Supporting Information

Proof-Reading Thioesterase Boosts Activity of Engineered Nonribosomal Peptide Synthetase

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Supporting Methods

Cloning

Most inserts and vectors were assembled using In-Fusion doning (Takara Bio) for transformation of E. coli Stellar cells. In-Fusion cloning was performed following the supplier’s instructions. Primers for PCR amplification of inserts (Table S1) were designed with 15-20 bp long overlaps complementary to the insertion position in the vector DNA and ordered as synthetic oligonucleotides (Eurofins GmbH). Competent cells were transformed by heat shock. All inserts were checked by sequencing (Genewiz). Restriction enzymes for linearization of vectors were purchased from New England Biolabs (NEB). PCR reactions were performed with Phusion or Q5 High-Fidelity polymerase (NEB).

Construction of pSU18(F)-grsBMtoL. The vector was prepared by linearization of plasmid pSU18-GrsB3 with XhoI and BamHI. The insert was prepared by digestion of pTrc99a-grsBMtol using the same set of enzymes followed by enzymatic ligation with the vector. pTrc99a-grsBMtol was kindly provided by Prof. Donald Hilvert (ETH Zurich).

Construction of pSU18(F)-grsTAB. The fragment containing genes grsT and grsA was amplified from A. migulaunus DSM2895 genomic DNA with primers grsTAB-EcoRBS-pSU18-f and grsTAB-EcoRBS-pSU18-r. The vector was prepared by linearizing pSU18_grsBmtoL with XhoI.

Construction of pSU18(F)-grsT and pSU18(F)-grsT(AUG). The gene grsT was amplified from pSU18(F)-grsTAB with the following two sets of primers: grsT-pSU18-F1 and grsT-pSU18-R1 for pSU18(F)-grsT; grsT-AUG-pSU18-F and grsT-pSU18-R1 for pSU18(F)-grsT(AUG). The vector was amplified from pSU18(F)-grsTAB with primers pSU18-F1, pSU18-R1 for both constructs. In pSU18(F)-grsT(AUG), the native start codon AUG of grsT is mutated to AUG.

Construction of pOPINM-grsT. The grsT gene was amplified from pSU18(F)-grsTAB with primers grsT-pOPINM-F1, and grsT-pOPINM-R1. The vector pOPINM (Addgene 26044) encoding an N-terminal MBP-tag was double digested with KpnI and HindIII.

Construction of pSU18(F)-MBP-grsT. The grsT gene was amplified from pOPINM-grsT with primers His6-MBP-grsT-pSU18-F, grsT-extra-pSU18-R. The vector was amplified from pSU18(F)-grsTAB with primers pSU18-extra-F1, pSU18-R1.

Construction of pSU18(F)-GST-grsT. The grsT gene was amplified from pOPINM-grsT with primers His6-GST-grsT-pSU18-F and grsT-extra-pSU18-R. The vector was amplified from pSU18(F)-grsTAB with primers pSU18-extra-F1, and pSU18-R1.

Construction of pSU18(F)-SUMO-grsT. The encoding the SUMO protein was amplified from plasmid hH3_1_A29C_H6Sumo (courtesy of M. Müller, Kings College London) with primers pSU18F-SUMO-F1 and pSU18F-SUMO-R2. The vector was amplified from pSU18(F)-GST-grsT with primers pSU18F-GST-F2 and pSU18-R1.

Construction of pSU18(F)-MBP-grsT-S95A. For the construction of grsT-S95A, the gene was amplified in two fragments with primers grsT/S95A-pSU18-P1-F1, grsT/S95A-pSU18-P1-R1, grsT/S95A-pSU18-P2-F1, and grsT/S95A-pSU18-P2-R1. The vector was linearized with KpnI and Pmel.

Construction of pTrc99a-His-GrsB12-Dc. Genes encoding docking domains from the Inx NRPS system of Xenorhabdus Innesi (DSM 16336) were doned from previously prepared plasmids. Sequences encoding the Dc domain and a His-tag were added in two steps. First, the sequence encoding the Dc domain from inxD was amplified using primers GsB12-Dc-F1 and His-GsB12-Dc-R1 with pSU18-Dn-GsB2-P (DcΔ2) as template. The vector was prepared by digestion of pTrc99a-GsBMtol with EcoRI and SalI. Assembly yielded plasmid pTrc99a-GsB12-Dc-2. Second, a sequence encoding a His-tag was added at the N-terminal of grsB12. For this purpose, two synthetic inserts were prepared from oligos His-pOPINM-P1-F, and His-pOPINM-P1-R for insert 1, and His-pOPINM-P2-F, and His-pOPINM-P2-R for insert 2. Double stranded DNA was prepared by denaturation at 95 °C for 5 min and then annealing at 4 °C for several hours in DNA assembly buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4). In-Fusion doning of the two inserts into vector pTrc99a-GsB12-Dc-linearized with Xhol and NruI yielded pTrc99a-His-GsB12-Dc.

Construction of pTrc99a-Dn-GrsB34. The sequence encoding the Dn domain was amplified using DN-GsB34-F2 and DN-GsB34-R1 as primers and pSU18_Bx-DnGrsB34-Zif268 as template and assembled with plasmid pTrc99a-GsBMtol linearized with Xhol and EcoNI. The linearized fragment was further digested with NruI to yield pTrc99a-Dn-GsB34.

Construction of pSU18(F)-grsT(S95A)AB. For the construction of pSU18(F)-grsT(S95A)AB, the gene was amplified in two fragments with primers grsT(S95A)AB-Ins1_F1, grsT(S95A)AB-Ins1_R1 using pSU18(F)-MBP-grsT-S95A as template and primers grsT(S95A)AB-Ins2_F1 and grsT(S95A)AB-Ins2_R1 using pSU18(F)-grsTAB as template. The vector, pSU18(F)-grsTAB, was linearized with Aval and BsaHI.

