Table of Contents

Strains and culture conditions

Saccharomyces cerevisiae was grown in YPAD liquid medium (20 g glucose, 10 g yeast extract, 20 g peptone, 40 mg adenine sulfatedehydrate, 1 L water) at 30 °C. *Escherichia coli* K12 (DSM 18039) and *E. coli* BL21 (DE3) were grown in LB medium at 37 °C. *Bacillus amyloliquefaciens* FZB42 was grown in 1 GYM medium (5.0 g peptone, 4.0 g meat extract, 10.0 mg $MnSO_4$, 1 L water, pH 7.0) at 30 °C. *Chitinophaga pinensis* (DSM 2588) was grown in 67 GYM liquid medium (3.0 g casitone, 1.0 g yeast extract, 1.36 g $CaCl_2 \cdot H_2O$ extract, 1 L water, pH 7.2) at 22 °C. *Streptomyces collinus* Tü365 (DSM 40733), *Streptomyces venezuelae* (ATCC 10712), *Streptomyces avermitilis* (DSM 46492), *Streptomyces clavuligerus* (ATCC 27064), *Streptomyces albus* J 1074 and *Streptomyces ambofaciens* (ATCC 23877) were grown in 65 GYM liquid medium (4.0 g glucose, 4.0 g yeast extract, 4.0 g malt extract, 1 L water, pH 7.2) at 30 °C.

Gene cloning

For *b3974* gene cloning (coding for *E. coli* CoaA), genomic DNA (gDNA) of *E. coli* K12 was isolated from freshly grown cultures in liquid medium (100 mL). The cultures were centrifuged at 8000 x g and the supernatant was discarded. The cells were resuspended in SET buffer (5 mL; 75 mMNaCl, 25 mM EDTA, 20 mMTrisHCl, pH 8.0), then lysozyme solution (100 μL; 50 mg mL^{-1}) was added and the mixture was incubated for 30 min at 37 °C. To this mixture, proteinase K solution (100 μL; 50 mg mL^{-1}) and 10 % SDS (600 μL) were added and the incubation was carried on for 1 h at 55 °C. Phenol/chloroform/isoamyl alcohol (25:24:1, 5 mL) was added, the phases were mixed, followed by centrifugation at top speed (14600 rpm) for 5 min. The aqueous layer was transferred to a fresh tube and the DNA was precipitated by the addition of 3 M NaOAc (0.1 vol) and ethanol (0.6 vol). The DNA was spun down, washed with 70 % ethanol, centrifuged again and dried overnight. The dry DNA was dissolved in nuclease free water at a concentration of approximately 1000 ng/μL. The same protocol was used for isolation of gDNA from the other source organisms as listed in Table S1.

Table S1. Genes cloned in this study for heterologous protein expression.

Accession number	Gene locus tag	Source organism
Coenzyme A biosynthesis		
NP_418405 (CoaA)	*b3974*	*Escherichia coli* K12
NP_418091 (CoaD)	*b3634*	*Escherichia coli* K12
NP_414645 (CoaE)	*b0103*	*Escherichia coli* K12
PPTases		
ABV89947 (Sfp)	*sfp*	*Bacillus amyloliquefaciens* FZB42
AGS67172 (KirP)	*b446_01700*	*Streptomyces collinus* Tü 365
BAC69459	*saverm_1748*	*Streptomyces avermitilis* MA-4680
CCA59476	*sven_6190*	*Streptomyces venezuelae* ATCC 10712
AKK16885 (EntD)	*u069_c1137*	*Escherichia coli* K12
AGI92022	*xnr_5716*	*Streptomyces albus* J 1074
AGS72899	*b446_30475*	*Streptomyces collinus* Tü 365
EFG07676	*sclav_2604*	*Streptomyces clavuligerus* ATCC 27064
WP_012791066	*cpin_rs16975*	*Chitinophaga pinensis* DSM 2588
WP_012793840	*cpin_rs31010*	*Chitinophaga pinensis* DSM 2588
CPs		
CAJ89357 (AntG)	*saml0370*	*Streptomyces ambofaciens* ATCC 23877
AKK17307 (AcpP)	*u069_c1559*	*Escherichia coli* K12
AAB36564 (JadC)	*acp*	*Streptomyces venezuelae* ATCC 10712

Polymerase chain reactions (PCR) were performed to amplify the target genes for gene clonings according to a standard 3-step PCR protocol provided by the supplier of Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). The *b3974* gene (NP_418405) was amplified by PCR using gDNA from *E. coli* K12 and primers NP_418405_Fw and NP_418405_Rv. PCR conditions were: initial denaturation at 98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66 °C, 30 s; 72 °C, 30 s; repeated 35 times; final elongation at 72 °C, 2 min. The obtained products were elongated with homology arms by PCR using the primers NP_418405_LFw and NP_418405_LRv (for *b3974*). Information about the primers used for other gene amplifications by PCR are provided in Table S2, and the PCR conditions are detailed in Table S3.

Table S2. Primers used for gene cloning.

Primer	Sequence[a]
NP_418405_Fw	ATGAGTATAAAAGAGCAAACGTTAATGACG
NP_418405_Rv	TTATTTGCGTAGTCTGACCTCTTCTAC
NP_418405_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGAGTATAAAAGAGCAAACGTTAATGACG
NP_418405_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTTATTTGCGTAGTCTGACCTCTTCTAC
NP_418091_Fw	ATGCAAAAACGGGCGATTTATCC
NP_418091_Rv	CTACGCTAACTTCGCCATCAG
NP_418091_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGCAAAAACGGGCGATTTATCC
NP_418091_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTCTACGCTAACTTCGCCATCAG
NP_414645_Fw	ATGAGGTATATAGTTGCCTTAACGG
NP_414645_Rv	TTACGGTTTTTCCTGTGAGACAAAC
NP_414645_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGAGGTATATAGTTGCCTTAACGG
NP_414645_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTTACGGTTTTTCCTGTGAGACAAAC
ABV89947_Fw	ATGAAGATTTACGGAATTTATATGGACCGC
ABV89947_Rv	TTATAAAAGCTCTTCGTACGAGACCATTG
ABV89947_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGAAGATTTACGGAATTTATATGGACCGC
ABV89947_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTTATAAAAGCTCTTCGTACGAGACCATTG
AGS67172_Fw	GTGATCACGGGATCCGTCGG
AGS67172_Rv	TCACCGTCCTCCTGCGTAG
AGS67172_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAGTGATCACGGGATCCGTCGG
AGS67172_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTCACCGTCCTCCTGCGTAG
BAC69459_Fw	GTGATCGAGGAGCTGCTCCC
BAC69459_Rv	TCAGTGGTGCGGCACCG
BAC69459_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAGTGATCGAGGAGCTGCTCCC
BAC69459_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTCAGTGGTGCGGCACCG
CCA59476_Fw	ATGCTGTCGACCCTGCTGCC
CCA59476_Rv	TCATGCCGCCTCCCGGG
CCA59476_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGCTGTCGACCCTGCTGCC
CCA59476_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTCATGCCGCCTCCCGGG
AKK16885_Fw	AACGCCTTATCCGGCCTACA
AKK16885_Rv	TTAATCGTGTTGGCACAGCGT
AKK16885_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAAACGCCTTATCCGGCCTACA
AKK16885_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTTAATCGTGTTGGCACAGCGT
AGI92022_Fw	ATGCTGAAGACGATCCTGCCCG
AGI92022_Rv	CTACGGCCGCGGGACGG
AGI92022_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGCTGAAGACGATCCTGCCCG
AGI92022_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTCTACGGCCGCGGGACGG
AGS72899_Fw	GTGATCGAGGAGCTGCTCCCG
AGS72899_Rv	TCAGCGGTGCGGGACGG
AGS72899_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAGTGATCGAGGAGCTGCTCCCG
AGS72899_LRv	TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTCAGCGGTGCGGGACGG
EFG07676_Fw	ATGGCGACCGAGTCCCC
EFG07676_Rv	TCACTGCGGGGTTTGGACC
EFG07676_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGGCGACCGAGTCCCC

EFG07676_LRv	TCTCAGTGGTGGTGGTGGTGCTCGAGTTCACTGCGGGGTTTGGACC
WP_012791066_Fw	ATGGTGTACGTTTATTACTGTGAAAACAGCCC
WP_012791066_Rv	TCAGGATGCAATGATATCCTCCGTACATAAGG
WP_012791066_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGGTGTACGTTTATTACTGTGAAAACAGCCC
WP_012791066_LRv	TCTCAGTGGTGGTGGTGGTGCTCGAGTTCAGGATGCAATGATATCCTCCGTACATAAGG
WP_012793840_Fw	ATGCCATTAATACGTACCATACAAATAAACCCCG
WP_012793840_Rv	TTAATGTCCTGAAGCTGCGTTCTCTG
WP_012793840_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGCCATTAATACGTACCATACAAATAAACCCCG
WP_012793840_LRv	TCTCAGTGGTGGTGGTGGTGCTCGAGTTTAATGTCCTGAAGCTGCGTTCTCTG
CAJ89357_Fw	ATGAGCACCATCAGGGAGTTGCTG
CAJ89357_Rv	TCACGCACCGCCGCTC
CAJ89357_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGAGCACCATCAGGGAGTTGCTG
CAJ89357_LRv	TCTCAGTGGTGGTGGTGGTGCTCGAGTTCACGCACCGCCGCTC
CAJ89357_S34A_Fw	GCATCGAT**GCG**GGGGACCTCG
CAJ89357_S34A_Rv	GGTCCCC**CGC**ATCGATGCCGCT
AKK17307_Fw	ATGAGCACTATCGAAGAACGCG
AKK17307_Rv	TTACGCCTGGTGGCCGTTG
AKK17307_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGAGCACTATCGAAGAACGCG
AKK17307_LRv	TCTCAGTGGTGGTGGTGGTGCTCGAGTTTACGCCTGGTGGCCGTTG
AAB36564_Fw	ATGAGCAGCAAGACCTTCACCCTC
AAB36564_Rv	TCAGGCGGCGGCGACGG
AAB36564_LFw	GGCAGCCATATGGCTAGCATGACTGGTGGAATGAGCAGCAAGACCTTCACCCTC
AAB36564_LRv	TCTCAGTGGTGGTGGTGGTGCTCGAGTTCAGGCGGCGGCGACGG

[a] Homology arms for recombination in yeast fitting to pYE-Express are underlined. Exchanged nucleotides in mutational primers are highlighted in bold

Table S3. PCR conditions used for gene cloning.

