A Mechanism Based Crosslinker for Acyl Carrier Protein Dehydratases

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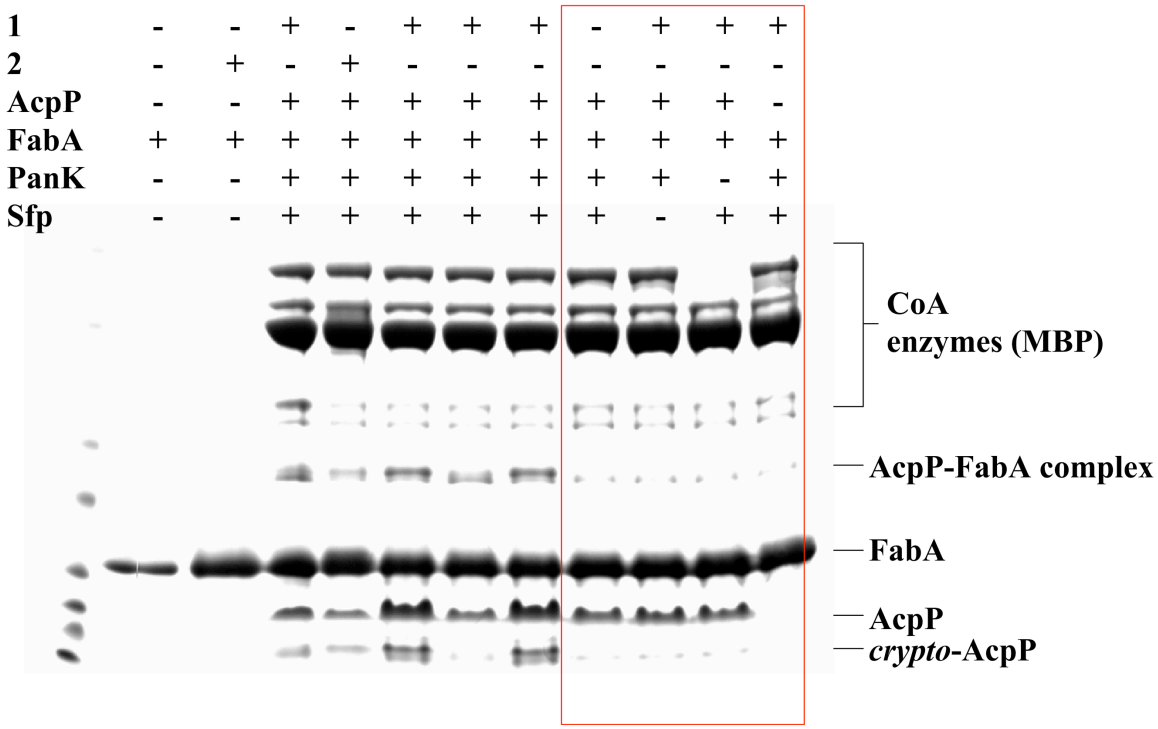


Figure S1: Evaluating the necessity of reaction components (AcpP, PanK, Sfp) for AcpP-FabA (ACP-DH) crosslinking. Each reaction component is necessary for formation of crosslinked complex. Lanes 2-3: FabA. Lanes 4-8: positive crosslinking. Lane 9: pantetheine analogue **1** omitted. Lane 10: Sfp omitted. Lane 11: PanK omitted. Lane 12: AcpP omitted.

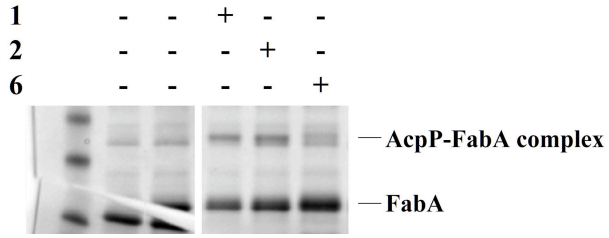


Figure S2: Comparison of AcpP-FabA crosslinking using chemoenzymatic labels **1**, **2**, and **6**. Pantetheine analogues **1** and **2** show approximately equivalent crosslinking (based on density of crosslinked band) while analogue **6** produces a crosslinked species that is notably more diffuse.

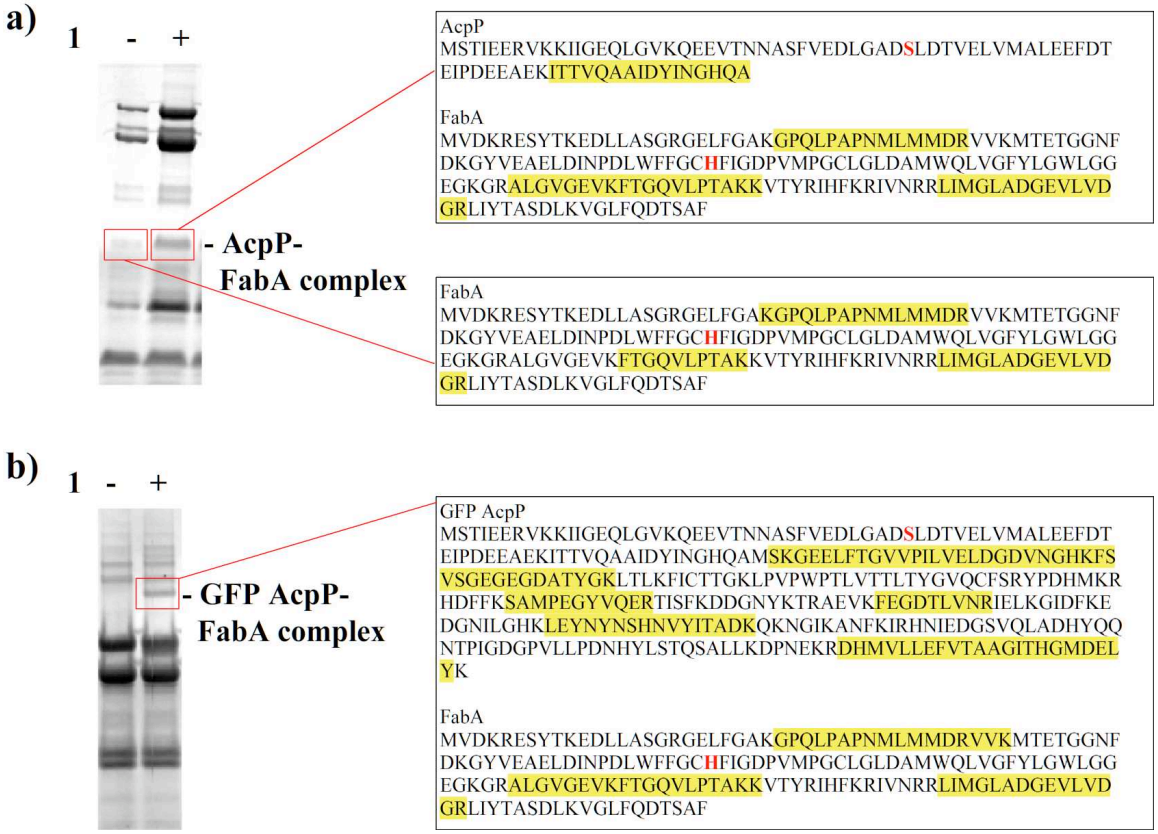


Figure S3: MALDI-TOF/TOF analysis of ACP-DH crosslinking. Peptides containing residues highlighted in yellow were identified by tryptic digest and MS analysis of the SDS-PAGE slice specified (boxed in red). (a) AcpP-FabA crosslinked complex. Peptides from both AcpP and FabA were identified in the crosslinked band (right lane), while peptides containing catalytic active site residues were *not* observed, consistent with their covalent modification. When crosslinking probe **1** was omitted (left lane), only peptides corresponding to FabA were observed, consistent with the presence of a persistent disulfide at this molecular weight (see main text for details). (b) Because of the low number of peptides formed upon tryptic digest of AcpP, the GFP AcpP-FabA crosslinked complex was subjected to an identical analysis. Peptides from both GFP AcpP and FabA were identified in the crosslinked band (right lane), while peptides containing catalytic active site residues were *not* observed, again consistent with their covalent modification.

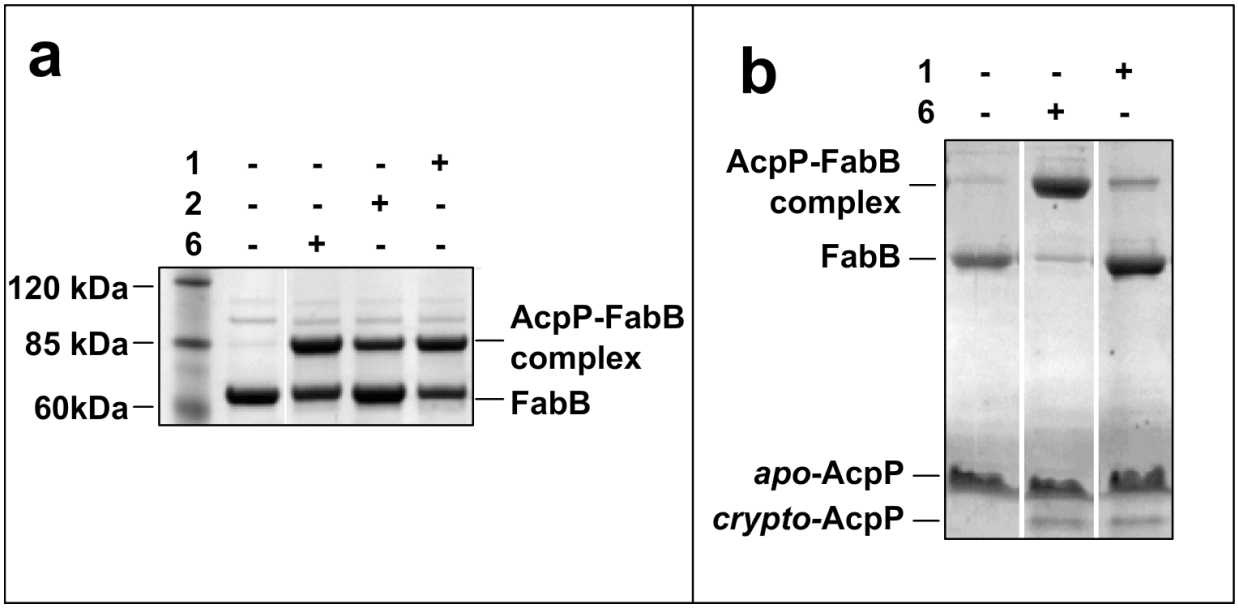


Figure S4. Evaluation of thioesters **1**, **2**, and **6** as ACP-KS crosslinking reagents. (a) AcpP modified by thioesters **1**-**2** and **6** forms a covalent crosslink with the *E. coli* FAS KS enzyme FabB. (b) Reaction of FabB with purified *apo*-AcpP (lane 1), 2-octynoyl-AcpP (lane 2), and 3-decynoyl-AcpP (lane 3). The lower amount of ACP-KS crosslinking observed using purified 3-decynoyl-AcpP compared to that seen in the one-pot generation of this species (seen in 3a) suggests the non-enzymatically generated, highly reactive 2,3-allenic thioester is subject to rapid degradation in solution.

