Supporting Information

Developing Crosslinkers Specific for Epimerization Domain in Initiation Modules to Evaluate Mechanism

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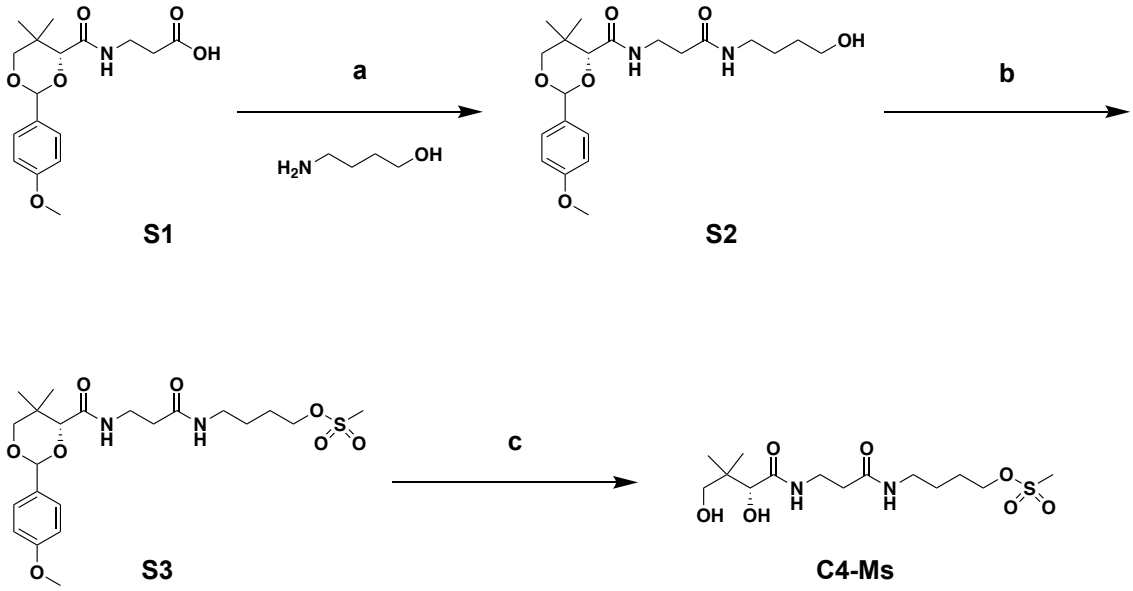
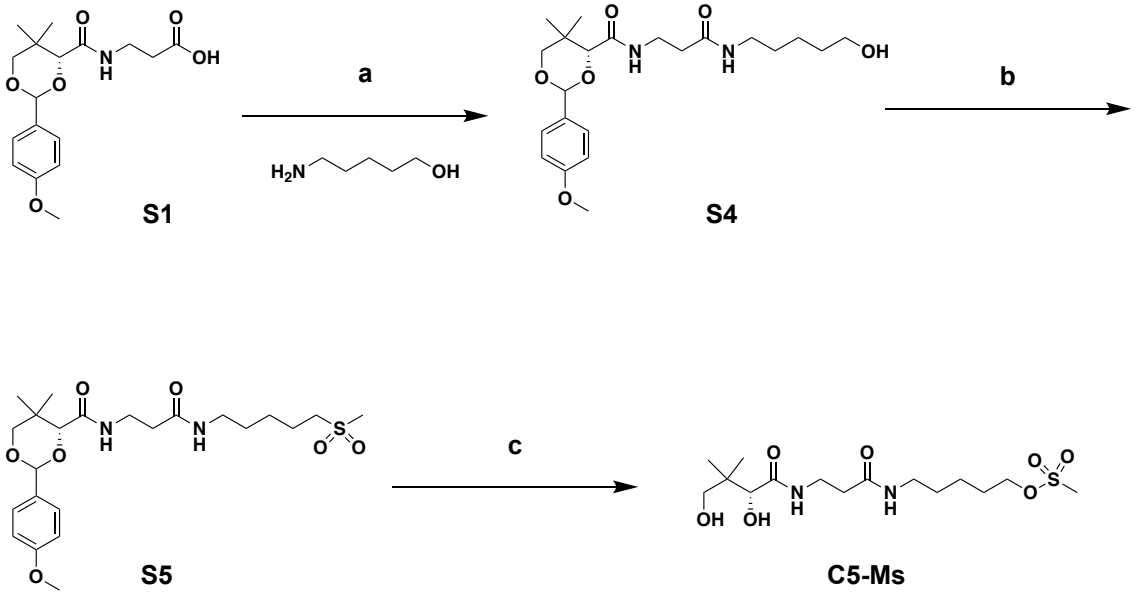
A. Synthetic Methods

A.1. General Synthetic Methods. All commercial reagents were used as provided unless otherwise indicated. Compound **S1** is a known compound. This compound was prepared according to published literature procedures.[1] All reactions were carried out under a nitrogen atmosphere in dry solvents with oven-dried glassware and constant magnetic stirring unless otherwise noted. ^{1}H-NMR spectra were recorded at 500 MHz. ^{13}C-NMR spectra were recorded at 125 MHz on JEOL NMR spectrometers and standardized to the NMR solvent signal as reported by Gottlieb.[2] Multiplicities are given as s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dt = doublet of triplets, tt = triplet of triplets, dq = doublet of quartets, dp = doublet of pentets, qt = quartet of triplets, ddt = doublet of doublet of triplets, ddq = doublet of doublet of quartets, and m = multiplet using integration and coupling constants in Hertz. TLC analysis was performed using Silica Gel 60 F254 plates (Merck) and visualization was accomplished with ultraviolet light (λ = 254 nm) and/or the appropriate stain [phosphomolybdic acid, iodine, ninhydrin, and potassium permanganate]. Silica gel chromatography was carried out with SiliaFlash F60 230-400 mesh (Silicycle), according to the method of Still.[3] Mass spectral data were obtained using a LCMS-IT-TOF mass spectrometer (Shimadzu).

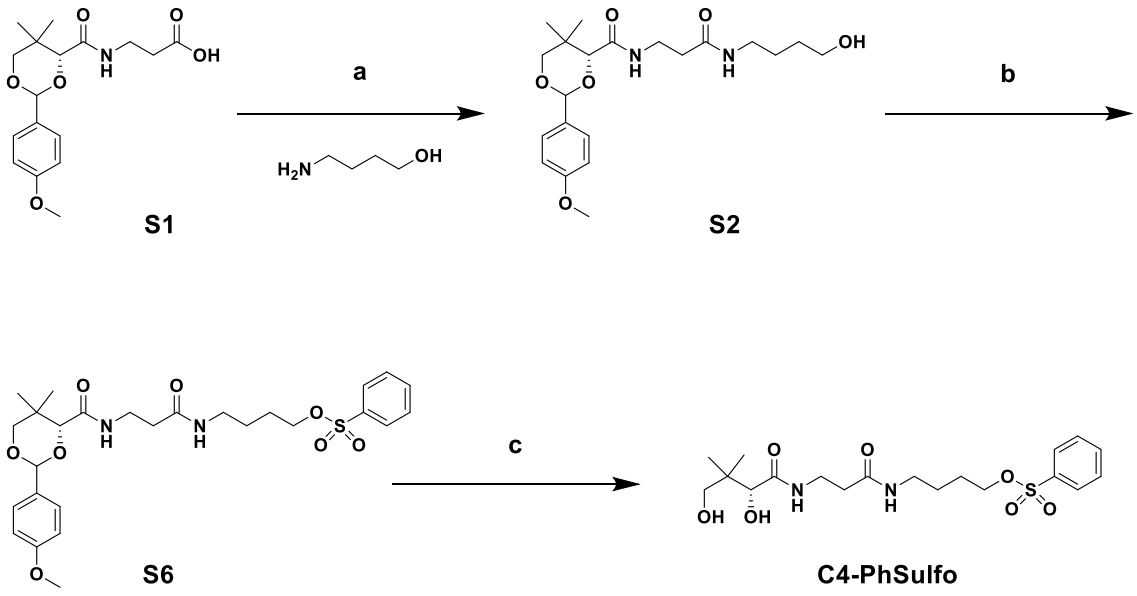
A.2. Chemical Synthetic Schemes



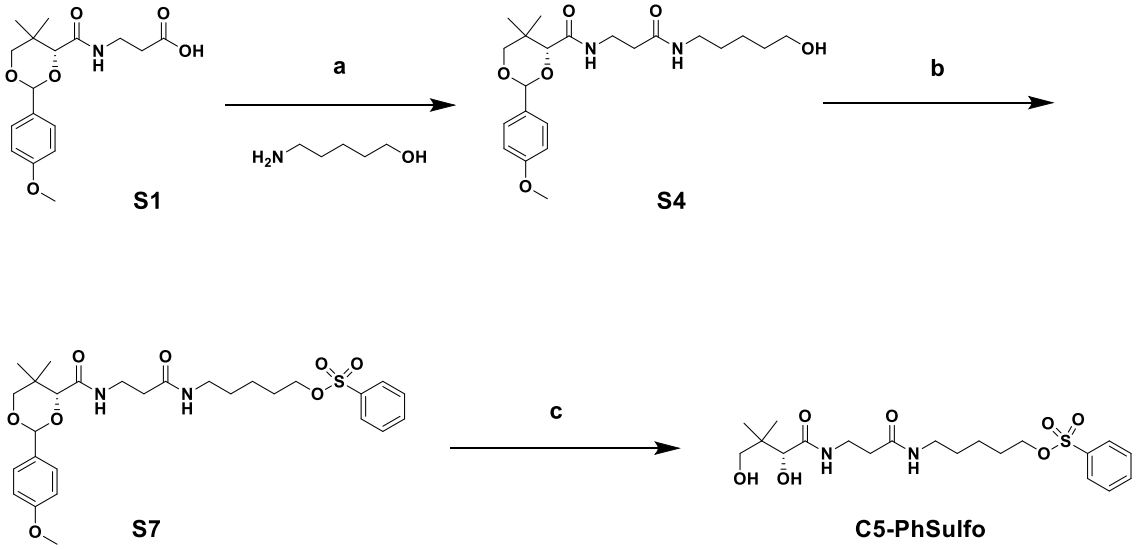
Scheme S1. Synthetic route to C4-Ms (1). *Reagents and conditions*: [a] 4-Amino-1-butanol, HATU, HOBt, DIEA, CH_2Cl_2 rt, 97%; [b] Methanesulfonyl chloride, Et_3N, CH_2Cl_2 rt, 99%; [c] 1 M aq. HCl, THF, rt, 99%.