Accession number	PCR conditions
NP_418405	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66 °C, 30 s; 72 °C, 30 s; repeated 35 times; final elongation at 72 °C, 2 min.
NP_418091	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 67 °C, 30 s; 72 °C, 15 s; repeated 35 times; final elongation at 72 °C, 2 min.
NP_414645	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 65 °C, 30 s; 72 °C, 20 s; repeated 35 times; final elongation at 72 °C, 2 min.
ABV89947	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 68 °C, 30 s; 72 °C, 30 s; repeated 35 times; final elongation at 72 °C, 2 min.
AGS67172	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 30 s; repeated 35 times; final elongation at 72 °C, 2 min.
BAC69459	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 25 s; repeated 35 times; final elongation at 72 °C, 2 min.
CCA59476	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 25 s; repeated 35 times; final elongation at 72 °C, 2 min.
AKK16885	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 25 s; repeated 35 times; final elongation at 72 °C, 2 min.
AGI92022	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 25 s; repeated 35 times; final elongation at 72 °C, 2 min.
AGS72899	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 25 s; repeated 35 times; final elongation at 72 °C, 2 min.
EFG07676	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 25 s; repeated 35 times; final elongation at 72 °C, 2 min.
WP_012791066	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 20 s; repeated 35 times; final elongation at 72 °C, 2 min.
WP_012793840	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 20 s; repeated 35 times; final elongation at 72 °C, 2 min.
CAJ89357	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 65 °C, 30 s; 72 °C, 10 s; repeated 35 times; final elongation at 72 °C, 2 min.
AKK17307	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 10 s; repeated 35 times; final elongation at 72 °C, 2 min.
AAB36564	98 °C, 60 s; 3-step cycle: 98 °C, 10 s; 66.7 °C, 30 s; 72 °C, 10 s; repeated 35 times; final elongation at 72 °C, 2 min.

Homologous recombination in yeast was then carried out using the elongated PCR products in combination with the pYE-Express shuttle vector[1] (linearised via digestion with HindIII and EcoRI), through the standard protocol using LiOAc, polyethylene glycol and salmon sperm DNA.[2] Transformed yeast cultures were grown on SM-URA agar plates (425 mg yeast nitrogen base, 1.25 g ammonium sulphate, 5 g glucose, 192.5 mg yeast synthetic medium supplements without uracil, 5 g agar, 250 mL water) at 28 °C for 3 days and colonies were collected to obtain the recombinant plasmids using the Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research, Irvine, CA, USA). The isolated plasmids were used for electroporation of *E. coli* BL21 (DE3) electrocompetent cells, which were grown overnight at 37 °C on LB agar plates. Single colonies were picked and inoculated in LB medium (10 mL) with kanamycin (10 μL; 50 mg mL^{-1}) and grown for 12 h to isolate plasmid DNA. The correct insertion of the desired gene was checked by PCR and by sequencing to obtain the plasmid pYE-NP_418405. The other constructed plasmids were named accordingly pYE-“accession no.”.

Site-directed mutagenesis

The coding sequence of AntG-S34A was obtained by site-directed mutation. Therefore, two overlapping gene fragments carrying the mutation in the overlapping sequence were obtained by PCR using plasmid pYE-CAJ89357 as a template. The first fragment was obtained using primers CAJ89357_LFw and CAJ89357_S34A_Rv and the following temperature program: initial denaturation at 98 °C for 30 s; 3-step cycle: 98 °C for 10 s, 65 °C for 30 s, 72 °C for 10 s; repeated 35 times; final elongation at 72 °C for 2 min. The second fragment was obtained using primers CAJ89357_S34A_Fw and CAJ89357_LRv and the following temperature program: initial denaturation at 98 °C for 30 s; 3-step cycle: 98 °C for 10 s, 65 °C for 30 s, 72 °C for 10 s; repeated 35 times; final elongation at 72 °C for 2 min. The two fragments were isolated through a plasmid purification kit (Wizard SV Gel and PCR Clean-Up System; Promega, Madison, Wisconsin, USA) and mixed for a third PCR using the following temperature program: initial denaturation at 98 °C for 30 s; 3-step cycle: 98 °C for 10 s, 65 °C for 30 s, 72 °C for 10 s; repeated 5 times; final elongation at 72 °C for 2 min. At this stage the primers CAJ89357_LFw and CAJ89357_LRv were added and the PCR was continued using the following temperature program: initial denaturation at 98 °C for 30 s; 3-step cycle: 98 °C for 10 s, 65 °C for 30 s, 72 °C for 10 s; repeated 35 times; final elongation at 72 °C for 2 min.

Gene expression and protein purification

E. coli BL21 (DE3) cells harboring the corresponding pYE-Express derived plasmids were used to inoculate small scale cultures in LB medium (10 mL) supplied with kanamycin (50 μg/mL final concentration), which were grown with shaking at 37 °C overnight. The small scale cultures were used to inoculate expression cultures (1/100) in LB medium with kanamycin (50 μg/mL final concentration) and the cells were grown with shaking at 37 °C until OD_{600} = 0.4 – 0.6 was reached. The cultures were cooled to 18 °C, before IPTG (0.4 mM final concentration) was added to induce expression. The cultures were shaken at the same temperature overnight and then centrifuged (3500 x g, 40 min, 4 °C). The medium was discarded and the cell pellet was resuspended in binding buffer (10 mL/L culture; 20 mM Tris-HCl, 1 M NaCl, 10% glycerol, 30 mM imidazole, pH 8.0, 4 °C). The cells were lysed by ultrasonication (10 x 1 min). The cell debris was spun down (14600 x g, 10 min, 4 °C) and the soluble protein fraction was filtrated and loaded onto a Ni^{2+}-NTA affinity chromatography column (Ni-NTA superflow, Qiagen, Venlo, Netherlands). The bound target protein was washed with washing buffer (2 x 10 mL/L culture; 20 mM Tris-HCl, 1 M NaCl, 10% glycerol, 30 mM imidazole, pH 8.0, 4 °C) and desorbed from the stationary phase with elution buffer (1 x 10 mL/L culture; 20 mM Tris-HCl, 1 M NaCl, 10% glycerol, 500 mM imidazole, pH 8.0, 4 °C) with fractionation. The buffer was replaced by incubation buffer (50 mM HEPES, 10 mM $MgCl_2$, pH 7.9) through repeated centrifugation using an ultrafiltration centrifugal tube. The protein concentration was determined through Bradford assay[3] and adjusted to 10 mg/mL.

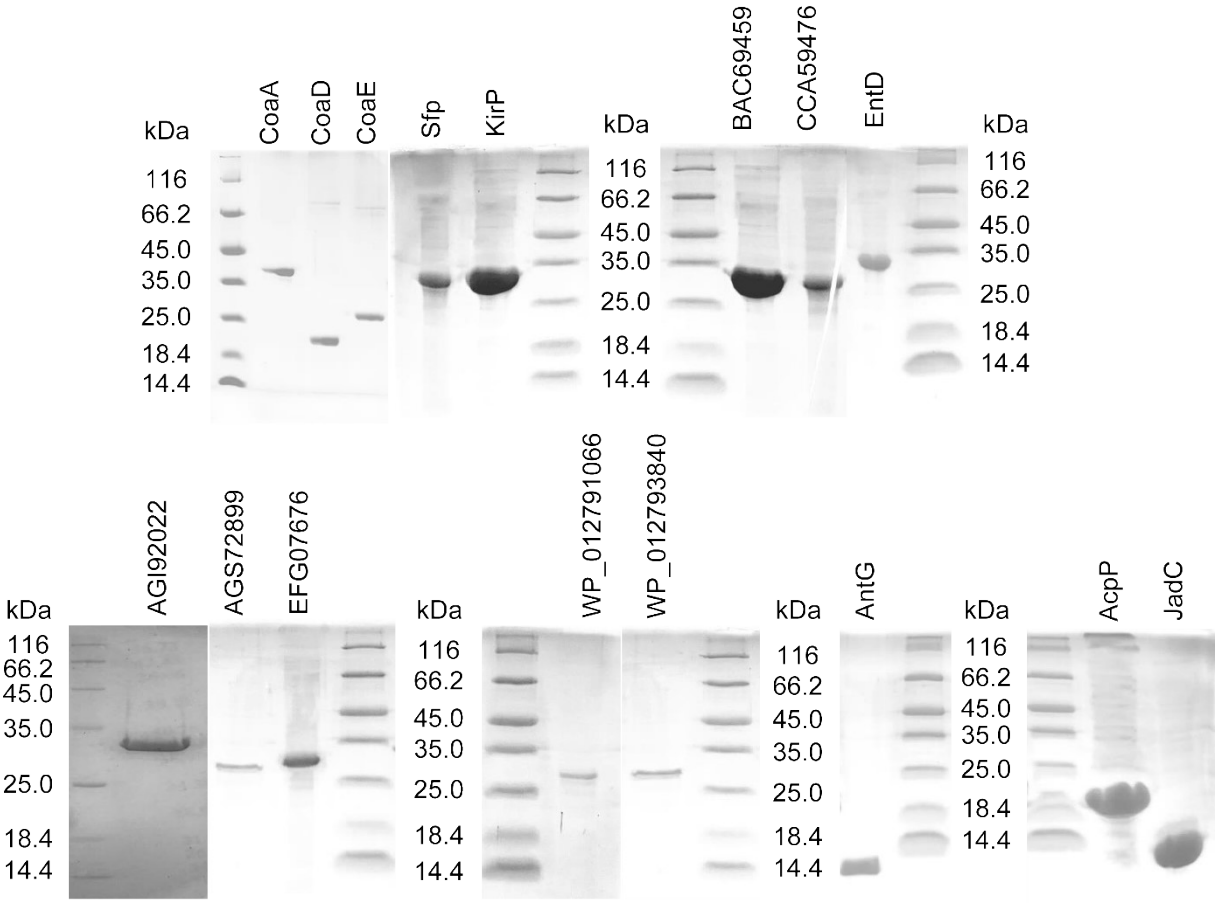

Figure S1. SDS PAGE analysis of purified recombinant proteins used in this study.

PPTase activity assays

Activity assays were conducted in a total volume of 1 mL containing the following components: HSCoA analog **5** or **9** (1 mM), ATP (9 mM), purified Sfp (1 µM), and purified AntG (250 µM) in a reaction buffer composed of HEPES (50 mM) and $MgCl_2$ (10 mM) at pH 7.9.[4] The reactions were incubated at 30 °C for 30 min. To the reaction with HSCoA analog **5**, compound **8** (5 µL, 50 mg/mL in DMSO), $CuSO_4$ (1 mg in 10 µL reaction buffer) and sodium ascorbate (1 mg in 10 µL reaction buffer) were added, followed by incubation at 30 °C for another 30 min. Both samples were analysed by SDS-PAGE (Figure S2). Attachment of the HSCoA analog **5** to AntG was also demonstrated by MALDI-ToF (Figure S15).

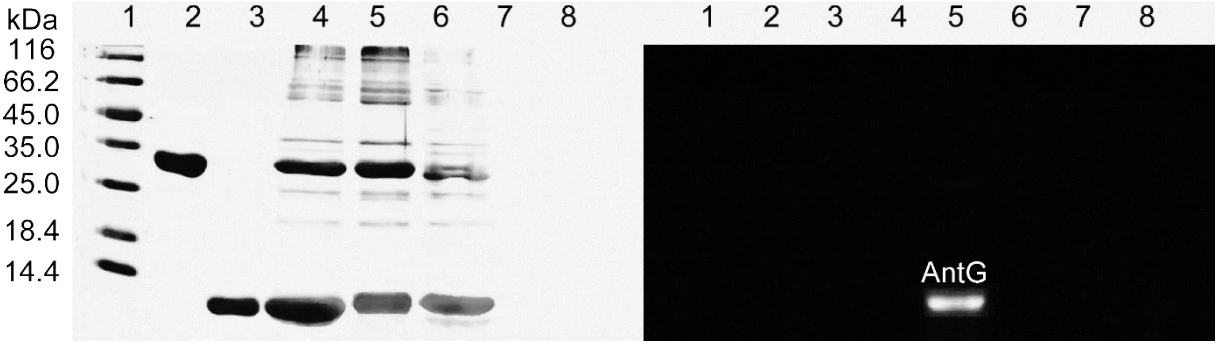

Figure S2. Activity assay for the PPTase-CP combination Sfp + AntG. Lane 1: protein marker; lane 2: purified Sfp; lane 3: purified AntG; lane 4: purified Sfp + AntG + **5**; lane 5: purified Sfp + AntG + **5** + **8** after CuAAC; lane 6: purified Sfp + AntG + **9** after CuAAC; lane 7: **8**; lane 8: **9**. The left picture shows the protein gel after coomassie brilliant blue staining, the right picture shows the same gel before staining under UV irradiation (λ = 360 nm).