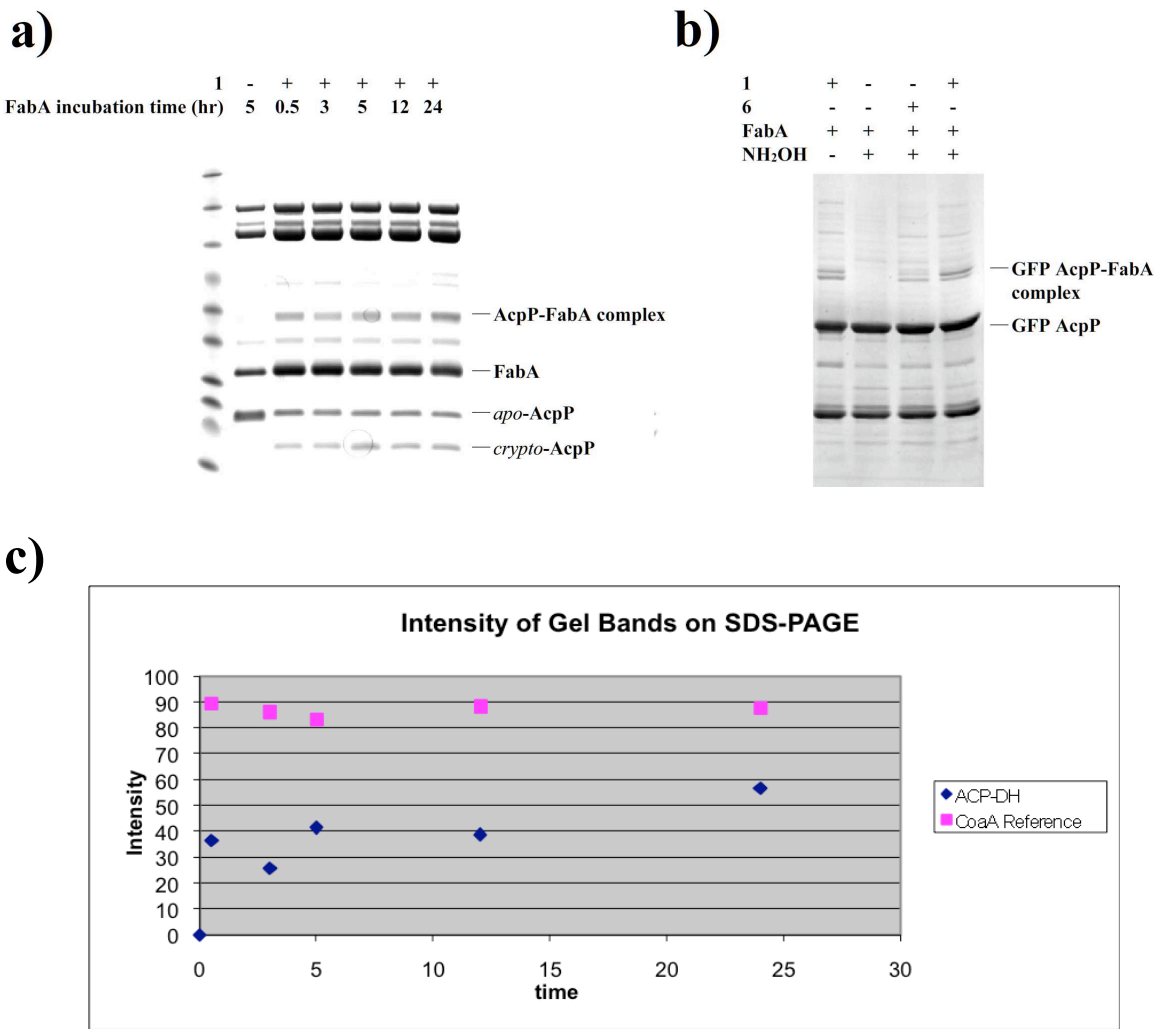


Figure S5. Assessing the stability of ACP-DH crosslinked complexes. (a) Monitoring hydrolysis of ACP-DH complex over time by SDS-PAGE. AcpP was incubated with **1**, Sfp, PanK, PPAT, DPCK for the time specified (30 or 60 min – CP load) to form *crypto*-AcpP modified by **1**. Recombinant FabA was then added to the unpurified one-pot reaction, and the mixture was incubated for the time specified (0.5 – 24 hours – FabA incubation time). SDS-PAGE indicates little change in the AcpP-FabA complex over the time period monitored. (b) Monitoring stability of ACP-DH complex to thioester hydrolyzing agents. GFP AcpP was incubated with **1**, Sfp, PanK, PPAT, DPCK to form *crypto*-AcpP modified by **1** or **6**, and then purified by Ni^{2+}-NTA affinity chromatography (see Supporting Methods). Upon elution from the resin, recombinant FabA was added to isolated *crypto*-AcpP (400 mM imidazole fraction) and allowed to incubate for 1 hour, followed by addition of hydroxylamine (final concentration 2 mM) and incubation for a further 3 hours. GFP AcpP-FabA complex is still observed by SDS-PAGE after hydroxylamine addition. (c) Analysis of hydrolysis from ACP-DH complex. Bands from the gel shown in a) are quantified using ImageJ (NIH). Intensity values compare ACP-DH complex (diamonds) to a reference band of MBP-CoaA (squares).

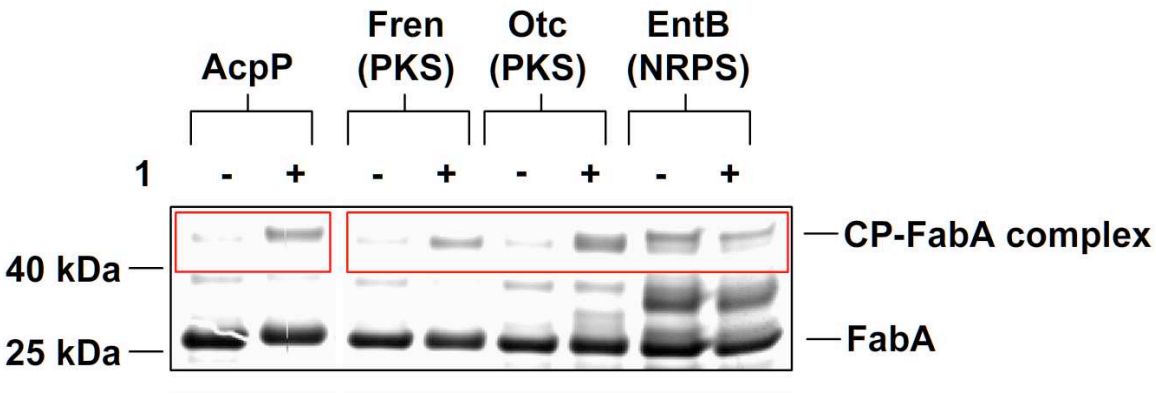


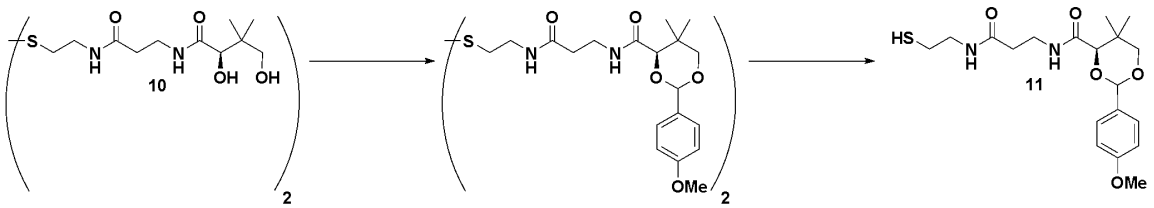
Figure S6. Effect of carrier protein identity on CP-FabA crosslinking. 3-decynoyl pantetheine **1** was used to modify the carrier proteins AcpP (FAS), Fren (PKS), Otc (PKS), and EntB (NRPS). Upon addition of FabA, probe-specific crosslinking is observed with AcpP, Fren, and Otc, indicating the preferential interaction of FabA with 3-decynoyl ACPs.

