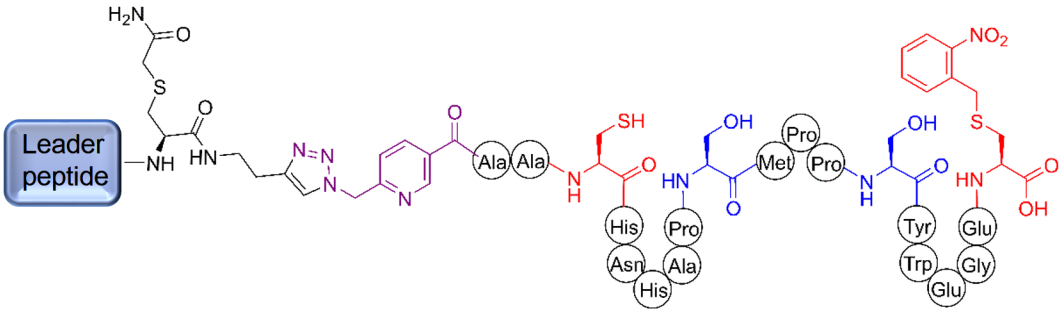
addition. The reaction mixture was desalted, reduced with TCEP and purified by RP-HPLC using a C_5 Phenomenex column using a gradient of 2% to 100% solvent A over 45 min with a flow rate of 8 mL/min. The product eluted with a R_t of 21 min, 48% solvent A. Yield: 0.42 mg (45%)



Peptide 13. Peptide **57** (0.81 mg, 0.09 µmol) and peptide **55** (0.25 mg, 0.1 µmol) were mixed and to the reaction was added three batches of 40 µL of freshly prepared catalyst solution with an interval of one hour between additions. The reaction was stirred for another 2 h after the third addition of catalyst solution. The reaction mixture was desalted, reduced with TCEP and purified by RP-HPLC using a C_5 Phenomenex column using a gradient of 2% to 100% solvent A over 45 min with a flow rate of 8 mL/min. The product **25** eluted with a R_t of 21.1 min (48% solvent A). Yield: 0.56 mg (54%).

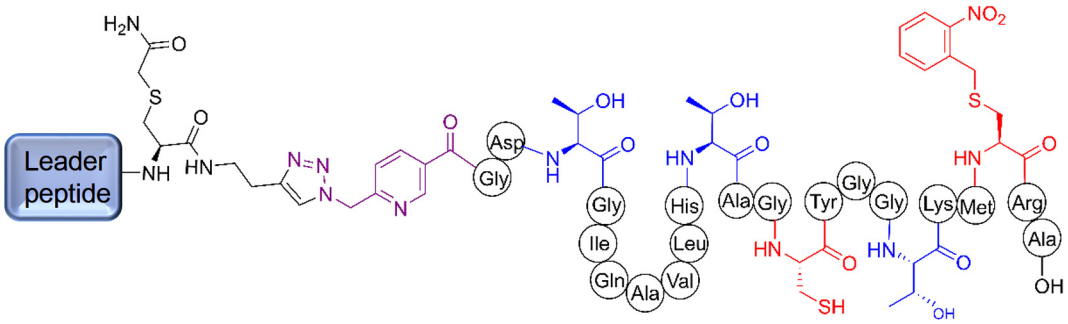


Peptide 14. Peptides **57** (440 µg, 0.05 µmol) and **47** (130 µg, 0.05 µmol) were mixed together and two batches of 40 µL of catalyst solution were added. The reaction mixture was desalted using a C_4 SPE column and lyophilized to generate precursor to **14** with thioethyl protected Cys19 (550 µg). The product was reduced with TCEP as described in General Methodology and further purified by RP-HPLC using the standard gradient to yield pure **14** (430 µg, 76%).

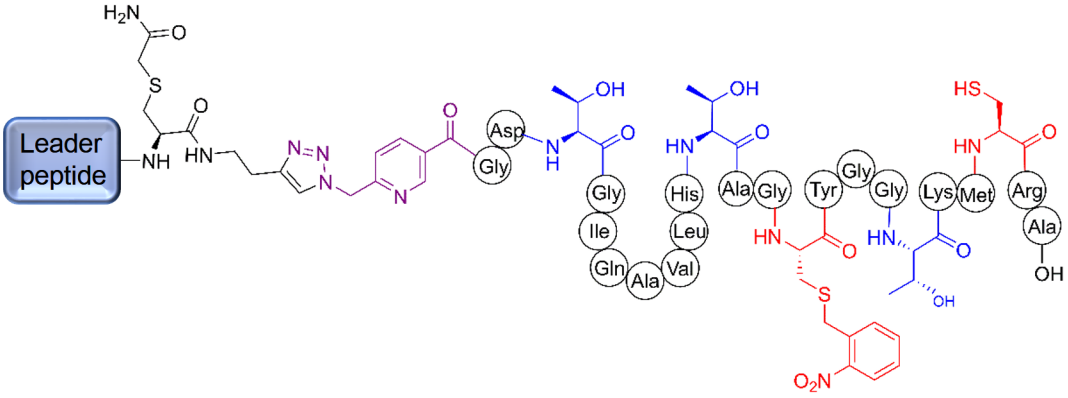


Peptide 15. Peptide **57** (925 µg, 0.1 µmol) was mixed with **48** (320 µg, 0.13 µmol) and the two batches of 40 µL of catalyst solution were added. The reaction mixture was desalted using a C_4 SPE column and lyophilized to yield crude precursor to **15** with thioethyl protected Cys3 (1.1 mg). MS (MALDI-TOF) indicated complete

conversion to product. The crude precursor to **15** was reduced with TCEP followed by RP-HPLC purification with a C_5 Phenomenex Luna column using the standard conditions to yield pure **15** (0.64 mg, 55%). R_t 20.8 min, eluting at 47% solvent A. LR-MS (MALDI-TOF) calculated 11466 ($M+H^+$), observed 11472.

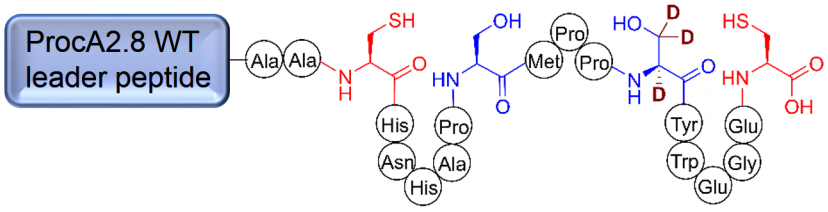


Peptide **16**. Peptide **57** (0.79 mg, 0.09 µmol) and peptide **50** (0.3 mg, 0.11 µmol) were mixed and three batches of 40 µL of freshly prepared catalyst solution were added with an interval of one hour between additions. The reaction mixture was desalted using a C_4 SPE column and lyophilized, reduced with TCEP and purified by RP-HPLC. The gradient was 2% to 100% solvent A over 45 min with a flow rate of 8 mL/min. The product **16** eluted with an R_t of 21.5 min. MALDI-TOF MS calculated 11694, observed 11693. Yield: 0.33 mg (33%).



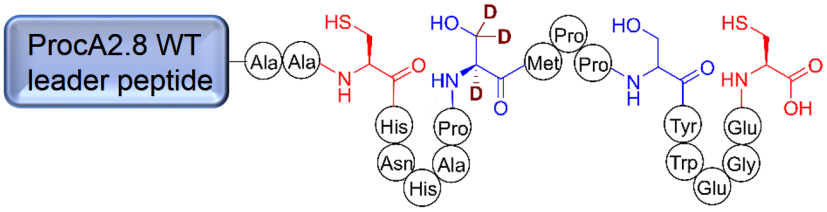
Peptide **17**. Peptide **57** (0.84 mg, 0.09 µmol) and peptide **51** (0.36 mg, 0.14 µmol) were mixed and three batches of 40 µL of freshly prepared catalyst solution were added with an interval of one hour between additions. The reaction was stirred for 2.5 h after the third addition of catalyst solution. The reaction mixture was desalted using a C_4 SPE column to yield peptide precursor with thioethyl protected Cys, which was reduced with TCEP and purified by RP-HPLC using a C_5 Phenomenex column using the standard gradient and a 8 mL/min flow rate. The product **7** eluted with a R_t of 21.4 min. MALDI-TOF MS calculated 11694, observed 11694. Yield: 0.52 mg (49%).

Peptides Generated by Native Chemical Ligation



Peptide **4**. Native chemical reaction was performed in a reaction volume of 500 µL. The final concentration of components are given in parenthesis. In an eppendorf tube, 250 µL of a 1

mM solution of peptide **2** (0.5 mM) was mixed with 25 µL of 20 mM TCEP (1 mM), 125 µL of 1 M NaP_i, pH 7.2 (250 mM), 50 µL of 1 M MESNa (100 mM), 25 µL of 10 mM EDTA (0.5 mM), and finally 25 µL of a 1 mM solution of peptide **1** (0.05 mM). The reaction was stirred at room temperature for 20 h when MALDI-TOF MS of crude product indicated the consumption of peptide **1**. The reaction mixture was acidified with TFA to pH ~ 6.8 and purified using an analytical HPLC column (C_4 Vydac) using a gradient of 2% solvent A (80% MeCN, 0.086% TFA in H_2O), 98% solvent D (0.1% TFA in H_2O) to 80% solvent A over 45 min using a flow rate of 1 mL/min. The product **4** eluted with a R_t of 28.2 min (50% solvent A), while starting synthetic peptide eluted earlier (R_t of 18 min, 32% solvent A). Yield: 0.13 mg (48%). MALDI-TOF MS calculated 10918 ($M+H^+$), observed 10921 ($M+H^+$), 11099 (phosphogluconylation of His-tag).



Peptide **5**. Native chemical reaction was performed in a total reaction volume of 500 µL. The final concentrations of components are given in parenthesis. In an eppendorf tube, 250 µL of 1 mM solution of peptide **3** (0.5 mM) was mixed with 25 µL of 20 mM TCEP (1 mM), 125 µL of 1 M NaP_i, pH 7.2 (250 mM), 50 µL of 1 M MESNa (100 mM), 25 µL of 10 mM EDTA (0.5 mM), and finally 25 µL of 1 mM solution of peptide **1** (0.05 mM). The reaction was stirred at room temperature for 20 h when MALDI-TOF MS analysis indicated consumption of peptide **1**. The reaction mixture was acidified with TFA to pH ~ 6.8 and purified using an analytical HPLC column (C_4 Vydac) using a gradient of 2% solvent A (80% MeCN, 0.086% TFA in H_2O), 98% solvent D (0.1% TFA in H_2O) to 80% solvent A in 45 min using a flow-rate of 1 mL/min. The product **5** eluted with a R_t of 28.2 min (50% solvent A), while starting synthetic peptide eluted earlier (R_t of 18 min, 32% solvent A). Yield: 0.1 mg (37%). MALDI-TOF MS calculated 10918 ($M+H^+$), observed 10925 ($M+H^+$), 11104 (phosphogluconylation of His-tag).

Purification of ProcM

The enzyme was overexpressed as reported earlier, with the exception that ProcM gene was cloned in a pRSFDuet vector instead of pET28b vector as originally reported.[9] After IMAC purification, ProcM was was further purified by size-exclusion chromatography (Superdex 200 resin, 120 mL column volume) using an FPLC (Akta P-920) and was obtained as a mixture of monomer and dimer/trimer; when separated, both were active. A brief procedure for ProcM purification is as follows. All steps were carried out in the cold room (4 °C) or on an ice-bath. Crude cell lysate (from 3 L culture) was suspended in 50 mL of ProcM start buffer (1 M NaCl, 20 mM Tris, pH 8.0), along with protease inhibitor (Roche cOmplete), and lysed by passing through a French-press and centrifuged (14,000 x g, 30 min). The supernatant was filtered through 0.45 µm syringe filters and loaded onto a Ni-HiTrap column equilibrated with 4 column volumes (CV) of ProcM start buffer. The column was washed by 6 CV of ProcM wash buffer (1 M NaCl, 20 mM Tris, pH 8.0, 30 mM imidazole) and then the column was attached to the FPLC and the protein was eluted using wash buffer and elute buffer (EB: 1 M NaCl, 20

mM Tris, pH-8.0, 200 mM imidazole). The following gradient was applied: 6 CV of 5% EB, then 6 to 10 CV of 5% to 40% EB, 10 to 12 CV: 40% to 100% EB, 12 to 18 CV: 100% EB. The fractions containing the protein were 8 CV to 14 CV, as monitored by absorbance at 280 nm. The purified protein was concentrated using an Amicon ultrafilter (50 kDa cut-off) and desalted/purified by gel-filtration on FPLC (using ProcM start buffer (1 M NaCl, 20 mM Tris, pH-8.0) at a flow rate of 1 mL/min. The aggregated protein (40 mL to 55 mL elution volume) was discarded and the monomer and oligomer fractions (60 mL to 85 mL elution volume) were collected and concentrated using 30 kDa cut-off Amicon centrifugation filters to a concentration of ca. 60 μM to 250 μM, depending on the batch.

Purification of ProcM in D_2O Containing Buffer

The monomer-oligomer mixture obtained from size-exclusion chromatography (15 mL), as mentioned in the previous section, was concentrated to 1 mL by ultrafiltration (2,300 x g), and then 10 mL of 1 M NaCl, 50 mM HEPES, pH 8.0 in D_2O was added and the protein was again concentrated to 1 mL by centrifugation. Further addition of 10 mL of 1 M NaCl, 50 mM HEPES, pH 8.0 in D_2O to the concentrated protein solution followed by ultrafiltration to generated a stock of 84 μM ProcM (10 mg/mL, D_2O:H_2O over 99:1). The solution was aliquoted in fractions of 25 μL.

General Procedure for Heterologous Expression of Precursor Peptides

ProcA2.8, ProcA3.3, ProcA2.8-S9T, ProcA2.8-S13T, and ProcA3.3-T11S were generated by the following general methodology.

E. coli BL21 cells were transformed with pET-15b plasmid encoding the desired gene and the transformed cells were spread on an LB + Amp (100 μg/ mL) agar plate and the plate was incubated overnight at 37 °C. Cells from single colonies were used to inoculate starter cultures (2 x 25 mL) in LB + Amp (100 μg/mL) and incubated at 37 °C in a shaker for 16 h. The starter cultures were centrifuged (4,300 x g, 10 min) and the LB media was replaced with fresh LB and was used to inoculate a large scale culture (2 x 1.5 L) of LB + Amp (100 μg/mL). The flasks were incubated at 37 °C with shaking until an $OD_{600\ nm}$ of 0.6 was reached (typically 3-4 h). The culture was induced with IPTG (0.5 mM final concentration) and further incubated at 37 °C in a shaker for 3 h when a final $OD_{600\ nm}$ in the range of 1.0 - 2.5 was reached. The cells were harvested by centrifugation (10,500 x g, 15 min), flash frozen and stored at −80 °C for future use. The cells were thawed on ice and suspended in 30 mL of LanA start buffer (20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and lysed by sonication (4.4 s pulse, 9.9 s off, 35% amplitude, pulse time 6 min). The cell lysate was centrifuged (22,800 x g, 30 min) and the supernatant, referred as 'soluble fraction' was filtered through a 0.45 μm filter. The cell residue was resuspended in 25 mL of Lan buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole), sonicated, and centrifuged as described above. The supernatant referred to as 'insoluble fraction' was filtered through a 0.45 μm filter. A 5 mL Ni-His trap column charged with $NiSO_4$ was equilibrated with two column volumes (CV) of LanA start buffer after which the 'soluble fraction' was loaded at flow rate of 2 mL/min. The column was then equilibrated with two CV of Lan buffer 1 and loaded with the 'insoluble fraction' at 2 mL/min. The column was washed with two CV of Lan buffer 1 and two CV of Lan buffer 2 (4 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 300 mM NaCl, 30 mM imidazole) and eluted with

LanA elution buffer (4 M guanidine hydrochloride, 20 mM Tris, pH 7.5 at 25 °C, 100 mM NaCl, 1 M imidazole) where 10 mL of the elution fraction was collected as fraction 1 and 5 mL was collected as fraction 2. Both fractions were desalted using C_4 SPE columns and lyophilized.