Construction of pSU18(F)-grsTAB12-B34. For the construction of pSU18(F)-grsTAB12-B34, two fragments were amplified with primers GrsTAB-Dc-F1 and GrsTAB-Dc-R1 using pTrc99a-His-GsB12-Dc as template and primers GrsTAB-Dn-F1 and GrsTAB-Dn-R1 using pTrc99a-GsB34-Dn as template. The vector, pSU18(F)-grsTAB, was linearized with Aval and BsaHI.
Construction of pSU18(F)-grsT(95A)AB12-B34. The fragment encoding the split site in GrsB was amplified from the plasmid pSU18(F)-grsTAB12-B34 with primers GrsTAB-DC-F1 and GrsTAB-DN-R1. The vector pSU18-grsT(95A)AB was linearized with Aval and BsaHI.

Protein Expression and Purification

**Strains.** The *E. coli* strain HM0079 (GT869 nrdD::sfp Spcr)^4 was used for protein production if not stated otherwise. The strain carries a genomically integrated *sfp* gene encoding an unspecific phosphopantetheine transferase. For production of TEIIs, *E. coli* BL21(DE3) from New England Bioslabs was used.

**Purification protocol.** For heterologous expression of proteins in *E. coli*, a preculture was prepared by inoculating a single colony of the desired strain in 3 mL LB medium supplemented with the appropriate antibiotic followed by overnight incubation at 37 °C with 230 rpm shaking. Of the preculture, 500 µL was used to inoculate 400 mL of liquid 2YT medium containing appropriate antibiotics in a 2 L Erlenmeyer flask. The main culture was incubated at 37 °C, 230 rpm until the OD600 reached 1.0. At this point, the temperature was reduced to 18 °C and after 30 min further incubation, a final concentration of 250 µM isopropyl-β-D-thiogalactopyranosid (IPTG, Thermo Fisher Scientific) was added followed by incubation overnight at 18 °C, 230 rpm. Cells were harvested by centrifugation at 6,000 x g for 10 min at 6 °C. The cell pellet was resuspended in 25 mL of low imidazole buffer (50 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 2 mM TCEP, pH 7.4) followed by addition of 100 µL of protease inhibition cocktail (P8849, Sigma-Aldrich). Protease inhibition cocktail was not added to TEII proteins. The cell suspension was sonicated on ice for 5 min at an amplitude of 40% in 2 s intervals with 3 s breaks with a UP200ST ultrasonic processor (Hielcher). The cell lysate was centrifuged at 19,000 x g for 30 min at 4 °C. The supernatant from the cell lysate was added to an Econo column (Bio-Rad) loaded with 2 mL of Ni-IDA beads (Rotigrase-His/Ni Beads, Carl Roth) and equilibrated with low imidazole buffer. The desired His-tagged protein was eluted in 4 fractions of 750 µL of high imidazole buffer (50 mM Tris-HCl, 0.5 M NaCl, 300 mM imidazole, 2 mM TCEP, pH 7.4) and samples from the last 3 elution steps were pooled and collected. Eluted protein was then buffer exchanged into protein storage buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5) by centrifugation at 4,000 x g and 4 ºC in Vivaspin filters (Sartorius) with a molecular weight cut-off threefold smaller than the size of the protein. The protein concentration was calculated from the OD280 measured using a Take3 Plate on an Epoch2 microplate spectrophotometer (BioTek Instruments) with calculated extinction coefficients (Benchling). For storage, 5% glycerol (Carl Roth) was added to the protein samples which were flash frozen in liquid nitrogen and stored at -80 ºC until use. Protein purity was analysed by SDS-PAGE using Bolt 4-12% Bis-Tris Plus Gels (Fisher Scientific) with MES-SDS running buffer. Sample was loaded in Bolt LDS sample buffer supplied with Bolt reducing agent. Triple Color Protein Standard II (Serva) was run alongside the protein samples as a size standard. The gels were run at 200 V for 22 min and stained with Quick Coomassie stain (Serva).

**Michaelis-Menten Kinetics**

Hydrolytic activity towards acyl-CoA substrates was measured by quantifying the product CoA. The assay was performed with 2.5 mM of Ni-IDA purified TEII at a range of substrate concentrations in acyl-CoA hydrolysis assay buffer (50 mM HEPES, 5 mM MgCl₂, 25 mM NaCl, pH 7.5, 1 mM tris[2-carboxyethyl] phosphine hydrochloride [TCEP-HCl, Iris Biotech]). Substrates (acetyl-CoA trilithium salt [AppliChem], n-propionyl CoA lithium salt [Sigma], n-butanoyl CoA lithium salt [Fisher], L-phenylalanine CoA [synthesised in our lab]) dissolved in ddH₂O were added to start the reaction, which was incubated at 25 °C. Aliquots were taken at 0, 20, 40, and 60 min and quenched by adjusting to 5% trichloroacetic acid (TCA, Roth). Reaction mixtures were then centrifuged at 20,000 x g for 5 min and the supernatant was collected for quantification of CoA by liquid chromatography-tandem mass-spectrometry (UPLC-MS/MS). The enzymatic rate was calculated by performing a linear fit to the CoA concentration over time and subtracting the background hydrolysis rate which was measured by conducting the hydrolysis assay in the absence of enzyme. The Michaelis-Menten parameters were determined using an R script (Table S4). Only *kcat/Km* values are reported because substrate saturation was not reached in most cases and the individual rate constants are extrapolated (Figure S6). All measurements were done with biological and technical duplicates. The error on *kcat/Km* was calculated by error propagation from the error of fit of the individual rate constants using the following standard equation:

\[
\Delta \left( \frac{k_{cat}}{K_m} \right) = \frac{k_{cat}}{K_m} \sqrt{\left( \frac{\Delta k_{cat}}{k_{cat}} \right)^2 + \left( \frac{\Delta K_m}{K_m} \right)^2}
\]

**Assays with GrsA-H753A**

A plasmid encoding for GrsA-H753A with inactivated E-domain was previously constructed by introducing the His753Ala mutation.⁵ Diketopiperazine (DKP) formation with the knockout variant GrsA-H753A was performed with 5mM ATP, 1 mM TCEP, 1.5 µM GrsB1 and 150 nM GrsA-H753A diluted in protein storage buffer with 0.1 mg mL⁻¹ bovine serum albumin (BSA) in 1× DKP assay buffer (50 mM HEPES, 10 mM MgCl₂, 75 mM NaCl, pH 7.5). A concentration of GrsA-H753A was chosen where the enzyme is limiting for the overall rate of peptide formation (Figure S7). As substrates, 0.5 mM L-Phe (Carl Roth), 0.5 mM of D-Phe (Acros) and 0.3 mM of L-Pro (Carl Roth) were added. Samples were prepared in duplicates. To one set of samples, 3 µM MBP-GrsT was added and to the other set the equivalent amount of 1× DKP assay buffer. Samples were incubated at 37 °C and aliquots were collected at 5 min intervals. Aliquots
were quenched by heat shock at 95 °C for 2 min and the denatured proteins were pelleted by centrifugation at 20,000 x g for 5 min. Supernatants were collected and the l-Phe-l-Pro-DKP (FP-DKP) and d-Phe-l-Pro-DKP (DP-DKP) formation was quantified by UPLC-MS/MS.