Determination of binding of the HSCoA analog 5 to the conserved serine residue of AntG

To determine if the HSCoA analog **5** specifically binds to the conserved serine residue of AntG and to exclude an only non-covalent interaction between **5** and AntG, the AntG-S34A enzyme variant was obtained through site-directed mutagenesis as described above.

Activity assays were conducted in a total volume of 1 mL containing the following components: HSCoA analog **5** (1 mM), ATP (9 mM), purified Sfp (1 µM), and purified AntG or AntG-S34A (250 µM) in a reaction buffer composed of HEPES (50 mM) and $MgCl_2$ (10 mM) at pH 7.9.[4] The reactions were incubated at 30 °C for 30 min. To each reaction, compound **8** (5 µL, 50 mg/mL in DMSO), $CuSO_4$ (1 mg in 10 µL reaction buffer) and sodium ascorbate (1 mg in 10 µL reaction buffer) were added, followed by incubation at 30 °C for another 30 min. Both samples were analysed by SDS-PAGE (Figure S3).

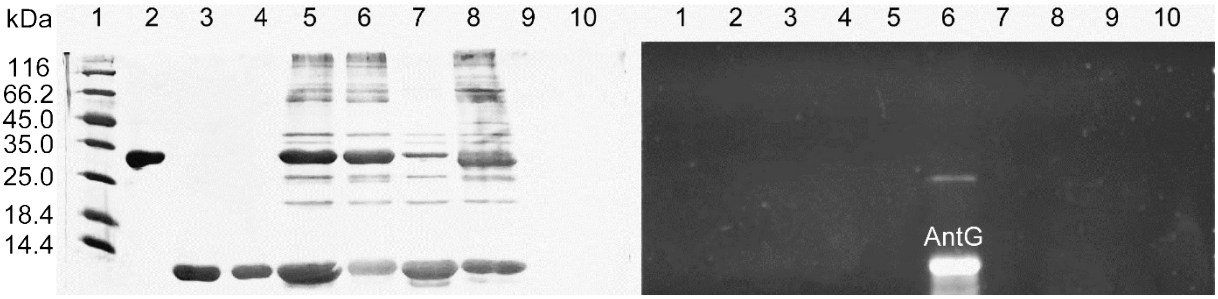

Figure S3. Activity assay for the PPTase-CP combinations Sfp + AntG and Sfp + AntG-S34A. Lane 1: Protein marker; lane 2: purified Sfp; lane 3: purified AntG; lane 4: purified AntG-S34A; lane 5: Sfp + AntG + **5**; lane 6: Sfp + AntG + **5** + **8** after CuAAC; lane 7: Sfp + AntG-S34A + **5**; lane 8: Sfp + AntG-S34A + **5** + **8** after CuAAC; lane 9: **8**; lane 10: **9**.

Screening of different PPTase-CP combinations

To investigate the potential of different PPTases to activate CPs, activity assays were performed with different enzyme combinations (Table S4).

Table S4. Screening of different PPTase-CP combinations.

PPTase	**CP**	**result shown in**
Sfp	AntG, AcpP or JadC	Figure S5
KirP	AntG, AcpP or JadC	Figure S6
BAC69459	AntG, AcpP or JadC	Figure S7
CCA59476	AntG, AcpP or JadC	Figure S8
EntD	AntG, AcpP or JadC	Figure S9
AGI92022	AntG, AcpP or JadC	Figure S10
AGS72899	AntG, AcpP or JadC	Figure S11
EFG07676	AntG, AcpP or JadC	Figure S12
WP_012791066	AntG, AcpP or JadC	Figure S13
WP_012793840	AntG, AcpP or JadC	Figure S14

Activity assays were conducted in a total volume of 1 mL containing the following components: HSCoA analog **5** (1 mM), ATP (9 mM), purified PPTase (1 µM), and purified CP (250 µM) in a reaction buffer composed of HEPES (50 mM) and $MgCl_2$ (10 mM) at pH 7.9.[4] The reactions were incubated at 30 °C for 30 min. To each reaction, compound **8** (5 µL, 50 mg/mL in DMSO), $CuSO_4$ (1 mg in 10 µL reaction buffer) and sodium ascorbate (1 mg in 10 µL reaction buffer) were added, followed by incubation at 30 °C for another 30 min. All samples were analysed by SDS-PAGE (Figures S5 – S14). Attachment of the HSCoA analog was also demonstrated by MALDI-ToF (Figures S15 – S17).

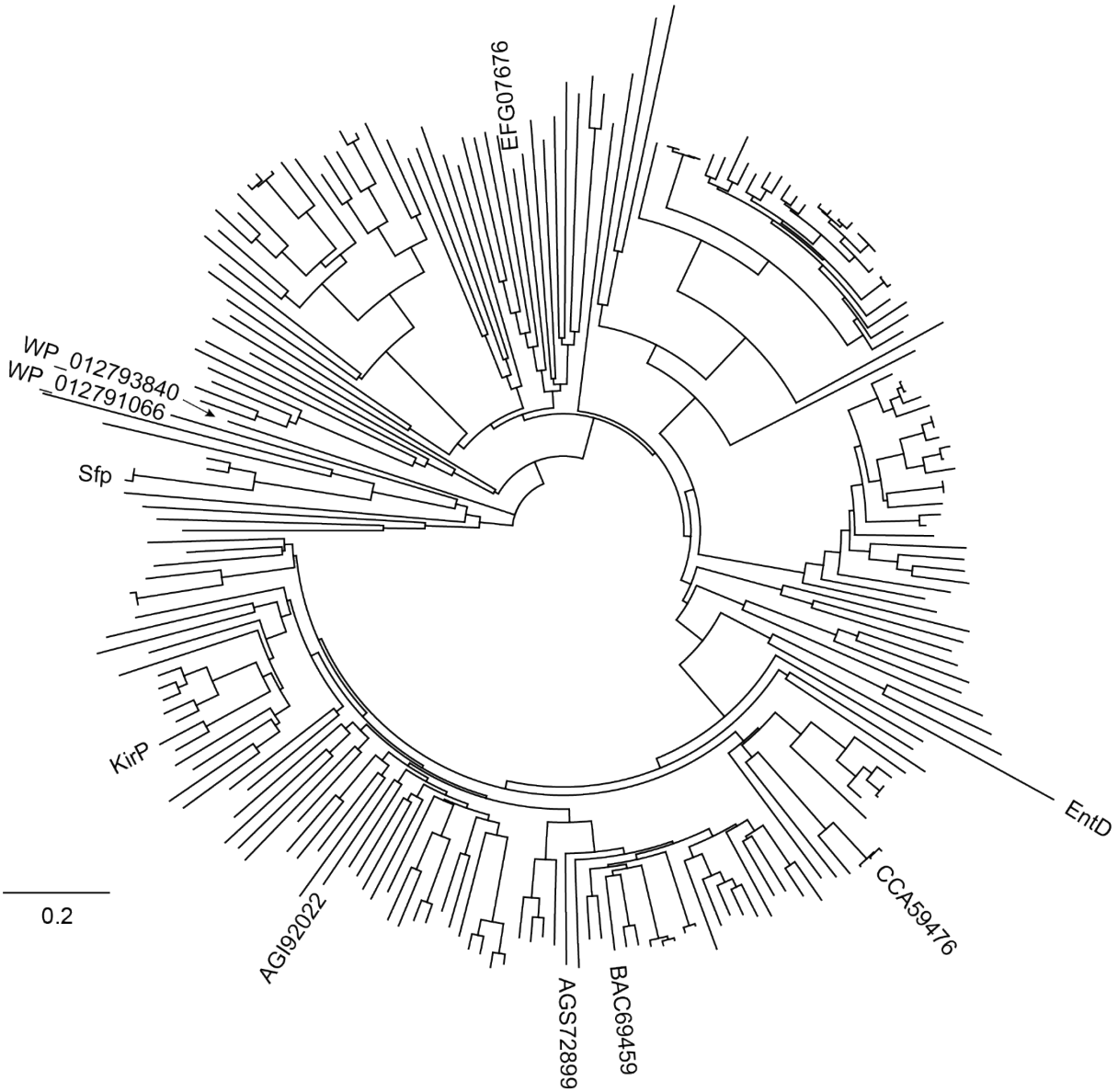

Figure S4. Phylogenetic tree constructed from the amino acid sequences of 219 PPTase homologs. For enzymes that were investigated in this study protein names or accession numbers are given at the corresponding tips. The scale bar indicates substitutions per site.

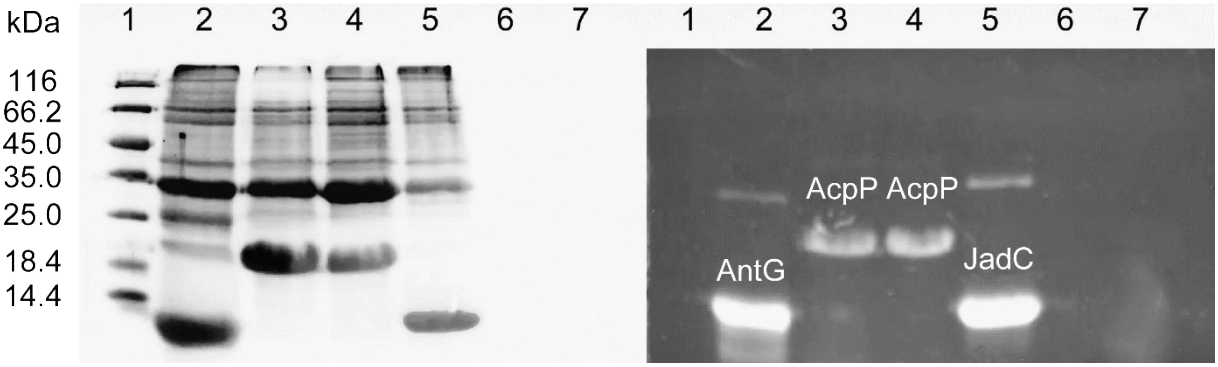

Figure S5. Lane 1: Protein marker; lane 2: Sfp + AntG + **5** + **8** after CuAAC; lanes 3 and 4: Sfp + AcpP + **5** + **8** after CuAAC; lane 5: Sfp + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