General synthetic procedures and materials

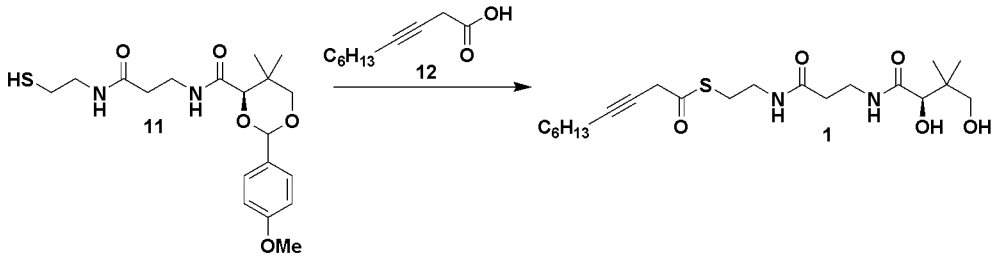
All commercial reagents (Sigma-Aldrich, Spectrum, MP Biomedicals, Alfa Aesar, TCI America, Acros) were used as provided unless otherwise indicated. 2-octynoyl-N-pantetheine **7**,[1] 2-bromo-hexanoyl-N-pantetheine **8**,[1] 3-decynoic acid **12**,[2] 3-decynoyl chloride **14**,[3] acetonide protected-S-pantetheine **13**,[4] *p*-methoxybenzylidene (PMB) protected pantothenic acid **16**,[5,6] PMB-protected N-pantetheine **20**,[6] and 7-dimethyl-aminocoumarin-4-acetic acid-cystamine **23**[7]were each prepared according to published literature procedures. 2-octynoyl chloride **21** was prepared from the commercially available 2-octynoic acid using oxalyl chloride in a procedure analogous to that for preparation of 3-decynoyl chloride.[3] All reactions were carried out under argon atmosphere in dry solvents with oven-dried glassware and constant magnetic stirring unless otherwise noted. Triethylamine (TEA), N-methyl morpholine (NMM), and ethyl-*N,N*-diisopropylamine (DIPEA) were dried over sodium and freshly distilled prior to use. ^{1}H-NMR spectra were taken at 300, 400, or 500 MHz and ^{13}C-NMR spectra were taken at 100.6 or 75.5 MHz on Varian NMR spectrometers and standardized to the NMR solvent signal as reported by Gottlieb[8]. Multiplicities are given as s=singlet, d=doublet, t=triplet, q=quartet, p=pentet, dd=doublet of doublets, bs=broad singlet, bt=broad triplet, m=multiplet using integration and coupling constant in Hertz. TLC analysis was performed using Silica Gel 60 F254 plates (EM Scientific) and visualization was accomplished with UV light (λ=254 nm) and/or the appropriate stain (iodine, 2,4-dinitrophenylhydrazine, cerium molybdate, ninhydrin). Silica gel chromatography was carried out with Silicycle 60 Angstrom 230-400 mesh according to the method of Still.[9] Electrospray (ESI) and fast atom bombardment (FAB) mass spectra were obtained at the UCSD Mass Spectrometry Facility using a Finnigan LCQDECA mass spectrometer and a ThermoFinnigan MAT- 900XL mass spectrometer, respectively.

Synthetic Procedures and Spectroscopic Data for Chemoenzymatic Crosslinking Probes 1-9

(a) Synthesis of 3-decynoyl-S-pantetheine (1)



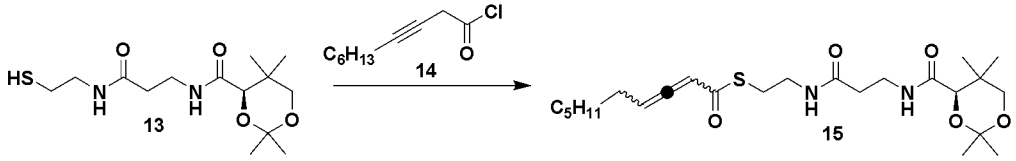
D-pantethine monohydrate **10** (1144 mg, 2.0 mmol was dissolved in DMF (20 mL) with stirring at room temperature. To this mixture was added *p*-anisaldehyde dimethyl acetal (0.68 mL, 4.0 mmol) followed by a solution of 4M HCl in dioxanes (0.1 mL). After stirring overnight the solvent was removed under reduced pressure to yield a crude oil which was further purified by flash chromatography (CH_2Cl_2 to 5% MeOH/ CH_2Cl_2) to yield PMB-pantethine-disulfide (948 mg, 60% for first step) as a white foam. In order to access the reduced thiol, this product was dissolved in distilled MeOH (50 mL) and cooled to 0°C. Sodium borohydride (1.0 g, 27 mmol) was then carefully added in four portions producing vigorous bubbling. After this initial stage of the reaction had subsided, the reaction vessel was placed under argon and stirred for two hours at room temperature. The reaction was quenched by dropwise addition of water using an addition funnel, and neutralized with HCl. The reaction mixture was then combined with brine (100 mL) and extracted three times with ethyl acetate (200 mL). The extracts were combined, washed again with brine, and the solvent removed under reduced pressure giving the free thiol **11** as a white foam (929 mg, 59% over two steps) with a characteristic faint, putrid odor. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J*= 8.4 Hz, 2H), 7.06 (bt, 1H), 7.00 (bt, 1H), 6.88 (d, *J*=8.4 Hz, 2H), 5.42 (s, 1H), 4.03 (s, 1H), 3.78 (s, 3H), 3.67 (d, *J*=11.2 Hz, 1H), 3.61 (d, *J*=11.2 Hz, 1H), 3.47 (m, 4H), 2.71 (t, *J*=6.0 Hz, 2H), 2.41 (t, *J*=6.4 Hz, 2H), 1.06 (s, 3H), 1.04 (s, 3H). ^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 172.0, 169.9, 160.5, 130.2, 127.6, 113.7, 101.3, 83.8, 78.4, 55.4, 42.0, 36.2, 34.4, 33.2, 24.5, 22.0, 19.4. HRMS (EI) (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{28}O_5N_2S_1$, 396.1719, found 396.1714.



3-decynoic acid **12** (218 mg, 1.3 mmol) was dissolved in THF (4 mL) and cooled to 0°C with stirring. N,N'-dicyclohexylcarbodiimide (267 mg, 1.3 mmol) was then added and stirred for 10 minutes before addition of thiol **11** (126 mg, 0.32 mmol) The reaction was allowed to slowly warm to room temperature and stirred for 15 hours. The reaction was filtered twice to remove urea byproducts and the solvent removed under reduced pressure. Resuspension in CH_2Cl_2 followed by flash chromatography (CH_2Cl_2 to 2% MeOH/ CH_2Cl_2) yielded the

coupled, PMB-protected thioester as a brown oil contaminated with 3-decynoic acid (3:1 product:acid as judged by ^{1}H-NMR). This material was dissolved in 1N HCl:THF, 1:8 (30 ml) and stirred until the starting material was consumed as shown by TLC (2,4-dinitrophenyl-hydrazine visualization). The reaction was then neutralized by addition of AG-1-X8 Strong Basic anionic exchange resin. After filtration, the solvent was removed under reduced pressure, and the reaction mixture was resuspended and purified by flash chromatography (1:6 EtOAc/hexanes to 2:1 EtOAc/hexanes) to afford compound **1** as an oil (120 mg, 88% over two steps). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.40 (bt, 1H), 6.40 (bt, 1H), 3.99 (s, 1H), 3.57-3.37 (m, 6H), 3.03 (m, 2H), 2.42 (t, *J*=5.0 Hz, 2H), 2.21 (t, *J*=5.0 Hz, 2H), 1.53-1.24 (m, 8H), 1.00 (s, 3H), 0.91 (s, 3H), 0.88 (t, *J*=7.0 Hz, 3H).^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 196.4, 173.9, 171.9, 87.0, 77.8, 76.9, 71.1, 39.6, 35.9, 35.4, 35.1, 31.5, 29.0, 28.8, 28.7, 22.8, 22.8, 21.8, 20.6, 19.0, 14.3. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{21}H_{36}O_5N_2S_1$, 428.2339, found 428.2342.

(b) Synthesis of 2,3-decadienoyl-S-pantetheine (2)

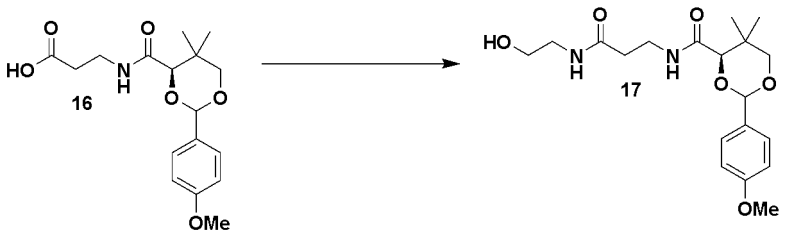


3-decynoyl chloride **14** (41 mg, 0.22 mmol) was added dropwise to a stirring solution of acetonide protected-S-pantetheine **13** (55 mg, 0.17 mmol) and triethylamine (0.082 mL, 0.6 mmol) in THF (5 mL) prechilled to 0^{o}C. After stirring for 1 hour, the reaction was diluted in EtOAc (50 mL) and washed with citric acid (1x50 mL), saturated $NaHCO_3$ (2x50 mL), and brine (1x100 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Resuspension and purification by flash chromatography (1:6 EtOAc/hexanes to 2:1 EtOAc/hexanes) afforded the protected allene **15** (50 mg, 63%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.03 (bt, 1H), 6.56 (bt, 1H), 5.79 (m, 1H), 5.71 (q, *J*=6.8 Hz, 1H), 4.01 (s, 1H), 3.62 (d, *J*=11.6 Hz, 1H), 3.52-3.35 (m, 4H), 3.21 (d, *J*=11.6 Hz, 1H), 2.96 (q, *J*=6.4 Hz, 2H), 2.38 (t, *J*=6.0 Hz, 2H), 2.12 (m, 2H), 1.45-1.18 (m, 14H), 0.97 (s, 3H), 0.91 (s, 3H), 0.83 (bt, 3H). $[M+H]^+$ calcd for $C_{24}H_{40}O_5N_2S_1$, 468.2658, found 468.2652.

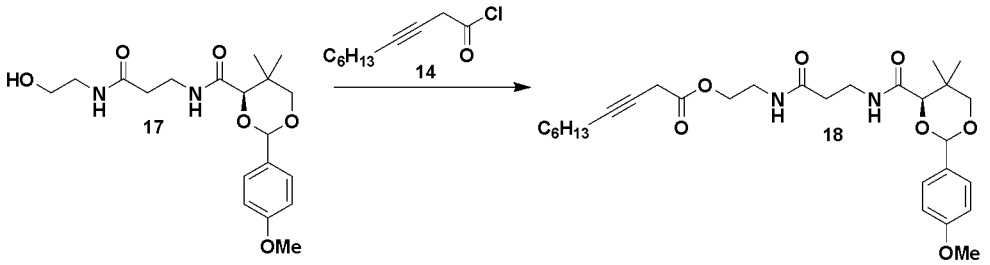
Allenic acetonide **15** was dissolved in 1N HCl:THF, 3:8 (6 ml) and stirred until the benzylidene protected starting material was consumed as shown by TLC (2,4-dinitrophenylhydrazine visualization). AG-1-X8 Strong Basic anionic exchange resin was added to neutralize the solution. After removal of solvent under reduced pressure, the reaction mixture was purified by flash chromatography (1:1 EtOAc/hexanes to EtOAc to 2%MeOH/EtOAc) to afford **2** as a mixture of diasteromeric allenes (38 mg, 63% over two steps). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.44 (bt, 1H), 6.57 (bt, 1H), 5.83 (m, 1H), 5.76 (q, *J*=5.2 Hz, 1H), 3.97 (s, 1H), 3.52-3.32 (m, 6H), 2.99 (m, 2H), 2.41 (m, 2H), 2.17 (m, 2H), 1.49-1.26 (m, 8H), 0.97 (s, 3H), 0.89 (s, 3H), 0.862 (bt, 3H). ^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 211.6, 191.2, 191.1, 174.1, 172.0, 98.8, 96.5, 77.7, 71.0, 40.0, 39.5, 35.9, 35.5,

31.7, 29.9, 28.9, 28.8, 28.3, 27.9, 22.8, 21.7, 21.6, 20.6, 19.0, 14.3. HRMS (EI) (*m/z*): $[M+H]^+$ calcd for $C_{21}H_{36}O_5N_2S_1$ 428.2345, found 428.2348.