Assays with sdVGrsA-STAP

The DKP formation assay was performed in 1 x DKP assay buffer with 2.5 µM sdVGrsA-STAP, 2.5 µM GrsB1, 5 mM ATP, 1 mM TCEP, and 1 mM l-Val (Carl Roth). The reaction was started by addition of 0.2 mM l-Phe (Carl Roth) and 0.2 mM l-Pro (Carl Roth). Reactions were prepared with two replicates. To one set of reactions, 5 µM MBP-GrsT was added and 5 µM MBP-GrsT-S95A, as the negative control, was added to the second set. Separate aliquots were prepared for each replicate and time point (0, 15, 30, 60, 120, 240, and 330 min). Samples were incubated at 33 °C. The enzymes were quenched by heat shock at 95 °C for 3 min and then centrifuged at 20,000 x g for 5 min. Supernatants were collected and the Phe-l-Pro-DKP and Val-l-Pro-DKP formation was measured and quantified using UPLC-MS/MS.

Large-Scale Purification of Gramicidin S

The wild-type producer of GS, A. migulanus DSM 5759 was streaked out on a LB agar plate. The plate was incubated overnight at 42 °C. A pre-culture was made by inoculating a single colony in LB followed by overnight incubation at 42 °C. The next day, 500 mL YP medium (50 g/L bacto-peptone and 50 g/L yeast extract) was inoculated with 500 µL of the pre-culture followed by incubation at 42 °C for 5 d without shaking. The culture was centrifuged for 15 min at 7,500 x g and 4 °C. The supernatant was discarded, and the cell pellet was re-suspended in 50 mL pre-extraction solution (150 mM NaCl, 20 mM HCl). The suspension was incubated at 60 °C for 3 h and 50 mL ethanol was added followed by stirring at room temperature for 1 h. The suspension was centrifuged at 7,500 x g for 5 min at room temperature. The cell pellet was discarded, and the supernatant concentrated on a rotary evaporator followed by overnight lyophilization. GS was purified from the crude extract by semipreparative HPLC on a Shimadzu HPLC (LC-8A pump system) equipped with an SPD-M20A diode array detector and a Phenomenex Synergi Hydro-RP 80 (250 x 10 mm, C18) column. The mobile phase was composed of H2O containing 0.1% TFA (solvent A) and acetonitrile (solvent B). The following gradient with a total run time of 28 min was used for separation: 5% B for 5 min, 5% to 50% B in 30 min, 50% to 100% B in 1 min, hold at 100% for 5 min, 100% to 5% B in 1 min, hold at 5% for 5 min. GS eluted at 15.07 min. Solvent was removed on a rotary evaporator followed by overnight lyophilisation.

In vitro Production of Gramicidin S

For in vitro GS production with split GrsB, the proteins GrsB12-Dc and Dn-GrsB34 were used at a concentration of 1.3 mg mL⁻¹ each, which would correspond to a concentration of 5 µM if the proteins were homogenous. GrsA was used at a concentration of 1 µM along with either MBP-GrsT (2.5 µM) or MBP-GrsT-S95A (2.5 µM) or buffer. For the reaction with intact GrsB, 2.6 mg mL⁻¹ GrsB was used instead of GrsB12-Dc and Dn-GrsB34. The assay was performed at 30 °C in assay buffer (50 mM HEPES, 0.5 mM MgCl₂, 100 mM NaCl, pH 8.0) with 5 mM ATP, 0.5 mM TCEP, 5 mM l-Pro, l-Val, l-Orn, l-Leu and 0.5 mM l-Phe as substrates. Samples were quenched by adding 1 volume MeOH and centrifuged at 20,000 x g for 5 min. The supernatant was collected and diluted with 50% ethanol containing 0.1% formic acid. GS was quantified using UPLC-MS/MS. Other compounds were measured with the same column as GS.

In vivo Production of Gramicidin S

For in vivo GS production, E. coli strain HM0079 was transformed with plasmids carrying four different versions of the GS cluster: i) pSU18-grsTAB, ii) pSU18-grsT(S95A)AB, iii) pSU18-grsTAB12-B34, and iv) pSU18-grsT(S95A)AB12-B34. The pre-cultures were prepared by inoculating LB medium (3 mL in 13 mL culture tubes) containing chloramphenicol (25 µg/mL) with single colonies from each strain and incubating at 37 °C and 200 rpm overnight. Main cultures were prepared by inoculating LB medium (3 mL in 13 mL culture tubes) containing chloramphenicol (25 µg/mL) and ornithine (6 mM) with the precultures (1/500, v/v). Main cultures were incubated at 30 °C and 230 rpm. From the main culture, a 500 µL sample was removed after 24 h for GS quantification. This sample was centrifuged (11,000 x g, 4 min, RT) and the supernatant removed. The cell pellet, which contains most of the GS, was resuspended in 70% ethanol, followed by sonication for 10 min and further incubation at 60 °C for 30 min. The cell debris was removed by centrifugation (19,000 x g, 4 min, RT). The supernatant was further diluted 50-fold in 50% ethanol containing 0.1% formic acid and the concentration of GS was quantified using UPLC-MS/MS in comparison with a standard purified from A. migulanus. Concentrations were calculated assuming the GS was homogenously distributed in the culture volume.