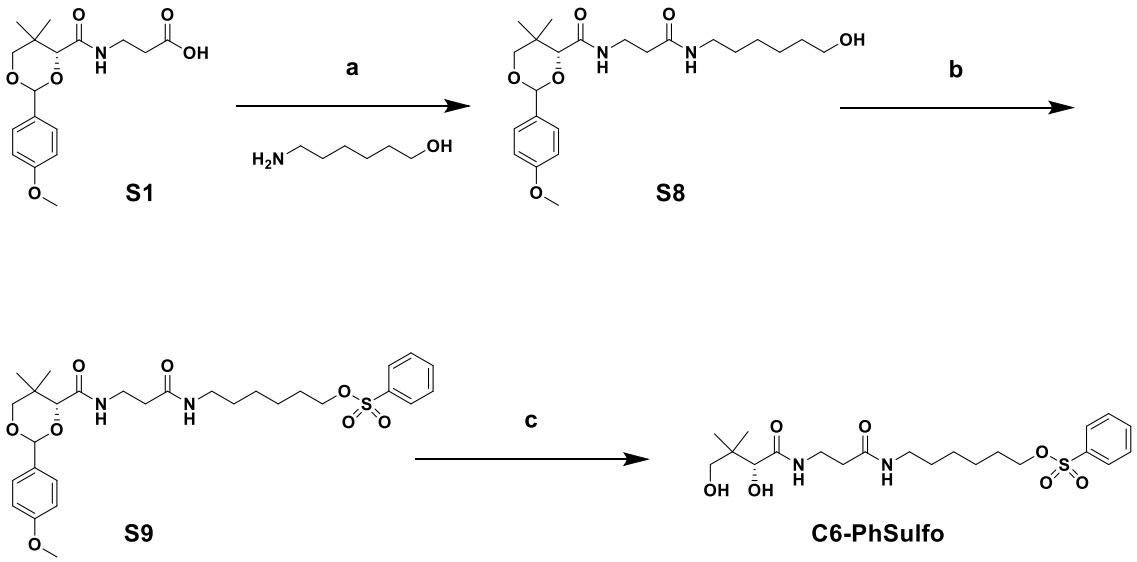
Scheme S2. Synthetic route to C5-Ms (2). *Reagents and conditions*: [a] 5-Amino-1-pentanol, HATU, HOBt, DIEA, CH_2Cl_2 rt, 89%; [b] Methanesulfonyl chloride, Et_3N, CH_2Cl_2 rt, 93%; [c] 1 M aq. HCl, THF, rt, 76%.



Scheme S3. Synthetic route to C4-PhSulfo (3). *Reagents and conditions:* [a] 4-amino-1-butanol, HATU, HOBt, DIEA, CH_2Cl_2, rt, 97%; [b] Benzenesulfonyl chloride, Et_3N, CH_2Cl_2 rt, 93%; [c] 80% aq. acetic acid, rt, 46%.



Scheme S4. Synthetic route to C5-PhSulfo (4). *Reagents and conditions:* [a] 5-amino-1-pentanol, HATU, HOBt, DIEA, CH_2Cl_2, rt, 89%; [b] Benzenesulfonyl chloride, Et_3N, CH_2Cl_2 rt, 90%; [c] 80% aq. acetic acid, rt, 63%.

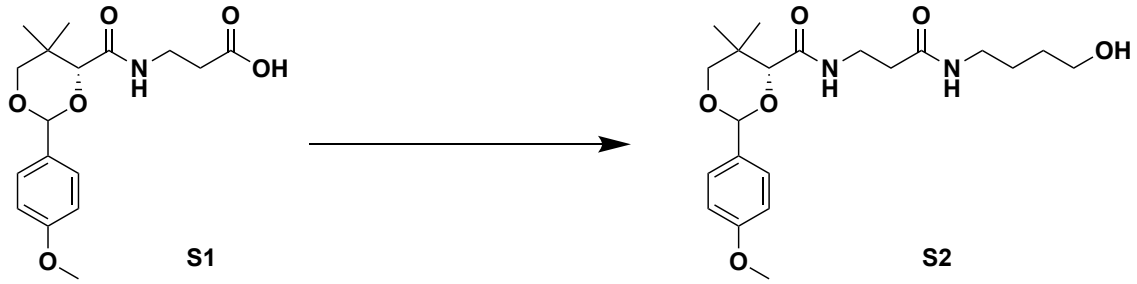


Scheme S5. Synthetic route to C6-PhSulfo (5). *Reagents and conditions:* [a] 6-amino-1-hexanol, HATU, HOBt, DIEA, CH_2Cl_2, rt, 84%; [b] Benzenesulfonyl chloride, Et_3N, CH_2Cl_2 rt, 93%; [c] 80% aq. acetic acid, rt, 69%.

A.3. Synthesis of Probes 1-5.

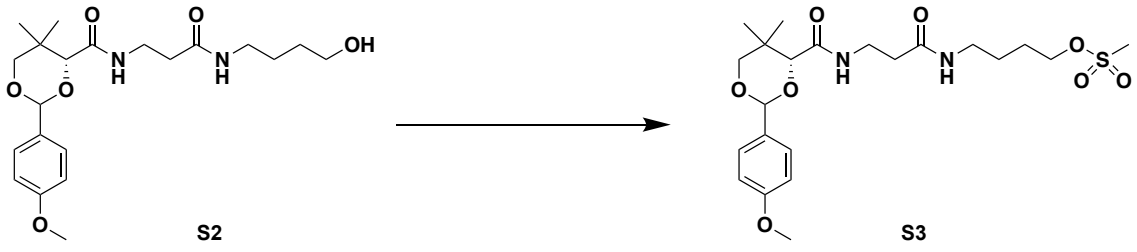
Chemical Synthesis of C4-Ms (1) Compound numbers in bold refer to the structures shown in Scheme S1.

Compound S2



HATU (449 mg, 1.18 mmol), 1-hydroxybenzotriazole (180 mg, 1.18 mmol), and *N*-ethyldiisopropylamine (124 µL, 0.71 mmol) were added to a solution of compound **S1** (200 mg, 0.59 mmol) in CH_2Cl_2 (20 mL). The solution was stirred at room temperature for 10 min and 4-amino-1-butanol (66 µL, 0.71 mmol) was then added. After 12h, the reaction mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (EtOAc to 95:5 EtOAc/MeOH) to afford compound **S2** as a colorless oil (242 mg, 97%). ^{1}H NMR (500 MHz, CD_3OD): δ 7.45 (d, J = 8.9 Hz, 2H), 6.92 (d, J = 8.9 Hz, 2H), 5.51 (s, 1H), 4.13 (s, 1H), 3.79 (s, 3H), 3.69 (q, J = 10.1 Hz, 2H), 3.56–3.40 (m, 4H), 3.19–3.11 (m, 2H), 2.40 (t, J = 6.6 Hz, 2H), 1.58–1.46 (m, 4H), 1.10 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 173.5, 171.6, 161.6, 131.8, 128.8, 114.5, 102.6, 85.0, 79.2, 62.4, 55.7, 40.2, 36.4, 36.3, 34.0, 30.9, 26.8, 22.2, 19.7. HRMS (ESI+): $[M+H]^+$ calcd for $C_{21}H_{32}N_2NaO_6$, 431.2158; found, 431.2010.

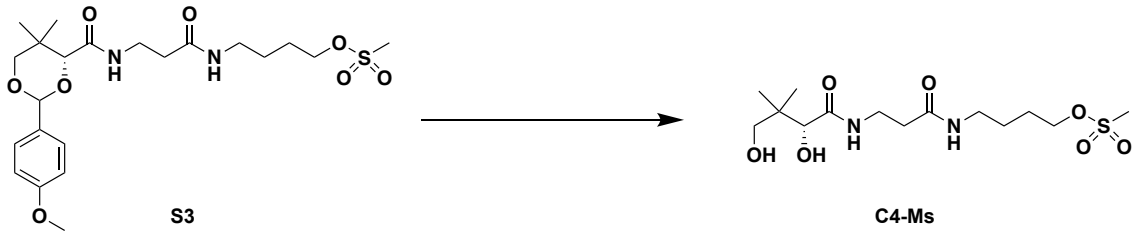
Compound S3



Methanesulfonyl chloride (45 µL, 0.58 mmol) and triethylamine (101 µL, 0.73 mmol) were added to a solution of compound **S2** (123 mg, 0.29 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at 0 °C. After 30 min, the reaction mixture was diluted with CH_2Cl_2. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The residue was

purified by flash chromatography (EtOAc) to afford compound **S3** as a white solid (155 mg, 99%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 5.43 (s, 1H), 4.16 (t, *J* = 6.3 Hz, 1H), 4.04 (s, 2H), 3.78 (s, 3H), 3.69–3.60 (m, 2H), 3.56–3.42 (m, 2H), 3.28–3.16 (m, 2H), 2.95 (s, 3H), 2.41 (t, *J* = 6.3 Hz, 2H), 1.78–1.66 (m, 2H), 1.64–1.50 (m, 2H), 1.06 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.2, 169.7, 160.2, 130.1, 127.6, 113.7, 101.3, 83.8, 78.4, 69.7, 55.4, 38.7, 37.3, 36.0, 34.9, 33.1, 26.5, 25.6, 21.9, 19.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_{22}H_{35}N_2O_8S$, 487.2114; found, 487.2101.