ProcA2.8 precursor peptide: The desalted peptide was injected onto a Phenomenex Luna C_5 column and eluted with a gradient of 2% to 67% solvent A in 30 min, and a flow rate of 7 mL/min. R_t 22 - 24.5 min. 13.3 mg of crude peptide yielded 10.8 mg of purified peptide. MALDI-TOF MS calculated 10,915 ($M+H^+$), observed 10,917. Peptide sequence:
GSSHHHHHHSSGLVPRGSHMSEEQLKAFLTKVQADTSLQEQLKIEGADVVAIAKAAGFSITTED
LNSHRQNLSDDELEGVAGGAACHNHAPSMPPSYWEGEC

ProcA3.3 precursor peptide: The desalted peptide was purified using a Phenomenex Luna C_5 column with a gradient of 2% to 65% solvent A over 65 min with a flow rate of 7 mL/min. The fraction eluting with a R_t of 44.8 min (46% solvent A) was pure product. MALDI-TOF MS calculated 10,934 ($M+H^+$), observed 10,927.
Peptide sequence:
GSSHHHHHHSSGLVPRGSHMSEEQLKAFIAKVQGDSSLQEQLKAEGADVVAIAKAAGFTIKQQ
DLNAAASELSDEELEAASGGGDTGIQAVLHTAGCYGGTKMCRA

ProcA2.8-S13T precursor peptide: Desalted peptide was used without further purification. MALDI-TOF MS calculated 10,936 ($M+H^+$), observed 10,934. Yield: 50 mg from 2 L of culture.
Peptide sequence (mutation underlined):
GSSHHHHHHSSGLVPRGSHMSEEQLKAFLTKVQADTSLQEQLKIEGADVVAIAKAAGFSITTED
LNSHRQNLSDDELEGVAGGAACHNHAPSMPP**T**YWEGEC

ProcA2.8-S9T precursor peptide: Desalted peptide was used without further purification. MALDI-TOF MS calculated 10,936 ($M+H^+$), observed 10,932. Yield: 49 mg from 1.5 L of culture.
Peptide sequence (mutation underlined):
GSSHHHHHHSSGLVPRGSHMSEEQLKAFLTKVQADTSLQEQLKIEGADVVAIAKAAGFSITTED
LNSHRQNLSDDELEGVAGGAACHNHAP**T**MPPSYWEGEC

ProcA3.3-T11S precursor peptide: Desalted peptide was used without further purification. MALDI-TOF MS calculated 10,920 ($M+H^+$), observed 10,920. Yield: 57 mg from 1.5 L of culture.
Peptide sequence (mutation underlined):
GSSHHHHHHSSGLVPRGSHMSEEQLKAFIAKVQGDSSLQEQLKAEGADVVAIAKAAGFTIKQQ
DLNAAASELSDEELEAASGGGDTGIQAVLH**S**AGCYGGTKMCRA

General Procedure for Selective Thioether Formation by ProcM

Substrates with one Cys protected with an o-nitrobenzyl group was treated with ProcM under the following typical conditions (any changes from these standard conditions are noted in the individual procedures). The assays were carried out in 200 μL scale containing HEPES (50 mM, pH 8.0), TCEP (0.2 mM), ATP (10 mM), $MgCl_2$ (10 mM), ProcM (20 μM), substrate (50 μM), and allowed to react at

room temperature for 16-20 h. ProcM was removed from the assay mixtures by ultrafiltration through 50 kDa molecular weight cut-off filters (Amicon) by centrifugation (14,000 x g, 10 min). To improve recovery of processed peptide, water (50 μL) was added to the retentate, and the solution was filtered by centrifugation (14,000 x g, 10 min). This process was repeated twice. The combined filtrates were collected and desalted on a C_4 solid phase extraction column (Vydac BioSelect). The SPE column was first wetted with 2 column volumes (CV) of 80% MeCN in H_2O. The column was washed with 0.1% trifluoroacetic acid (TFA) in H_2O (3 CV) and then the assay product was diluted with 0.1 % TFA in H_2O (1:1) and passed through the column. The column was further washed with 0.1 % TFA in H_2O (3 CV) and finally the product was eluted with 80% MeCN in H_2O (2 x 1 mL). The eluents were lyophilized to yield the processed peptide with selectively formed thioether rings and one o-nitrobenzyl protected cysteine.

The lyophilized peptide obtained from the previous step was dissolved in 0.1% formic acid in H_2O and subjected to UV (365 nm) irradiation for 4 h, using a portable UV lamp source (Spectroline ENF-240C). After irradiation, the solution was lyophilized.

General Procedure for Probing Enzymatic vs. Non-Enzymatic Cyclization

Non-enzymatic cyclization: The peptide obtained from the previous step was incubated at room temperature in solution containing all components used in standard ProcM assay except the enzyme. A typical non-enzymatic assay solution comprised HEPES (50 mM), ATP (10 mM), TCEP (0.2 mM), $MgCl_2$ (10 mM), and substrate (ca. 50 μM) with a final pH of 8.0. A second assay was conducted at higher pH 8.5, and assays were kept at room temperature for 16 h.

Enzymatic cyclization: Control assays of the substrate generated in the previous step in the presence of ProcM (20 μM) were also set up under identical conditions and incubated at room temperature for 16 h. The enzymatic and the non-enzymatic cyclization assays were carried out and analyzed in parallel.

To probe the extent of cyclization of the second thioether ring, iodoacetamide (IAA) assays were performed on the products of the non-enzymatic and the enzymatic assays. The pH of the assay solution was raised to 8.5 by the addition of NH_4HCO_3 buffer (100 mM), TCEP (0.5 mM) and excess IAA (ca. 20 mM) was added, and the assay was incubated at room temperature for 3 h, before analysis by MALDI-TOF MS.

General Procedure for the Incorporation of Deuterium in the ProcM Product

ProcM assays were conducted at room temperature for 20 h with the following compositions in D_2O: HEPES (50 mM, pH 8.0), TCEP (0.5 mM), ATP (10 mM), $MgCl_2$ (10 mM), ProcM (10 μM), and (50 μM) substrate. ProcM was removed by centrifugation through Amicon 50 kDa MWCO filters (14,000 x g, 20 min), and the flow through was desalted using C_4 SPE column and lyophilized. MALDI-TOF analysis demonstrated the incorporation of one deuterium per Lan/MeLan.

General Procedure for D-H Exchange Assays

Each lyophilized deuterium labeled peptide was dissolved in H_2O and ProcM assays were conducted at room temperature for 20 h in aqueous solution with the following composition: HEPES (50 mM, pH 8.0), TCEP (0.5 mM), ATP (10 mM), $MgCl_2$ (10 mM), ProcM (10 μM), and substrate (50 μM). Control assays in which the substrates were treated with all components except ProcM were also performed. MALDI-TOF MS analysis was carried out after digesting the peptide with GluC by adding 1 μL of GluC (20 μg/μL) or 1 μL of LysC (30 U/mL) to 20 μL of assay mixture. Simulation of extent of exchange was carried out using mmass software.[10]

General Procedure for Trapping Partially Dehydrated Peptides Containing Deuterium Labeled Ser/Thr to Probe Directionality of Dehydration

Substrates assembled by CuAAC with deuterium labeled Ser/Thr were treated with ProcM under the following conditions unless otherwise noted. A typical assay volume of 20 μL contained HEPES (50 mM, pH 8.0), ATP (10 mM), TCEP (0.5 mM), $MgCl_2$ (10 mM), ProcM (2 μM), and substrate (50 μM). The assays were kept at room temperature and after each time-point, one assay sample was filtered through 50 kDa cut-off filters by centrifugation to remove ProcM (14,000 x g, 10 min). The chosen time-points were 10 min, 30 min, and 1 h. An assay with higher ProcM concentration (10 μM), and longer incubation (9 h) was also performed.

Substrates assembled by expressed protein ligation (EPL) with deuterium labeled Ser/Thr were treated with ProcM under the following conditions unless otherwise noted. A typical assay volume of 20 μL contained HEPES (50 mM, pH 8.0), ATP (10 mM), TCEP (0.5 mM), $MgCl_2$ (10 mM), ProcM (2 μM), and substrate (100 μM). The assays were kept at room temperature and after each time-point, one assay sample was filtered through a 50 kDa cut-off filter by centrifugation (14,000 x g, 10 min). The chosen time-points were 5 min, 10 min, and 30 min.

The filtered peptides were digested with endo-proteinase GluC (1 μg/μL) or LysC (0.3 U/μL), as noted, and then analyzed by MALDI-TOF MS and ESI-MS.

General Procedure for Trapping Partially Cyclized Species to Probe Directionality of Cyclization

ProcA precursor peptides were modified by ProcM at room temperature in solutions containing HEPES (50 mM, pH 8.0), ATP (10 mM), TCEP (1.0 mM), $MgCl_2$ (10 mM), ProcM (5 μM), and substrate (100 μM). After each time point (15 min, 30 min, 1 h), 100 μL aliquots were taken and filtered through 50 kDa cut-off filters by centrifugation (14,000 x g, 10 min). The filtrates were digested with GluC (1 μg/μL) for 1 h. The endoproteinase digested filtrate (16 μL) was then diluted with 24 μL of 200 mM NH_4HCO_3 (pH 8.8), 1 μL of TCEP (10 mM), and 10 μL of iodoacetamide (10 mg/mL) was added. The solution was incubated at room temperature for 2 h. The samples were frozen and stored at – 80 °C, until analyzed by ESI LC-MS and tandem MS. Prior to loading the samples onto the LC system, 0.1% formic acid was added (1:1) to lower the pH to ca. 6.5.

Molecular Cloning of His-Tag-ProcA3.2-Leader-Intein-CBD

The DNA sequence encoding the ProcA3.2 leader peptide was cloned between NdeI and SapI restriction sites of a pTXB1 vector to position the ProcA3.2 leader gene before the gene encoding intein and chitin binding domain (CBD). pET28b encoding the ProcA3.2 precursor peptide gene was used as template.[9] The primers were 5'-GGT GGT CAT ATG ATG TCA GAA GAA CAA CTC AAG GCA TTT ATT G-3' (ProcA3.2-leader_NdeI_FP), and 5'-GGT GGT TGC TCT TCC GCA TCC CCC AGC CAC ACC TTC 3' (ProcA3.2-leader_SapI_RP). The generated pTXB1 plasmid encoding 'ProcA3.2-leader_intein_CBD' was used as template to insert 'ProcA3.2-leader_intein_CBD' between the NdeI and BamHI restriction sites of a pET15b plasmid after the DNA sequence encoding the His-tag. The primers used were 5'-GGT GGT CAT ATG TCA GAA GAA CAA CTC AAG GCA TTT ATT G-3' (His-tag_ProcA3.2-leader_intein_CBD_NdeI_FP) and 5'-GGT GGT GGA TCC TCA TTG AAG CTG CCA CAA GG-3' (His-tag_ProcA3.2-leader_intein_CBD_BamHI_RP) to generate the final pET15b construct of 'His-tag_ProcA3.2-leader_intein_CBD'.

Mutagenesis of ProcA Genes

The first residue of the core peptide is designated as 1 and the last residue of the leader peptide is designated as −1. Mutation of the *C*-terminal Gly of ProcA3.2 leader to Lys in 'His-tag_ProcA3.2-leader_intein_CBD' was performed by QuikChange site directed mutagenesis. The primers were 5'-GAA GGT GTG GCT GGG AAA TGC ATC ACG GGA GAT G-3' (His-tag_ProcA3.2-leader-G–1K_intein_CBD_FP) and 5'-CCC AGC CAC ACC TTC CAG CTC ATC ATC-3' (His-tag_ProcA3.2-leader-G–1K_intein_CBD_RP), which were used to generate pET15b construct encoding 'His-tag_ProcA3.2-leader-G–1K_intein_CBD'.

The ProcA2.8 and ProcA3.3 Ser/Thr mutants were generated using the following primers by QuikChange site directed mutagenesis based on a modified protocol.[11]

Primer Name	**Primer Sequences (5'-3')**
ProcA2.8_S9T_FP	GTC ATA ACC ATG CTC CAA CCA TGC CTC CAT CCT ATT G
ProcA2.8_S9T_RP	CAA TAG GAT GGA GGC ATG GTT GGA GCA TGG TTA TGA C
ProcA2.8_S13T_FP	CCA TCT ATG CCT CCA ACC TAT TGG GAG GGT G
ProcA2.8_S13T_RP	CAC CCT CCC AAT AGG TTG GAG GCA TAG ATG G
ProcA3.3_T11S_FP	GGT GCT GCA CAG CGC TGG ATG TTA C
ProcA3.3_T11S_RP	GTA ACA TCC AGC GCT GTG CAG CAC C

Figures

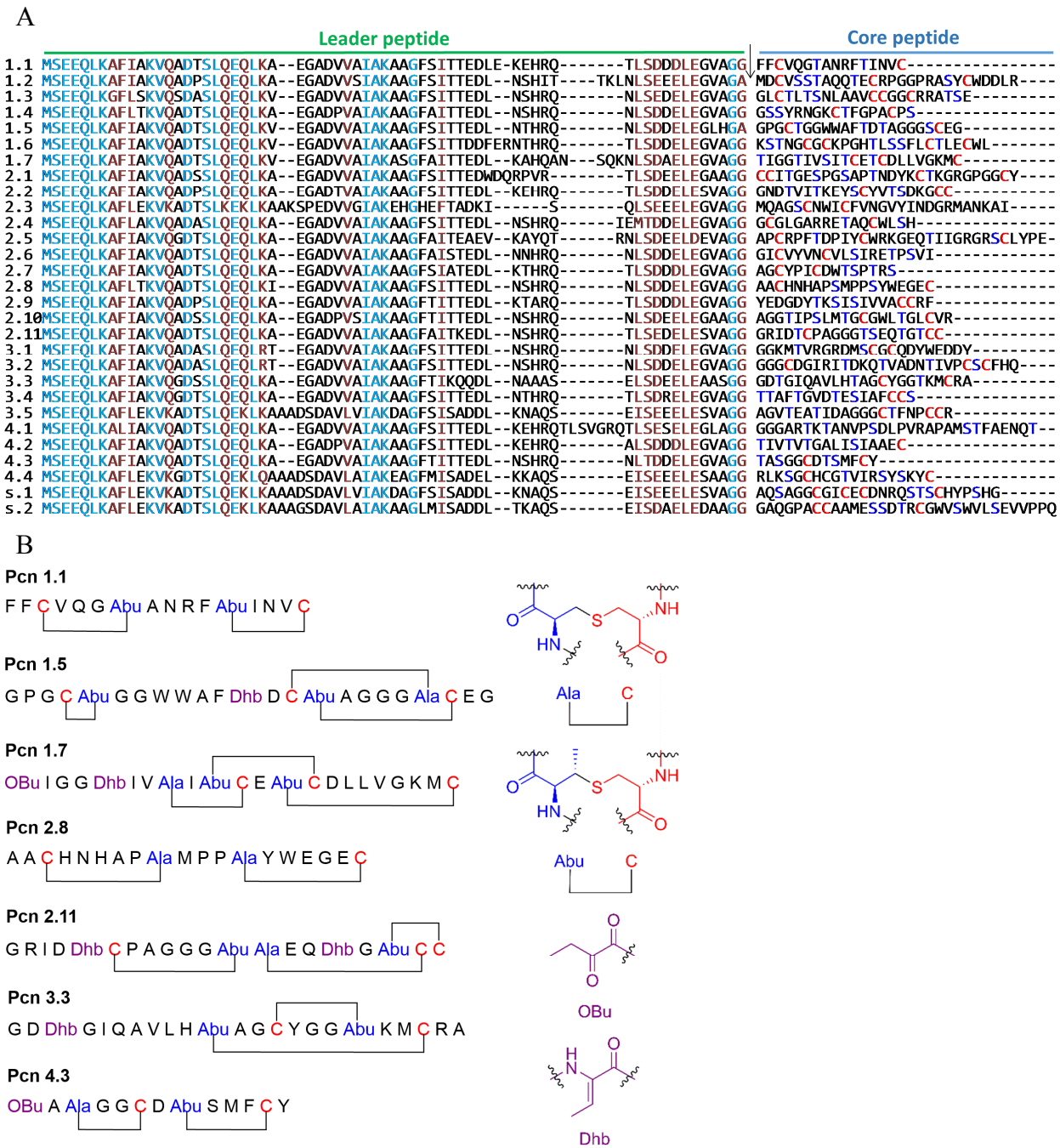


Figure S1. (A) Sequences of 29 precursor peptides encoded by the genome of *Prochlorococcus* MIT 9313.[12] Highly conserved leader peptide and diverse core peptides are presented. Of the 29 substrates, the 16 investigated to date are all substrates of ProcM.[9] Fully conserved residues are in bluish-green, and highly conserved residues are brown. (B) Structure of several representative prochlorosins after maturation shows the diverse possible ring topologies. Ser/Thr is shown in blue, Cys in red, dehydrated residues and residue after hydrolysis of *N*-terminal dehydrobutyrine in purple. Pcn = prochlorosin.