Triple Quad UPLC-MS/MS General Conditions

UPLC-MS/MS analysis was performed on a Waters ACQUITY H-class UPLC system coupled to a Xevo TQ-S micro (Waters) tandem quadrupole instrument under conditions optimized for each analyte (Table S2). The injection volume was 2 µL. The flow rate for all compounds was 0.5 mL min⁻¹. Acetonitrile (B) and water with 50 mM ammonium acetate (A) or water with 0.1% formic acid (A) were used as strong and weak eluent, respectively. Acetonitrile was used as the needle wash between the samples. Data acquisition and quantification were done using the MassLynx software (version 4.1). MS/MS analyses were performed using an ESI source in positive ion mode. Nitrogen was used as desolvation gas and argon as collision gas. The following source parameters were used: capillary voltage 0.5 kV, desolvation temperature 600 °C, desolvation gas flow 1000 L h⁻¹. All peptide standards were custom synthesized on a 5–9 mg scale.
Untargeted HRMS General Conditions
For untargeted high-resolution mass spectrometric analysis, samples were 2-fold diluted in MeOH and analysed on an UltiMate3000 UHPLC system (Thermo Fisher Scientific, Germany) coupled to a Thermo Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a reversed-phase UPLC column (Nucleodur HTec C18, 1.8 μm particle size, 2 × 100 mm, Macherey-Nagel). Mobile phases consisted of 0.1% (v/v) formic acid in water (Eluent A) and 0.1% (v/v) formic acid in acetonitrile (Eluent B). The column temperature was maintained at 25 °C, and the UPLC system was set to operate at a flow rate of 0.4 mL min⁻¹ with an injection volume of 2 μL. Chromatographic separation was obtained as follows: 5% eluent B starting conditions for 0.5 min, an increase to 100% eluent B in 6.5 min followed by an isocratic hold for 3 min, and a step decrease to 5% eluent B in 0.01 min followed by an isocratic hold for 2.99 min. High-resolution mass spectra were acquired in positive ion mode over a mass range of 200–1000 m/z. The data were analysed using the software CompoundDiscoverer (3.3) from Thermo Fisher Scientific and compared using differential analysis. Due to identical retention times, the initial identification of a mass feature with an m/z value of 215.1392 (corresponding to protonated PV) was attributed as in-source fragment formed during the ionisation of the protonated version of the peptide iP (m/z value of 362.2075). Fragmentation (with a normalised collision energy of 20%) of the feature corresponding to iP validated this idea and showed the m/z value of 215.1392 as y-ion obtained from the fragmentation of protonated iP (Figure S9C).

UPLC-HRMS/MS Analysis of Tryptic Digests
A reaction was conducted with 5 μM GrsB12 and 2.5 μM MBP-GrsT or MBP-GrsT-S95A in assay buffer for 2 h at 37 °C. To obtain tryptic peptides, 25 μL of this reaction were diluted with 5 μL acetonitrile. Subsequently, CaCl₂ (final 2 mM) and modified trypsin (sequencing grade, Promega) were added in a 20:1 (protein:trypsin) ratio with respect to total mass of protein to be digested. The final reaction volume was adjusted to 50 μL with H₂O, the assay incubated at 37 °C for 150 min and subjected to UPLC-HRMS/MS analysis.

Samples digested with trypsin were directly analysed on an UltiMate3000 UHPLC system (Thermo Fisher Scientific, Germany) coupled to a Thermo Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a reversed-phase UPLC column (Hypersil GOLD C8, 1.9 μm particle size, 2.1 × 100 mm, Thermo Fisher Scientific). Mobile phases consisted of 0.1% (v/v) formic acid in water (Eluent A) and 0.1% (v/v) formic acid in acetonitrile (Eluent B). The column temperature was maintained at 25 °C, and the UPLC system was set to operate at a flow rate of 0.3 mL min⁻¹. With an injection volume of 2 μL. Chromatographic separation was obtained as follows: 2% eluent B starting conditions for 2 min, an increase to 10.4% eluent B in 4.5 min followed by an increase to 50% eluent B in 30.5 min followed by an increase to 90% eluent B in 2 min followed by an isocratic hold by an increase to 2% eluent B in 1 min followed by an isocratic hold for 4 min. High-resolution mass spectra were acquired in positive ion mode over a mass range of 500–3000 m/z, with a resolution of 120 000. To observe pantetheine (Pant) ejection ions, fragmentation with a normalised collision energy of 25% was performed in positive ion mode with an isolation window of 0.4 m/z and a resolution of 60,000.

Chemical Synthesis of l-Phe-CoA
Boc-Phe-OH (17.4 mg, 65.72 μmol, 1 molar equiv; BACHEM) was mixed with PyBOP (52 mg, 98.58 μmol, 1.5 molar equiv; Carbolution) and K₂CO₃ (34 mg) in 500 μl acetonitrile. To this solution, CoA lithium salt (52 mg, 65.72 μmol, 1 molar equiv; Carbolution) was added in 500 μl water was added and the reaction was stirred at room temperature for 2 hours. The crude mixture was directly deprotected using 95% trifluoroacetic acid (TFA, 950 μl). 2.5% water (25 μl) and 2.5% TIPS (25 μl). The reaction mixture was allowed to stir at room temperature for 1 h. TFA was removed on a rotary evaporator. The crude product was dissolved in 1 mL water/acetonitrile (1:1) and directly purified by preparative HPLC and analysed using NMR and HRMS.

HPLC Purification of l-Phe-CoA
A Shimadzu HPLC (LC-8A pump system) equipped with an SPD-M20A diode array detector and a Phenomenex Synergi Hydro-RP 80 (250 × 21.2 mm, 10 μm) column was used for the purification of l-Phe-CoA. The mobile phase was composed of H₂O containing 0.1% TFA (solvent A) and acetonitrile (solvent B). The following gradient with a total run time of 47 min was used for separation: 5% B for 5 min, 5% to 50% B in 30 min, 50% to 100% B in 1 min, hold at 100% for 5 min, 100% to 5% B in 1 min, hold at 5% for 5 min. l-Phe-CoA eluted at 15.15 min. Solvent was removed on a rotary evaporator followed by overnight lyophilisation.