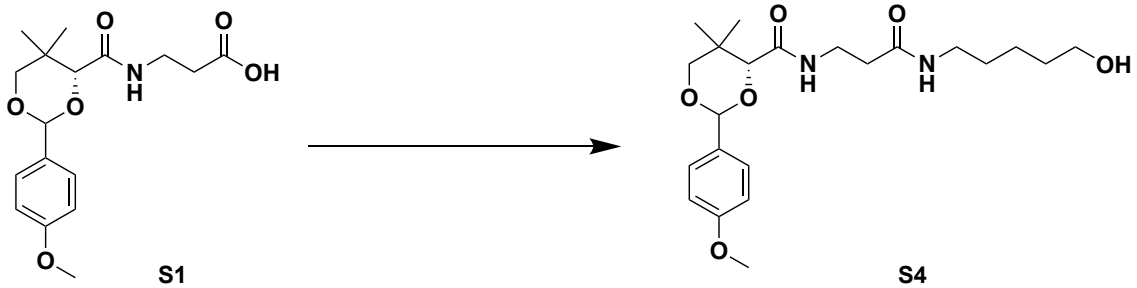
C4-Ms (1)



Compound **S3** (88 mg, 0.18 mmol) was dissolved in 1 M aq. HCl (3 mL) and THF (3 mL) and stirred until the starting material was consumed as shown by TLC. The reaction was then neutralized by addition of AG-1-X8 strong basic anionic exchange resin. After filtration, the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (95:5 EtOAc/acetone to acetone) to afford compound **C4-Ms (1)** as a colorless oil (67 mg, 99%). ^{1}H NMR (500 MHz, $(CD_3)_2CO$): δ 4.25 (t, *J* = 6.3 Hz, 2H), 3.93 (s, 1H), 3.55–3.38 (m, 4H), 3.24 (q, *J* = 6.3 Hz, 2H), 3.09 (s, 3H), 2.41, (t, *J* = 6.3 Hz, 2H), 1.80–1.72 (m, 2H), 1.64–1.56 (m, 2H), 0.94 (s, 3H), 0.86 (s, 3H). ^{13}C NMR (125 MHz, $(CD_3)_2CO_2$): δ174.1, 171.8, 77.4, 70.8, 40.1, 39.0, 37.0, 36.2, 35.9, 27.2, 26.4, 21.8, 20.6. HRMS (ESI+): $[M+H]^+$ calcd for $C_{14}H_{29}N_2O_7S$, 369.1695; found, 369.1621.

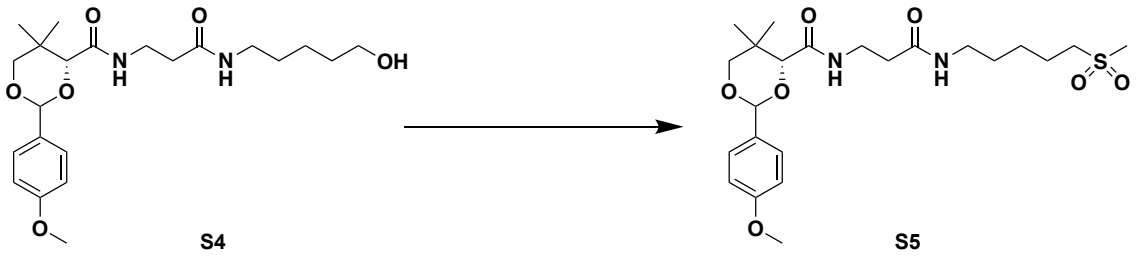
Chemical Synthesis of C5-Ms (2) Compound numbers in bold refer to the structures shown in Scheme S2.

Compound S4



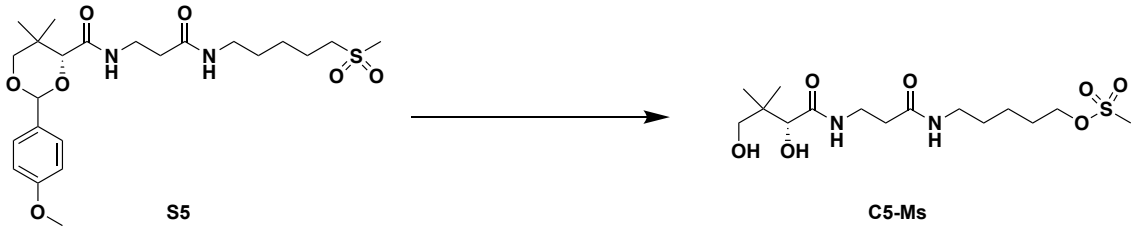
HATU (2 g, 4.74 mmol), 1-hydroxybenzotriazole (726 mg, 4.74 mmol), and *N*-ethyldiisopropylamine (825 µL, 4.74 mmol) were added to a solution of compound **S1** (800 mg, 2.37 mmol) in CH_2Cl_2 (100 mL). The solution was stirred at room temperature for 10 min and 5-amino-1-pentanol (292 mg, 2.84 mmol) was then added. After 10 h, the reaction mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (EtOAc to 90:10 EtOAc/MeOH) to afford compound **S4** as a colorless oil (890 mg, 89%). ^{1}H NMR (500 MHz, CD_3OD): δ 7.45 (d, J = 8.6 Hz, 2H), 6.93 (d, J = 8.6 Hz, 2H), 5.53 (s, 1H), 4.14 (s, 1H), 3.80 (s, 3H), 3.75–3.65 (m, 2H), 3.56–3.40 (m, 4H), 3.18–3.10 (m, 2H), 2.40 (t, J = 6.6 Hz, 2H), 1.56–1.44 (m, 4H), 1.40–1.31 (m, 2H), 1.10 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 173.5, 171.6, 161.7, 131.8, 128.8, 114.5, 102.6, 85.1, 79.3, 62.8, 40.4, 36.4, 36.3, 34.0, 33.2, 30.2, 24.3, 22.2, 19.7. HRMS (ESI+): $[M+H]^+$ calcd for $C_{22}H_{35}N_2O_6$, 423.2495; found, 423.2501.

Compound S5



Methanesulfonyl chloride (325 µL, 4.2 mmol) and triethylamine (731 µL, 5.25 mmol) were added to a solution of compound **S4** (890 mg, 2.10 mmol) in CH_2Cl_2 (50 mL). The solution was stirred at 0 °C. After 2 h, the reaction mixture was diluted with CH_2Cl_2. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The residue was purified by flash chromatography (EtOAc) to afford compound **S5** as a white solid (975 mg, 93%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 5.43 (s, 1H), 4.17 (t, J = 6.3 Hz, 2H), 4.04 (s, 1H), 3.79 (s, 3H), 3.66 (q, J = 11.2 Hz, 2H), 3.59–3.41 (m, 2H), 3.18 (q, J = 6.7 Hz, 2H), 2.96 (s, 3H), 2.38 (t, J = 6.3 Hz, 2H), 1.70 (p, J = 7.0 Hz, 2H), 1.48 (p, J = 7.3 Hz, 2H), 1.38 (p, J = 7.3 Hz, 2H), 1.06 (d, J = 5.7 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.9, 169.6, 160.3, 130.2, 127.6, 113.8, 101.3, 83.8, 78.5, 69.9, 55.4, 39.1, 37.4, 36.1, 35.0, 33.1, 28.9, 28.7, 22.8, 21.9, 19.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_{23}H_{37}N_2O_8S$, 501.2271; found, 501.2235.

C5-Ms (2)

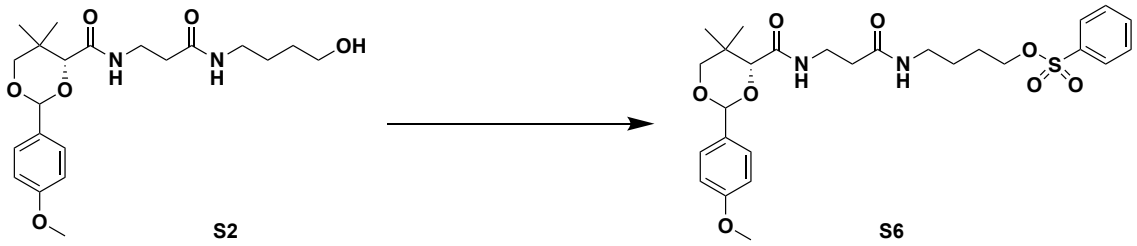


S5 C5-Ms

Compound **S5** (975 mg, 1.95 mmol) was dissolved in 1 M aq. HCl (10 mL) and THF (10 mL) and stirred until the starting material was consumed as shown by TLC. The reaction was then neutralized by addition of AG-1-X8 strong basic anionic exchange resin. After filtration, the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (90:10 EtOAc) to afford compound **C5-Ms (2)** as a colorless oil (565 mg, 76%). ^{1}H NMR (500 MHz, $(CD_3)_2CO$): δ 4.27–4.17 (m, 3H), 3.61 (d, J = 5.1 Hz, 1H), 3.56–3.35 (m, 4H), 3.20 (q, J = 6.5 Hz, 2H), 3.08 (s, 3H), 2.39 (q, J = 6.3 Hz, 2H), 1.75 (p, J = 7.0 Hz, 2H), 1.53 (p, J = 7.2 Hz, 2H), 1.44 (p, J = 7.2 Hz, 2H), 0.94 (s, 3H), 0.86 (s, 3H). ^{13}C NMR (125 MHz, $(CD_3)_2CO$): δ 174.1, 171.6, 77.5, 71.1, 70.8, 40.2, 39.4, 37.0, 36.2, 35.9, 29.8, 29.5, 23.5, 21.9, 20.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{15}H_{30}N_2NaO_7S$, 405.1671; found, 405.1653.

Chemical Synthesis of C4-PhSulfo (3) Compound numbers in bold refer to the structures shown in Scheme S3.