(c) Synthesis of 3-decynoyl-O-pantetheine (3)

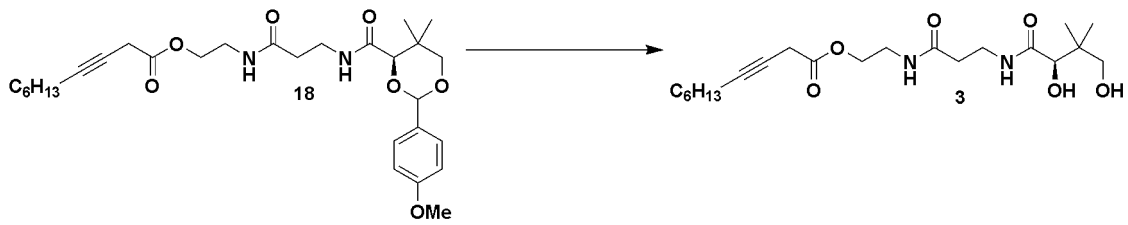


PMB-pantothenic acid **16** (2.36 g, 7.0 mmol), ethanolamine (0.843 mL, 14.0 mmol), DIPEA (16.3 mL, 14.0 mmol), and HOBt (2.63 g, 17.5 mmol) were dissolved in DMF with stirring and cooled to 0 ^oC. EDC (2.67 g 14 mmol) was added in one portion and the reaction was allowed to slowly warm to RT and stir for 24 hrs. The solvent was removed under reduced pressure, taken up in EtOAc (100 mL), and extracted w/ saturated $NaHCO_3$ (3x75 mL), water (1x50 mL), and brine (1x75 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvent removed under reduced pressure.to provide PMB-oxypantetheine **17** (2.37 g, 89%) as a white foam. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J*= 9.0 Hz, 2H), 7.09 (bt, 1H), 6.90 (d, *J*=8.5 Hz, 2H), 6.84 (bt, 1H), 5.44 (s, 1H), 4.05 (s. 1H), 3.79 (s, 3H), 3.68 (d, *J*=11.5 Hz, 1H) 3.64-3.60 (m, 3H), 3.52-3.49 (m, 2H), 3.33 (t, *J*=5.5 Hz, 2H), 2.42 (t, *J*=6.5 Hz), 1.08 (s, 3H), 1.05 (s, 3H). ^{13}C-NMR (100.6 MHz, $CDCl_3$) δ 172.1, 170.1, 160.5, 130.3, 127.8, 113.9, 101.5, 84.0, 78.6, 61.9, 55.6, 42.6, 36.2, 35.4, 33.3, 22.1, 19.4. HRMS (EI) (*m/z*) $[M]^+$ calcd for $C_{19}H_{28}O_6N_2$, 380.1942, found 380.1947.



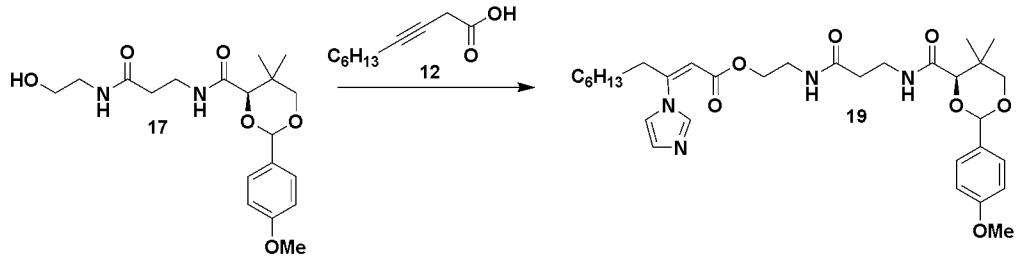
A solution of PMB-oxypantetheine **17** (200 mg, 0.53 mmol) and pyridine (0.05 mL, 0.54 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a stirring solution of 3-decynoyl chloride **14** (50 mg, 0.27 mmol) in CH_2Cl_2 (5 mL) prechilled to 0^oC. The addition step was visibly exothermic and caused the reaction to turn a deep brown/black color. After stirring for 1.5 hours, the reaction was quenched with MeOH (1 mL), diluted in EtOAc (75 mL) and washed with brine (1x100 mL), saturated $NaHCO_3$ (2x75 mL), and brine again (1x100 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Resuspension and purification by flash chromatography (1:1 EtOAc/hexanes to EtOAc) afforded a mixture the protected pantetheine analogue **18** (18.5 mg, 13%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.42 (d, *J*= 8.8 Hz, 2H), 7.08 (bt, 1H), 6.91 (d, *J*=8.8 Hz, 2H), 6.18 (bt, 1H), 4.16 (t, *J*=4.8 Hz, 2H), 4.07 (s, 1H), 3.82 (s, 3H), 3.70 (d, *J*=11.2 Hz, 1H), 3.66 (d, *J*=11.2 Hz, 1H), 3.54-3.51 (m, 4H), 3.27 (t, *J*=2.8 Hz, 2H), 2.44 (t, *J*=6.2 Hz, 2H), 2.18 (m, 2H), 1.61-1.25 (m, 8H), 1.09 (s, 6H), 0.88 (t, *J*=7.2 Hz, 3H). ^{13}C-NMR (100.6 MHz, $CDCl_3$) δ 171.3, 169.8, 168.3, 160.4, 130.3, 127.7, 113.9, 101.5, 84.0, 78.6, 64.3, 63.8, 55.5, 38.9, 38.7,

36.2, 35.1, 33.3, 31.8, 31.5, 29.2, 28.1, 26.2, 22.8, 22.1, 19.0, 14.3. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{29}H_{42}O_7N_2$, 530.2987, found 530.2993.



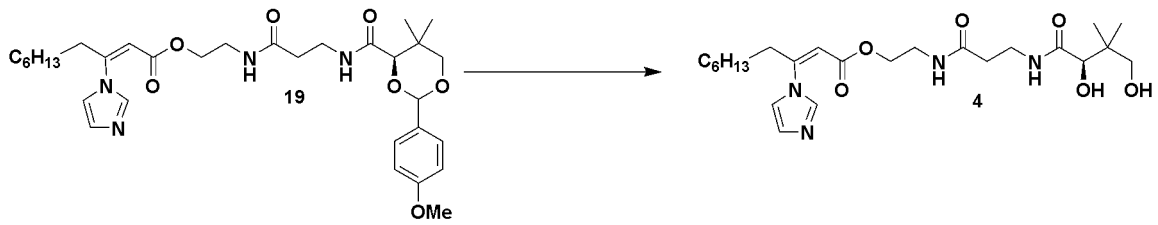
Product **18** (18.5 mg, 0.03 mmol) was dissolved in 1N HCl:THF, 1:3 (6 ml) and stirred until the benzylidene protected starting material was consumed as shown by TLC (2,4-dinitrophenylhydrazine visualization). The reaction was neutralized by dropwise addition of 1M NaOH, diluted in brine (50 mL) and extracted with CH_2Cl_2 (3x50 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Resuspension and purification by flash chromatography (EtOAc to 5% MeOH/EtOAc) afforded **3** as an oil (8.5 mg, 59%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.36 (bt, 1H), 6.29 (bt, 1H), 4.27-4.17 (m, 2H), 4.00 (s, 1H), 3.63-3.41 (m, 6H), 3.28 (s, 2H), 2.49-2.43 (m, 2H), 2.22-2.18 (m, 2H), 1.52-1.25 (m, 8H), 1.02 (s, 3H), 0.89 (s, 3H), 0.88 (t, *J*=7.5 Hz, 3H). ^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 173.9, 171.9, 169.7, 84.6, 77.9, 71.2, 64.3, 39.6, 39.0, 36.0, 35.3, 31.6, 29.9, 28.9, 26.2, 22.8, 21.9, 20.6, 19.0, 14.3. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{21}H_{35}O_5N_2$, 412.2568, found 412.2575.