HRMS Analysis of l-Phe-CoA
HPLC purified l-Phe-CoA was analysed with a Thermo Vanquish Horizon UHPLC system (Thermo Fisher Scientific, Germany) coupled to a Thermo Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany) connected to a reversed-phase UPLC column (Phenomenex Kinetex C8, 2.6 μm particle size, 2.1 × 100 mm, 100 Å pore size). Mobile phases consisted of 0.1% (v/v) formic acid in water (eluent A) and 0.1% (v/v) formic acid in acetonitrile (eluent B). The column temperature was maintained at 25 °C, and the UPLC
system was set to operate at a flow rate of 0.4 mL min\(^{-1}\) with an injection volume of 2 \(\mu\)L. Chromatographic separation was obtained as follows: 10% eluent B starting conditions for 1 min, an increase to 98% eluent B in 4 min followed by an isocratic hold for 3 min, and a decrease to 10% eluent B in 0.1 min followed by an isocratic hold for 2.9 min. The high-resolution mass spectra were acquired at positive and negative ion full scan mode at the mass range of 120-1800 m/z.

**HRMS:** Calculated for C\(_{30}\)H\(_{46}\)N\(_8\)O\(_{17}\)P\(_3\)S \([\text{M+H}]^+\), m/z = 915.1915; found: m/z = 915.1879

**NMR Analysis**

**1H NMR (500 MHz, DMSO-d\(_6\)):** \(\delta\) (ppm) = 8.62 (s, 1H), 8.58 (s, 1H), 8.32 (s, 1H), 8.15 (br, 1H), 7.75 (br, 1H), 7.36-7.30 (m, 2H), 7.30-7.24 (m, 3H), 5.99 (d, \(J = 5.25\) Hz, 1H), 5.26 (s, 1H), 4.82 (br, 1H), 4.73 (br, 1H), 4.51 (br, 1H), 4.48 (br, 1H), 4.40 (br, 1H), 4.23 - 4.12 (m, 2H), 4.11 - 4.03 (m, 1H), 3.93 - 3.83 (m, 1H), 3.76 (s, 1H), 3.59 - 3.51 (m, 1H), 3.37 - 3.13 (m, 5H), 3.11 (m, 1H) 3.02 - 2.92 (m, 2H), 2.27 (br, 2H), 0.95 (s, 3H), 0.77 (s, 3H).

**13C NMR (500 MHz, DMSO-d\(_6\)):** \(\delta\) (ppm) = 196.2, 172.1, 170.8, 158.2, 158.0, 148.8, 140.3, 134.2, 129.6, 128.6, 127.3, 118.8, 87.3, 73.7, 73.4, 72.6, 72.0, 59.4, 38.6, 37.6, 37.0, 35.2, 34.7, 28.4, 21.2, 19.1.
$^1$H-NMR (Phe-CoA)

$^{13}$C-NMR (Phe-CoA)
Supporting Figures

Figure S1. SDS-PAGE Analysis of Expression and Purification of GrsT variants. The desired protein bands in elution fractions are marked with black arrows.

A. Influence of the start codon. 1: Total protein GrsT, 2: total protein GrsT-AUG, 3: pellet GrsT, 4: pellet GrsT-AUG, 5: eluate GrsT, 6: eluate GrsT-AUG.

B. Comparison of GST and MBP tag. 1: Total protein GST-GrsT, 2: total protein MBP-GrsT, 3: pellet GST-GrsT, 4: pellet MBP-GrsT, 5: eluate GST-GrsT, 6: eluate MBP-GrsT.