Compound S6

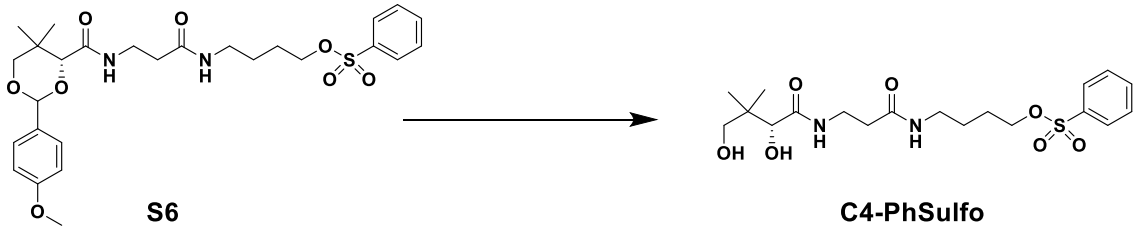


S2 S6

Benzenesulfonyl chloride (47 µL, 0.37 mmol) and triethylamine (102 µL, 0.73 mmol) were added to a solution of compound **S2** (100 mg, 0.25 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at rt overnight. The reaction mixture was diluted with CH_2Cl_2 and H_2O. The organic phase was separated, washed with brine, dried and concentrated under reduced pressure. The oil was purified by flash chromatography (CH_2Cl_2:EtOAc = 5:3) to afford compound **S6** as an oil (125 mg, 93%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.89 (dd, J = 8.2, 1.4 Hz, 2H), 7.69 – 7.62 (m, 1H), 7.59 – 7.52 (m, 2H), 7.45 – 7.39 (m, 2H), 7.05 – 6.98 (m, 1H), 6.96 – 6.88 (m, 2H), 6.12 (s, 1H), 5.46 (s, 1H), 4.08 (s, 1H), 4.03 (q, J = 6.1 Hz, 2H), 3.81 (d, J = 0.9 Hz, 3H), 3.74 – 3.63 (m, 2H), 3.52 (p, J = 6.6 Hz, 2H), 3.25

– 3.11 (m, 2H), 2.41 (q, J = 7.3, 6.2 Hz, 2H), 1.65 (dq, J = 8.2, 6.1 Hz, 2H), 1.52 (q, J = 7.1 Hz, 2H), 1.09 (d, J = 4.2 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 169.9, 160.4, 133.9, 129.4, 128.0, 127.6, 113.9, 101.5, 83.9, 78.6, 70.3, 55.5, 38.8, 36.4, 35.1, 33.2, 26.4, 25.7, 22.0, 19.3. HRMS (ESI+): $[M+H]^+$ calcd for $C_{27}H_{36}N_2O_8S$, 549.2192; found, 549.2262.

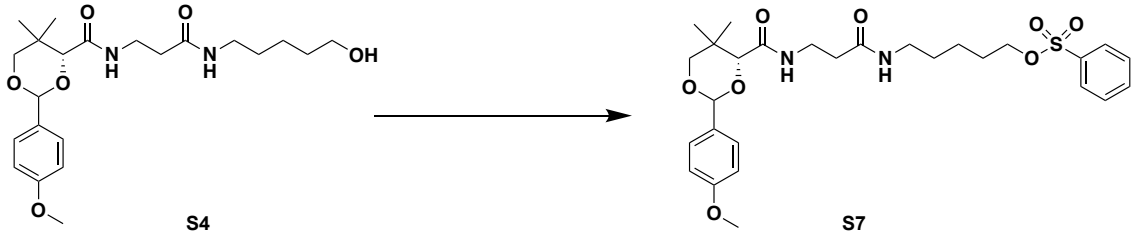
C4-BPhSulfo (3)



Compound **S6** (39 mg, 0.07 mmol) was dissolved in 80% aq. acetic acid (1.5 mL) and stirred until the starting material was consumed as shown by TLC. The solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (EtOAc:CH_2Cl_2 = 5:3) to afford compound **C4-PhSulfo (3)** as a colorless oil (14 mg, 46%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.95 – 7.85 (m, 2H), 7.67 (t, J = 7.4 Hz, 1H), 7.57 (t, J = 7.6 Hz, 2H), 7.38 (q, J = 6.2 Hz, 1H), 6.26 (t, J = 5.8 Hz, 1H), 4.06 (t, J = 6.1 Hz, 2H), 3.99 (s, 1H), 3.55 (q, J = 6.0 Hz, 2H), 3.48 (s, 2H), 3.23 (ddq, J = 19.2, 13.6, 6.8 Hz, 2H), 2.43 (q, J = 6.0 Hz, 2H), 1.67 (ddd, J = 16.4, 9.9, 6.7 Hz, 2H), 1.56 (p, J = 6.5, 6.0 Hz, 2H), 1.00 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.8, 171.7, 135.9, 134.1, 129.5, 127.9, 71.1, 70.6, 44.7, 39.5, 38.9, 36.0, 35.4, 29.9, 27.0, 26.5, 25.8, 21.6, 20.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{19}H_{30}N_2O_7S$, 431.1774; found, 431.1847.

Chemical Synthesis of C5-PhSulfo (4) Compound numbers in bold refer to the structures shown in Scheme S4.

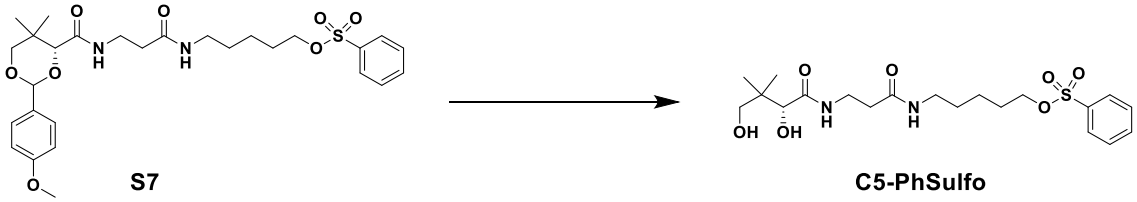
Compound S7



Benzenesulfonyl chloride (45 μL, 0.36 mmol) and triethylamine (99 μL, 0.71 mmol) were added to a solution of compound **S4** (100 mg, 0.24 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at rt overnight. The reaction mixture was diluted with CH_2Cl_2 and H_2O. The organic phase was separated, washed with brine, dried and concentrated in

vacuo. The oil was purified by flash chromatography (CH_2Cl_2:EtOAc = 5:3) to afford compound **S7** as an oil (117 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.93 – 7.85 (m, 2H), 7.69 – 7.61 (m, 1H), 7.56 – 7.51 (m, 2H), 7.40 (d, *J* = 8.2 Hz, 2H), 7.04 (t, *J* = 6.3 Hz, 1H), 6.89 (d, *J* = 8.1 Hz, 2H), 6.20 (t, *J* = 5.7 Hz, 1H), 5.44 (s, 1H), 4.05 (s, 1H), 4.00 (t, *J* = 6.3 Hz, 2H), 3.79 (s, 3H), 3.71 – 3.61 (m, 2H), 3.50 (dp, *J* = 20.3, 6.9 Hz, 2H), 3.17 – 3.10 (m, 2H), 2.38 (t, *J* = 6.2 Hz, 2H), 1.61 (p, *J* = 6.6 Hz, 2H), 1.40 (p, *J* = 7.1 Hz, 2H), 1.31 (q, *J* = 6.9, 5.7 Hz, 2H), 1.06 (d, *J* = 3.2 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.9, 169.7, 160.3, 136.1, 133.9, 130.2, 129.4, 127.9, 127.6, 113.8, 101.4, 83.9, 78.5, 70.6, 55.4, 39.2, 36.2, 35.1, 33.2, 28.9, 28.5, 22.8, 21.9, 19.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_{28}H_{38}N_2O_8S$, 563.2349; found, 563.2415.

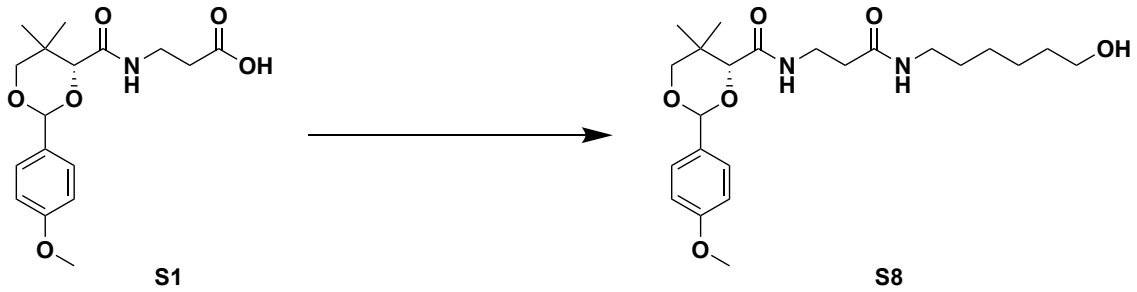
C5-PhSulfo (4)



Compound **S7** (24 mg, 0.04 mmol) was dissolved in 80% aq. acetic acid (1 mL) and stirred until the starting material was consumed as shown by TLC. The solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (EtOAc:CH_2Cl_2 = 5:3) to afford compound **C5-PhSulfo (4)** as a colorless oil (12 mg, 63%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.92 – 7.89 (m, 2H), 7.66 (d, *J* = 7.5 Hz, 1H), 7.57 (dd, *J* = 8.5, 7.2 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 1H), 5.95 (s, 1H), 4.05 (t, *J* = 6.2 Hz, 2H), 4.00 (s, 1H), 3.58 (q, *J* = 6.2 Hz, 2H), 3.53 – 3.47 (m, 2H), 3.22 (dq, *J* = 19.2, 6.8 Hz, 2H), 2.46 – 2.41 (m, 2H), 1.69 (d, *J* = 6.9 Hz, 2H), 1.52 – 1.45 (m, 2H), 1.38 (tt, *J* = 9.4, 5.6 Hz, 2H), 1.02 (s, 3H), 0.92 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.6, 171.5, 154.1, 136.0, 134.1, 129.4, 128.0, 77.9, 77.7, 71.1, 70.8, 39.5, 39.3, 36.0, 35.4, 28.9, 28.6, 22.9, 21.8, 20.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{20}H_{32}N_2O_7S$, 445.1930; found, 445.2002.