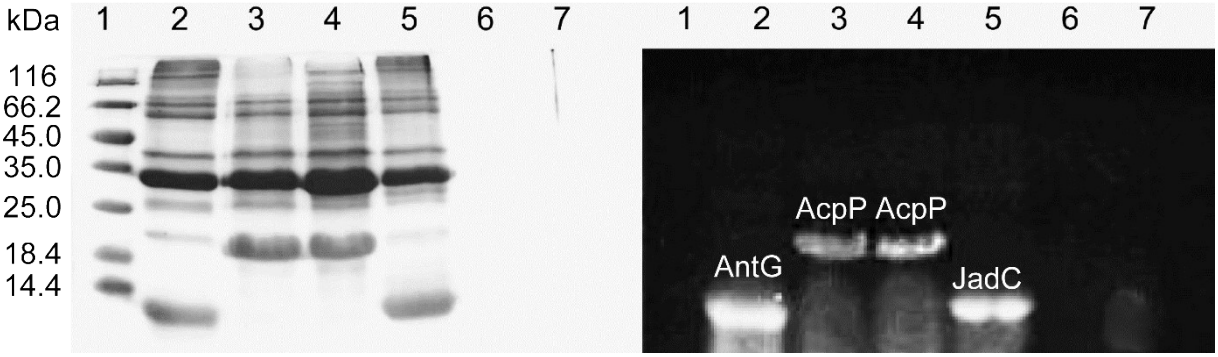

Figure S6. Lane 1: Protein marker; lane 2: KirP + AntG + **5** + **8** after CuAAC; lanes 3 and 4: KirP + AcpP + **5** + **8** after CuAAC; lane 5: KirP + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

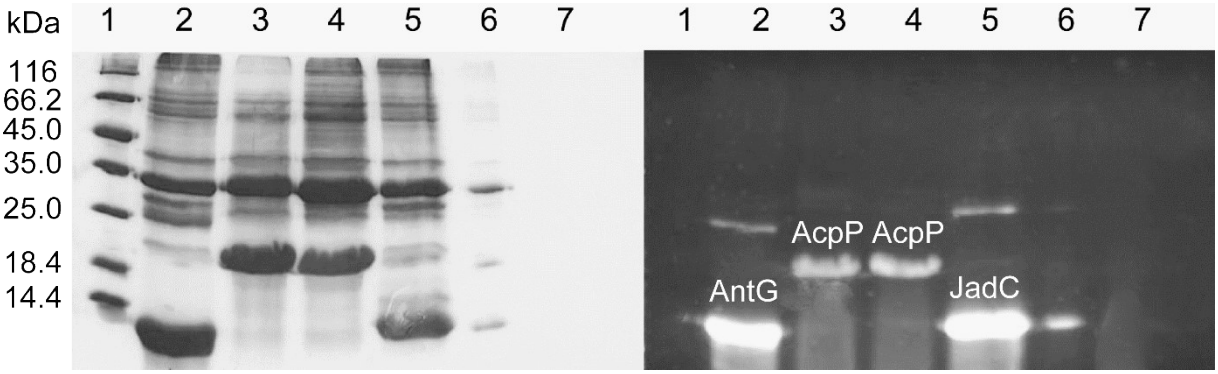

Figure S7. Lane 1: Protein marker; lane 2: BAC69459 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: BAC69459 + AcpP + **5** + **8** after CuAAC; lane 5: BAC69459 + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

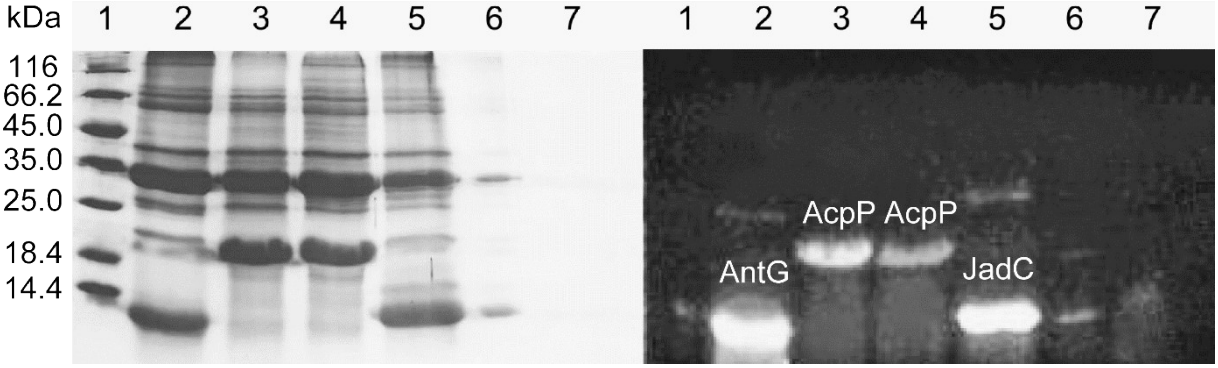

Figure S8. Lane 1: Protein marker; lane 2: CCA59476 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: CCA59476 + AcpP + **5** + **8** after CuAAC; lane 5: CCA59476 + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

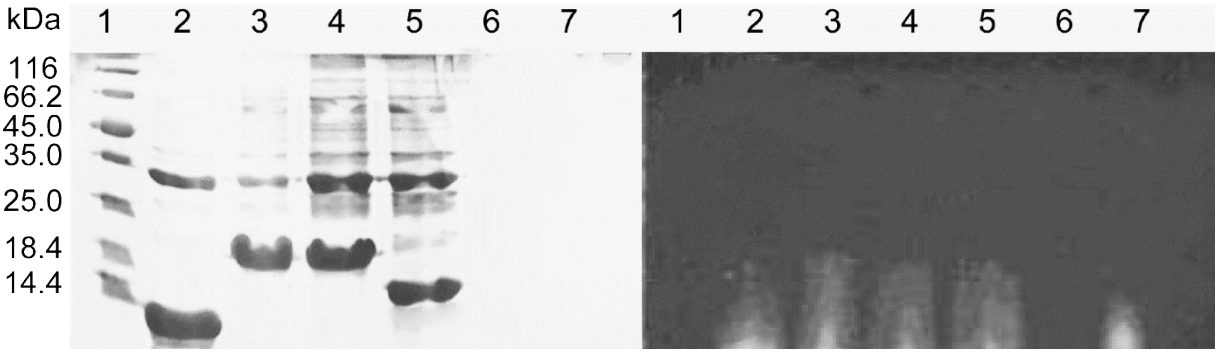

Figure S9. Lane 1: Protein marker; lane 2: EntD + AntG + **5** + **8** after CuAAC; lanes 3 and 4: EntD + AcpP + **5** + **8** after CuAAC; lane 5: EntD + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

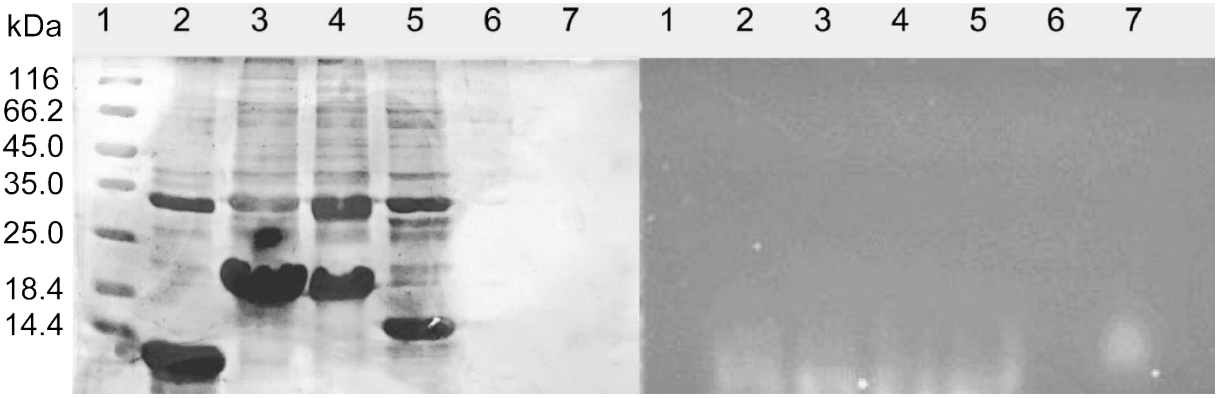

Figure S10. Lane 1: Protein marker; lane 2: AGI92022 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: AGI92022 + AcpP + **5** + **8** after CuAAC; lane 5: AGI92022 + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

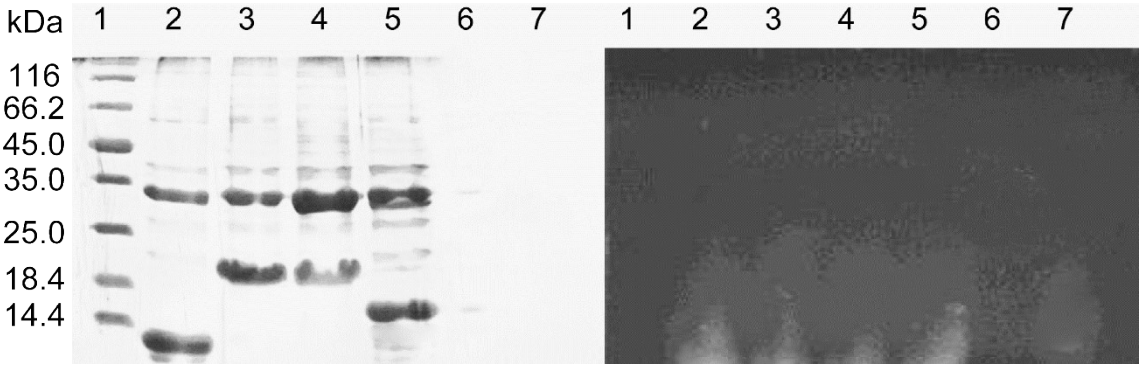

Figure S11. Lane 1: Protein marker; lane 2: AGS72899 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: AGS72899 + AcpP + **5** + **8** after CuAAC; lane 5: AGS72899 + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

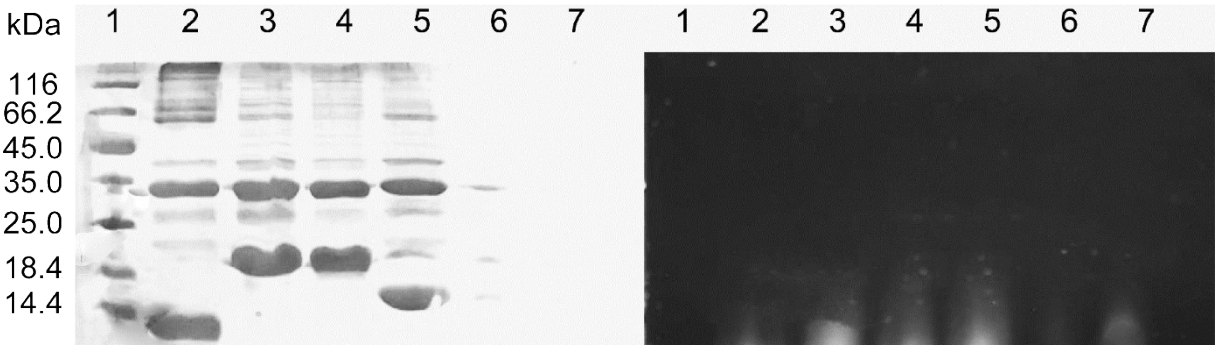

Figure S12. Lane 1: Protein marker; lane 2: EFG07676 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: EFG07676 + AcpP + **5** + **8** after CuAAC; lane 5: EFG07676 + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