(d) Synthesis of (*Z*)-2-decenoyl-3-imidazole-O-pantetheine (4)



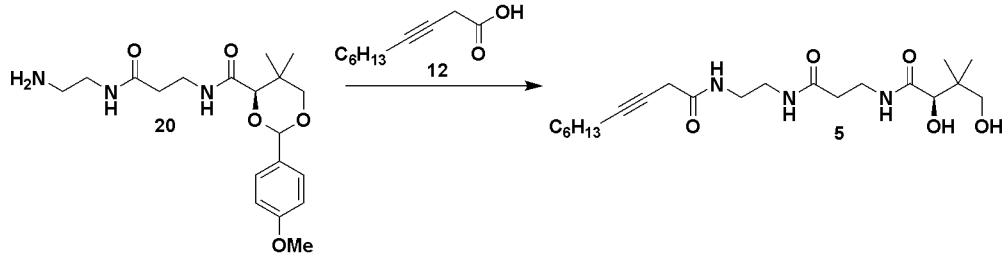
Carbonyl diimidazole (136 mg, 1 mmol) was added to a stirring solution of 3-decynoic acid **12** (169 mg, 1 mmol) dissolved in dry DMF (10 mL). The reaction was heated at 50°C for 20 minutes, during which time the reaction mixture turned a pale orange. To this mixture was added dropwise a solution of PMB-oxypantetheine **17** (760 mg, 2 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (101 mg, 1 mmol) in DMF (1 mL). After stirring overnight the solvent was removed under reduced pressure, and the reaction mixture resuspended and purified by flash chromatography (2:1 EtOAc/hexanes to 5% MeOH/EtOAc) to afford recovered PMB-oxypantetheine starting material (590 mg) as well as compound **19** as an oil (221 mg, 42%).^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.41 (d, *J*=8.1 Hz, 2H), 7.18 (s, 1H), 7.13 (s, 1H), 7.03 (bt, 1H), 6.90 (d, *J*=8.0 Hz, 2H), 6.41 (bt, 1H), 6.92 (s, 1H), 5.44 (s, 1H), 4.19 (t, *J*=5.5 Hz, 2H), 4.05 (s, 1H), 3.80 (s, 3H), 3.66 (d, *J*=15.5 Hz, 1H), 3.63 (d, *J*=15.5 Hz, 1H), 3.57-3.49 (m, 4H), 3.11 (t, *J*=8.0 Hz, 2H), 2.44 (t, *J*=6.0 Hz, 2H), 1.51 (p, *J*=8.0 Hz, 2H), 1.37-1.22 (m, 6H), 1.21 (s, 3H), 1.06 (s, 3H), 0.85 (t, *J*=6.5 Hz, 3H). ^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 171.2, 169.8, 165.7, 160.5, 153.9, 135.5, 131.2, 130.3, 127.7, 117.0, 114.0, 107.1, 101.6, 84.0, 78.7, 63.4, 55.6, 38.8, 36.3, 35.1, 33.3, 31.9, 29.9, 29.6,

29.2, 28.5, 22.8, 22.1, 19.3, 14.3. HRMS (EI) (*m/z*): $[M+H]^+$ calcd for $C_{32}H_{46}O_7N_4$, 598.3367, found 598.3364.



Product **19** (28 mg, 0.05 mmol) was dissolved in 1N HCl:THF, 1:4 (10 ml) and stirred until the starting material was consumed as shown by TLC (2,4-dinitrophenylhydrazine visualization). The reaction was then neutralized by addition of AG-1-X8 Strong Basic anionic exchange resin. After filtration, the solvent was removed under reduced pressure, and the reaction mixture was resuspended and purified by flash chromatography (EtOAc to 5% MeOH/EtOAc) to afford compound **4** as an oil (11 mg, 49%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.46 (bt, 1H), 7.20 (s, 1H), 7.11 (s, 1H), 6.87 (bt, 1H), 5.96 (s, 1H), 4.20-4.19 (m, 2H), 3.97 (s, 1H), 3.58-3.43 (m, 6H), 3.10 (t, *J*=8.0 Hz, 2H), 2.45 (t, *J*=6.5 Hz, 2H), 1.50 (p, *J*=7.5 Hz, 2H), 1.37-1.24 (m, 6H), 0.97 (s, 3H), 0.88 (s, 3H), 0.85 (t, *J*=6.5 Hz, 3H).^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 174.2, 171.9, 165.8, 153.8, 136.2, 130.9, 117.8, 107.4, 77.7, 71.1, 63.4, 39.5, 38.9, 36.1, 35.4, 31.9, 30.0, 29.6, 29.1, 28.5, 22.8, 21.6, 20.7, 14.3. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{24}H_{40}O_6N_4$, 480.2942, found 480.2941.

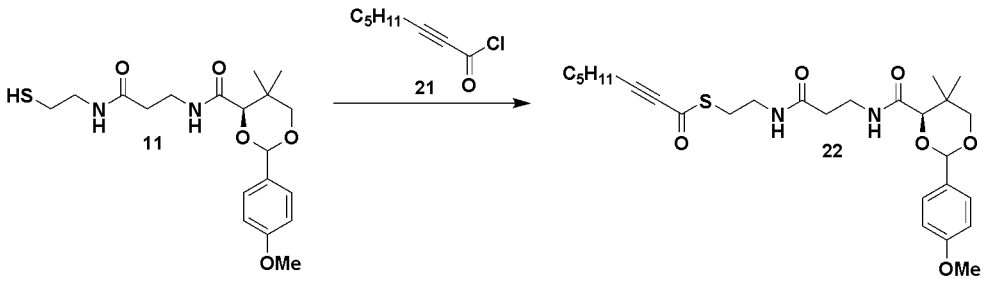
(e) Synthesis of 3-decynoyl-N-pantetheine (5)



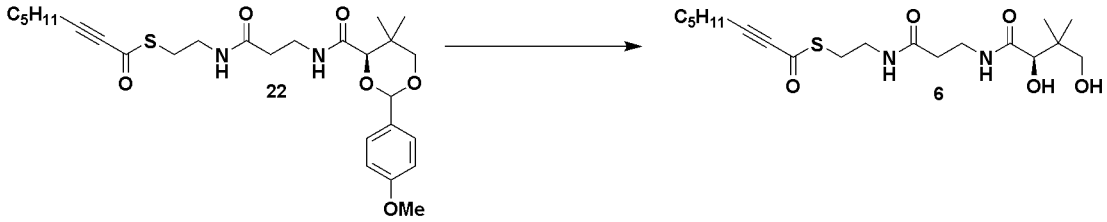
PMB-pantetheine amine **20** (89 mg, 0.23 mmol), 3-decynoic acid **12** (116 mg, 0.69 mmol), DIPEA (0.08 mL, 0.47 mmol), and HOBt (89 mg, 0.58 mmol) were dissolved in DMF (4 mL) with stirring and cooled to 0 ^{o}C. EDC (89 mg 0.47 mmol) was added in one portion and the reaction was allowed to slowly warm to RT and stir for 24 hrs. The solvent was removed under reduced pressure, taken up in EtOAc (75 mL), and extracted w/ water (1x50 mL), saturated $NaHCO_3$ (3x75 mL), and brine (1x50 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvent removed under reduced pressure. This material was resuspended and run through a silica gel plug (CH_2Cl_2 to 3% MeOH/ CH_2Cl_2) to afford the crude coupled material. compound **19** as an oil (78 mg). The coupled material (78 mg) was then dissolved in 1N HCl:THF, 1:1 (8 ml) and stirred until the starting material was consumed as shown by TLC (2,4-dinitrophenylhydrazine visualization). The reaction was then neutralized by addition of AG-1-X8 Strong Basic anionic exchange resin. After filtration, the solvent was removed under reduced pressure, and the reaction mixture was resuspended and purified by flash chromatography (CH_2Cl_2 to 3% MeOH/ CH_2Cl_2) to afford compound **5** as an oil (59 mg, 62% over two steps). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.45 (bt, 1H), 7.2 (bt, 1H), 7.15 (bt, 1H), 3.97 (s, 1H), 3.57-3.27 (m, 6H), 3.15 (t, *J*=2.4 Hz, 2H), 2.41

(m, 2H), 2.20 (t, *J*=6.8 Hz, 2H), 1.49 (p, *J*=7.2 Hz, 2H), 1.38-1.24 (m, 6H), 0.96 (s, 3H), 0.89 (s, 3H), 0.87 (t, *J*=6.8 Hz, 3H). ^{13}C-NMR (100.5 MHz, ($CDCl_3$) δ 174.4, 172.5, 169.7, 86.9, 77.6, 72.6, 70.9, 40.2, 39.9, 39.5, 36.2, 35.5, 31.5, 28.9, 28.8, 27.9, 22.8, 21.6, 20.8, 19.0, 14.3. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{21}H_{37}O_5N_3$, 411.2728, found 411.2728.

(f) Synthesis of 2-octynoyl-S-pantetheine (6)



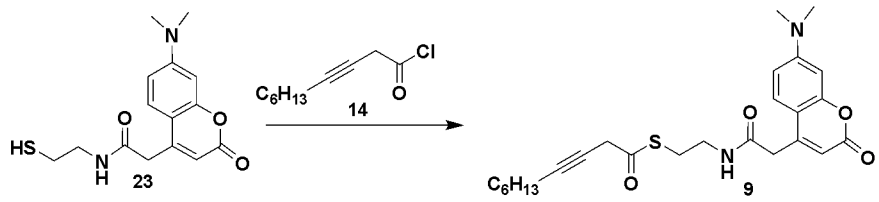
A solution of reduced thiol **11** (300 mg, 0.76 mmol) and triethylamine (0.51 mL, 3.7 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a stirring solution of 2-octynoyl chloride **21** (150 mg, 0.94 mmol) in CH_2Cl_2 (5 mL) prechilled to 0^{o}C. The addition step was visibly exothermic and caused the reaction to turn a pale orange/red color. After stirring for 1 hour, the reaction was quenched with MeOH (1 mL), diluted in EtOAc (75 mL) and washed with brine (1x100 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Resuspension and purification by flash chromatography (1:1 EtOAc/hexanes to 2:1 EtOAc/hexanes) resulted in recovery of PMB-pantetheine **22** (200 mg, 0.51 mmol) as well as isolation of **21** (40 mg, 10%) as a crude oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J*=8.8 Hz, 2H), 7.02 (bt, 1H), 6.90 (d, *J*=8.4 Hz, 2H), 6.35 (bt, 1H), 5.45 (s, 1H), 4.07 (s, 1H), 3.81 (s, 3H), 3.69 (d, *J*=11.6 Hz, 1H), 3.64 (d, *J*=12.0 Hz, 1H), 3.54-3.36 (m, 4H), 3.02 (t, *J*=6.4 Hz, 2H), 2.42-2.35 (m, 4H), 1.58 (p, *J*=7.2 Hz, 2H), 1.39-1.29 (m, 4H), 1.09 (s, 3H), 1.08 (s, 3H), 0.89 (t, *J*=7.6 Hz, 3H). ^{13}C-NMR (100.5 MHz, $(CD_3)_2SO$) δ 176.6, 171.4, 169.8, 160.4, 130.4, 127.7, 113.9, 101.6, 97.0, 84.1, 78.8, 78.7, 55.6, 39.4, 36.1, 35.1, 33.3, 31.2, 29.5, 27.4, 22.3, 22.1, 19.4, 19.3, 14.1. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{27}H_{38}O_6N_2S_1$, 518.2451, found 518.2455.