C. Comparison of SUMO and MBP tag. 1: Total protein SUMO-GrsT, 2: total protein MBP-GrsT, 3: pellet SUMO-GrsT, 4: pellet MBP-GrsT, 5: eluate SUMO-GrsT, 6: eluate MBP-GrsT.
Figure S2. Homology Model of GrsT. The protein sequence of GrsT was submitted to the SWISS-MODEL server for homology modelling. The template with the highest GMQE score was chosen from the template list given by SWISS-MODEL. The GrsT tertiary structure was then modelled by SWISS-MODEL based on the template structure RifR (PDB ID: 3FLA), a TEII from the rifamycin NRPS/PKS biosynthetic pathway. The structural model of GrsT (red) and the template (blue) were visualized using PyMOL. Sequence alignment and homology modelling confirmed GrsT as a TEII candidate. With RifR, GrsT shares 34% of sequence identity. The alignment to RifR confirms the presence of the conserved amino acid residues Ser95, Asp202, and His230 (yellow sticks) in a suitable arrangement to form the catalytic triad of a TEII. Hence, homology modeling identified Ser95 as the core catalytic residue.
Figure S3. SDS-PAGE Analysis of Heterologously Expressed and Purified GrsB1, GrsA-H753A, GrsA and MBP-GrsT-S95A. On each lane, 3-5 µg of protein was loaded. M: SERVA triple color protein standard III, 1: GrsB1, 2: GrsA-H753A, 3: GrsA, and 4: MBP-GrsT-S95A. The bands corresponding to the desired protein are marked with black arrows.
Figure S4. SDS-PAGE Analysis of 3C Protease Digestion of MBP-GrsT. The digestion reaction to remove the MBP tag was set up with 1 U 3C protease for every 100 µg of the fusion protein in protein storage buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5) at 4 °C for 16 h. To remove the MBP-tag carrying the N-terminal His<sub>6</sub>-tag, the protein was applied to Ni-IDA beads twice and the flow-through containing untagged GrsT was collected. The protein was buffer exchanged with protein storage buffer using Vivaspin columns. M: SERVA triple colour protein standard III; T: total protein after digestion by 3C protease; E1: eluate from first Ni-IDA column purification; E2: eluate from second Ni-IDA column purification; F1: flow-through from first Ni-IDA column purification; F2: flow-through from second Ni-IDA column purification; P: protein sample after buffer exchange. Expected sizes: fusion protein MBP-GrsT (72 kDa); cleaved His<sub>6</sub>-MBP tag (42 kDa), marked with dashed arrow; GrsT (30 kDa) marked with a plain arrow. The gel indicates that little uncleaved MBP-GrsT remains in the final sample P, but there is a sizeable contamination with MBP. To gauge the purity of the cleaved protein GrsT, the density of lane P was integrated using the ImageJ software. The relative densities of the bands are 20% (His<sub>6</sub>-MBP-tag) and 80% (GrsT).
Figure S5. Temperature-Rate Profile of GrsT. Acetyl-CoA hydrolysis was measured with freshly prepared 2.5 μM MBP-GrsT at different temperatures (20 °C, 25 °C, 30 °C, 35 °C, 40 °C) with 1 mM acetyl-CoA as substrate. This substrate concentration is sub-saturating (Figure S6A). Experiments were performed with biological replicates. At each temperature, 4 time points were taken (0 min, 20 min, 40 min and 60 min). At each time point, aliquots were taken and quenched with 5% trichloroacetic acid followed by centrifugation at 20,800 x g for 5 min. The concentration of the hydrolysis product CoA was measured and quantified by UPLC-MS/MS.
Figure S6. Michaelis-Menten Kinetics for Acyl-CoA Hydrolysis. Saturation kinetics are shown for the following enzyme/substrate combinations: A. MBP-GrxT/acyetyl CoA, B. MBP-GrxT/propionyl CoA, C. MBP-GrxT/butyryl CoA, D. MBP-GrxT, L-Phe-CoA, E. GrxT/acyetyl CoA. The error bars indicate standard errors of two biological replicates. The background hydrolysis rate of each substrate in the absence of enzyme has been subtracted. In all panels except (D), $k_{cat}$ and $K_M$ values are extrapolated because substrate saturation was not reached.
Figure S7. DKP Formation with Different Concentrations of GrsA-H753A. To evaluate the concentration range within which GrsA-H753A will behave as the rate limiting enzyme, DKP formation was determined with different concentrations of GrsA-H753A and 2.5 µM of GrsB1. As substrates, d-Phe and L-Pro (1 mM) were added to the reaction samples in DKP assay buffer, supplemented with 1 mM ATP, and incubated for 1 h at 37 °C. Assay samples were quenched by heat shock at 90 °C for 2 min. fP-DKP was quantified by UPLC-MS/MS in the supernatant after centrifugation of the samples at 20,000 x g for 5 min. Error bars represent the standard deviation from technical duplicates.
Figure S8. DKP Formation Assay with GrsA-H753A in Presence and Absence of MBP-GrsT. Error bars represent the standard deviation from biological duplicates. Data for LL-DKP are identical to those shown in Figure 2A.
Figure S9. Untargeted High-Resolution Mass Spectrometric Analysis. A. Comparison of assays performed with intact and split GrsB in the presence of MBP-GrsT. Features that are upregulated in the respective assays with intact (blue) or split (green) GrsB are annotated with the corresponding putative peptides. The peptide fPV shows in-source fragmentation which yields an m/z value that corresponds to protonated PV. The peptide PVOL was detected as doubly charged species ([M+2H]²⁺). B. Comparison of assays performed with split GrsB containing inactive (KO) or active MBP-GrsT (WT). Features that are upregulated in the respective assays with inactive (blue) or active (green) MBP-GrsT are annotated with the corresponding putative peptides. C. MS/MS spectrum of the m/z identified as fPV as obtained with a normalised collision energy of 20%. The obtained fragments are annotated according to the standard nomenclature of peptide fragment ions.
Figure S10. Optimal Concentration of MBP-GrsT for GS Formation Using Split GrsB. TEIs are expected to have a concentration optimum above which the undesired hydrolysis of on-pathway intermediates slows down synthesis. To find this optimum, the protein GrsA (1 µM), GrsB12-Dc (5 µM) and Dn-GrsB34 (5 µM) were added to the assay buffer (50 mM HEPES, 0.5 mM MgCl₂, 100 mM NaCl, pH 8.0) with 5 mM ATP, 0.5 mM TCEP, 5 mM L-Pro, L-Val, L-Orn, L-Leu and 0.5 mM L-Phe as substrates. Protein MBP-GrsT was added in concentrations of 0, 0.5, 1.0, 2.5, 5.0, and 7.5 µM. The assay was performed at 30°C for 1 h and stopped by adding 1 volume MeOH. The samples were centrifuged at 20,000 x g for 5 min and the supernatant was diluted with 50% ethanol (0.1% formic acid). GS was quantified using UPLC-MS/MS. A shallow optimum for GS production at 2.5 µM MBP-GrsT was found. Error bars represent the standard deviation for two biological replicates.
Figure S11. The Yield of Peptides Produced by Intact and Split GrsB in vitro. For each peptide, the bar on the left represents the sample with inactive MBP-GrsT-S95A and the bar on the right represents the sample with active MBP-GrsT. The concentration of fP-DKP, PV, fPV, PVOL, fPVOL, and GS has been determined by UPLC-MS/MS. Error bars represent the standard deviation from biological duplicates. The data shown in Figures 3C and 3D are identically repeated in this plot.
Figure S12. Heterologous production of GS in E. coli. Biosynthesis of GS was performed using four different plasmids encoding intact or split GrsB and active or inactive GrsT. The concentration of GS has been determined by UPLC-MS/MS in comparison with an authentic standard. Error bars represent the standard deviation from three biological replicates. Active GrsT seems to increase the average production of GS by 1.5 (intact GrsB) and 3.0-fold (split GrsB), but the statistical significance of the effect is low indicated by p-values of 0.23 and 0.18, respectively.
Figure S13: MS/MS Spectrum of the PV Loaded Peptide ($m/z$ [M+2H]$^{2+}$ calc.: 1155.5712; found: 1155.5697) obtained after tryptic digest of GrsB12. Fragmentation as obtained with a normalised collision energy of 25% shows the characteristic Pant ejection ion ($m/z$ [M+H]$^+$ calc.: 457.2479; found: 457.2470) for the peptide PV loaded onto the second phosphopantetheinyl arm of the GrsB12 dimodule.
Figure S14: GS Formation Time Course Recorded for Both Intact and Split GrsB *in vitro*. The concentration of GS has been determined by UPLC-MS/MS. Error bars represent the standard deviation from biological duplicates.
### Table S1. List of Primers.