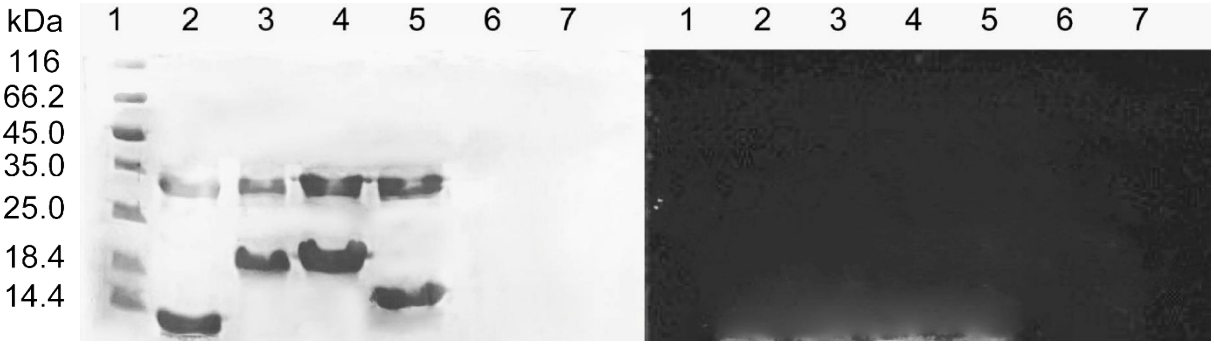

Figure S13. Lane 1: Protein marker; lane 2: WP_012791066 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: WP_012791066 + AcpP + **5** + **8** after CuAAC; lane 5: WP_012791066 + JadC + **5** + **8** after CuAAC; lane 6: **8**; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

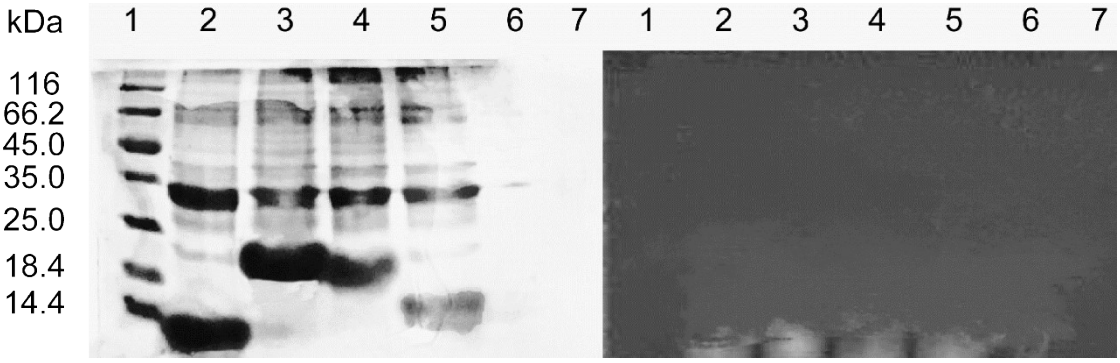

Figure S14. Lane 1: Protein marker; lane 2: WP_012793840 + AntG + **5** + **8** after CuAAC; lanes 3 and 4: WP_012793840 + AcpP + **5** + **8** after CuAAC; lane 5: WP_012793840 + JadC + **5** + **8** after CuAAC; lane 6: **8**. ; lane 7: **9**. AcpP from *E. coli* (MW 8,640 Da) is known to run with an apparent MW of ~18,000 Da.[5]

Determination of CP activation by MALDI-ToF MS

Activity assays were conducted using the same conditions as outlined above for all CP-PPTase combinations in this study that were successful according to the fluorescence assay. Subsequently, the incubation buffer of the reaction mixture was exchanged through repeated centrifugation using an ultrafiltration centrifugal tube (3 kDa cut-off), followed by the addition of NH_4OAc buffer (100 mM). Through this method, 5 sequential 10-fold dilutions were achieved. The protein solution was analysed using a MALDI ultrafleXtreme ToF/ToF (Bruker Daltonik, Bremen).

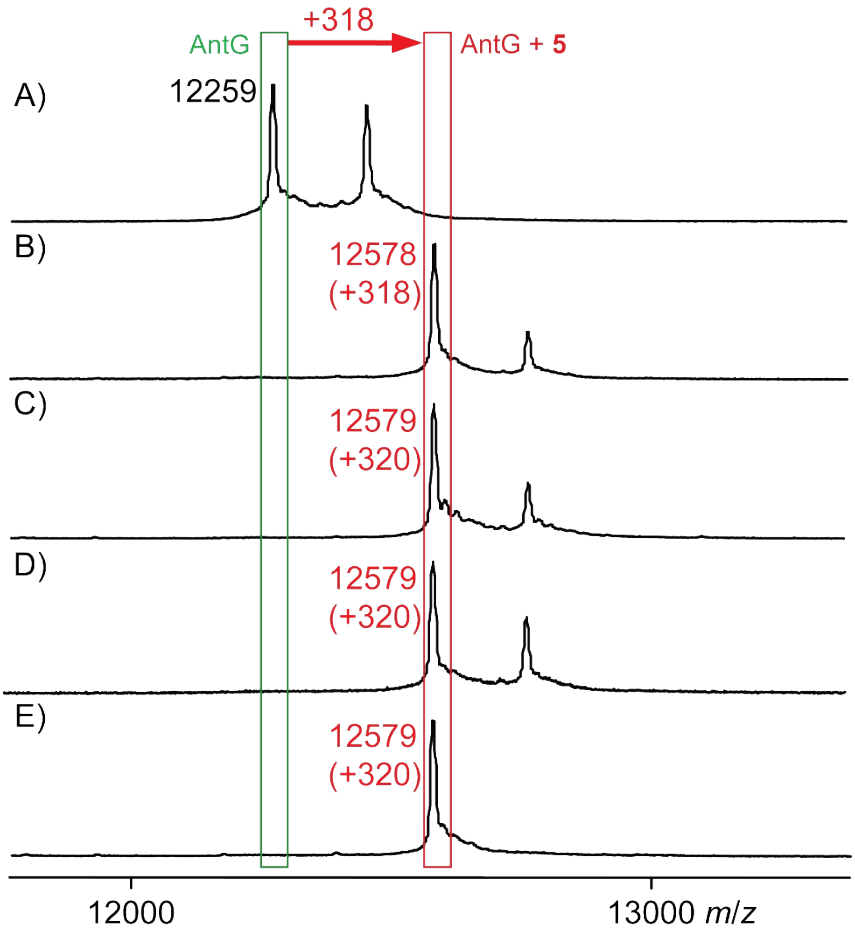

Figure S15. Determination of the activation of AntG by PPTases by mass spectrometry (MALDI-ToF). A) purified AntG (calculated for $[M–Met]^+$ *m*/*z* 12254, found *m*/*z* 12259; B) purified Sfp + AntG + **5**; C) purified KirP + AntG + **5**; D) purified BAC69459 + AntG + **5**; E) purified CCA59476 + AntG + **5**. The red arrow indicates the expected mass increase after attachment of the HSCoA analog **5** (+318 Da), the red numbers in brackets next to each mass peak are the observed mass increases.

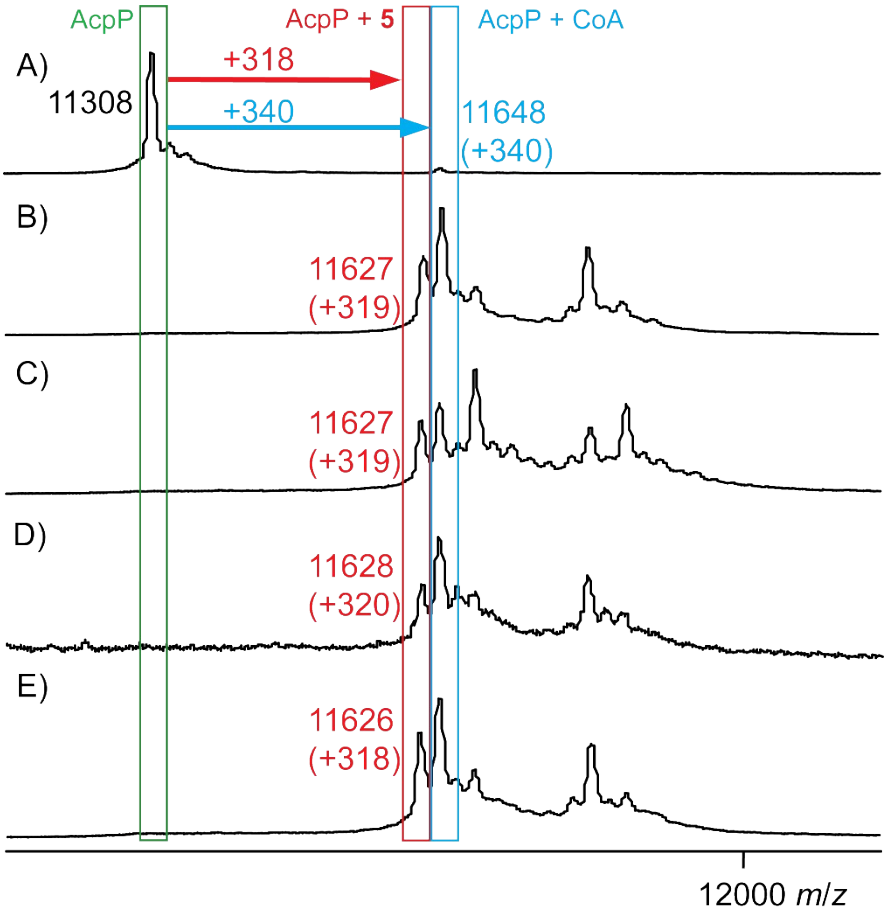

Figure S16. Determination of the activation of AcpP by PPTases by mass spectrometry (MALDI-ToF). A) purified AcpP (calculated for $[M–Met]^+$ *m*/*z* 11309, found *m*/*z* 11308; B) purified Sfp + AcpP + **5**; C) purified KirP + AcpP + **5**; D) purified BAC69459 + AcpP + **5**; E) purified CCA59476 + AcpP + **5**. The red arrow indicates the expected mass increase after attachment of the HSCoA analog **5** (+318 Da), the red numbers in brackets next to each mass peak are the observed mass increases. The blue arrow indicates the expected mass increase after attachment of HSCoA that supposedly happens intracellularly during protein expression in *E. coli* by ACPS.