Protected pantetheine **22** (28 mg, 0.06 mmol) was dissolved in 1N HCl:THF, 1:4 (5 ml) and stirred until the starting material was consumed as shown by TLC (2,4-dinitrophenylhydrazine visualization). The reaction was then neutralized by addition of saturated $NaHCO_3$ (10 mL), and extracted with EtOAc (3x25 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Resuspension and purification by flash chromatography (1:1 EtOAc/hexanes to 1% MeOH/EtOAc) resulted in isolation of **6** as an oil (20 mg, 91%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.48 (bt, 1H), 6.69 (bt,

1H), 4.1-4.03 (m, 1H), 3.99 (s, 1H), 3.57-3.36 (m, 6H), 3.13-3.04 (m, 2H), 2.43 (t, *J*=6.0 Hz, 2H), 2.37 (t, *J*=6.8 Hz, 2H), 1.62-1.55 (m, 2H), 1.41-1.29 (m, 4H), 0.98 (s, 3H), 0.94-0.88 (m, 6H). ^{13}C-NMR (75 MHz, ($CDCl_3$) δ 176.9, 174.2, 172.2, 97.5, 78.7, 78.5, 39.5, 39.4, 35.8, 35.5, 31.2, 29.4, 29.3, 27.4, 22.3, 21.7, 20.6, 19.3, 14.1. HRMS (EI) (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{32}O_5N_2S_1$, 400.2032, found 400.2026.

(g) Synthesis of 3-decynoyl-N-DMC-cystamine (9)



A solution of 7-dimethylamino-coumarin thiol **23** (23 mg, 0.08 mmol) and triethylamine (0.011 mL, 0.08 mmol) in DMF (1 mL) was added to a stirring solution of 3-decynoyl chloride **14** (70 mg, 0.375 mmol) in CH_2Cl_2 (5 mL) prechilled to 0^{o}C. Care must be taken that only a small amount of triethylamine is added to the reaction, to prevent chemical isomerization to the allenic thioester. After stirring for 1 hour, the reaction was quenched with MeOH (1 mL), diluted in EtOAc (75 mL) and washed with saturated Na_2HCO_3 (1x25 mL) and brine (1x100 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Resuspension and purification by flash chromatography (1:6 EtOAc/hexanes to 2:1 EtOAc/hexanes) resulted in isolation of fluorescent oil **9** (10 mg, 29%) as well as recovery of the disulfide of **22** (10 mg). Compound **9** can alternatively be prepared by coupling of 3-decynoic acid **12** and **22** by DCC without the addition of base (see synthetic notes for compound **1**).^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.43 (d, *J*= 8.8 Hz, 1H), 6.61 (d, *J*= 9.2 Hz, 1H), 6.51 (s, 1H), 6.09 (bt, 1H), 6.03 (s, 1H), 3.61 (s, 2H), 3.43 (t, *J*=5.6 Hz, 2H), 3.31 (s, 2H), 3.06 (s, 6H), 2.97 (t, *J*=6.0 Hz, 2H), 2.20 (m, 2H), 1.39-1.25 (m, 8H), 0.88 (m, 3H). ^{13}C-NMR (100.5 MHz, ($CD_3)_2SO$) δ 195.3, 168.7, 161.4, 156.1, 153.5, 151.7, 126.7, 110.2, 109.7, 108.9, 98.2, 86.2, 73.0, 45.1, 40.8, 40.2, 38.9, 31.7, 31.4, 28.9, 28.5, 22.7, 18.8, 14.6. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{25}H_{32}O_4N_2S_1$, 456.2077, found 456.2073.

Supporting Methods

Protein Expression and Materials

Recombinant *E. coli* CoA biosynthetic enzymes (pantothenate kinase, phosphopantetheine-adenyltransferase, dephospho-CoA kinase) were expressed and purified as MBP-fusions as described previously.[10] Recombinantally expressed Sfp (PPTase; *B. subtilis*) was overexpressed in *E. coli* as the native protein (untagged) and purified as described previously.[10] The carrier proteins AcpP (*E. coli* FAS), Fren (frenolicin PKS; *Streptomyces roseofulvus*), Otc (oxytetracycline PKS; *Streptomyces rimosus*), and EntB (enterobactin NRPS; *E. coli*) were overexpressed and purified as the 6xHis tagged constructs as described previously.[11,12] FabA (*E. coli* DH domain) and the GFP-AcpP fusion were produced and isolated as the 6xHis tagged constructs using the respective *E. coli* overexpression strains from the ASKA collection, kindly provided by Dr. Hirotada Mori at Keio University, Japan.[13] Briefly, proteins were expressed by growth of *E. coli* (K12, strain AG1) ASKA clones harboring the desired plasmid in Luria Bertani medium supplemented with 30 μg/ml chloramphenicol at 37°C. At OD 0.7 (600 nm) protein expression was induced using 1mM isopropyl β-D-1 thiogalactopyranoside, and cells were cultured with shaking for approximately 16 hr. Cells were pelleted and resuspended in lysis buffer (25 mM potassium phosphate, 100 mM NaCl, pH 7.4) and then lysed by passage through a French pressure cell. After treatment with DNAse cellular debris was separated from overexpressed enzyme by centrifugation and the supernatant was loaded onto a Ni^{2+}-NTA column and eluted with a gradient of imidazole (5-300 mM). Ni-NTA affinity chromatography and all steps following growth were performed at 4°C. Eluted proteins were visualized by SDS-PAGE with Blue-silver Coomassie stain[14] and quantitated by the method of Bradford.[15]

Procedures for ACP-DH Crosslinking

Crosslinking reactions consisted of two sequential steps. *First*, electrophilic pantetheine analogues (**1**, **2**, or **6**) were loaded onto AcpP through a one-pot chemoenzymatic reaction protocol which utilized the CoA biosynthetic enzymes (PanK, PPAT, DPCK) to form the corresponding CoA analogues in situ, followed by loading onto *apo*-AcpP by the promiscuous PPTase Sfp. Reagents and enzymes were added to 27 μL reactions in sequential order as follows. Final reaction concentrations: 55 mM potassium phosphate (pH 7.0), 27 mM ATP, 47 mM $MgCl_2$, 0.1 μg/μL MBP-PanK, 0.1 μg/μL MBP-PPAT, 0.1 μg/μL MBP-DPCK, 0.08 μg/μL Sfp (native), 0.37 μg/μL AcpP, 185 μM pantetheine analogue (**1**, **2**, or **6**). In negative controls pantetheine analogues **1**-**3** were replaced with vehicle DMSO. In all reactions DMSO concentrations were maintained at a level ≤ 1 %. For affinity purification of crosslinked complex, reactions were performed similarly on a 570 μL scale. For crosslinking studies of alternate carrier proteins (GFP AcpP, Fren, Otc, EntB), the corresponding carrier protein was substituted for native AcpP. After addition of all components reactions were vortexed and incubated at 37°C for 1-3 hours. In the *second* step of the reaction FabA (final concentration 0.1 μg/μL) was added, and reactions were further incubated for 3-12 hours before quenching with 5x SDS loading buffer (strong reducing) and analysis by SDS-PAGE. Gels were fixed in destain solution (50:43:7 MeOH:H_2O:AcOH) and washed twice with water prior to staining with blue-silver Coomassie stain.

Procedure for Affinity Purification of AcpP-FabA Complex and *crypto*-ACPs

For affinity purification of the AcpP-FabA complex (Figure 2d), crosslinking reactions were performed on a 570 µL scale using pantetheine analogues **1** or **6** as described above and incubated 3 hours. FabA was added after 3 hours and reactions were incubated overnight. Reactions were then added to 60 µL Ni^{2+}-NTA resin (pre-washed with 50 mM sodium phosphate, pH 7.0) and rotated at room temperature for 1 hour. Reactions were centrifuged, supernatant removed, and 1000 µL washing buffer 1 (50 mM sodium phosphate, pH 7.0) added. After gentle mixing for 5 minutes, reactions were centrifuged, the supernatant removed, and 1000 µL washing buffer 2 (5 mM imidazole, 50 mM sodium phosphate, pH 7.0) added. After gentle mixing for 5 minutes, reactions were centrifuged, the supernatant removed, and 100 µL elution buffer 1 (50 mM imidazole, 50 mM sodium phosphate, pH 7.0) added. After gentle mixing for 5 minutes, reactions were centrifuged, the supernatant removed, and 100 µL elution buffer 2 (400 mM imidazole, 50 mM sodium phosphate, pH 7.0) added. After gentle mixing for 5 minutes, reactions were centrifuged, the supernatant removed, and 100 µL rinsing buffer (2500 mM imidazole, 50 mM sodium phosphate, pH 7.0) added. After gentle mixing for 5 minutes, reactions were centrifuged, the supernatant removed. For affinity purification of *crypto*-GFP AcpPs modified by **1** or **6** (used in Figure 2e), reactions were performed as above and purified by Ni^{2+}-NTA chromatography *prior* to addition of FabA and incubation overnight. Following elution and crosslinking, all fractions were analyzed by SDS-PAGE, fixed, and visualized by staining with Blue-silver Coomassie stain.

Procedures for ACP-KS Crosslinking

ACP-KS crosslinking of AcpP and FabB was performed in a similar fashion as above, using a slightly modified version of the previously reported protocol. Briefly, to a buffered solution consisting of 55 mM potassium phosphate (pH 7.0), 50 mM $MgCl_2$, and 27 mM ATP was added 6xHis-PanK (0.1 µg/µL), 6xHis-PPAT (0.1 µg/µL), 6xHis-DPCK (0.1 µg/µL), Sfp (native) (0.08 µg/µL), and AcpP (0.37 µg/µL) to bring the total reaction volume to 27 µL. Note that 6xHis tagged CoA biosynthetic proteins are used in this protocol (in contrast to the MBP-tagged proteins of the ACP-DH crosslinking reaction) to facilitate visualization of the AcpP-FabB crosslinked complex (~ 70 kDa). Electrophilic pantetheine analogues (**1**, **2**, or **6**) were then added to a final concentration of 185 µM. In negative controls pantetheine analogues **1**-**3** were replaced with vehicle DMSO. In all reactions DMSO concentrations were maintained at a level ≤ 1 %. Reactions were incubated for 30 minutes at 37 °C, followed by addition of ketosynthase enzyme FabB (0.1 µg/µL). Reactions were further incubated for another hour before quenching with 5x SDS loading buffer (strong reducing) and analysis by SDS-PAGE. Gels were fixed in destain solution (50:43:7 MeOH:H_2O:AcOH) and washed twice with water prior to staining with blue-silver Coomassie stain.