| Primer names           | Sequence (5' - 3')                     |
|------------------------|---------------------------------------|
| pSU18-F-1              | GGA TCC AGA TCT CAC CAT CAC CAT TAA GC |
| pSU18-R-1              | AGC TGT TTC CTG TGT GAA ATT GTT ATC CGC |
| grsT-pSU18-F-1         | TCA CAC AGG AAA CAG CTG TGA CTT TTA TTT CAC AAG TAA ATA AAT GGT TGT TTA ATG C |
| grsT-pSU18-R-1         | TGA TGA GAT CTG GAT CCT ATT TTA GGA GCT AAT ACT AAA CGT AAT TGT TTA CAC ATG A |
| grsT-AUG-pSU18-F-1     | TCA CAC AGG AAA CAG CTA TGA CTT TTA TTT CAC AAG TAA ATA AAT GGT TTT TTA ATG C |
| grsT-pOPINM-F1         | CTG TTT CAG GGT ACC GTG ACT TTT ATT TCA CAA GTA AAT AAA TGG TTT GTT AAT GC |
| grsT-pOPINM-R1         | CTG GTG TAG AAA GCT TGG TTA TAT TAG AGG AGC TAA TAC TAA ACG TAA TGT TTT ACA CAT G |
| His-MBP-grsT-pSU18-F   | ACA CAG GAA ACA GCT ATG GCA CAC CAT CAC CAT CA |
| His-GST-grsT-pSU18-F   | TTC ACA CAG GAA ACA GCT ATG GCA CAT CAC CAT CAT C |
| grsT-extra-pSU18-R     | CTA ATT AAG CTT TTA AAC TGG TCT AAG AAG CTT GGT TAT ATT TTA GGA GC |
| pSU18-extra-F1         | GTT TAA ACG CTT AAT TAG CTG AGC TGG GAC TCC TGT T |
| pSU18-F-SUMO-F1        | CAC AGG AAA CAG CTA TGG GCA GCA GCC ATC ATC AT |
| pSU18F-SUMO-M20        | TTG TGA AAT AAA AGT CAC ACC AAT CTG TCC TGT AGC |
| pSU18F-grsT-F2         | GTG ACT TTT ATT ATC CAA GTA AAT AAA TGG TTT GTT AAT GC |
| grsT/S95A-pSU18-P1-F1  | TCT GGA ACT GTT CTG TCA GGG TAC CTT G |
| grsT/S95A-pSU18-P1-R1  | AAC TTA TTA ATG CTC CCA TGG CAT GCC |
| grsT/S95A-pSU18-P2-F1  | CAT GGG AGC ATT AAT AAG TTT TGA ACT GGC TCG |
| grsT/S95A-pSU18-P2-R1  | GCT CAG CTA ATT AAG CTA TAA AAC TGG TCT AAG AAG ATC CTC TCT T |
| GrsB12-Dc-F1           | TGG GTA TCA TGG GAA TTC TTA AAG CAG GGG CTT CAG TCT ACT TCT T |
| His-GrsB12-Dc-R1       | CAT GCC TGC AGG TCC ACT TAG ATG TCA CCT TTT AAG AGA GCT TGT GCA GTT TCT |
| His-pOPINM-P1-F        | TTC ACA CAG GAA ACT CGA GAT GCC ACA CCA TCA CCA CCA TCA CAG CAG |
|                         | GGG TAG TAC TAC ATT TAA AAA AGA AAT AGC TAA TAA TGG CAA CAG TCA TGG C |
| His-pOPINM-P1-R        | GAG ATA AAC GAT ACA TAT CCT GAA CAT GTT CTT TTT TTA ATG TAC TCA TAC CTC GTC TGT GAT GTT GTG CCA TCG GTG TCT GTG GTG AA |
| His-pOPINM-P2-F        | ATG TGT ACG TCT ATG TAT ATT ATG CCA GTC TCC TGT CGG TAA ATG TCT TGG C |
| His-pOPINM-P2-R        | CCA CGA TAC CCT CGA TCG CGA TAG ACA TTA AGG GGA AAT GCC GTG TCT TTT GAG CGG TAC CGC GTC TGG |
| GrsB34-F-2             | CAC ACA GGA AAC TCC AGA TGA AAG ATG CAG CAC AAA TTA TCT ACG A |
| GrsB34-R-1             | GAT TGC GAC TTA AGG CCG AGC CCA CCT GCT TCT CTA TC |
| grsTAB-EcRBS-pSU18-f   | CAA TTT CAC ACA GGA AAC AGC TGT GAC TTT TAT TTA AGT AAA TAA |
| grsTAB-EcRBS-pSU18-r   | GCT CTG TTT TAA ATG TAC TGA TCA TGC TAC GAG CGC TCT AAC TGA AAG CAG C |
| GrsTAB-Dc-F1           | AAC AGG TGG AGA AGA CCG CCG ACC ATG TGA CAG TGT GAT G |
| GrsTAB-Dn-R1           | AAT TCC TTC CTG TCC CGG GTA TTT TGT TAA AAG TAC ATT GTA CGC CCG TTA |
| grsT(S95A)AB-Ins1_F1   | ATA GTA GAA ATA GTA GCT GAG GAA ATA CAA CC |
| grsT(S95A)AB-Ins1_R1   | TCC AAA AGC AGT TGG GCA TCC A |
| grsT(S95A)AB-Ins2_F1   | GCC CAA TCA CTG CTT TGG GAG GAA |
| Primer                     | Sequence                                    |
|---------------------------|---------------------------------------------|
| grsT(S95A)AB-Ins2_R1      | CTG ATG GAT CGT CTT ATC ACG TGG A           |
| GrsTAB-Dn-F1              | TAA AAG GTG ACA TCT AAT ATA ATG TGT GGA ATT GTG AGC GGA TAA CA |
| GrsTAB-Dc-R1              | TTA GAT GTC ACC TTT TAA CAG AGC TTG TGC    |
| Compound | UPLC-MS/MS method |
|----------|------------------|
| Coenzyme A | Column: ACQUITY UPLC HSS T3, 1.8 μm particle size, 2.1 × 100 mm  
Elution profile: linear gradient of 2 to 30% B over 3.5 min followed by 2.2 min re-equilibration  
MRM transition: 768.1108 > 261.1667  
Calibration curve range: 0.04 μM to 50 μM  
Cone voltage: 40 V |
| Phe-Pro-DKP | Column: CORTECS UPLC C18, 1.6 μm particle size, 2.1 × 150 mm  
Elution profile: linear gradient of 20 to 95% B over 3 min (flow rate 0.3 mL min⁻¹)  
followed by 0.5 min wash and 2 min re-equilibration  
MRM transition: 245.0030 > 69.9310  
Calibration curve range: 0.001 µM to 1 µM  
Cone voltage: 56 V |
| Val-Pro-DKP | Column: CORTECS UPLC C18, 1.6 μm particle size, 2.1 × 150 mm  
Elution profile: linear gradient of 20 to 95% B over 3 min (flow rate 0.3 mL min⁻¹)  
followed by 0.5 min wash and 2 min re-equilibration  
MRM transition: 197.0904 > 69.9460  
Calibration curve range: 0.0006 µM to 2 µM  
Cone voltage: 24 V |
| GS | Column: ACQUITY UPLC CSH C18, 1.7 μm particle size, 2.1 × 50 mm  
Elution profile: linear gradient of 40 to 98% B over 1 min followed by 1.2 min re-equilibration  
MRM transition: 571.6957 > 70.0991  
Calibration curve range: 0.05 µM to 10 μM  
Cone voltage: 16 V |
| fPVOL | Column: Cortecs UPLC C18 column (1.6 µm, 2.1 x 50 mm, Waters)  
Elution profile: linear gradient of 2 to 50% B over 1.5 min (flow rate 0.5 mL min⁻¹)  
followed by 0.3 min wash and 1 min re-equilibration  
MRM transition: 295.19 > 120.08  
Calibration curve range: 0.32 nM to 200 nM  
Cone voltage: 40 V |
| PV | Column: ACQUITY UPLC HSS T3, 1.8 μm, 2.1 × 100 mm, Waters  
Elution profile: linear gradient of 2 to 50% B over 1.5 min (flow rate 0.5 mL min⁻¹)  
followed by 0.3 min wash and 1 min re-equilibration  
MRM transition: 215.14 > 70.07  
Calibration curve range: 1.6 nM to 1000 nM  
Cone voltage: 20 V |
| fPV | Column: Cortecs UPLC C18 column (1.6 µm, 2.1 x 50 mm, Waters)  
Elution profile: linear gradient of 2 to 50% B over 3.2 min (flow rate 0.5 mL min⁻¹)  
followed by 0.3 min wash and 1 min re-equilibration  
MRM transition: 362.30 > 215.14  
Calibration curve range: 1.6 nM to 1000 nM  
Cone voltage: 22 V |
| PVOL | Column: Cortecs UPLC C18 column (1.6 µm, 2.1 x 50 mm, Waters)  
Elution profile: linear gradient of 2 to 50% B over 3.2 min (flow rate 0.5 mL min⁻¹)  
followed by 0.3 min wash and 1 min re-equilibration  
MRM transition: 442.37 > 115.12  
Calibration curve range: 1.6 nM to 1000 nM  
Cone voltage: 42 V |
Table S3. Sequences of Proteins Used in this Study.