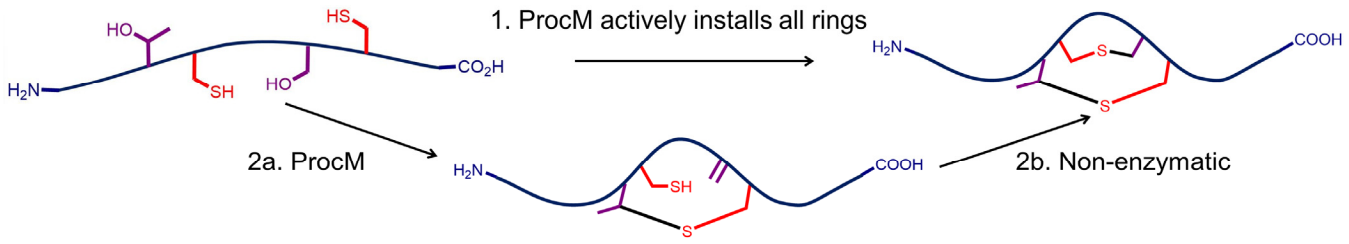


Figure S2. Scheme showing enzymatic vs. non-enzymatic cyclization during prochlorosin maturation. Either all the thioether rings are installed by active participation of ProcM (1) or ProcM installs a subset of rings (2a) and pre-organizes the substrate for non-enzymatic cyclization (2b).

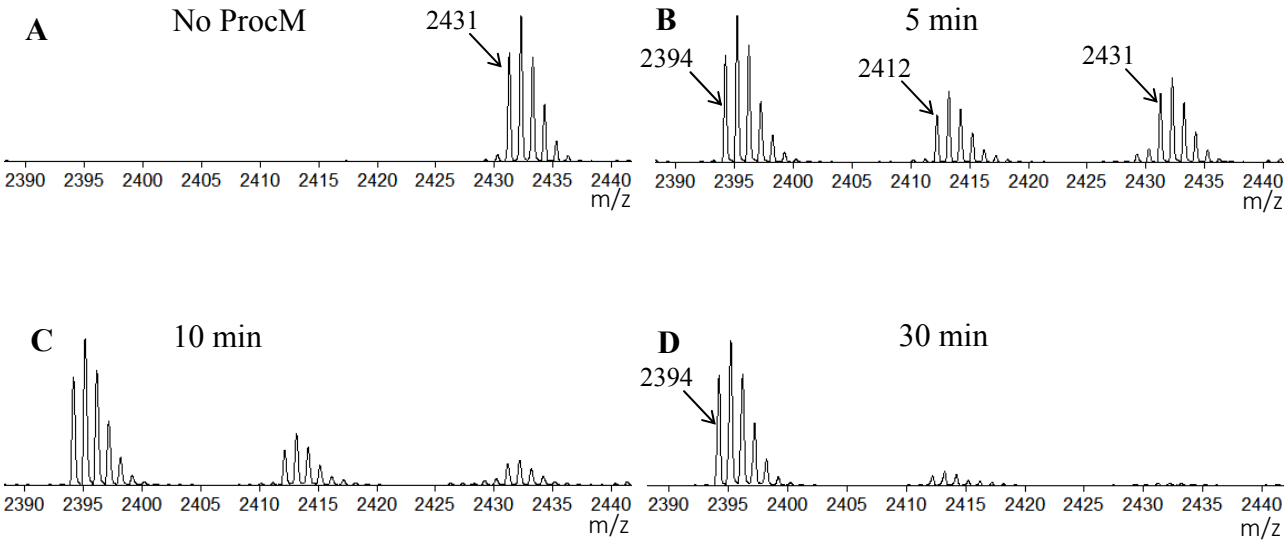


Figure S3. Directionality of dehydration of **4** (Figure 5A) by MALDI-TOF MS analysis of the ProcM assay at different time-points. After each time-point, ProcM was removed by ultrafiltration (14000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with GluC prior to MS analysis. (A) Control MS of substrate without ProcM treatment. (B)-(D) MALDI-TOF MS of assays in which substrate (100 μM) was incubated with ProcM (2 μM), ATP, and Mg^{2+} for (B) 5 min, (C) 10 min, and (D) 30 min.

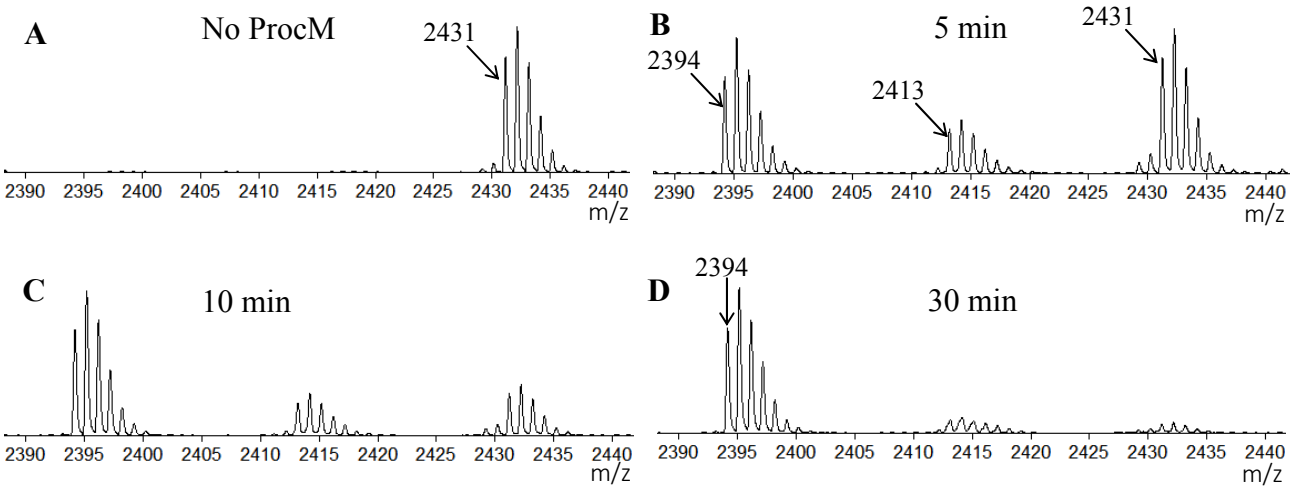


Figure S4. Directionality of dehydration of **5** (Figure 5C) by MALDI-TOF MS analysis of the ProcM assay at different time-points. After each time-point, ProcM was removed by ultrafiltration (14,000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with GluC prior to MS analysis. (A) Control mass spectrum of substrate without ProcM treatment. (B)-(D) MALDI-TOF MS of assays in which substrate (100 μM) was incubated with ProcM (2 μM), ATP, and Mg^{2+} for (B) 5 min, (C) 10 min, and (D) 30 min.

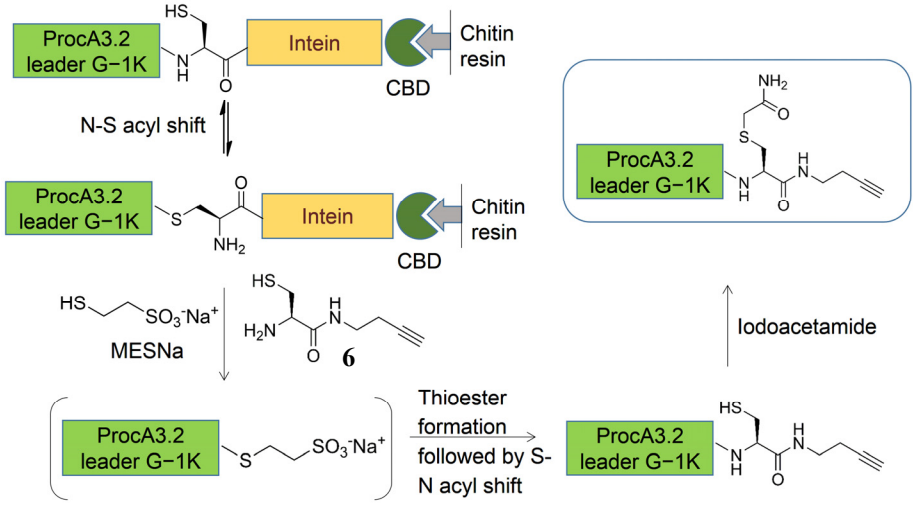


Figure S5. Scheme for generation of ProcA leader peptide with a *C*-terminal alkyne. The leader peptide was expressed as a fusion protein with an intein and chitin binding domain at its *C*-terminus. Chitin resin was used to affinity purify the fusion protein, which was cleaved from the resin by transthioesterification with MESNa. The *C*-terminal MESNa thioester of the leader peptide formed in-situ reacts with **6** (expressed protein ligation) to generate the leader peptide with a C-terminal alkyne. The introduced cysteine was capped with iodoacetamide.

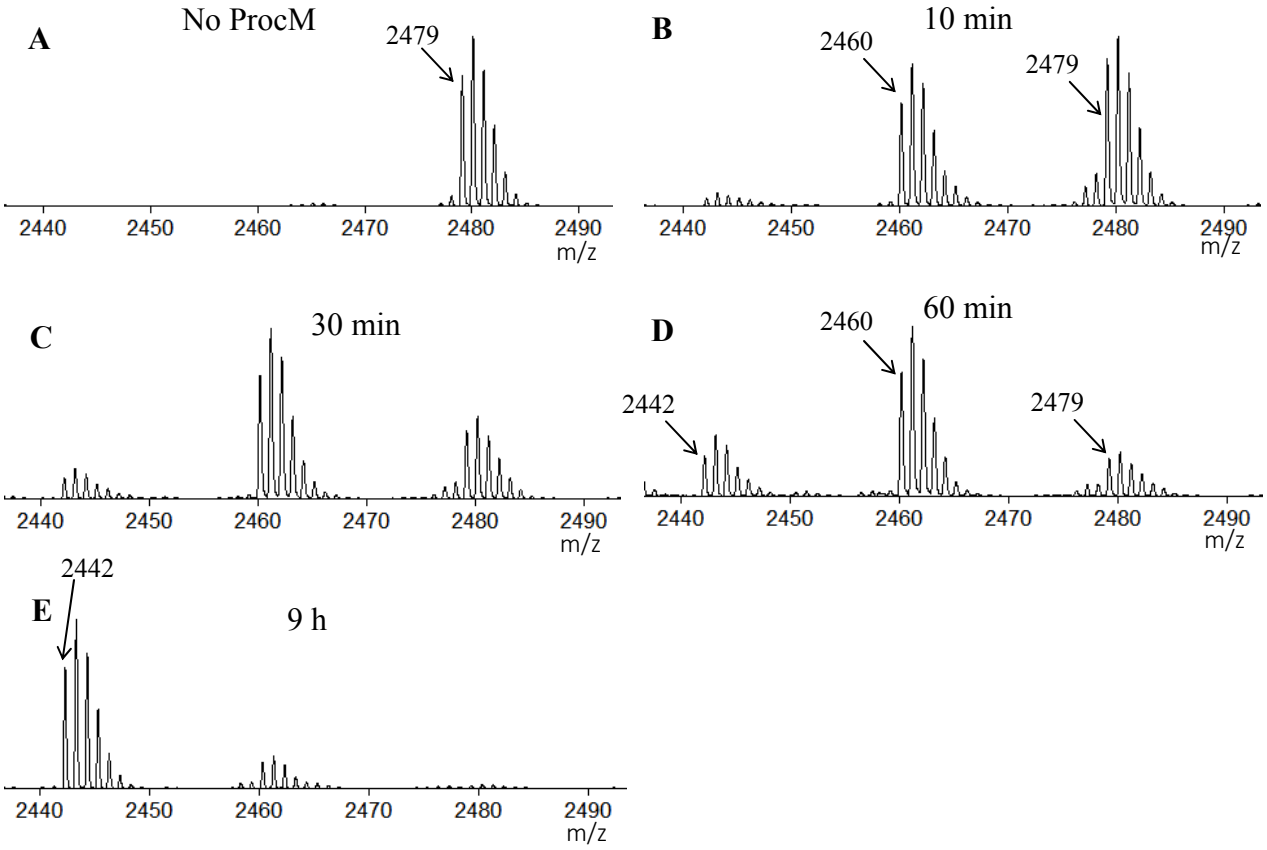


Figure S6. Directionality of dehydration of **9** (Figure 7A) by ProcM. After each time-point, ProcM was removed by ultrafiltration (14,000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with LysC prior to MALDI-TOF MS analysis. (A) MALDI-TOF mass spectrum of substrate without ProcM treatment. (B)-(D) Assays conducted by incubating substrate (50 μM) with ProcM (2 μM), ATP, and Mg^{2+} for (B) 10 min, (C) 30 min, and (D) 60 min. (E) MALDI-TOF MS of substrate (50 μM), ProcM (10 μM) and other standard assay components incubated for 9 h.

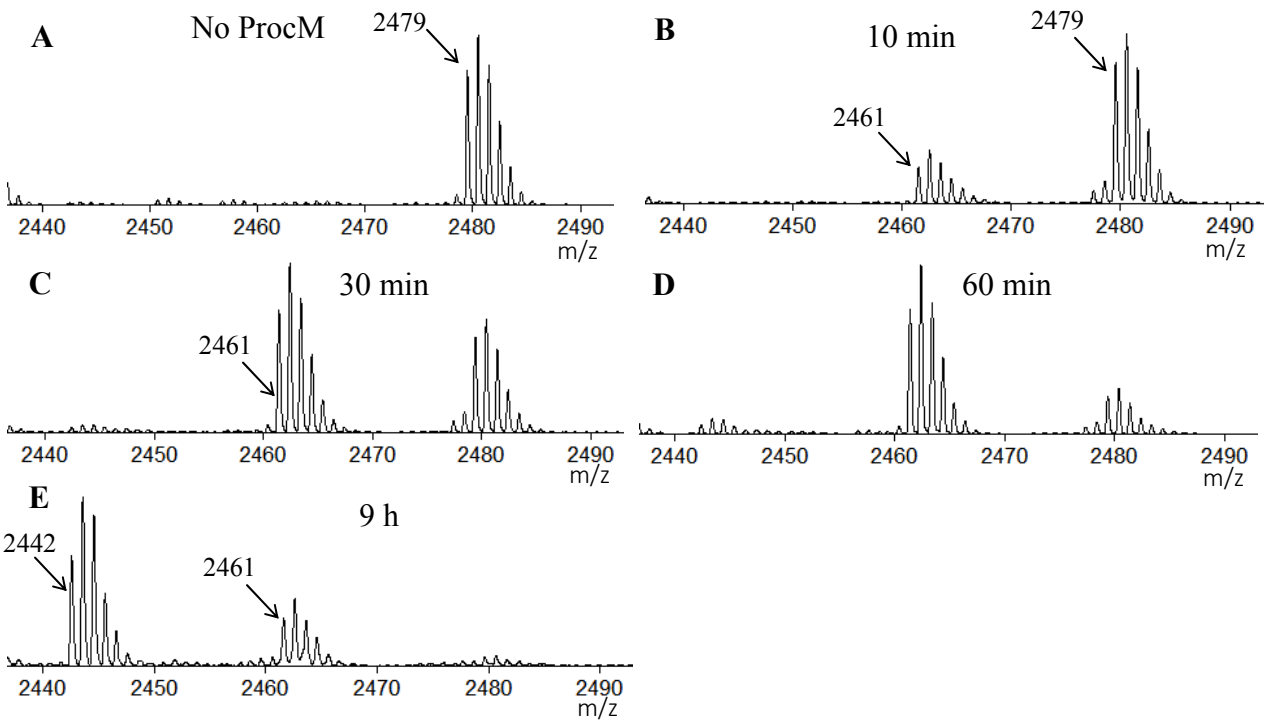


Figure S7. Directionality of dehydration of **10** (Figure 7C). After each time-point, ProcM was removed by ultrafiltration (14,000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with LysC (1.5 U/mL) prior to MS analysis. (A) MALDI-TOF MS of substrate without ProcM treatment.(B)-(D) Assays containing substrate (50 μM), ProcM (2 μM) and other standard components for (B) 10 min, (C) 30 min, and (D) 60 min. (E) MALDI-TOF MS of substrate (50 μM) incubated with ProcM (10 μM) and other standard components for 9 h.