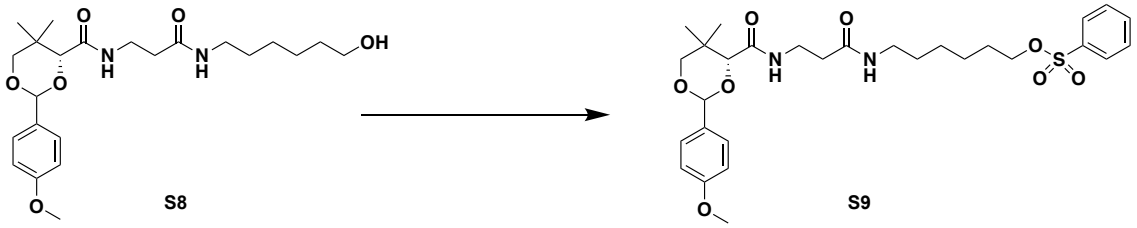
Chemical Synthesis of C6-PhSulfo (5) Compound numbers in bold refer to the structures shown in Scheme S5.

Compound S8



HATU (733 mg, 1.93 mmol), 1-hydroxybenzotriazole (261 mg, 1.93 mmol) and *N*-ethyldiisopropylamine (520 µL, 2.96 mmol) were added to a solution of compound **S1** (500 mg, 1.48 mmol) in CH_2Cl_2 (25 mL). The solution was stirred at room temperature for 10 min and 6-amino-1-hexanol (174 mg, 1.48 mmol) was then added. After 12 h, the reaction mixture was washed with 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (EtOAc to 95:5 EtOAc/MeOH) to afford compound **S8** as a colorless oil (543 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 – 7.41 (m, 2H), 7.02 (s, 1H), 6.97 – 6.90 (m, 2H), 5.95 (s, 1H), 5.46 (s, 1H), 4.08 (s, 1H), 3.82 (d, *J* = 1.7 Hz, 3H), 3.70 (dd, *J* = 13.9, 7.9 Hz, 2H), 3.62 (dd, *J* = 6.7, 6.1 Hz, 2H), 3.51 (ddt, *J* = 19.9, 13.1, 6.3 Hz, 1H), 3.20 (dq, *J* = 12.0, 6.9 Hz, 2H), 3.12 (s, 1H), 2.41 (t, *J* = 6.2 Hz, 2H), 1.64 (s, 2H), 1.59 – 1.28 (m, 10H), 1.09 (d, *J* = 2.2 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.1, 169.8, 160.3, 130.1, 127.7, 113.9, 101.3, 83.8, 78.5, 62.5, 55.3, 43.3, 39.4, 36.2, 35.3, 33.1, 32.4, 29.3, 26.5, 25.3, 22.0, 19.3, 12.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{23}H_{36}N_2NaO_6$, 459.2471; found, 459.2462.

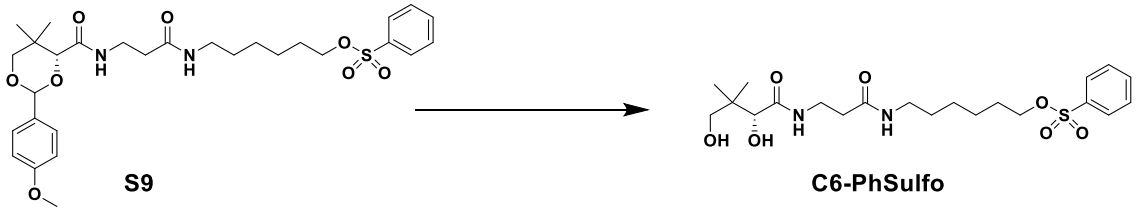
Compound S9



Benzenesulfonyl chloride (100 µL, 0.61 mmol) and triethylamine (170 µL, 1.22 mmol) were added to a solution of compound **S8** (177 mg, 0.41 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at rt overnight. The reaction mixture was diluted with CH_2Cl_2 and H_2O. The organic phase was separated, washed with brine, dried and concentrated in vacuo. The oil was purified by flash chromatography (CH_2Cl_2:EtOAc = 5:3) to afford compound **S9** as an oil (217 mg, 93%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 (d, *J* = 7.2 Hz, 2H), 7.68 – 7.62 (m, 1H), 7.55 (dd, *J* = 8.4, 7.0 Hz, 2H), 7.45 – 7.39 (m, 2H), 7.00 (s,

1H), 6.94 – 6.88 (m, 2H), 5.91 (s, 1H), 5.45 (s, 1H), 4.07 (s, 1H), 4.02 (t, *J* = 6.3 Hz, 2H), 3.81 (s, 3H), 3.68 (q, *J* = 11.4 Hz, 2H), 3.53 (dq, *J* = 19.8, 6.9 Hz, 1H), 3.16 (q, *J* = 6.7 Hz, 2H), 2.40 (t, *J* = 6.2 Hz, 2H), 1.68 (s, 2H), 1.65 – 1.59 (m, 2H), 1.41 (q, *J* = 7.2 Hz, 2H), 1.33 – 1.27 (m, 4H), 1.09 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.8, 169.7, 160.4, 133.9, 130.3, 129.4, 128.0, 127.6, 122.5, 113.9, 101.5, 84.0, 78.6, 70.8, 60.5, 55.5, 39.4, 36.4, 35.1, 33.2, 29.4, 28.8, 26.3, 25.1, 22.0, 21.2, 19.3, 14.3. HRMS (ESI+): $[M+H]^+$ calcd for $C_{28}H_{38}N_2O_8S$, 577.2505; found, 577.2580.

C6-PhSulfo (5)



Compound **S9** (82 mg, 0.14 mmol) was dissolved in 80% aq. acetic acid (2 mL) and stirred until the starting material was consumed as shown by TLC. The solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (EtOAc:CH_2Cl_2 = 5:3) to afford compound **C6-PhSulfo (5)** as a colorless oil (45 mg, 69%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 (dt, *J* = 7.2, 1.4 Hz, 2H), 7.69 – 7.63 (m, 1H), 7.56 (dd, *J* = 8.4, 7.0 Hz, 2H), 7.42 (t, *J* = 6.2 Hz, 1H), 6.18 (t, *J* = 5.9 Hz, 1H), 4.19 (d, *J* = 19.0 Hz, 1H), 4.04 (t, *J* = 6.3 Hz, 2H), 3.98 (s, 1H), 3.55 (qt, *J* = 6.2, 2.6 Hz, 2H), 3.48 (s, 2H), 3.18 (q, *J* = 6.7 Hz, 2H), 2.42 (t, *J* = 6.0 Hz, 2H), 1.93 (s, 2H), 1.70 – 1.60 (m, 2H), 1.45 (p, *J* = 7.1 Hz, 2H), 1.38 – 1.22 (m, 5H), 0.99 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.8, 171.6, 136.1, 134.0, 129.4, 127.9, 77.8, 71.1, 70.9, 39.5, 35.9, 35.4, 29.3, 28.8, 26.2, 25.1, 21.6, 20.6. HRMS (ESI+): $[M+H]^+$ calcd for $C_{21}H_{34}N_2O_7S$, 459.2087; found, 459.2155.

B. Chemical Biological Protocols

B.1. Protein expression and purification

Stocks of CoaA, CoaD, CoaE, and Sfp were prepared as described previously and stored at –80 °C.[4] Briefly, *apo*-TycA *E. coli* BL21(DE3) cells were grown in Terrific Broth at 37 °C with 30 μg/mL kanamycin and 100 μg/mL ampicillin (*apo* TycA). Cells were induced with 1 mM β-D-1-thiogalactopyranoside (IPTG) at OD_{600} of 0.8 and incubated at 16 °C for 16 h. Cells were spun down by centrifugation at 2,000 rpm for 30 min and the collected pellets were lysed by sonication, followed by another centrifugation at 10,000 rpm for 1 h to clear the lysate. The His_6-tagged protein was purified using a combination of Ni-NTA and size exclusion chromatography (Superdex 200 column, GE Healthcare). Purified proteins were concentrated to 8–10 mg/ml using a 100-kDa cut-off Amicon Ultra Centrifugation Filter (Millipore).

B.2. Preparation of crosslinked complexes

Crosslinking of TycA using probes 1 and 2 (Figure 3A)

One-pot reaction mixtures contained the following (final volume of 50 μL): 50 mM potassium phosphate buffer (pH 7.0), 8 mM ATP, 15 mM $MgCl_2$, 0.01 μg/μL MBP-CoaA, 0.01 μg/μL MBP-CoaD, 0.01 μg/μL MBP-CoaE, 0.008 μg/μL Sfp (native), and 1 μM of *apo*-TycA. The reactions were initiated by the addition of 1 mM C4-Ms or C5-Ms and incubated for 12 h at 37 °C. In negative controls, crosslinkers C4-Ms and C5-Ms were replaced with vehicle DMSO or Sfp was omitted. In all experiments, the total DMSO concentration was kept at 1.0%. Reactions were treated with 5× SDS-loading buffer (strong reducing) and the samples were heated at 95 °C for 5 min. Samples were separated by 1D SDS-PAGE. Gels were fixed in destain solution and washed with water prior to staining with bluesilver Coomassie stain.

Crosslinking of TycA using probes 3, 4, and 5 (Figure 3B)

The final reaction conditions for chemoenzymatic loading and crosslinking were: 12.5 mM $MgCl_2$, 4 mM ATP, 0.01 mg/ml maltose-binding protein-CoA biosynthetic enzyme A (MBP-CoaA), 0.01 mg/ml MBP-CoaD, 0.01 mg/ml MBP-CoaE, 0.04 mg/ml Sfp (native), 0.1 mM crosslinking probe dissolved in DMSO, and 1 mg/ml TycA in reaction buffer (50 mM HEPES, 250 mM NaCl, 0.5 mM TCEP, pH 7.4). After the addition of all components, reactions were gently mixed and incubated at room temperature for 12 hr. The loading and crosslinking activity was monitored by running the reaction on an 8% SDS-PAGE gel.