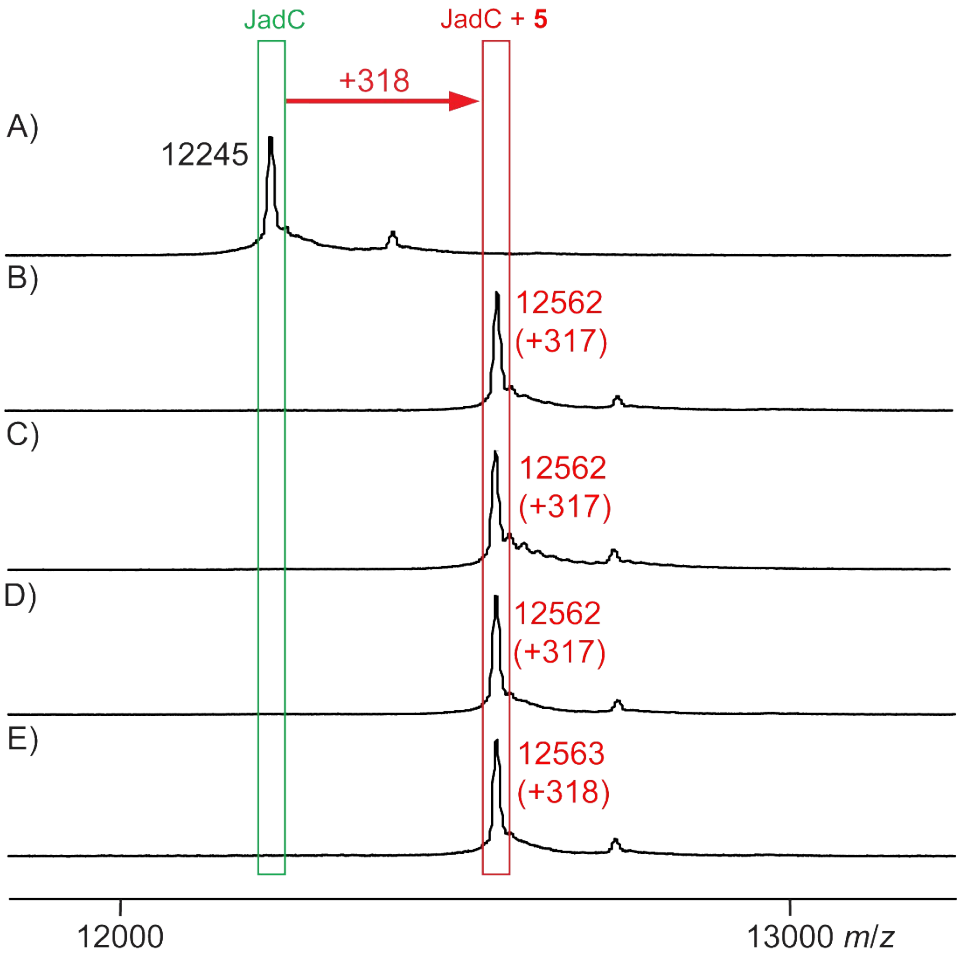

Figure S17. Determination of the activation of JadC by PPTases by mass spectrometry (MALDI-ToF). A) purified JadC (calculated for $[M–Met]^+$ *m*/*z* 12250, found *m*/*z* 12245; B) purified Sfp + JadC + **5**; C) purified KirP + JadC + **5**; D) purified BAC69459 + JadC + **5**; E) purified CCA59476 + JadC + **5**. The red arrow indicates the expected mass increase after attachment of the HSCoA analog **5** (+318 Da), the red numbers in brackets next to each mass peak are the observed mass increases.

Synthetic procedures

Synthesis of 2[6]

Propargylamine (220 mg, 4.00 mmol), pantothenate (876 mg, 4.00 mmol), EDAC (1.53 g, 8.00 mmol), and HOBt (1.53 g, 10.0 mmol) were combined and dissolved in dry DMF (10 mL). DIPEA (1.03 g, 8.00 mmol) was added, and the reaction was stirred overnight at room temperature. The solvent was removed under reduced pressure, and the resultant oil was purified by column chromatography (EtOAc to MeOH/EtOAc = 1/10) to yield **2** (820 mg, 3.20 mmol, 80%) as a yellow oil.

Compound 2. ^{1}H NMR (500 MHz, MeOD): δ_H 3.94 (d, $^{4}J_{H,H}$ = 2.6 Hz, 2H), 3.88 (s, 1H), 3.51 (ddd, $^{2}J_{H,H}$ = 13.4 Hz, $^{3}J_{H,H}$ = 6.6 Hz, $^{3}J_{H,H}$ = 6.6 Hz, 1H), 3.45 (ddd, $^{2}J_{H,H}$ = 13.5 Hz, $^{3}J_{H,H}$ = 6.7 Hz, $^{3}J_{H,H}$ = 6.7 Hz, 1H), 3.46 (d, $^{2}J_{H,H}$ = 11.0 Hz, 1H), 3.39 (d, $^{2}J_{H,H}$ = 11.0 Hz, 1H), 2.58 (t, $^{4}J_{H,H}$ = 2.6 Hz, 1H), 2.44 (t, $^{3}J_{H,H}$ = 6.6 Hz, 2H), 0.92 (s, 6H) ppm (Figure S18); ^{13}C NMR (126 MHz, MeOD): δ_C 176.1 (Cq), 173.4 (C_q), 80.5 (C_q), 77.3 (CH), 72.2 (CH), 70.4 (CH_2), 40.4 (C_q), 36.2 (CH_2), 36.2 (CH_2), 29.4 (CH_2), 21.3 (CH_3), 20.9 (CH_3) ppm (Figure S19). HRMS (ESI): $[M+H]^+$ calculated for $C_{12}H_{21}N_2O_4^+$ *m*/*z* 257.1496; found *m*/*z* 257.1498.

Synthesis of 5

To a 50 mL final assay volume, the following components were added in sequence: Substrate **2** (50 mg) was dissolved in reaction buffer (15 mL, 50 mM HEPES, 25 mM KCl, 10 mM $MgCl_2$, pH 7.5), ATP solution (7.5 mL, 60 mM ATP in reaction buffer, pH 7.5), a solution of the enzymes (CoaA, CoaD, and CoaE) (5 mL, 10 mg/mL in 50 mM HEPES, 25 mM NaCl, 10 mM $MgCl_2$, pH 8.0)) were added at 30 min intervals, and the reaction was left at 30 °C for 16 h.[7] The reaction was quenched by the addition of an equal volume of MeCN + 0.2% formic acid. The mixture was vortexed and centrifuged (14600 x g, 10 min, 4 °C). The supernatant was dried by lyophilisation and the residue was purified by HPLC to obtain **5** (145 mg, 0.20 mmol, 100%) as a colourless powder.

Compound 5. ^{1}H NMR (700 MHz, D_2O) δ 8.62 (s, 1H), 8.40 (br s, 1H), 6.18 (d, $^{3}J_{H,H}$ = 5.9 Hz, 1H), 4.91–4.84 (m, 2H), 4.58 (br s, 1H), 4.29–4.22 (m, 2H), 3.99 (s, 1H), 3.87 (t, $^{4}J_{H,H}$ = 2.5 Hz, 2H), 3.86–3.84 (m, 1H), 3.61 (dd, $^{3}J_{H,H}$ = 9.7 Hz,

$^{3}J_{H,H}$ = 2.5 Hz, 1H), 3.45 (t, $^{3}J_{H,H}$ = 6.5 Hz, 2H), 2.53 (t, $^{4}J_{H,H}$ = 2.5 Hz, 1H), 2.44 (t, $^{3}J_{H,H}$ = 6.5 Hz, 2H), 0.91 (s, 3H), 0.80 (s, 3H) (Figure S20). ^{13}C NMR (176 MHz, D_2O) δ 174.70 (C_q), 173.68 (C_q), 149.78 (C_q), 148.41 (C_q), 144.64 (CH), 142.40 (CH), 118.46 (C_q), 87.41 (CH), 83.3 (dd, $^{3}J_{C,P}$ = 2.5 Hz, $^{3}J_{C,P}$ = 3.8 Hz, CH), 79.37 (CH), 74.2(d, $^{2}J_{C,P}$ = 5.0 Hz, CH) 74.10 (CH), 73.80 (d, $^{2}J_{C,P}$ = 4.8 Hz, CH), 72.1 (dd, $^{2}J_{C,P}$ = 5.9 Hz, CH), 71.75 (CH), 65.1 (d, $^{2}J_{C,P}$ = 4.8 Hz, CH), 38.30 ((d, $^{3}J_{C,P}$ = 8.1 Hz, C_q), 35.26 (CH_2), 35.11 (CH_2), 28.70 (CH_2), 20.76 (CH_3), 18.22 (CH_3) (Figure S21).[8] HRMS (ESI): $[M-H]^-$ calculated for $C_{22}H_{33}N_7O_{16}P_3^-$ *m*/*z* 744.1202; found *m*/*z* 744.1204.

Synthesis of 8

To a mixture of 3-azidopropan-1-amine (20 mg, 0.20 mmol) and Et_3N (49 mg, 0.48 mmol) in CH_2Cl_2 (2 mL) was added 5-(dimethylamino)naphthalene-1-sulfonyl chloride (64 mg, 0.24 mmol) dropwise at 0 °C under Ar. Stirring was continued for 6 h. The mixture was poured onto H_2O (10 mL). The aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic layers were dried with $MgSO_4$ and concentrated to dryness. The residue was purified through silica gel column chromatography (petrol ether/EtOAc, 10:1) to afford *N*-(3-azidopropyl)-5-(dimethylamino)naphthalene-1-sulfonamide (**8**) as a yellow oil (61 mg, 0.18 mmol, 91%).

Compound 8. ^{1}H NMR (700 MHz, C_6D_6): δ_H 8.62 (br d, $^{3}J_{H,H}$ = 8.6 Hz, 1H), 8.42 (br d, $^{3}J_{H,H}$ = 8.6 Hz, 1H), 8.31 (dd, $^{3}J_{H,H}$ = 7.3 Hz, $^{4}J_{H,H}$ = 1.2 Hz, 1H), 7.40 (dd, $^{3}J_{H,H}$ = 8.5 Hz, $^{3}J_{H,H}$ = 7.6 Hz, 1H), 7.09 (dd, $^{3}J_{H,H}$ = 8.5 Hz, $^{3}J_{H,H}$ = 7.3 Hz, 1H), 6.86 (d, $^{3}J_{H,H}$ = 7.6 Hz, 1H), 4.41 (t, $^{3}J_{H,H}$ = 6.3 Hz, NH), 2.49 (s, 6H), 2.47 (td, $^{3}J_{H,H}$ = 6.6 Hz, $^{3}J_{H,H}$ = 6.6 Hz, 2H), 2.41 (t, $^{3}J_{H,H}$ = 6.6 Hz, 2H), 0.94 (tt, $^{3}J_{H,H}$ = 6.6 Hz, $^{3}J_{H,H}$ = 6.6 Hz, 2H) ppm (Figure S22);^{13}C NMR (176 MHz, C_6D_6): δ_C 152.3 (C_q), 136.1 (C_q), 130.5 (C_q), 130.4 (CH), 130.3 (CH), 130.0 (C_q), 128.4 (CH), 123.5 (CH), 119.8 (CH), 115.6 (CH), 48.4 (CH_2), 45.1 (2xCH_3), 40.6 (CH_2), 28.8 (CH_2) ppm (Figure S23). HRMS (ESI): $[M+H]^+$ calculated for $C_{15}H_{20}N_5O_2S^+$ *m*/*z* 334.1332; found *m*/*z* 334.1330.

Synthesis of fluorescent HSCoA analog (9).