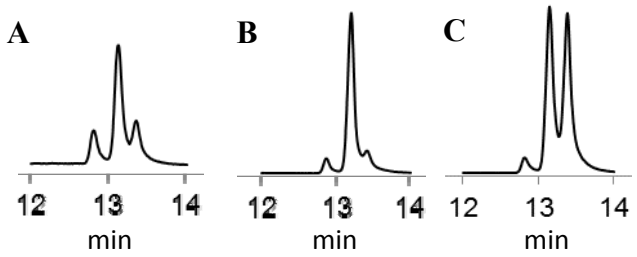


Figure S8. GC-MS analysis illustrating that triazole-linked ProcA2.8 **11** is processed by ProcM to generate Lan and MeLan with the same stereochemistry as in wt peptide. ProcM modified peptide was hydrolyzed in acid and the resulting amino acids were derivatized as reported previously (also as previously reported, this procedure leads to partial epimerization of Lan).[13] The derivatized material was analyzed by GC-MS using a chiral structure phase as previously reported.[13] Selected ion mode (SIM) trace for Lan (m = 365) of (A) modified ProcA2.8 core obtained (B) modified ProcA2.8 core spiked with synthetic DL-Lan standard (C) modified ProcA2.8 core spiked with synthetic DL-Lan and LL-Lan standards.

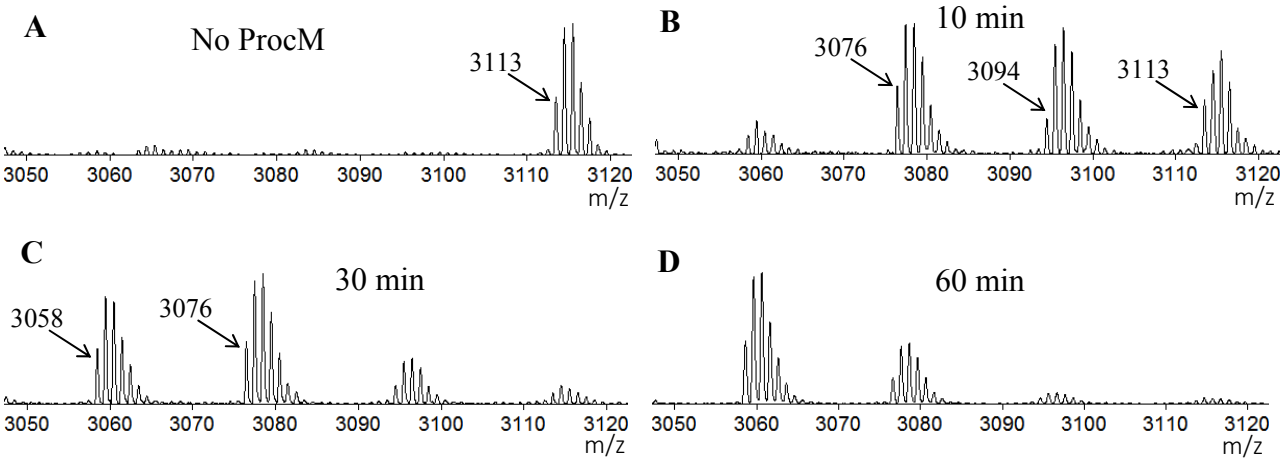


Figure S9. Directionality of dehydration of **12** (Figure 8A). After each time-point, ProcM was removed by ultrafiltration (14,000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with GluC prior to MS analysis. (A) MALDI-TOF MS of substrate (50 μM) without ProcM treatment. (B)-(D) MALDI-TOF MS of assays conducted with substrate (50 μM), ProcM (2 μM) with other standard components for (B) 10 min, (C) 30 min, and (D) 60 min.

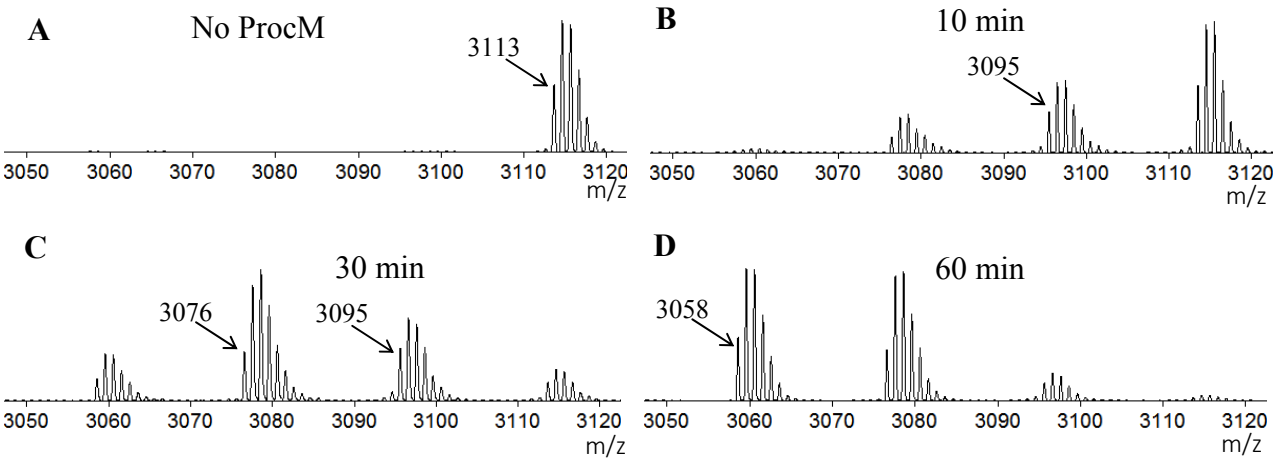


Figure S10. Directionality of dehydration of **13** (Figure 8C). After each time-point, ProcM was removed by ultrafiltration (14,000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with GluC prior to MS analysis. (A) Control MS of substrate (50 μM) without ProcM treatment. MALDI-TOF MS of assay conducted with substrate (50 μM), ProcM (2 μM) with other standard components for (B) 10 min, (C) 30 min, and (D) 60 min.

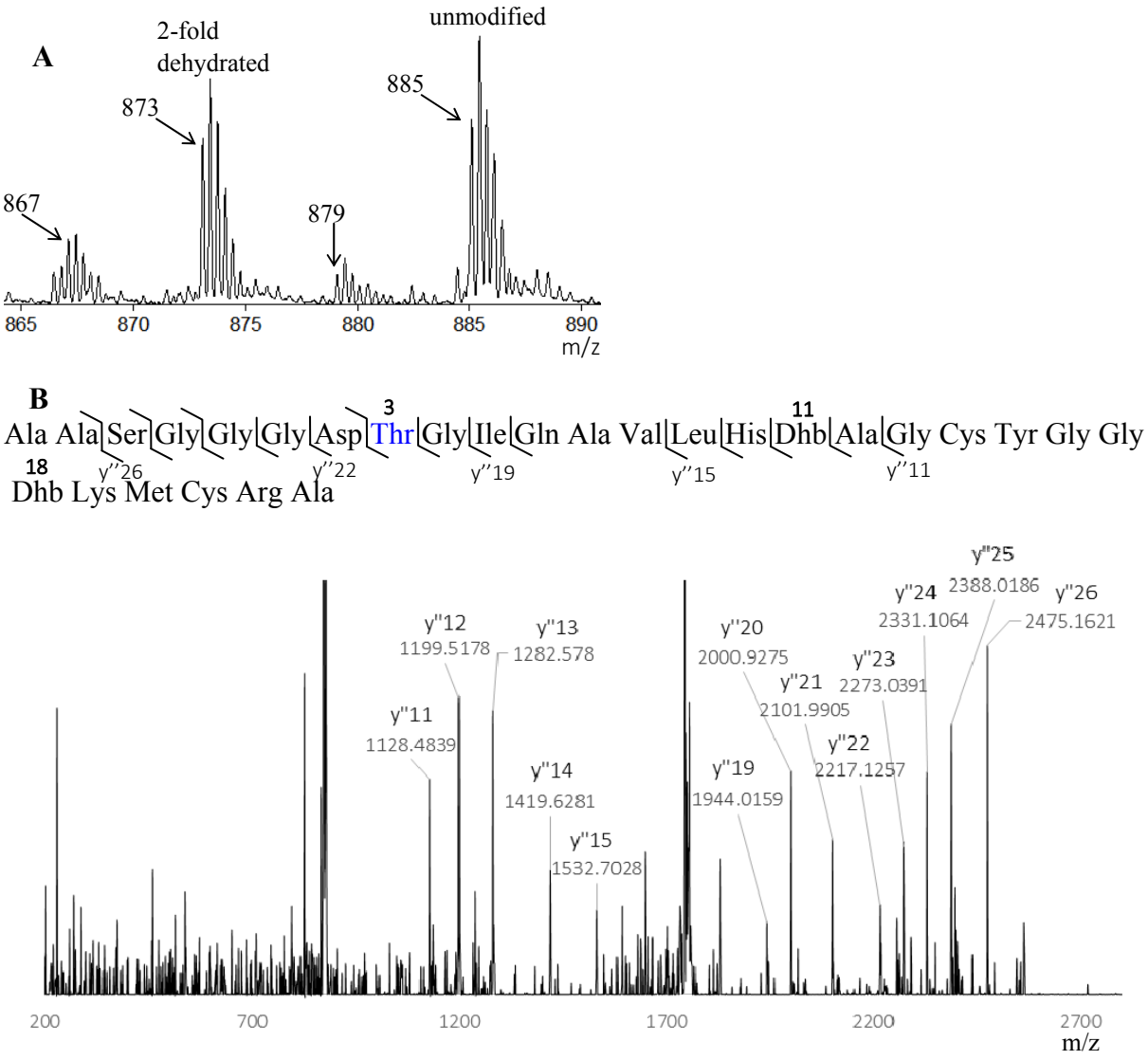


Figure S11. Tandem MS of partially dehydrated ProcA3.3 WT peptide to confirm *C*-to-*N*-terminal directionality. (A) ESI-MS on ProcA3.3 WT to trap partially dehydrated species generated by treating ProcA3.3 WT precursor (100 μM), ProcM (2 μM) using the standard ProcM assay conditions for 5 min at room temperature, after which ProcM was removed by ultrafiltration (14,000 x g, 10 min) through Amicon Ultra 50 kDa MWCO filters. The assay contents were digested with GluC prior to MS analysis. The 3^+ charged species are presented. (B) Tandem MS analysis on two-fold dehydrated species of m/z 873 (3^+ charged). Thr3 that escapes dehydration is labeled in blue.

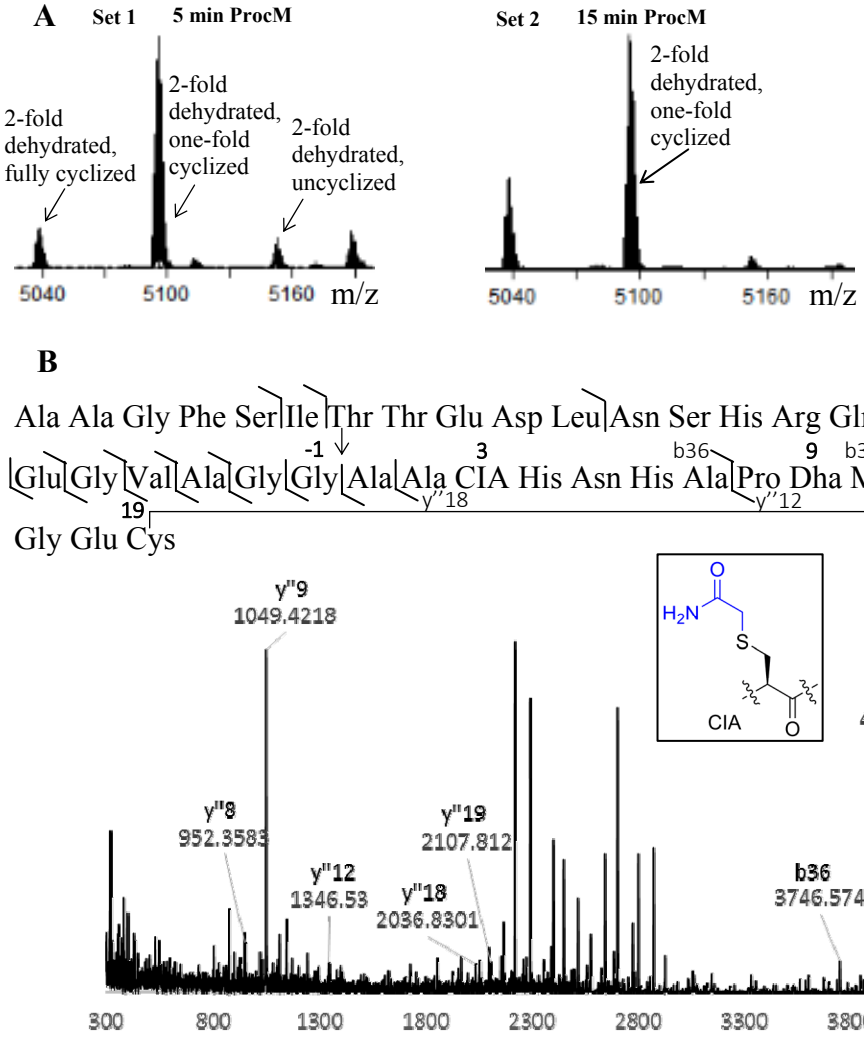


Figure S12. Directionality of cyclization of ProcA2.8 precursor peptide by ProcM. (A) Assay (100 µL) was performed for 15 min with His-tagged ProcA2.8 precursor peptide (100 µM), ProcM (5 µM), HEPES (50 mM, pH 8.0), ATP (10 mM), TCEP (0.5 mM), and $MgCl_2$ (10 mM). Then ProcM was removed by centrifugation through a 50 kDa cut-off filter. The filtrate was digested with LysC (3 U/mL) for 1 h, then the assay solution (16 µL) was diluted with 19 µL of NH_4HCO_3 (200 mM, pH 8.8), 5 µL of TCEP (10 mM), and 10 µL of IAA (10 mg/mL) and incubated for 2 h prior to MALDI-TOF MS analysis. The two-fold dehydrated species with full cyclization has an m/z of 5035.3 ($M+H^+$) and the two-fold dehydrated species with one iodoacetamide adduct has an m/z of 5092.3 ($M+H^+$). (B) ESI-MSMS was performed on fully dehydrated, one-fold cyclized species with one IAA adduct (m/z of 1019.2; 5^+ charge state) present at the 15 min time point.