Procedures for Activity Based Labeling of DH Domains by Fluorescent Suicide Substrate 9

For FabA (DH) activity based labeling experiments, fluorescent probe **9** (25 μM) was added to a 27 uL reaction containing FabA (0.1 μg/μL), Tris-HCl pH 6.8 (100 mM), and DTT (10 mM). Controls were performed by pre-denaturation of FabA by addition of 0.5% SDS. To assess the FabA active site modification properties of pantetheine analogues **1**-**8**, FabA was preincubated with each analogue for 30 minutes at 1 mM before addition of **9**. In all reactions DMSO concentrations were maintained at a level ≤ 3%. After reaction for 30 minutes FabA labeling reactions were quenched with 5x SDS-loading buffer (strong reducing) and subjected to SDS-PAGE. Probe **9** showed high off-site reactivity when heated prior to SDS-PAGE, so boiling of these samples was avoided. Fluorescent gel visualization was performed using a BioRad Fluor-S Gel Doc equipped with a 460 nm emission filter.

Procedures for MS Analysis Crosslinked Complex

Procedures for In Gel Digest of Crosslinked Complex

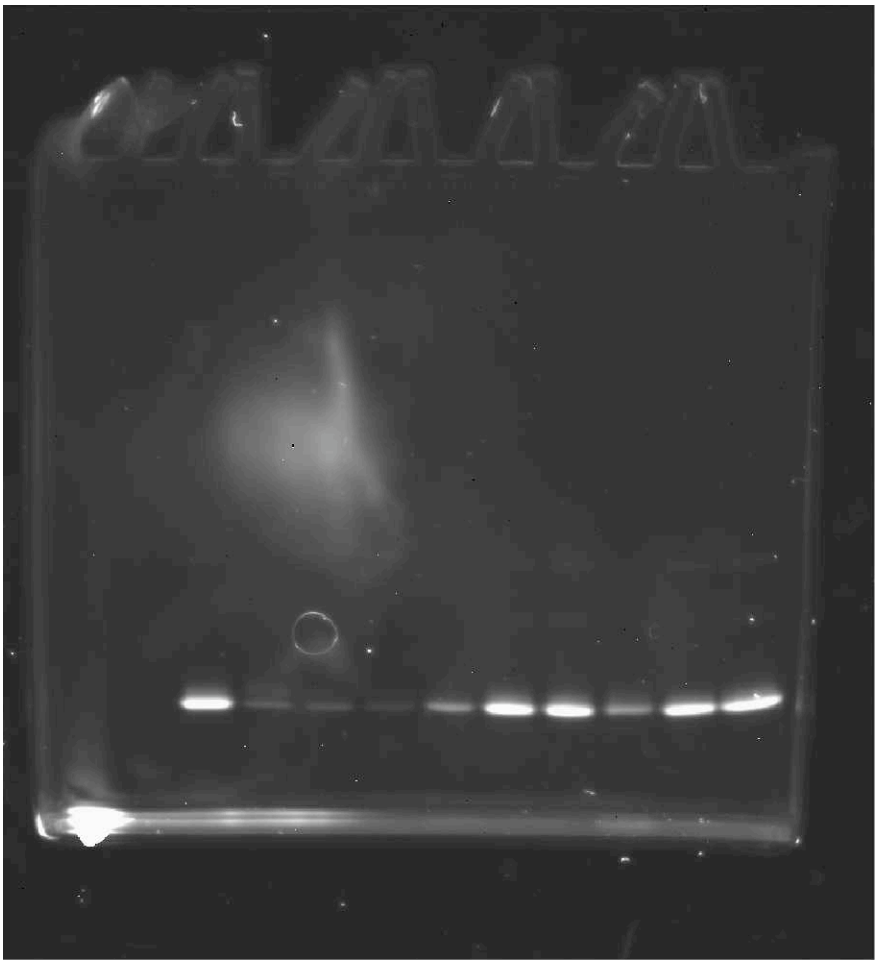
For MS analysis, proteins were separated by SDS-PAGE using 10% Bis-Tris NuPAGE Gels (Invitrogen). Gels were fixed in destain solution (50:43:7 MeOH:H_2O:AcOH) and washed twice with water prior to overnight staining with blue-silver Coomassie stain. Subsequently, the gel was washed twice with water (30 min each). Excised gel bands were cut into 1 mm cubes and washed for 10 min with vortexing with 1:1 H_2O/CH_3CN (40 μL) followed by CH_3CN (40 μL). After removal of the supernatant, 100 mM NH_4CO_3 (40 μL) was added and shaken for 5 min, followed by addition of CH_3CN (40 μL) and shaking for 10 min. The gel slice was then dried on a speed vacuum concentrator system. The gel slice was then resuspended in 100 mM NH_4CO_3 (100 μL) and TCEP added to a concentration of 10 mM and incubated at 55°C for 30 minutes. The supernatant was removed and the gel slice resuspended in 100 mM NH_4CO_3 (100 μL) and iodoacetamide added to a concentration of 55 mM. The reaction was covered with foil and shaken at room temperature for 30 minutes. After removal of the supernatant, 100 mM NH_4CO_3 (40 μL) was added and shaken for 5 min, followed by addition of CH_3CN (40 μL) and shaking for 10 min. The gel slice was then dried on a speed vacuum concentrator system. The gel slice was then resuspended by addition of 20 μL 10 ng/μL trypsin (Promega) in a 5 mM DTT, 25 mM NH_4CO_3, 5 mM $CaCl_2$ solution and incubated on ice 45 minutes. Additional trypsin was added if necessary, and the digest allowed to continue at 37°C overnight. The supernatant was then extracted and the gel slice washed multiple times (1:1 25 mM NH_4CO_3:CH_3CN, 1:1 5% formic acid: CH_3CN) with vortexing. Combining each wash provided ~ 100 μL post-digest supernatant. This material was then dried on a speed vacuum concentrator system and resuspended in 15 μL 5% formic acid for MALDI TOF/TOF analysis.

MALDI TOF/TOF Analysis of Crosslinked Bands

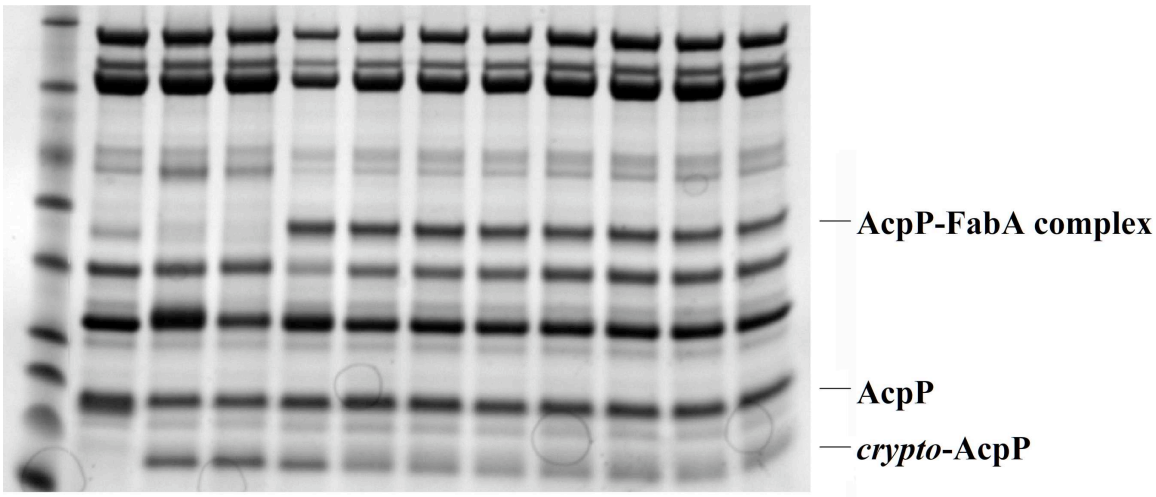
Each sample from the in gel digest (prepared above) was mixed 1:1 with α-cyano-4-hydroxycinnamic acid (Agilent) and spotted on a MALDI target plate. Peptides were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) on a 4800 MALDI

tandem time-of-flight mass spectrometer (Applied Biosystems). Briefly, peptide mass fingerprints were acquired with 500 shots in reflector positive mode from *m/z* 1000 – 3000. Peptide tandem mass spectra were acquired with 2000 shots in 2 kV positive MSMS mode. Peaks occurring with Minimim Chromatogram Peak Width of 2 fractions, a Minimum S/N filter of 40, and a Fraction-to-Fraction Precursor Mass Tolerance of 100 ppm were MSMS acquired in the order of Strongest Precursors First with a Max Precursors/Fraction at 8. Sodium and potassium adducts were excluded with an Adduct Tolerance at +/- 0.03 Da, and precursors < 200 resolution were excluded. A Plate Model and Default Calibration were performed for reflector positive mode as well as a Default calibration for 2 kV positive mode. MSMS spectra were assigned by database searching using Mascot 2.1 (Matrix Science) using Global Proteomics Sever 3.1 (Applied Biosystems). A custom database containing 57 sequences including the *E. coli* proteins AcpP, FabA, PanK, PPAT, DPCK, *B. subtilis* Sfp, the fusion tags GFPuv4 and MBP, and a number of commonly occurring protein contaminants (e.g. keratin) was searched. The MSMS ion search parameters identified tryptic peptides with up to 2 missed cleavages and used mass tolerances of 100 ppm (MS) and 0.10 Da (MSMS), with the constant modification carbamidomethylation (C) due to reductive alkylation by iodoacetamide. Common non-specific variable modifications that occur as a result of the in-gel digest assay were also included in the search parameters: deamidation (NQ), oxidation (M), propionamide (C), and pyro-glu (N-term Q). The search results indicated that individual ion scores > 31 indicate identity or extensive homology ($P < 0.05$).