To a mixture of the HSCoA analog **5** (20 mg, 0.03 mmol) in MeOH/H_2O (1:1, 3 mL) and *N*-(3-azidopropyl)-5-(dimethylamino)naphthalene-1-sulfonamide **8** (36 mg, 0.11 mmol) in CH_2Cl_2 (0.1 mL) was added $CuSO_4$ (1 mg, 0.01 mmol) and sodium ascorbate (1 mg, 0.01 mmol).[9] Stirring was continued for 1 h. The crude product was used for the enzyme reaction. HRMS (ESI): $[M-H]^-$ calculated for $C_{37}H_{52}N_{12}O_{18}P_3S^-$ *m*/*z* 1077.2462; found *m*/*z* 1077.2459.

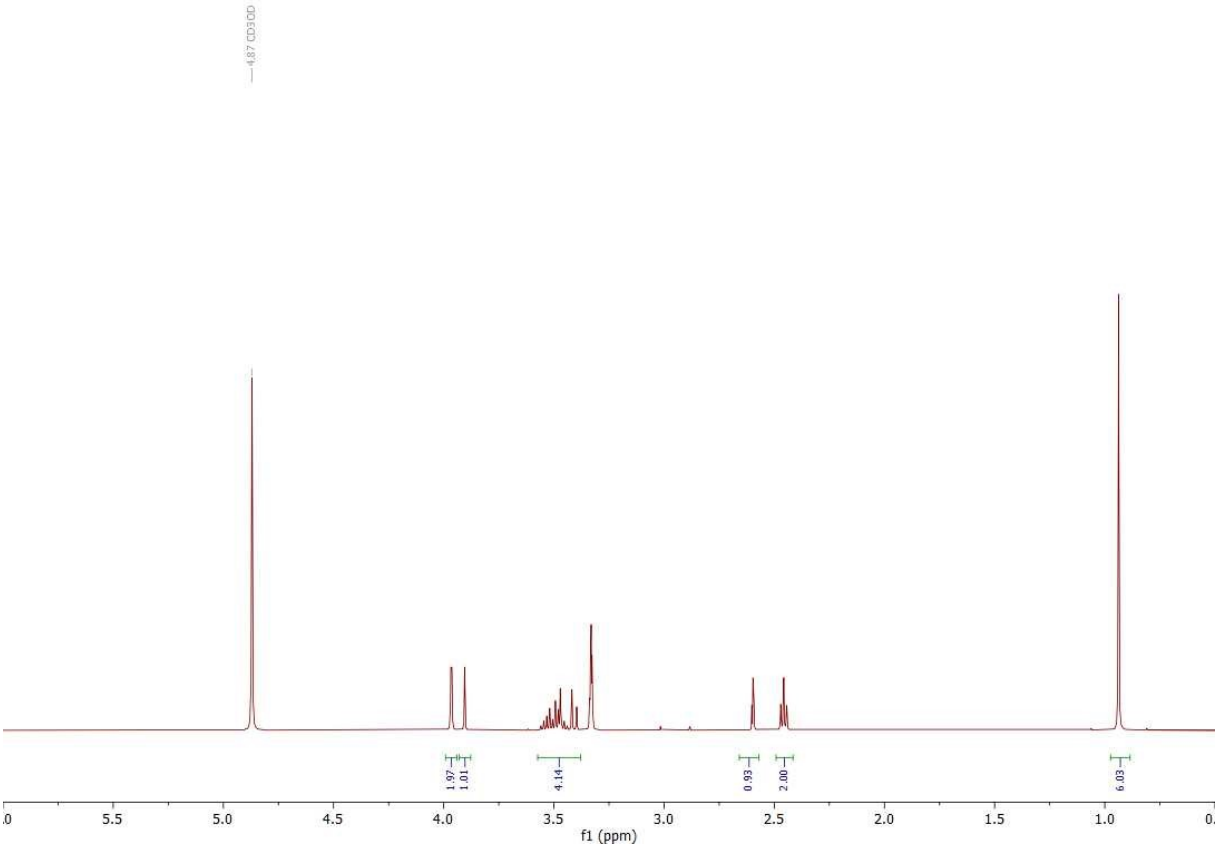

Figure S18. ^{1}H-NMR spectrum of **2** (500 MHz, MeOD).

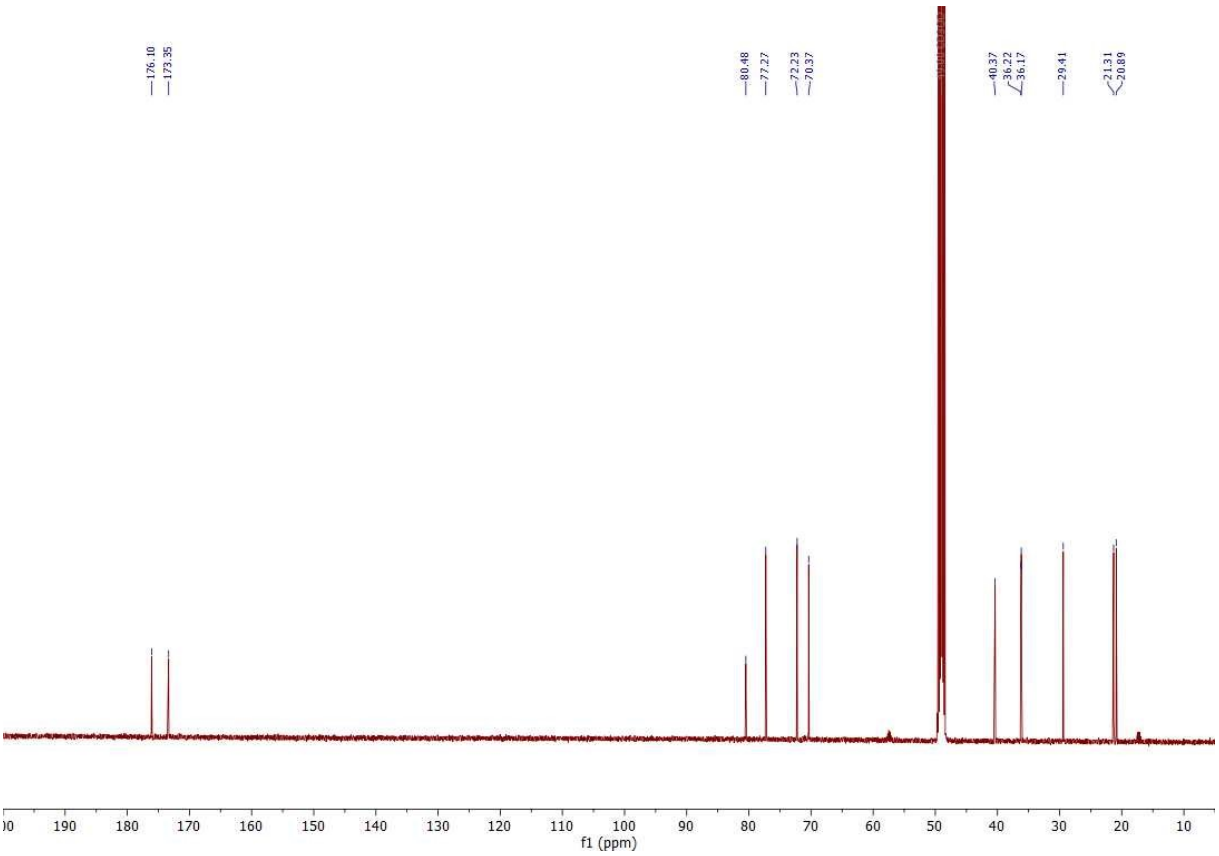

Figure S19. ^{13}C-NMR spectrum of **2** (126 MHz, MeOD).

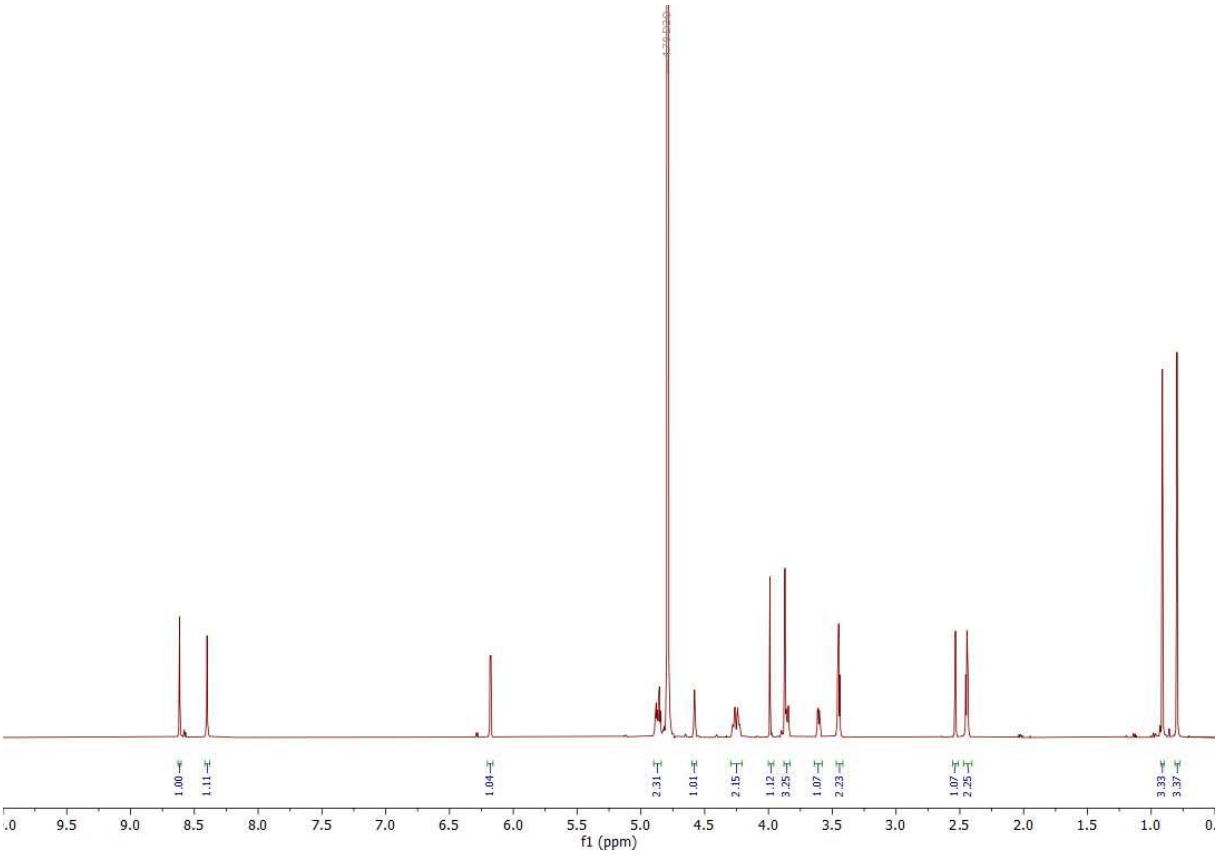

Figure S20. ^{1}H-NMR spectrum of **5** (700 MHz, D_2O).