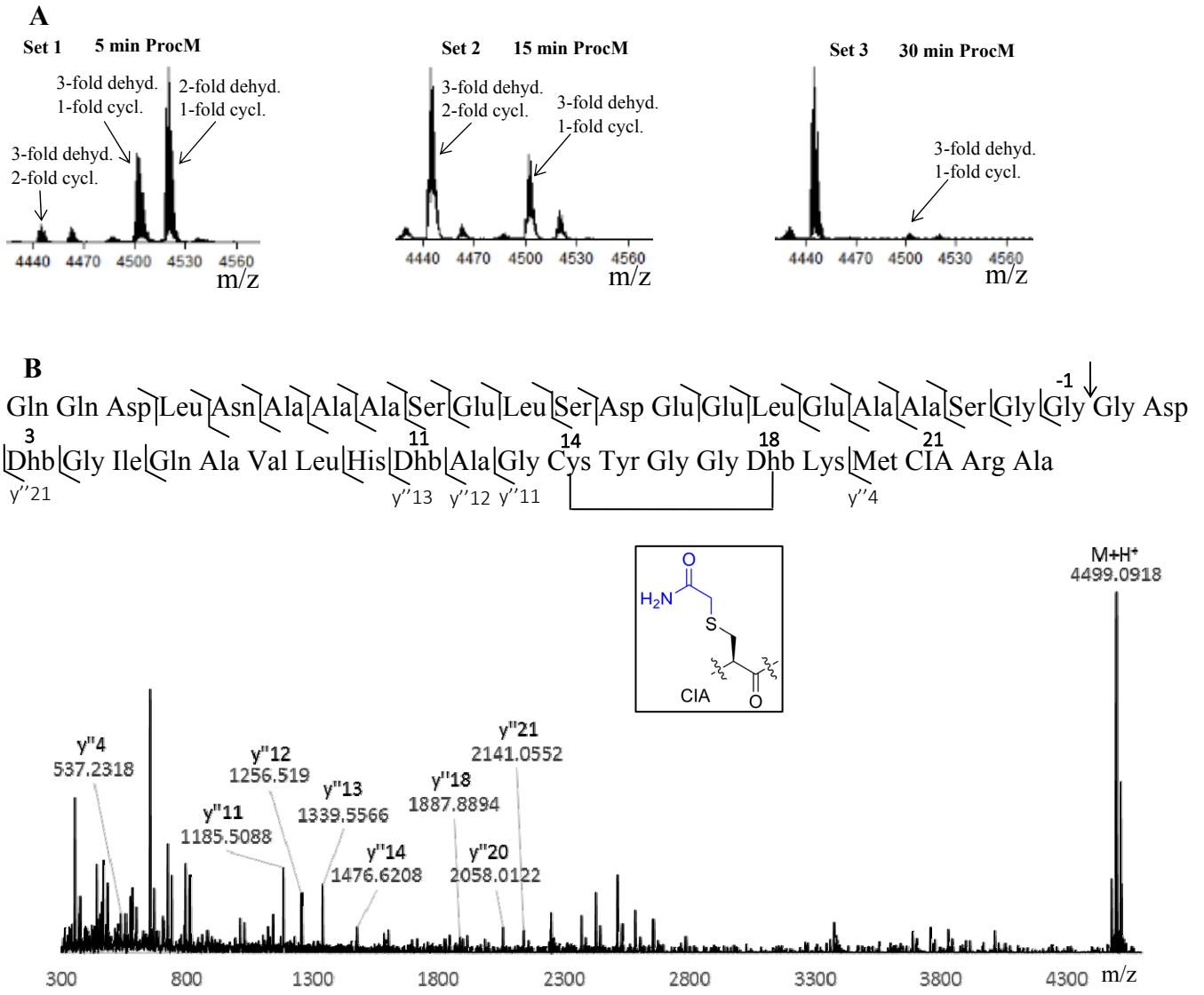


Figure S13. Directionality of cyclization of ProcA3.3 precursor peptide by ProcM. (A) Assay (100 µL) was performed with His-tagged ProcA3.3 precursor peptide (100 µM), ProcM (5 µM), HEPES (50 mM, pH 8.0), ATP (10 mM), TCEP (1 mM), and $MgCl_2$ (10 mM). ProcM was removed after 5, 15, and 30 min by centrifugation through a 50 kDa cut-off filter. The filtrate was digested with LysC (3 U/mL) for 1 h, and then the assay solution (16 µL) was diluted with 19 µL NH_4HCO_3 (200 mM, pH 8.8), 5 µL of TCEP (10 mM), and 10 µL of IAA (10 mg/mL) and incubated for 2 h prior to MALDI-TOF MS analysis. The three-fold dehydrated species with full cyclization has a m/z of 4442.4 ($M+H^+$) and the three-fold dehydrated species with one iodoacetamide adduct has a m/z of 4499.5 ($M+H^+$) (B) ESI-MSMS was performed on fully dehydrated, one-fold cyclized species with one IAA adduct (m/z of 1125.5; 4^+ charge state) generated from the 15 min time point.

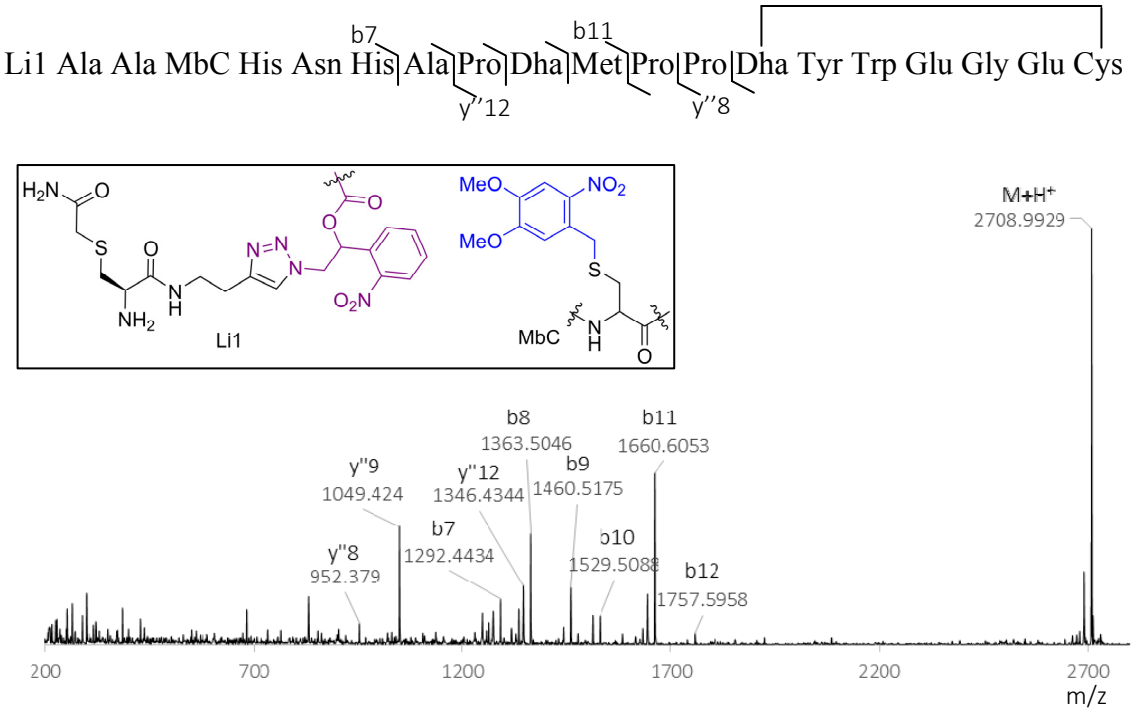


Figure S14. ESI-MSMS analysis of intermediate **18** (Figure 9A) after LysC cleavage, which established that ring-B is formed between Cys19 and Dha13.

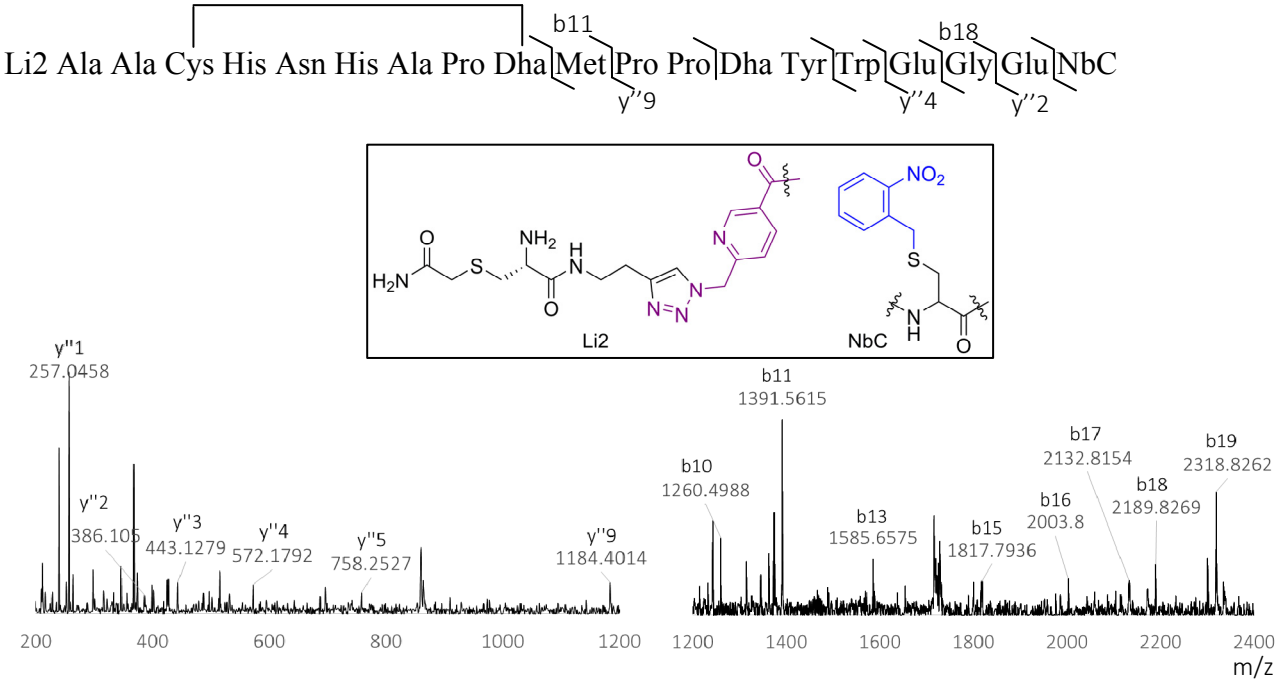


Figure S15. Tandem MS analysis on intermediate **19** (Figure 9C) after LysC cleavage, which established that ring-A is formed between Cys3 and Dha9.

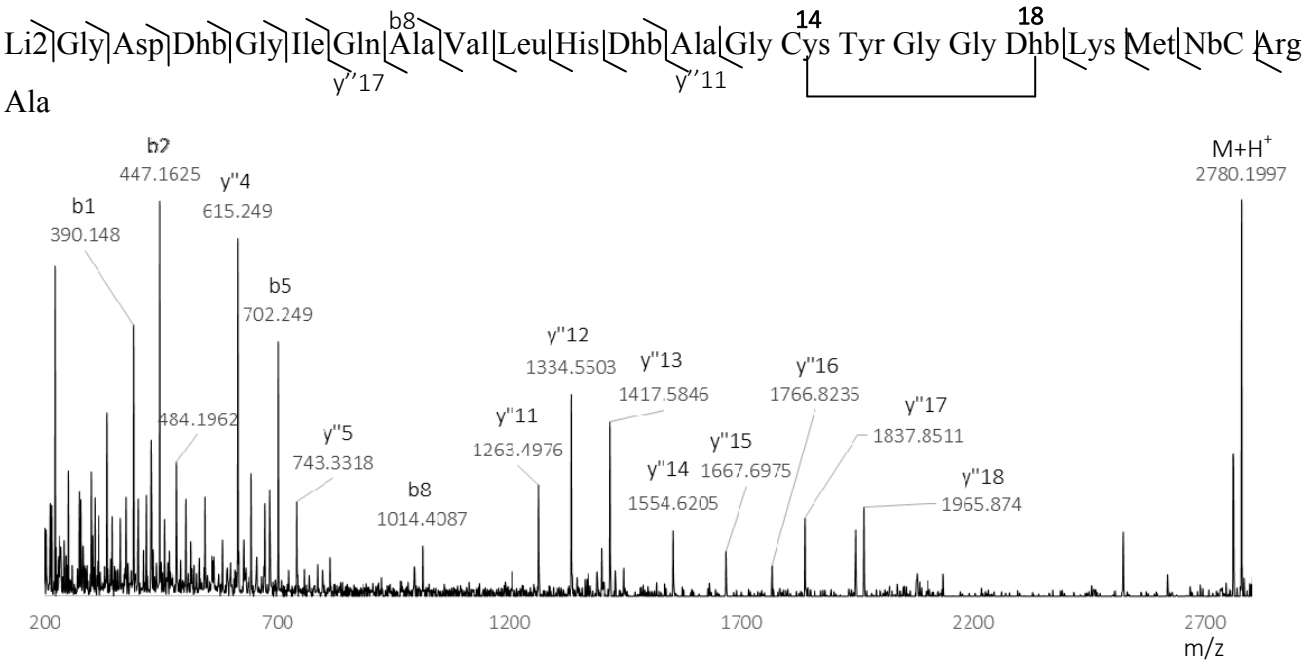
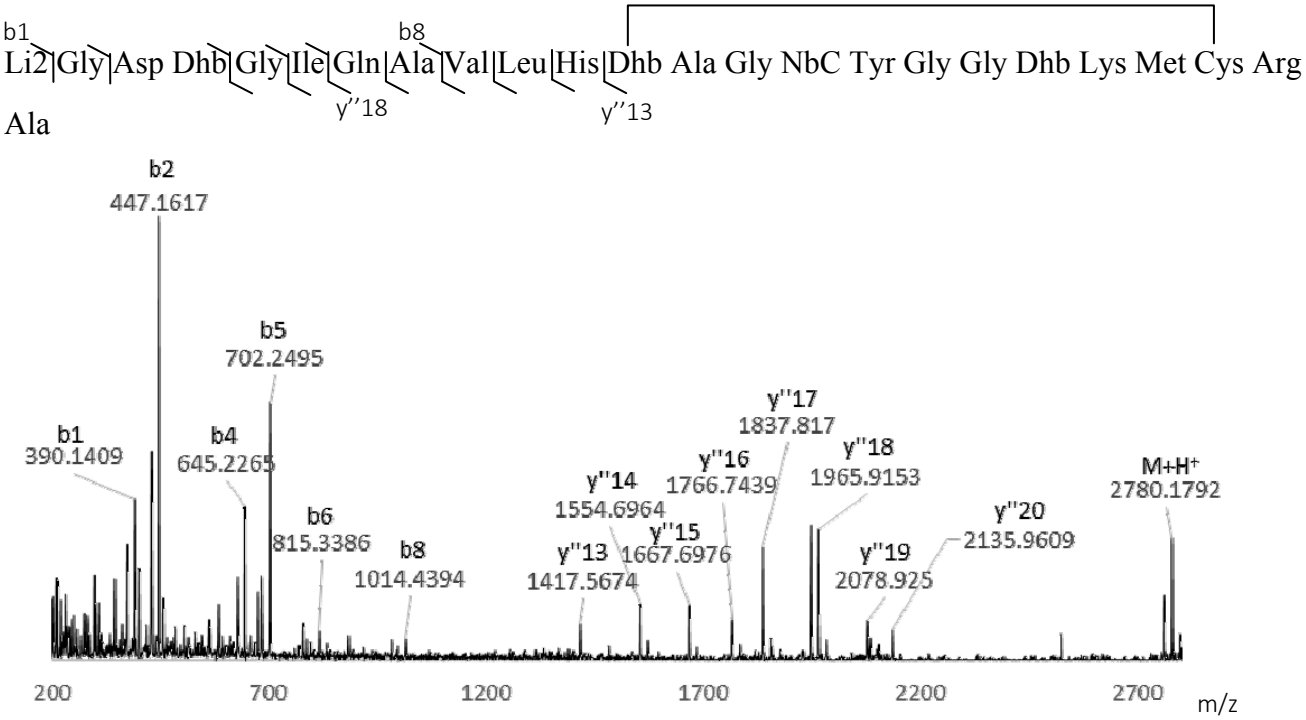


Figure S16. Tandem MS on intermediate **20** (Figure 10A) after LysC cleavage (parent ion m/z of 696), showing the formation of a MeLan between Cys14 and Dhb18. For the structure of Li2, see Fig. S15.