B.3. Protein labeling

One-pot reaction mixtures contained the following (final volume of 50 µL): 50 mM potassium phosphate buffer (pH 7.0), 8 mM ATP, 15 mM $MgCl_2$, 0.01 µg/µL MBP-CoaA, 0.01 µg/µL MBP-CoaD, 0.01 µg/µL MBP-CoaE, 0.008 µg/µL Sfp (native), and 1 µM of *apo*-TycA. The reactions were initiated by the addition of 500 µM C5-Ms and incubated for 12 h at 37 °C. In crosslinking experiments the total DMSO concentration was kept at 1.0%. For the labeling of TycA, one-pot reaction mixtures (45 µL) were treated with *L*-Phe-AMS-BPyne (1 µM from a 100 µM stock in DMSO). Inhibition studies were performed by pre-incubation of one-pot reaction mixtures (45 µL) with *L*-Phe-AMS (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature, respectively. In labeling experiments the total DMSO concentration was kept at 3.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice. To initiate the click reaction, rhodamine (Rh)-azide, TCEP, TBTA ligand, and $CuSO_4$ were added to provide final concentrations of 100 µM, 1 mM, 100 µM, and 1 mM, respectively. After 1 h at room temperature, 5× SDS-loading buffer (strong reducing) was added and the samples were heated at 95 °C for 5 min. Samples were separated by 1D SDS-PAGE and fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

B.4. Generation of mutant constructs

The His_6-tagged TycA in pQE60 plasmid was used as the DNA template for site-directed mutagenesis with the mutagenic primer listed in Table S1. PCRs were performed using varying annealing temperatures. TycA PCR products were digested with Dpn1 to eliminate template WT TycA. Digested PCR products were transformed into pREP4. Single colonies were isolated and their plasmids were extracted using GenElute miniprep kit (Sigma). Site-directed mutagenesis was verified by DNA sequencing (Genewiz).

Table S1. Primers used in site-directed mutagenesis

*Nucleotides that correspond to the mutated amino acid residues are bolded.

Mutant	Forward Primer	Reverse Primer
H743A	AATTCAT**GCG**TTGGTCGTG GATGGCATTTCCT	CAA**CGC**ATGAATTGCGAGAAACAAATG ATCGCC
D858A	CAAC**GCG**CTGTTGTTGGCG GCGC	AG**CGC**GTTGATTTCCGTTTGATAGGCCT GATTG
E870A	TTGCG**GCG**TGGAGCAAGCT TGCGCA	A**CGC**CGCAAAAGCCAAGCCGAGCG
S872A	GG**GCG**AAGCTTGCGCAA ATCGTCATTCATTTGG	GCTT**CGC**CCACTCCGCAAAAGCCAAGC
K873A	C**GCG**CTTGCGCAAATCGTC ATTCATTTGGAGGG	GCAAG**CGC**GCTCCACTCCGCAAAA GCC
Q876A	G**GCG**ATCGTCATTCATTTGG AGGGGCACG	GACGAT**CGC**CGCAAGCTTGCTCCACTC C
H880A	GTCATT**GCG**TTGGAGGGGC ACGGGC	CAA**CGC**AATGACGATTTGCGCAAGCTT GCTCC
E882A	TCATTTG**GCG**GGGCACGGG CGC	C**CGC**CAAATGAATGACGATTTGCGCAA GCT
H887A	GC**GCG**GACATCATCGAACA GGCAAACGTG	GTC**CGC**GCGCCCGTGCCCCT

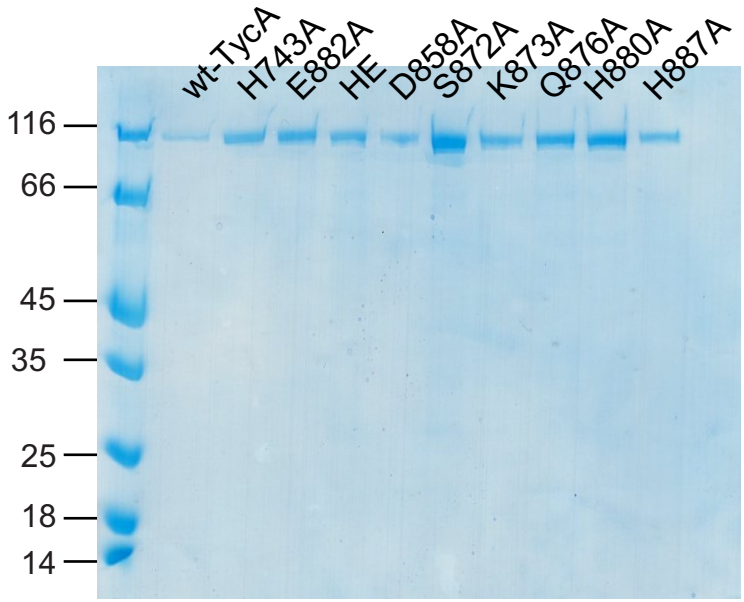


Figure S1. Expressed and purified TycA mutants on an 8% SDS-PAGE gel.

B.5. Experimental procedure for protein digestion followed by mass spectrometry

After SDS-PAGE, each band was excised and digested with a trypsin (TPCK treated; Worthington Biochemical Co., Lakewood, NJ) and endoproteinase Asp-N (Sequencing Grade, Roche, Basel, Switzerland) at 37 °C for 12 h. The resultant peptides were subjected to matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) on an ultrafleXtreme (Bruker Daltonics, Bremen, Germany) in a positive reflector mode using α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) as a matrix. Selected ion peaks were analyzed MS/MS in LIFT mode.

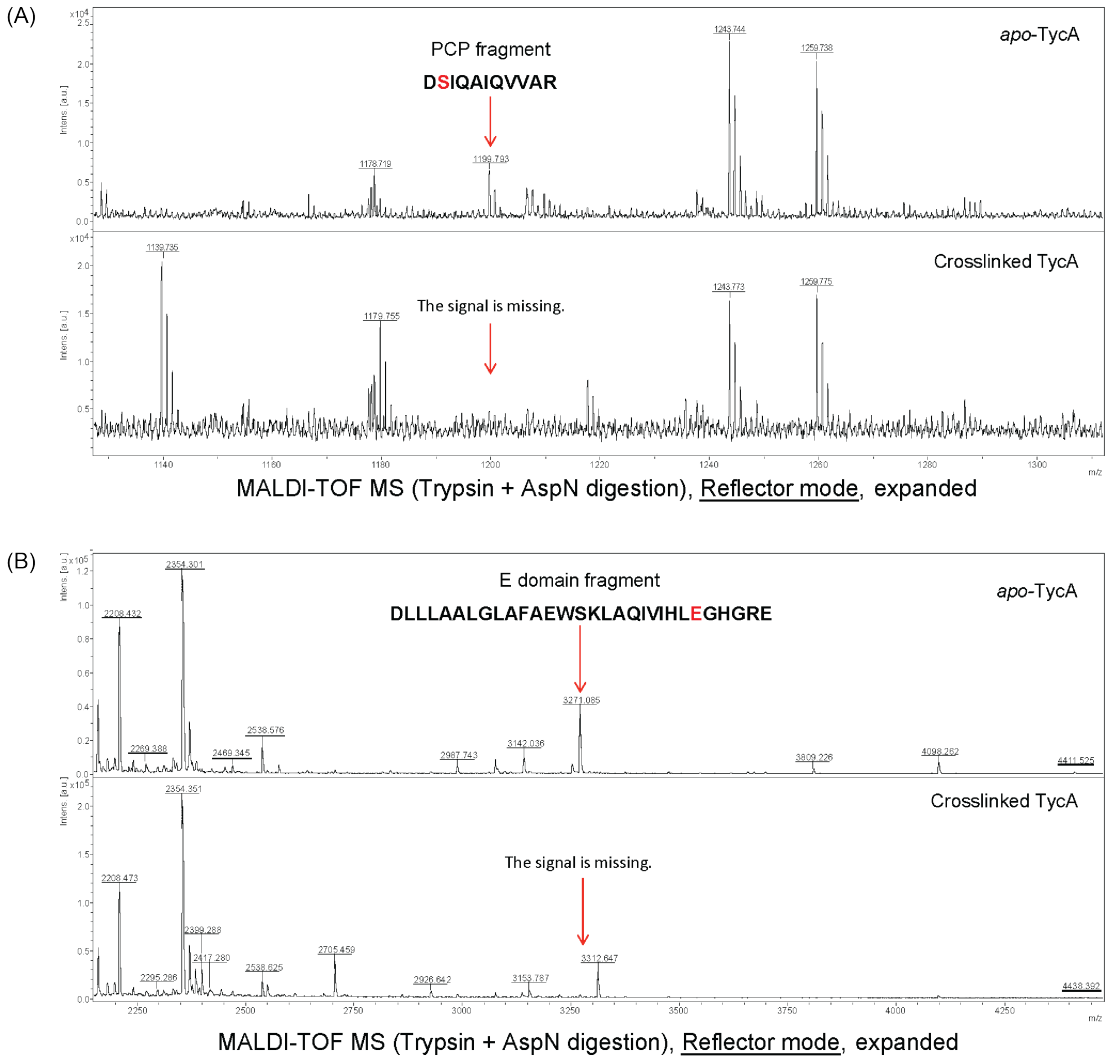


Figure S2. Crosslinking construct protease digestion mass spectrometry analysis compared with wild-type TycA. The top diagram (A) shows the PCP side of the fragment, and the diagram below (B) shows the E domain side of the fragment.