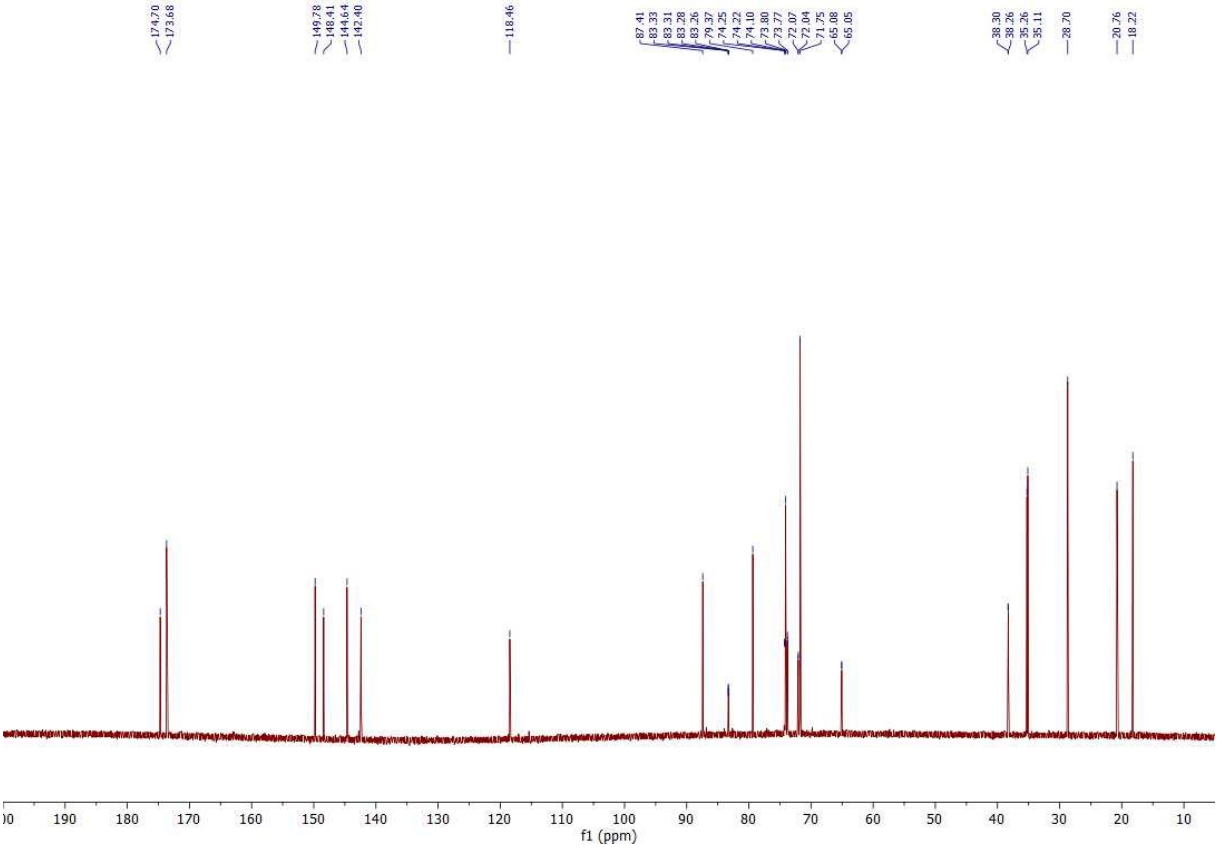

Figure S21. ^{13}C-NMR spectrum of **5** (176 MHz, D_2O).

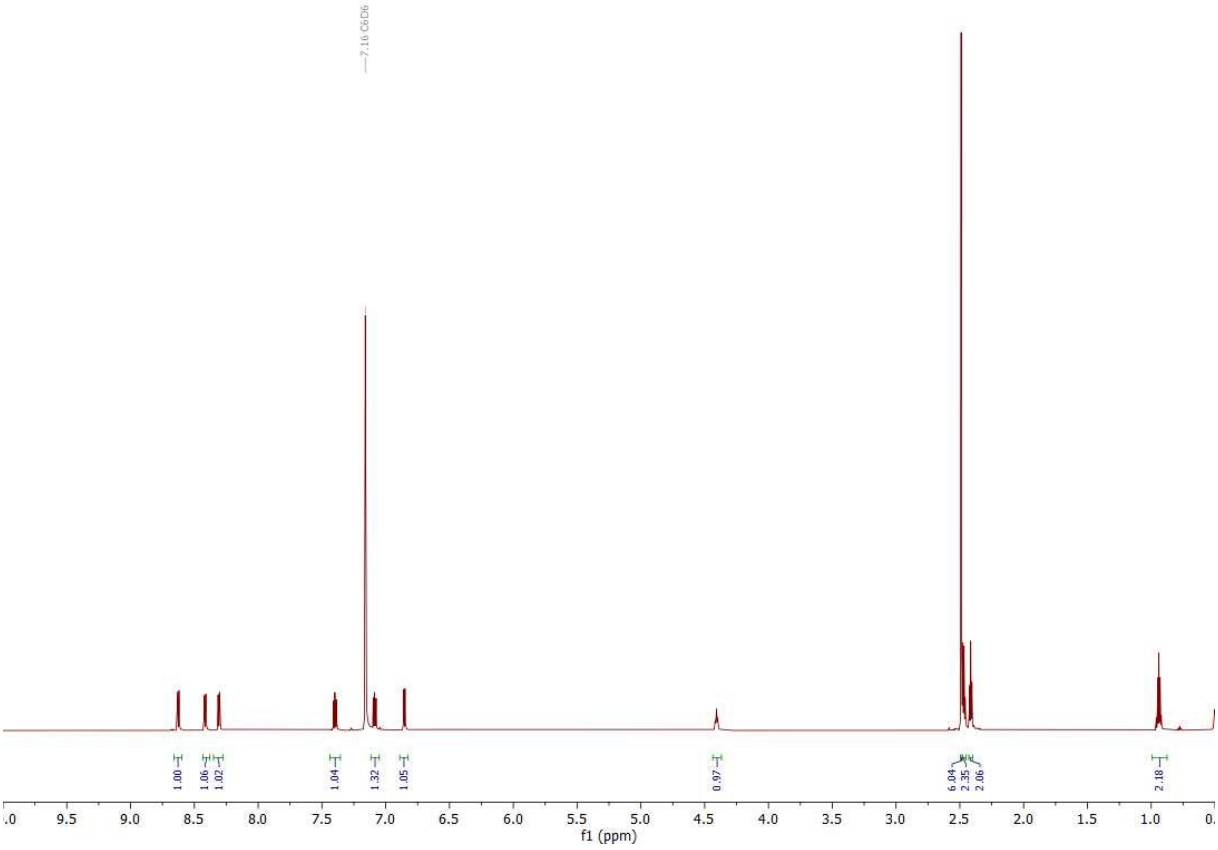

Figure S22. ^{1}H-NMR spectrum of **8** (700 MHz, C_6D_6).

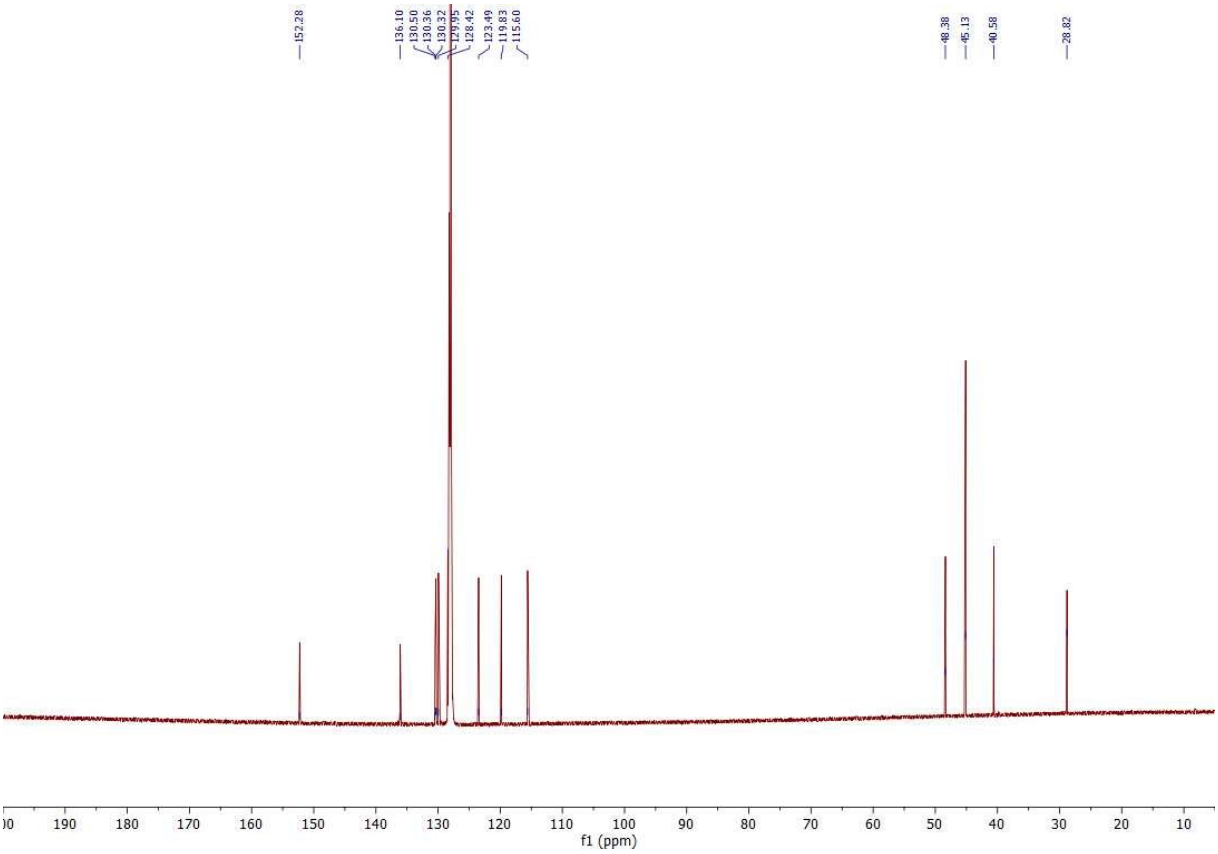

Figure S23. ^{13}C-NMR spectrum of **8** (176 MHz, C_6D_6).

References

1 J. S. Dickschat, K. A. K. Pahirulzaman, P. Rabe and T. A. Klapschinski, *ChemBioChem*, 2014, **15**, 810.

2 R. D. Giets, R. H. Schiestl, *Nat. Protoc.*, 2007, **2**, 31.

3 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.

4 V. Agarwal, S. Diethelm, L. Ray, N. Garg, T. Awakawa, P. C. Dorrestein and B. S. Moore, *Org. Lett.*, 2015, **17**, 4452.

5 J. P. Prince, J. R. Bolla, G. L. M. Fisher, J. Mäkelä, M. Fournier, C. V. Robinson, L. K. Arciszewska and D. J. Sherratt, *Nat. Commun.*, 2021, **12**, 6721.

6 J. L. Meier, A. C. Mercer, H. Rivera and M. D. Burkart, *J. Am. Chem. Soc.*, 2006, **128**, 12174.

7 R. B. Hamed, L. Henry, J. R. Gomez-Castellanos, A. Asghar, J. Brem, T. D. Claridge and C. J. Schofield, *Org. Biomol. Chem.*, 2013, **11**, 8191.

8 R. Sanichar and J. C. Vederas, *Org. Lett.*, 2017, **19**, 1950.

9 F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless and V. V. Fokin, *J. Am. Chem. Soc.*, 2005, **127**, 210.