A



B

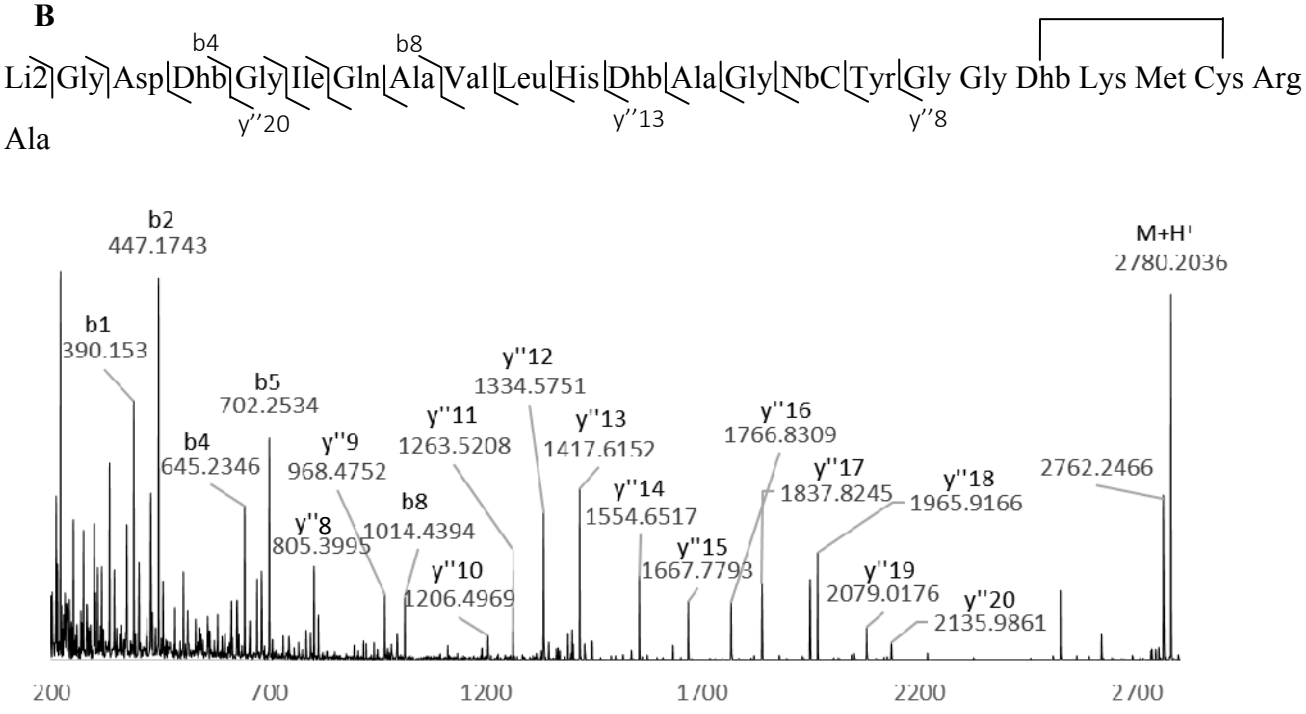
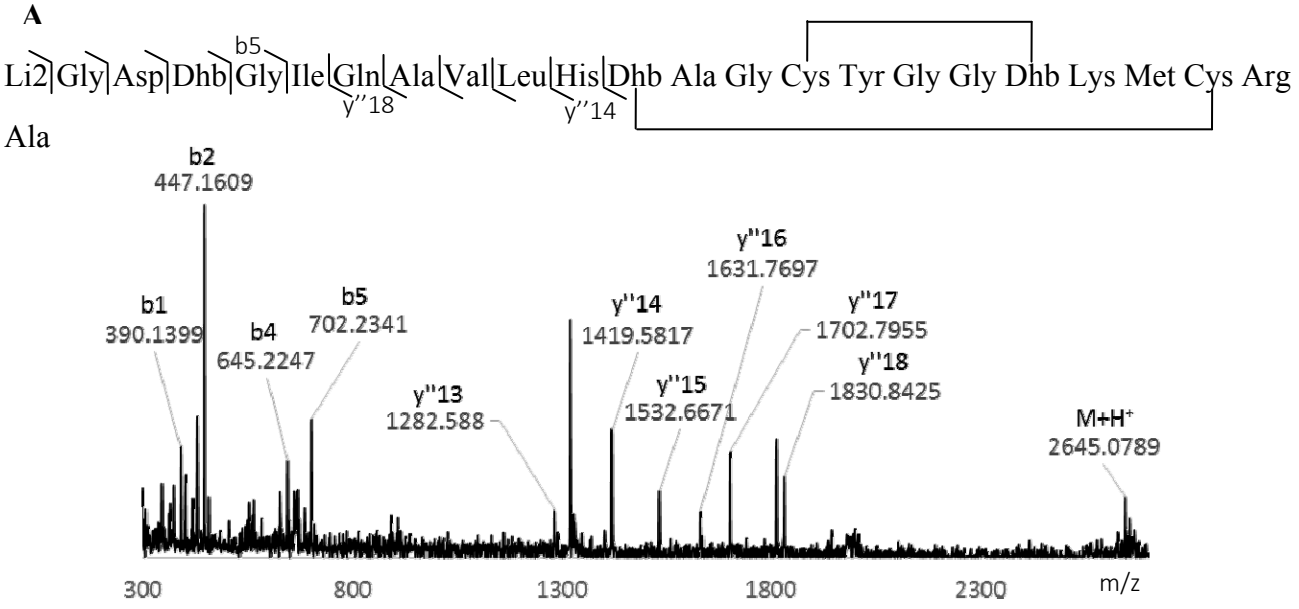


Figure S17. (A) Tandem-MS analysis of intermediate **25**, after LysC cleavage, and (B) tandem analysis of intermediate **21** after LysC cleavage. For the structure of Li2, see Fig. S15.

A



B

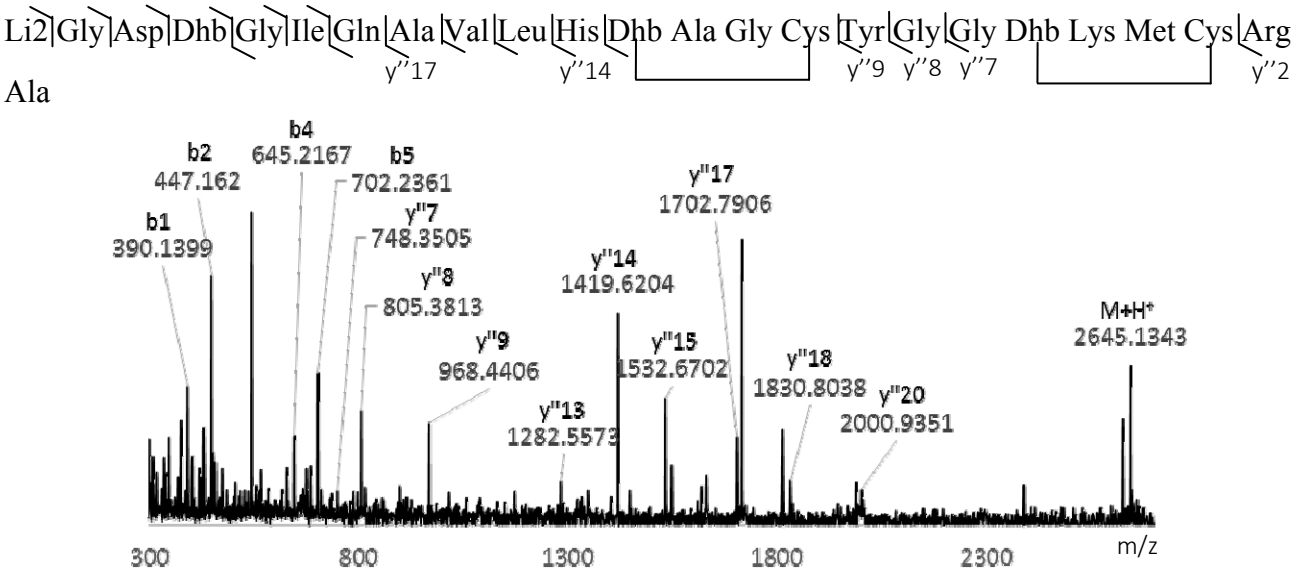


Figure S18. Tandem MS analysis of a mixture of cyclized material derived from enzymatic cyclization of a mixtures of **21** and **25** after photolysis. The cyclized mixture was separated on an analytical scale which confirmed (A) the native Pcn3.3 like overlapping ring-topology, and (B) the alternate non-overlapping ring topology. For the structure of Li2, see Fig. S15.

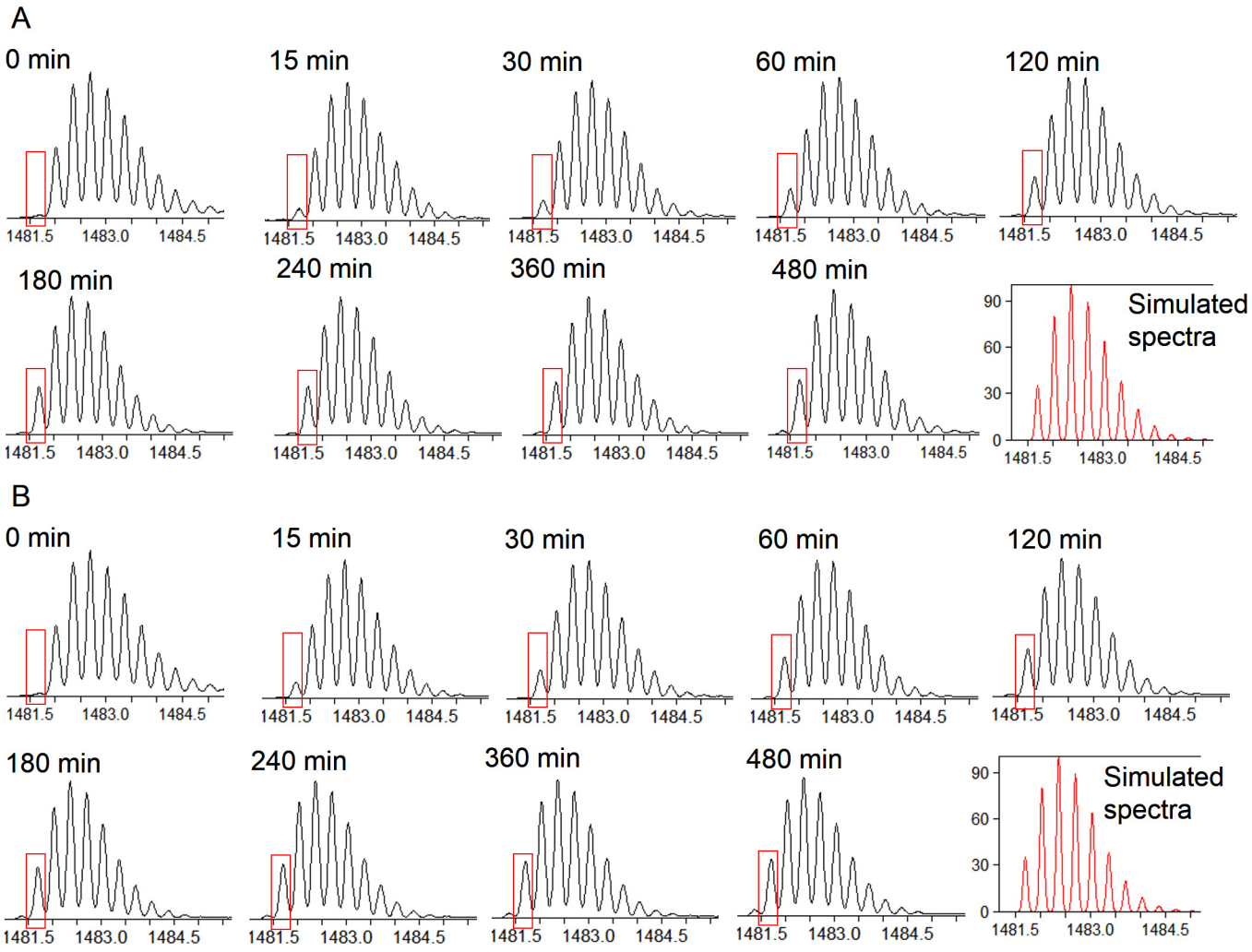


Figure S19. Two sets of assays were performed: (A) with 5 μM ProcM, and (B) with 10 μM ProcM. In both cases, the assay solution contained HEPES (50 mM, pH 8.0), TCEP (0.5 mM), ATP (10 mM), $MgCl_2$ (10 mM), and ProcM-modified ProcA3.3 containing two deuterium atoms (100 μM). Time points were taken at 15, 30, 60, 120, 180, 240, 360, and 480 min. The 0 min time point is taken as the substrate without ProcM. For each sample, ProcM was removed by filtration, and the filtrate was digested by LysC and analyzed by ESI-MS. The 3^+ charged fragment ion is shown to show the extent of D-H exchange with time. The red box shows the appearance of the new peak in isotopic pattern as the first D is exchanged by H. The spectra in red are the simulated 3^+ charged spectra for species with one D exchanged with H (m/z: 1481.67).

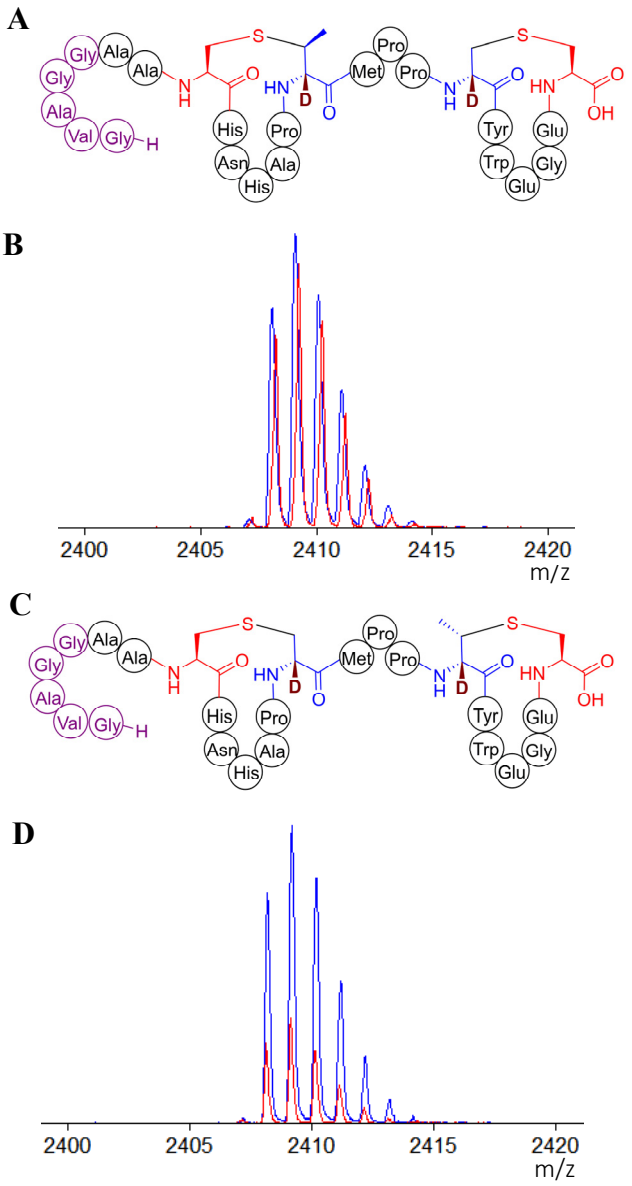


Figure S20. Investigation of D-H exchange in ProcA2.8-S9T and ProcA2.8-S13T mutant. (A) Structure of ProcA2.8-S9T incorporating two deuteriums and after GluC digestion. (B) MALDI-TOF MS analysis on D-H exchange assay when modified precursor peptide ProcA2.8-S9T incorporating two D was treated with ProcM in standard assay conditions in aqueous buffer (red trace) and without ProcM in standard conditions in aqueous buffer (blue trace). (C) Structure of ProcA2.8-S13T incorporating two deuteriums and after GluC digestion. (D) MALDI-TOF MS analysis on D-H exchange assay when modified precursor peptide ProcA2.8-S13T incorporating two D was treated with ProcM in standard assay conditions in aqueous buffer (red trace) and without ProcM in standard conditions in aqueous buffer (blue trace).