B.6. DKP Assay protocol and HPLC traces

TycA and its mutants were expressed and purified as previously described. $TycB_1$ was expressed and purified by using a previously published protocol[5]: pQE60 (Qiagen) harboring the genes were transformed into BL21 (DE3) cells. *tycB1* was co-transformed with *sfp* in pREP4. For protein production, two times yeast and tryptone medium (2xYT: 16 g L^{-1} tryptone, 10 g L^{-1} yeast extract and 5 g L^{-1} NaCl, 500 mL) with kanamycin sulfate (30 mg L^{-1}) and ampicillin (100 mg L^{-1}) was inoculated with an overnight culture (5 mL) of the expression strain. The culture was grown at 37°C to OD_{600} of 0.3, and then the temperature was reduced to 30 °C. The cells were then induced at an OD_{600} of 0.6–0.8 by adding IPTG (0.4 mM) and then were subsequently grown for an additional 2 h. Cells were spun down by centrifugation at 2,000 rpm for 30 min and the collected pellets were resuspended in lysis buffer (25 mM HEPES, 200 mM NaCl, 10mM imidazole, pH 8.0) lysed by sonication, followed by another centrifugation at 10,000 rpm for 1 h. The His_6-tagged protein was purified using a combination of Ni-NTA, dialyzed overnight into a new buffer (10 mM HEPES, 50 mM NaCl, 1 mM $MgCl_2$, and 5 mM dithiothreitol, DTT, at pH 7.5) at 4°C, and concentrated.

To ensure quantitative phosphopantetheinylation of the TycA and $TycB_1$ proteins, they were incubated with coenzyme A (CoA; 10 equiv) and Sfp (0.05 equiv) for 6 h at 20°C and then concentrated before being stored at -80°C.

The DKP assay was performed in accordance with a previously published protocol[5]: mixtures of *holo*-TycA (wt or mutant, 2.8μM), L-Phe (8.0 mM), and ATP (8.0 mM) as well as *holo*-$TycB_1$ (2.8 μM), L-Pro (8.0 mM), and ATP (8.0 mM), were made on a 100 μL scale in HEPES (50mM, pH 7.5), NaCl (300 mM), and $MgCl_2$ (10 mM). DKP formation was started by combining the two mixtures and incubated for 1 h at 37ºC. The reaction was then stopped by extraction with butan-1-ol/chloroform (4:1; 2x200μL). The combined organic phases were washed with NaCl (200 μL, 0.1M) and dried under reduced pressure using Savant Speed Vac Concentrator SVC-100H.

The dried extract was dissolved in methanol (25 μL 10%, v/v), and aliquots (15 μL) were loaded onto an Ascentis Express Peptide ES C_{18} column (15 cm x 4.6 cm; 2.7 μm particle size; Sigma-Aldrich) for HPLC quantification. Solvent A was 0.05% trifluoroacetic acid (TFA) in water; solvent B was 0.05% TFA in acetonitrile. Prior to loading of a sample, the column was equilibrated with 2 % B, and the following gradient was used at a flow rate of 1 mL min−1: 0–2 min, 2 % B; 2–10 min, 2–30 % B; 10–12 min, 30–95 % B; 12–

15 min, 95 % B; 15–17 min, 95–2 % B; 17–20 min, 2 % B (UV absorbance at 280, 254, 214 nm). Peaks were collected to screen for the mass of DKP ($C_{14}H_{16}N_2O_2$ cald: 245.13 $[M+H]^+$, obs.: 245.13). The yield of DKP was determined by the area of its respective peak at 214 nm.

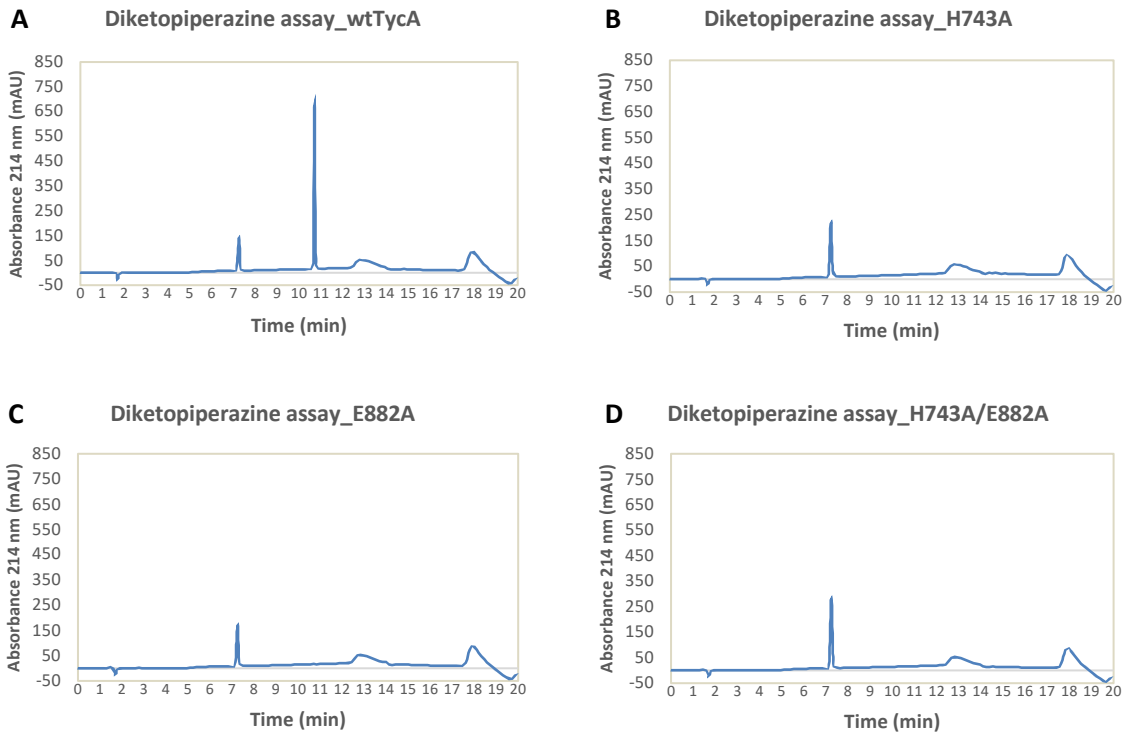


Figure S3. HPLC traces of % DKP formation with wild-type TycA and mutants. Raw HPLC trace of DKP formation with (A) wild-type TycA, (B) TycA H743A mutant, (C) TycA E882A mutant, and (D) TycA H743A/E882A double mutant. D-Phe-L-Pro diketopiperazine is observed at a retention time of 10.68 min. The peak around 7 min corresponds to Phe.

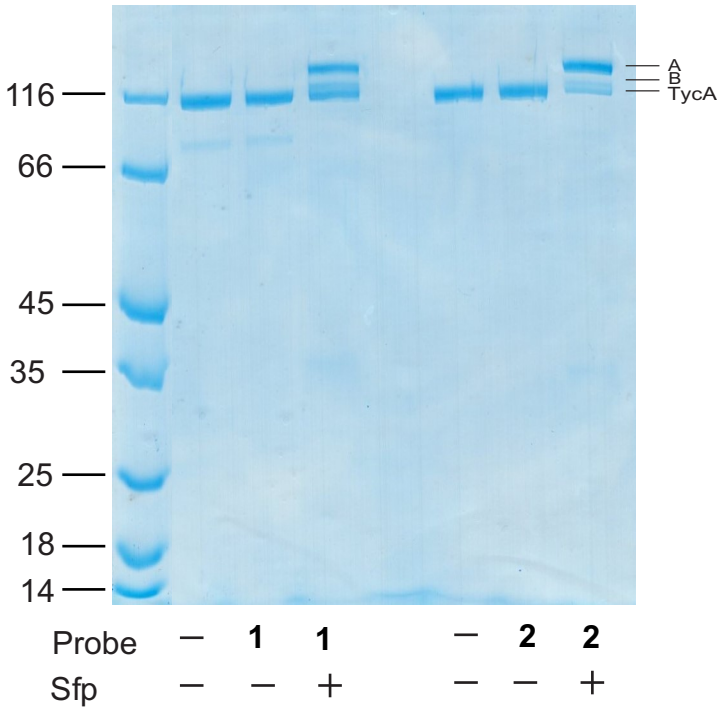


Figure S4. Full SDS-PAGE gel image of TycA crosslinking constructs with probes **1** and **2**. Full 8% SDS-PAGE gel image corresponding to the main text Figure 3A.

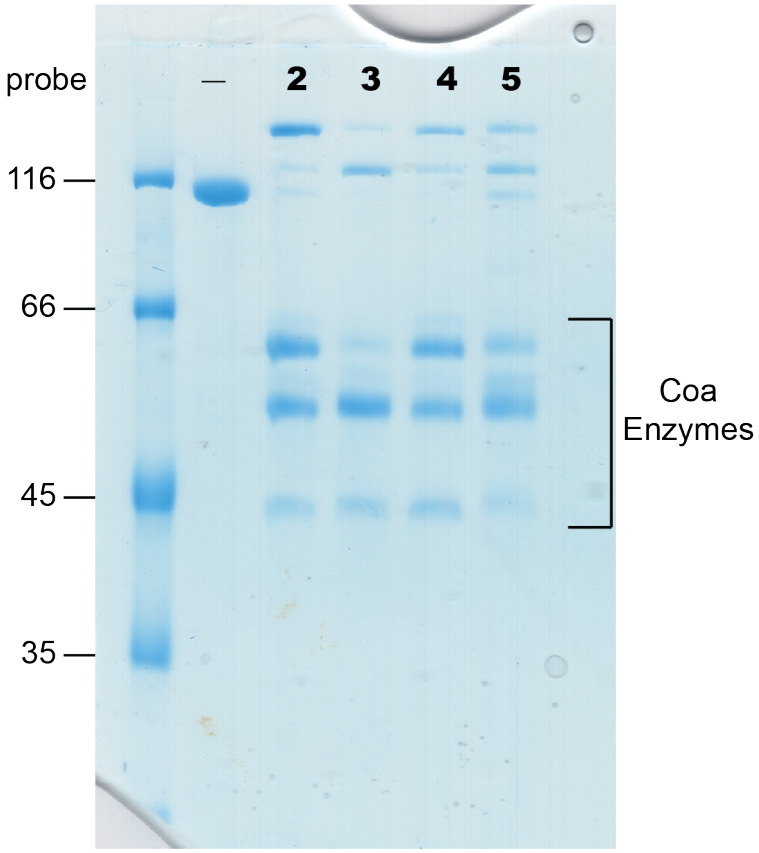


Figure S5. Full SDS-PAGE gel image of TycA crosslinking constructs comparing different probes. Full 8% SDS-PAGE gel image corresponding to the main text Figure 3B.