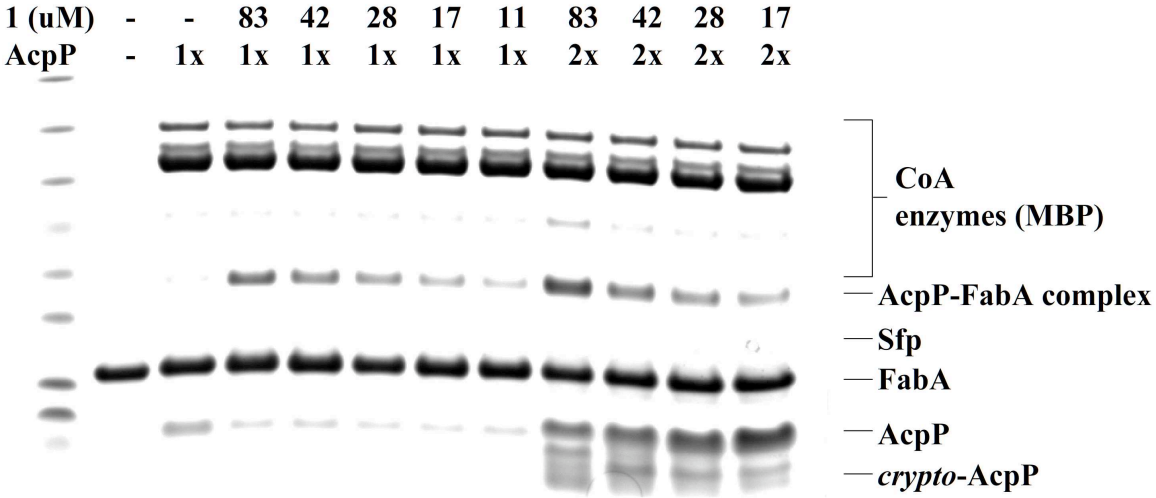
Full Gel Data for Figure 2.



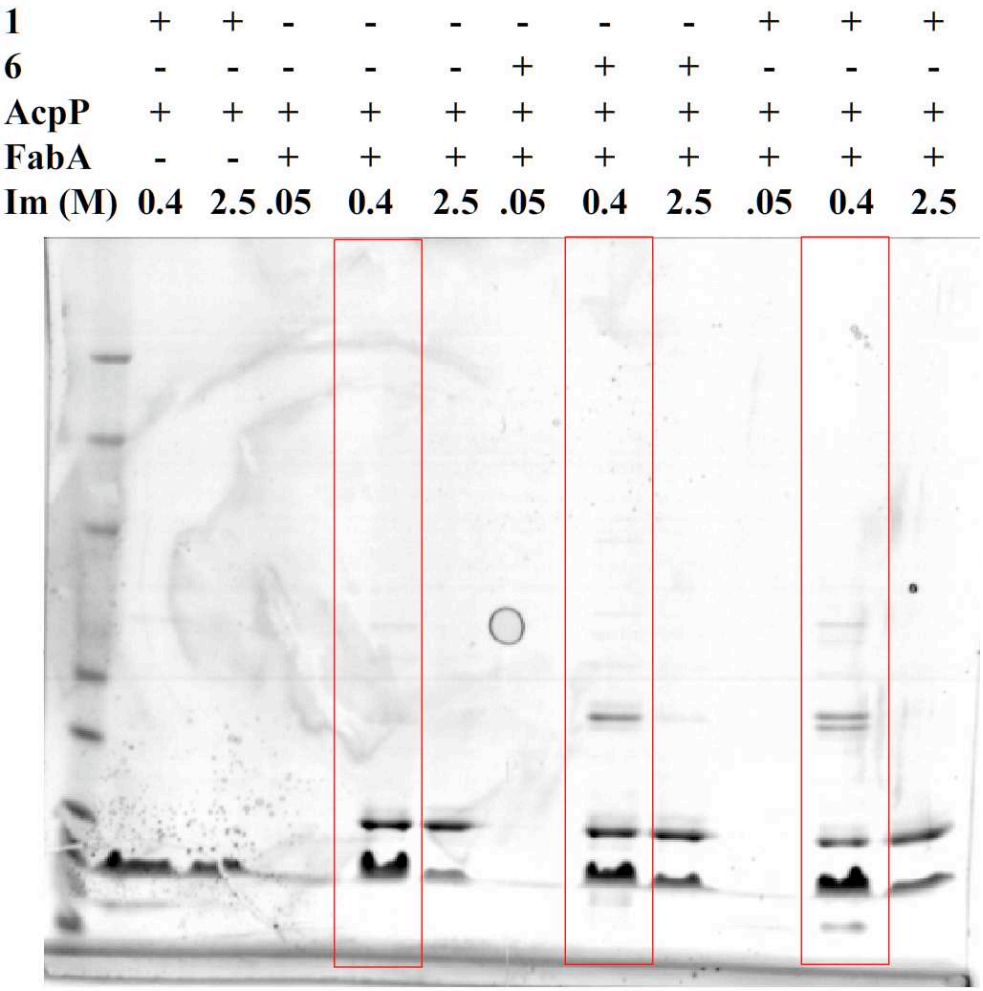
Full gel data for Figure 2a. See main text and "Procedures for Activity Based Labeling of DH Domains by Fluorescent Suicide Substrate **9**" (Supporting Methods) for full description.



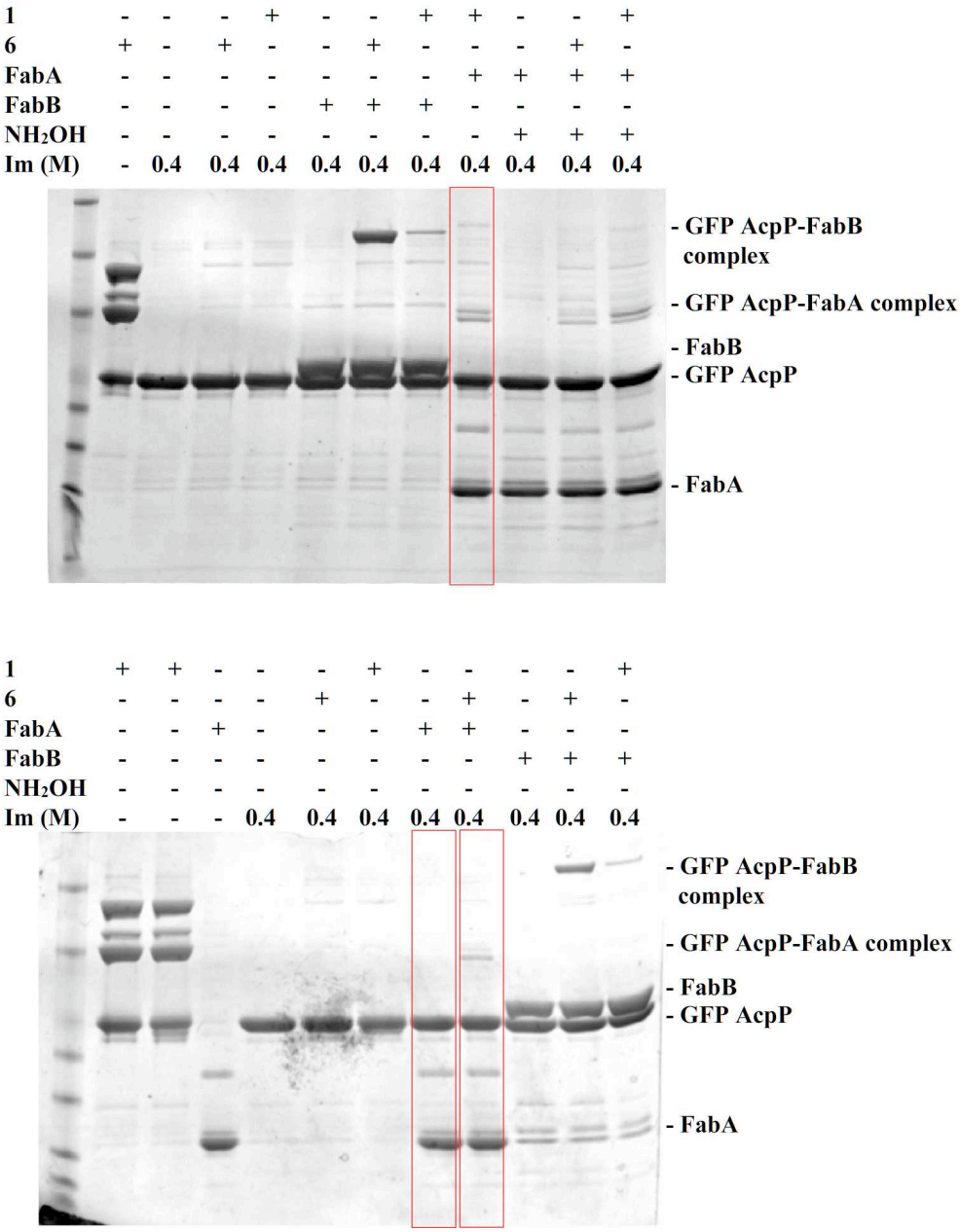
Full gel data for Figure 2b. Lane 1 is a molecular weight ladder, lanes 2-6 are depicted in Figure 2b. See main text for full description.



Full gel data for Figure 2c. Lane 1 is a molecular weight ladder, lanes 2-8 are depicted in Figure 2c. See main text for full description.



Full gel data for Figure 2d. Lanes boxed in red are depicted in Figure 2d. Addition of 0.05, 0.4, and 2.5 M imidazole (Im) was used to elute crosslinked AcpP-FabA complex from Ni^{2+}-NTA beads. Each fraction was then analyzed by SDS-PAGE. See "Procedure for Affinity Purification of AcpP-FabA Complex and *crypto*-ACPs" (Supporting Methods) and main text for full description.



Full gel data for Figure 2e. Lanes boxed in red are depicted in Figure 2e. Addition of 0.4 M imidazole (Im) was used to elute *crypto*-GFP AcpPs from Ni^{2+}-NTA beads following modification by **1** or **6** using the one-pot chemoenzymatic method. An aliquot of FabA was then added, followed by incubation overnight and analysis by SDS-PAGE. See "Procedure for Affinity Purification of AcpP-FabA Complex and *crypto*-ACPs" (Supporting Methods) and main text for full experimental details. When FabA is added to *crypto*-GFP AcpP **6**, a single crosslinked band is observed (bottom right red box). When FabA is added to *crypto*-GFP AcpP **1**, a two crosslinked bands are observed (bottom right red box), similar to when AcpP-FabA complex is purified (see Figure 2d and previous page).

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