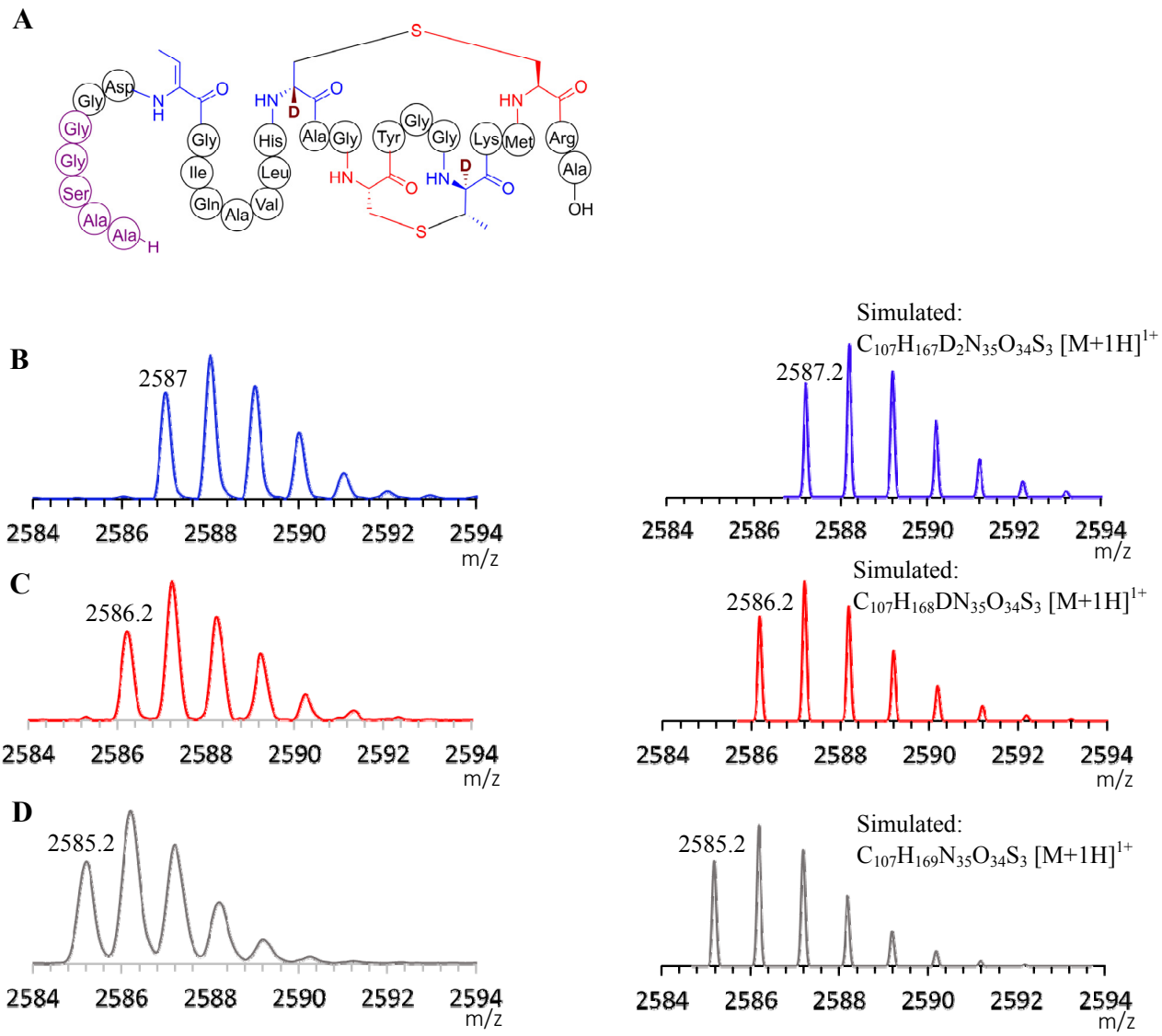


Figure S21. Investigation of D-H exchange in ProcA3.3-T11S. (A) Structure of the core region of ProcA3.3-T11S incorporating two deuterium atoms after GluC digestion. The amino acids remaining from the leader peptide are shown in magenta. (B) MALDI-TOF MS analysis after treatment of the modified ProcA3.3-T11S incorporating two deuterium atoms (100 μM) with ProcM (10 μM) in standard conditions in aqueous buffer (red trace), and in the absence of ProcM in standard ProcM assay conditions (blue trace). For comparison, also shown is the MALDI-TOF mass spectrum of unlabeled ProcA3.3-T11S modified by ProcM in aqueous buffer and then digested with GluC (gray trace). The corresponding simulated spectra are drawn to the right of the corresponding panels.

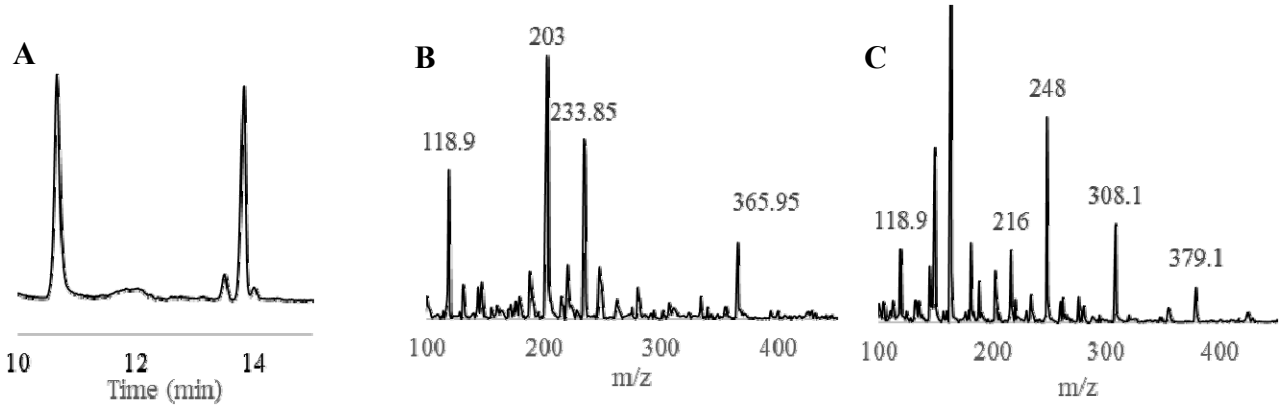


Figure S22. GC/MS analysis to identify the thioether residue in ProcA3.3-T11S mutant participating in DH exchange. (A) GC/MS traces for the hydrolyzed/derivatized Lan/MeLan residues obtained from ProcA3.3-T11S mutant with one α-deuterium exchanged with protium. (B) Mass spectrum of derivatized Lan showing fragments consistent with the Lan residue retaining the α-deuterium (m/z: 366, 203). (C) Mass spectrum of the derivatized MeLan showing fragments consistent with the MeLan residue not containing a deuterium atom (m/z: 379, 216).[14]

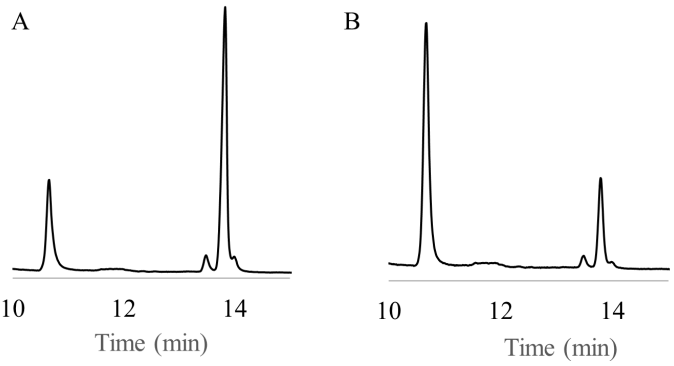
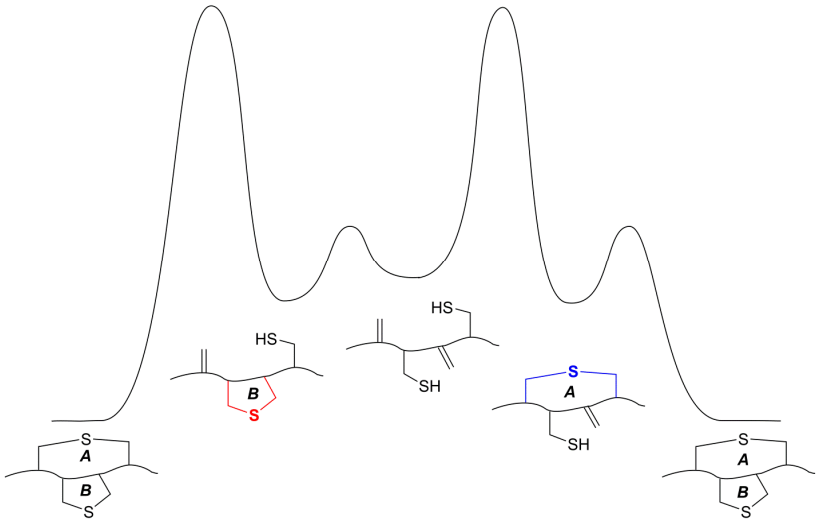


Figure S23. GC/MS traces for the co-injection of hydrolyzed/derivatized Lan/MeLan residues from Fig. S21 with (A) synthetic, derivatized (2*S*,6*R*)-Lan standard, and (B) synthetic, derivatized (2*S*,3*S*,6*R*)-MeLan standard.

A



B

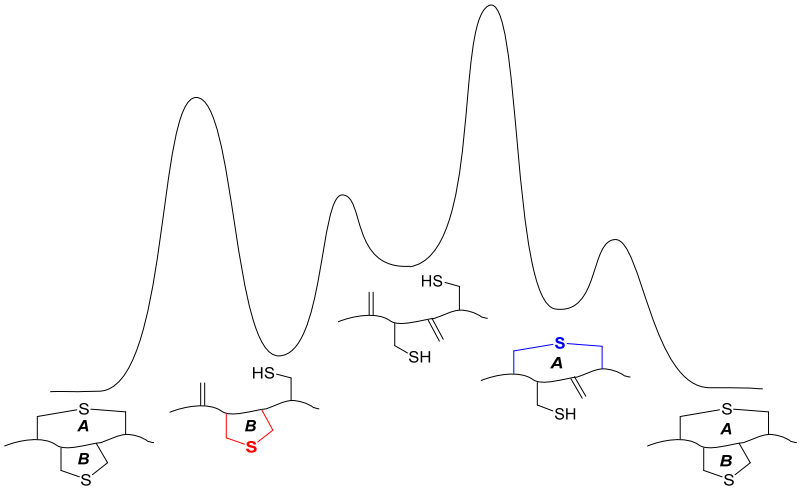


Figure S24. Schematic illustration of scenarios that can explain the experimental observations that an intermediate with the B-ring formed is observed upon cyclization of a linear peptide (shown in the center), and that the same B-ring undergoes exchange faster than the A-ring when starting with fully cyclized peptide (shown on the left and right). In this model, the transition state energies for forming the A- or B-ring are independent on whether the other ring is already installed. In panel A, the transition state energies for cyclization of the A-ring (or B-ring) are drawn the same regardless of whether the B-ring (or A-ring) is already formed. The energies for the peptides containing either the A- or B-rings are also arbitrarily drawn the same. In panel B, the barriers for cyclization of the A- and B-rings are drawn as independent of whether the other ring is already formed or not, but the transition state energies are not identical. Once again this scenario can explain the experimental data, but the difference with panel A is that product formation will preferentially go through the left hand pathway.

In either panel A or B, in the forward direction (i.e. starting with uncyclized peptide) only the intermediate with the B-ring can be detected and in the reverse direction, exchange of the proton in the B-ring will be faster than that of the A-ring.

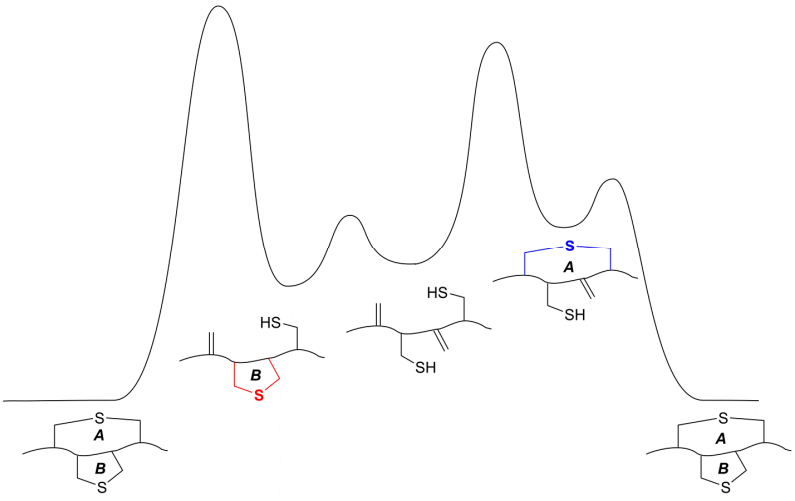
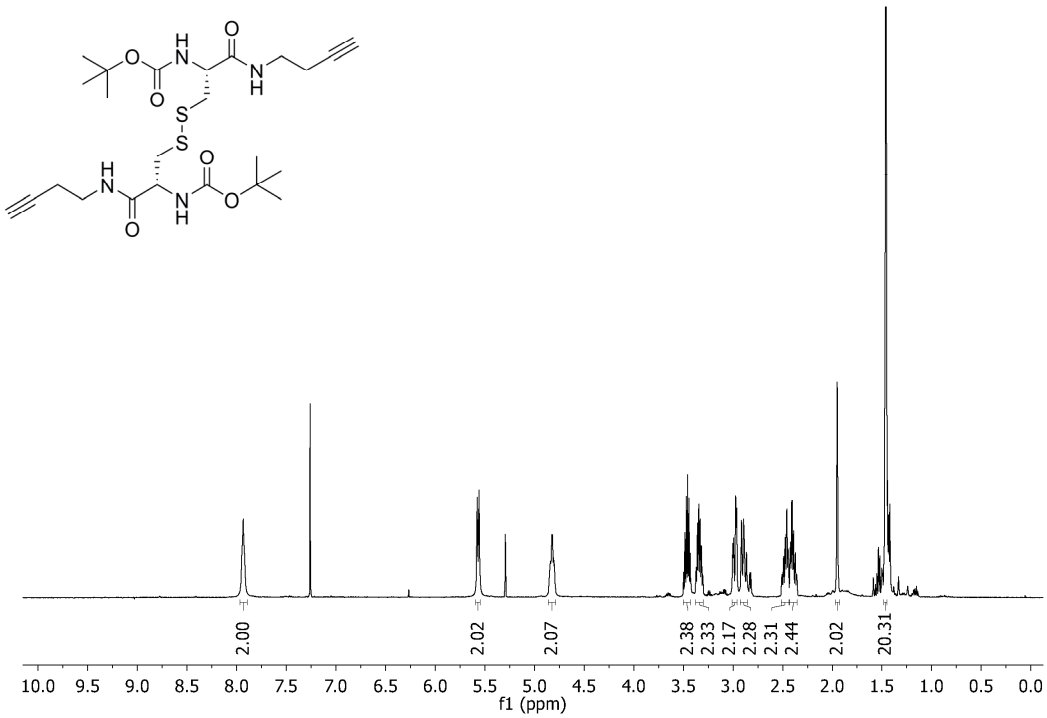
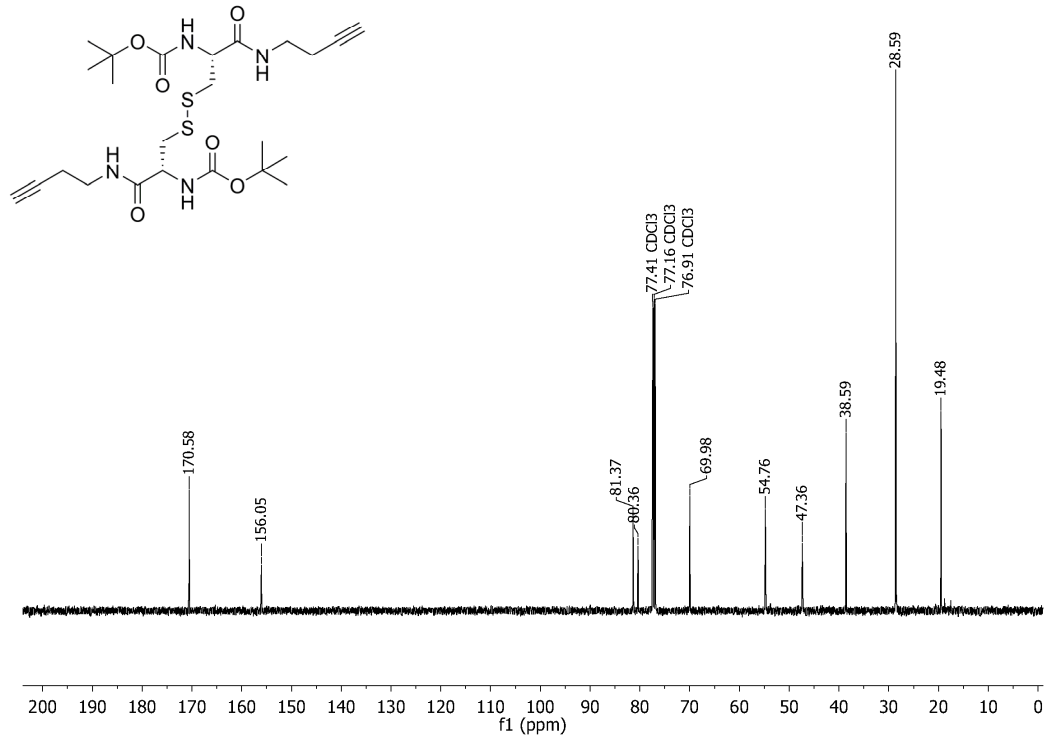


Figure S25. Alternative model to explain the experimental observations that an intermediate with the B-ring formed is observed upon cyclization of the linear peptide (shown in the center), and that the same B-ring undergoes exchange faster than the A-ring when starting with fully cyclized peptide (shown on the left and right). In this model, the intermediate with the B-ring formed is not a productive intermediate to product. It has to revert to the linear peptide, which in a much slower process forms the A-ring. Formation of the A-ring is then followed by fast closure of the B-ring. This scenario is reminiscent of the Curtin-Hammett principle in which two compounds are in equilibrium and for which the ratio by which the starting compound follows the two pathways to product is determined by the difference in the transition state energies.