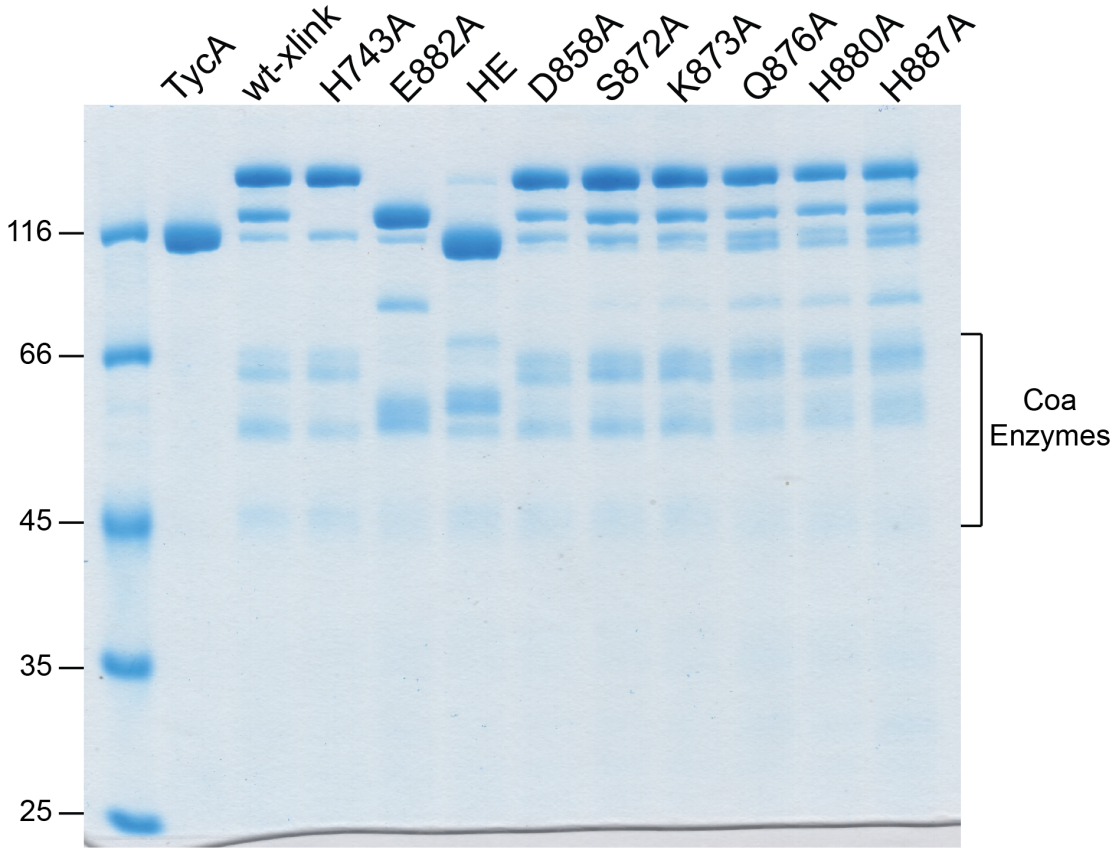


Figure S6. Full SDS-PAGE gel image of TycA mutant crosslinking constructs with probe **4**. Full 8% SDS-PAGE gel image corresponding to the main text Figure 4A. Additional bands for uncrosslinked TycA protein in rightmost lanes may be attributed to multiple native conformations of mutant proteins or their instability compared to wild-type and other mutant TycA proteins.

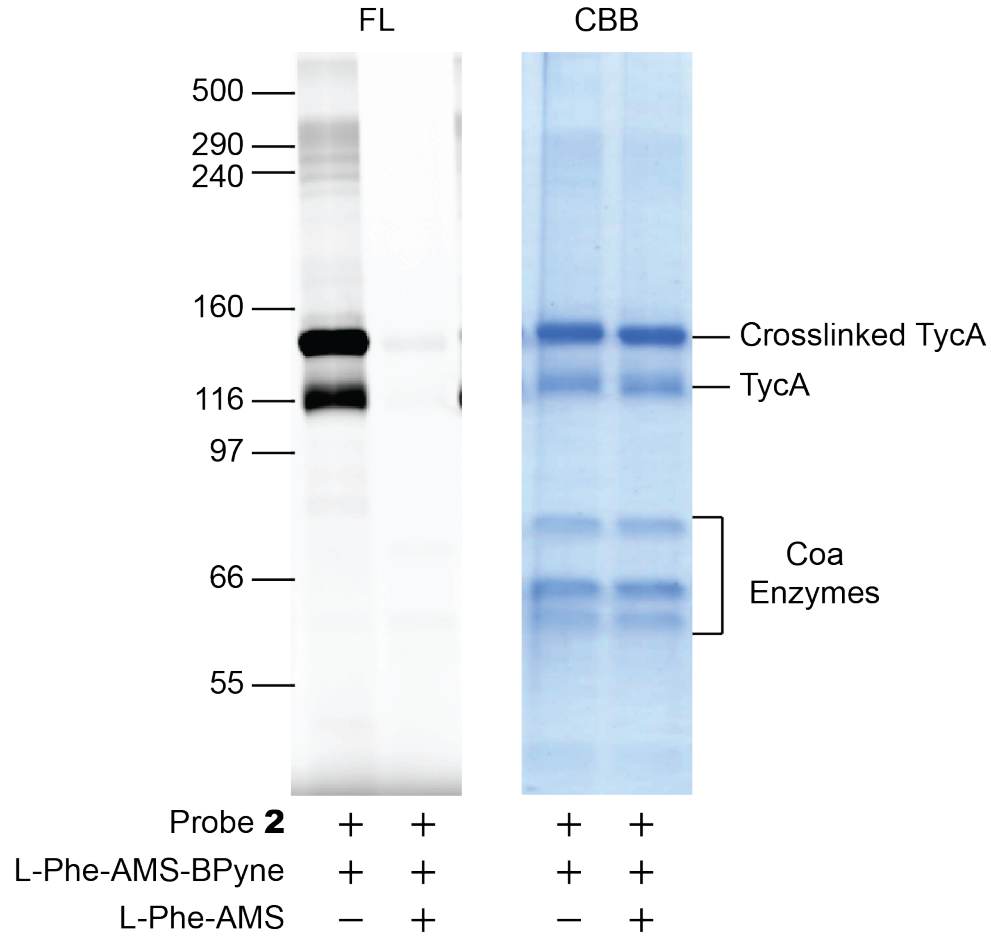


Figure S7. Full SDS-PAGE gel image of TycA crosslinking site validation using A domain labeling reagent. Full 8% SDS-PAGE gel image corresponding to the main text Figure 6C.

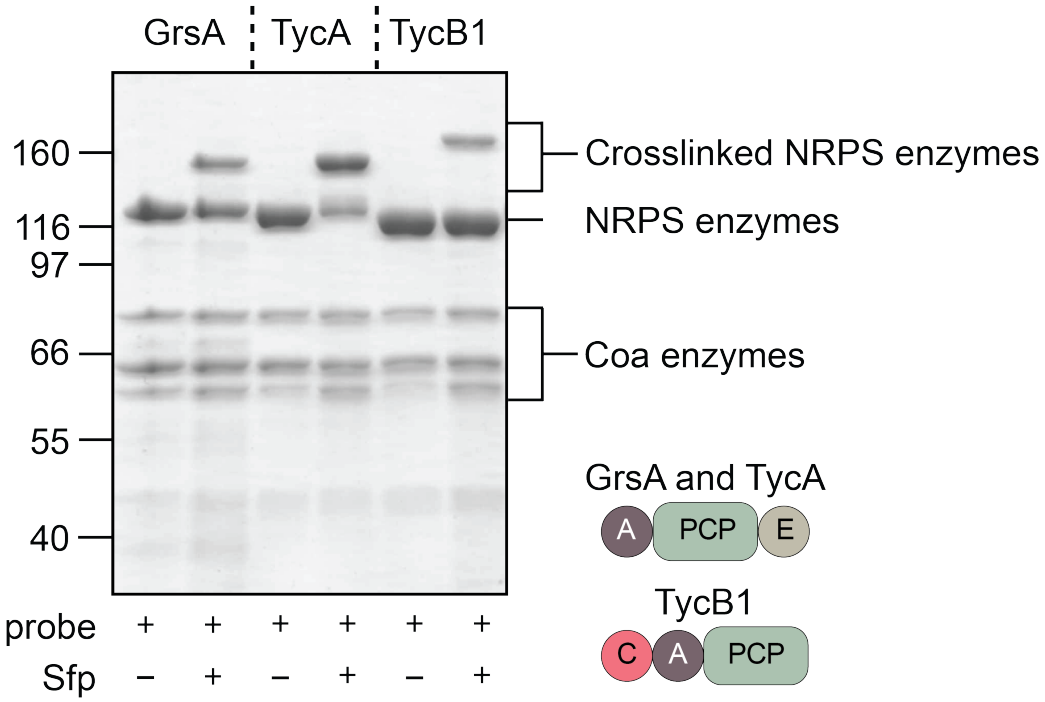


Figure S8. Full 8% SDS-PAGE gel image of probe **2** specificity with additional NRPS modules. TycA crosslinking compared with that of GrsA, the initiation module of gramicidin biosynthesis, and TycB1, the first module of TycB in tyrocidine biosynthesis. Gel shows the crosslinker's ability to crosslink both E and C domains.

C. References

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