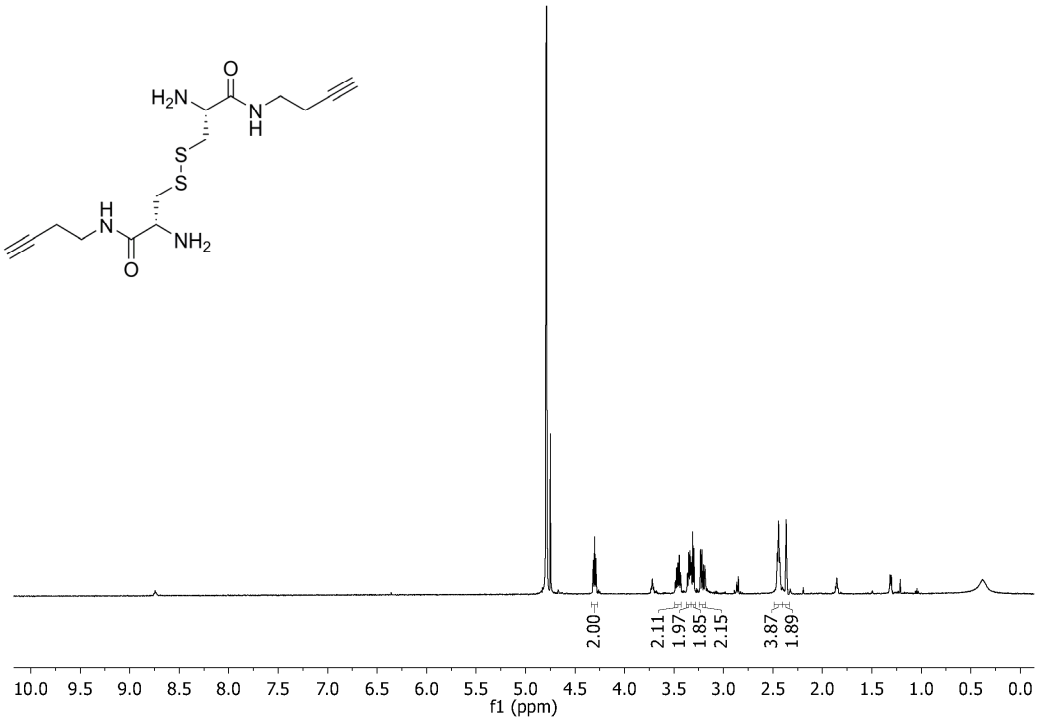
NMR Spectra of New Molecules

Compound 26.





Compound 27.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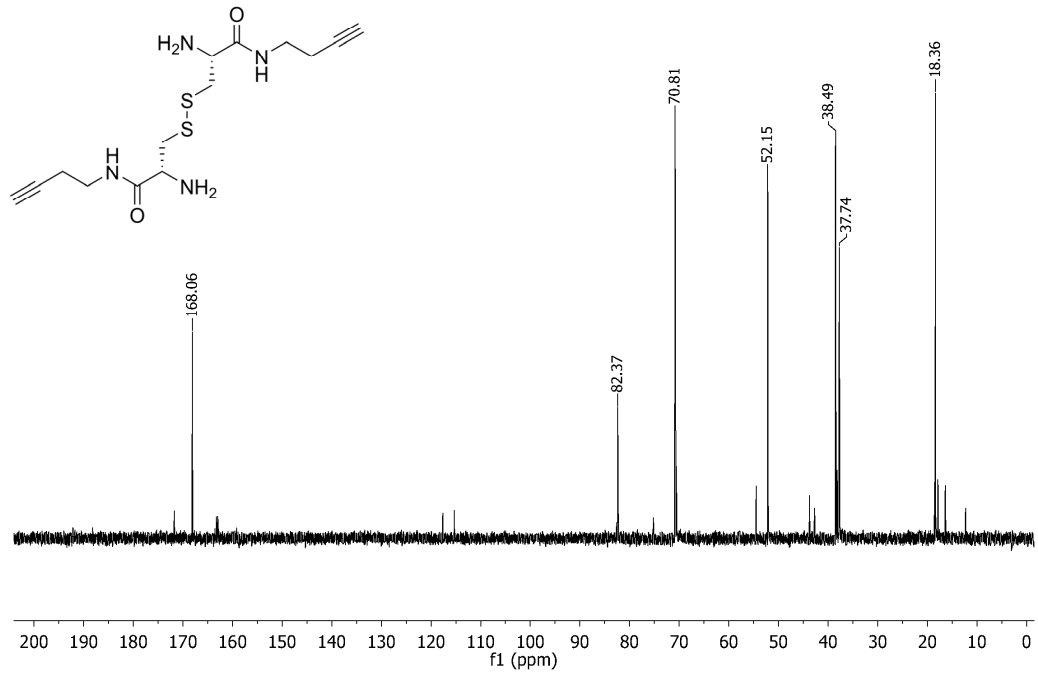
1.85
2.15
3.87
1.89
f1 (ppm)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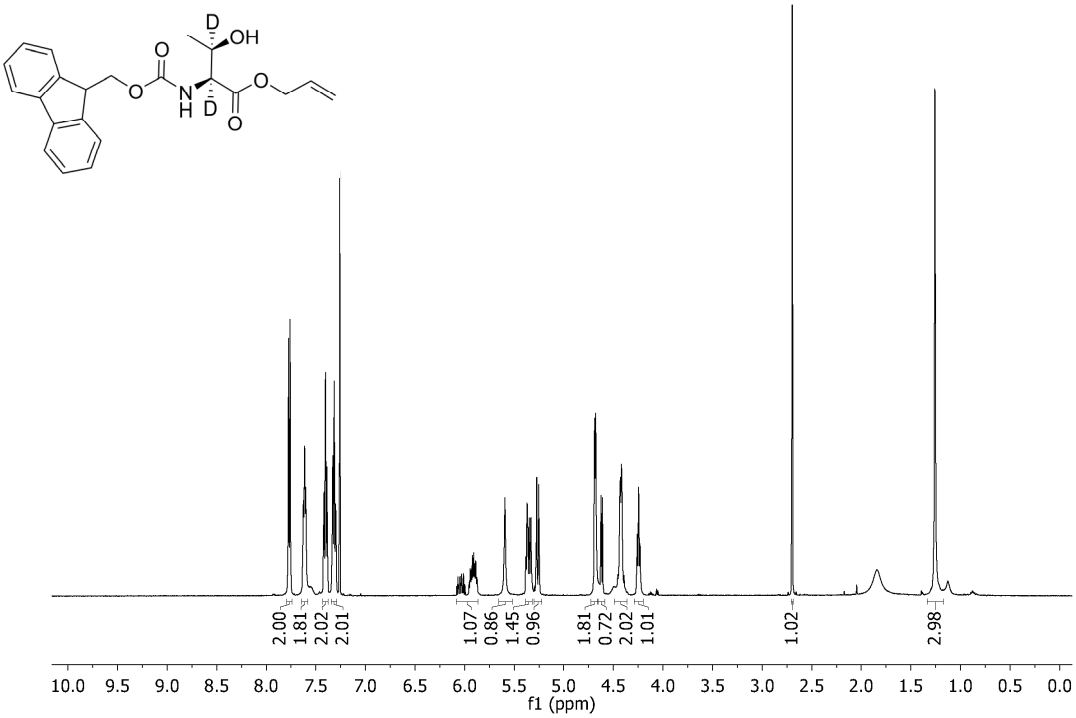
70.81
52.15
38.49
37.74
18.36
f1 (ppm)

Compound 44.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H
D
O
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2.02
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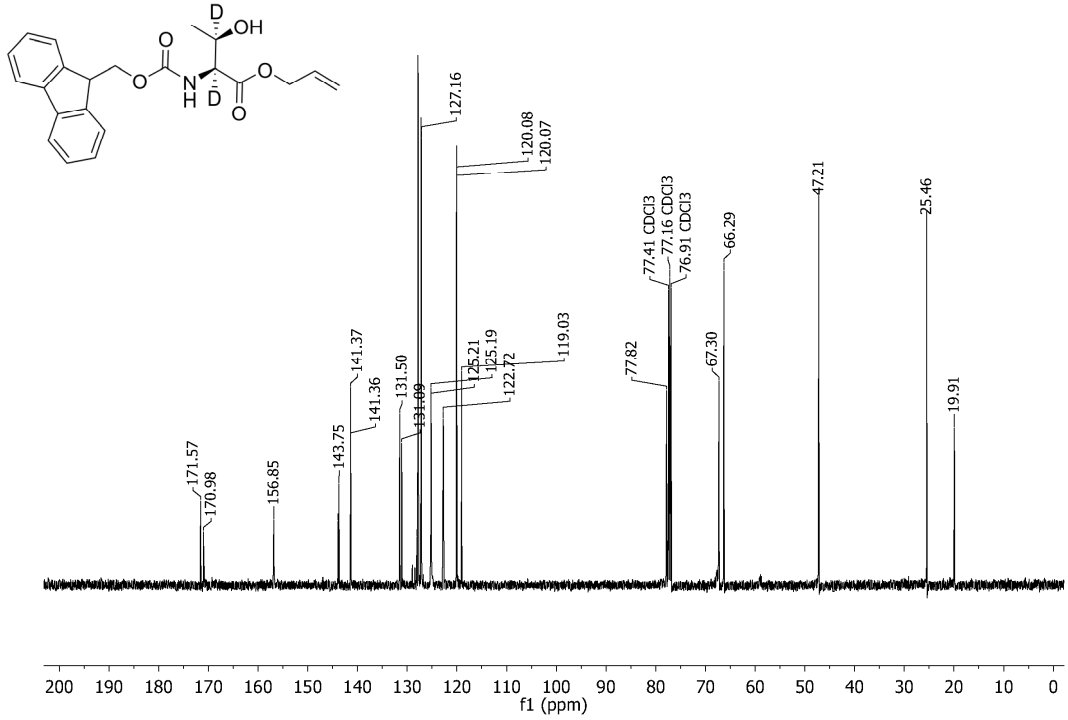
0.96
1.81
0.72
2.02
1.01
1.02
2.98
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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H
D
O
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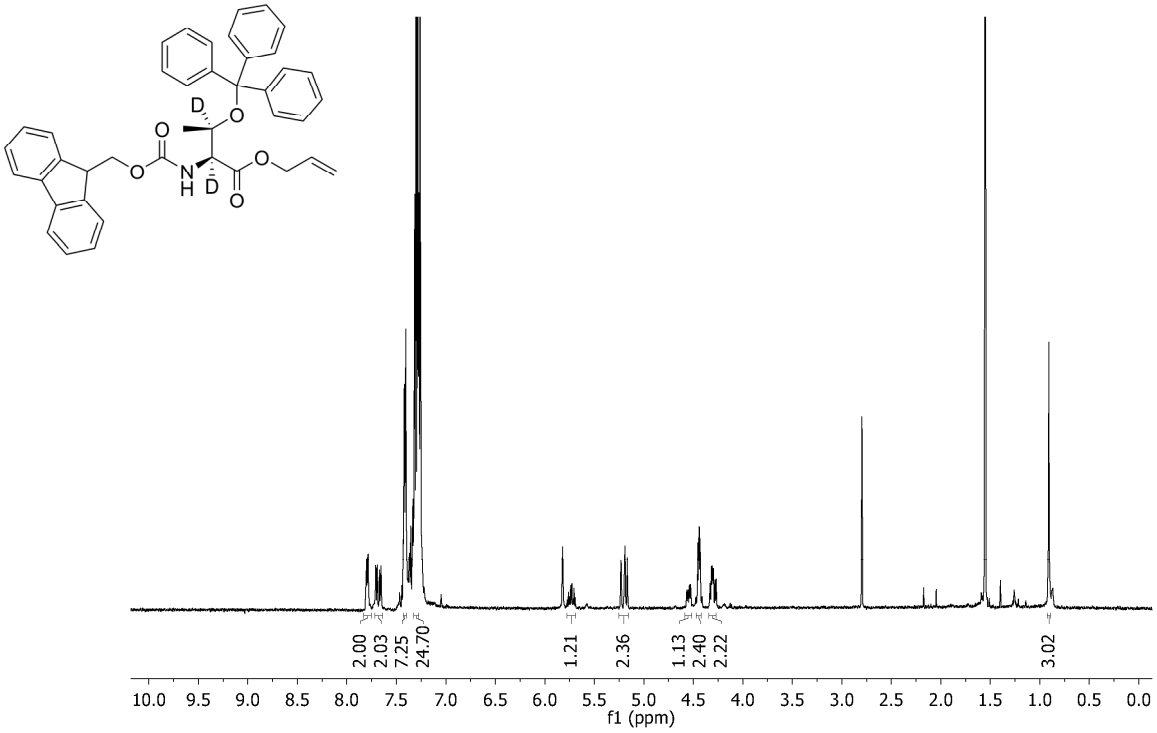
127.16
126.21
125.19
122.72
120.08
120.07
119.03
77.82
77.41 CDCl3
77.16 CDCl3
76.91 CDCl3
67.30
66.29
47.21
25.46
19.91
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

Compound 45.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1.21
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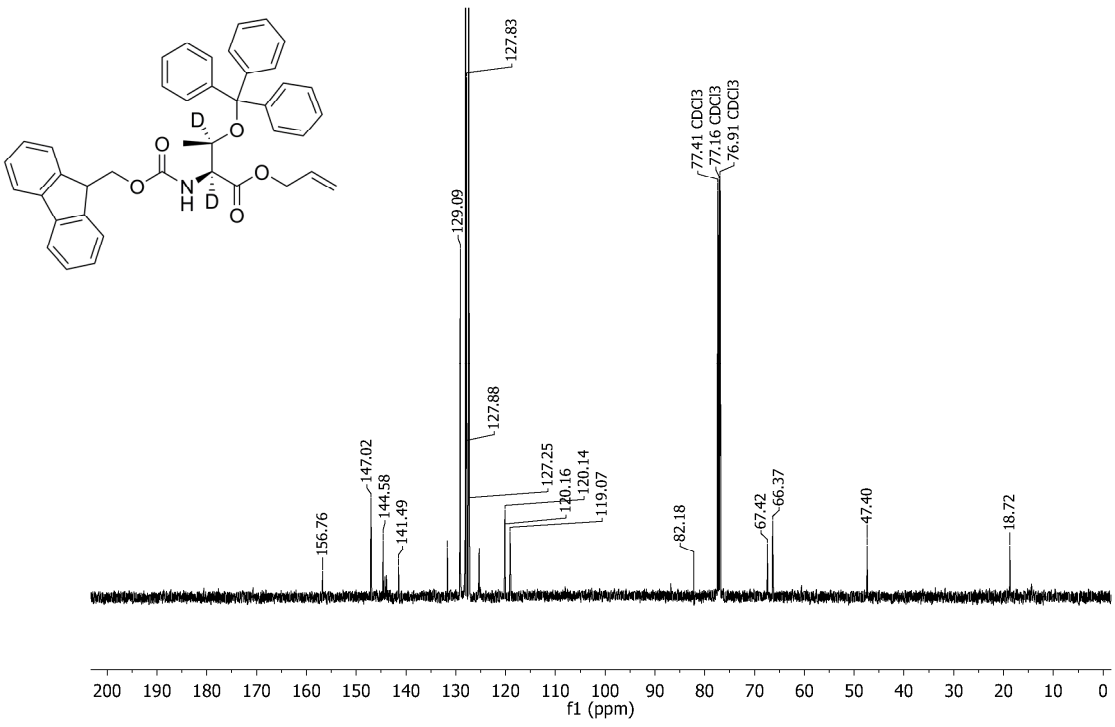
1.13
2.40
2.22
3.02
f1 (ppm)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119.07
156.76
82.18
67.42
66.37
47.40
18.72
f1 (ppm)

References

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