**MBP-GrsT**

MAHHHHHSSGMKIEEGKLIVWDGKNGLAEVGKKFEKDTGIKTVEHPDKLEKKFPQVAATGDGIDIIFWAHRDFGGYAGSGLLAETIPKAFQDKLYFTDARVYNGKLIAYPIAVEALSLYINKDLLPNPKTWEENPALDEKKAQGSKALMFQNFYPFRTWPLIAADDGAYFKYNQKDYKDVGQNAGAKAGLTFVLVDL1KNKHMNATDTSYIAEAANNKGETAMTINGPWAWSNIDTSKVNYGVTVPFTKPQFSPKFGVVLSSAGIASSPNKEAFLENYLMTDELEGAVNKDPLGAVALKSYEELAKDPRIAATMENAQKGEIMPINIQMAWFAYAVRTAVINASAAGRTQDVEALKDAATQSSGLERVFGTVTFISQVKNWFWANVNSA

**MBP-GrsT(S95A)**

MAHHHHHSSGMKIEEGKLIVWDGKNGLAEVGKKFEKDTGIKTVEHPDKLEKKFPQVAATGDGIDIIFWAHRDFGGYAGSGLLAETIPKAFQDKLYFTDARVYNGKLIAYPIAVEALSLYINKDLLPNPKTWEENPALDEKKAQGSKALMFQNFYPFRTWPLIAADDGAYFKYNQKDYKDVGQNAGAKAGLTFVLVDL1KNKHMNATDTSYIAEAANNKGETAMTINGPWAWSNIDTSKVNYGVTVPFTKPQFSPKFGVVLSSAGIASSPNKEAFLENYLMTDELEGAVNKDPLGAVALKSYEELAKDPRIAATMENAQKGEIMPINIQMAWFAYAVRTAVINASAAGRTQDVEALKDAATQSSGLERVFGTVTFISQVKNWFWANVNSA

**GrsB**

The sequence of GrsB encoded by plasmid pTrc99a-grsBMtol is identical to that in NCBI accession number WP_043064679.1, except for the GSRSHHHHHH* sequence appended at the C-terminus, and a Met to Leu mutation at position 3574 introduced previously for better protein production.

**GrsB12-Dc**

MSTFKKEHVSQDMRLSPMVEQGMLFHALLDKDKNATVLQMSIAIEGIVDLSELSSLNLIDRYDVFRTTFLHEKIQKQPLQVEKVFQQFDKDSLLEDEKREIAEQYQKQDGETVDFLIDRTDPMRMAIFQGTVGNYQMISWFIHLMDGCVFHFNDFGNYLSEKQKLQPLEAVQYQFIKWKLEQDKQEAIRYWKELMNQVSITLPKKAIAINNTTPEFRDKVTLQRTAILRIANQTSQVTLIQVFTGIVLQKYNSTNDVYGRVSSRPEISEGIEKVMFGIINTLPLNQTKQDSIEFLKTQNVHQLFVSQQHEFYYELIEQHNTWELQLNHIMVIVALQVEELQKNSIMQKVGTFRVNDKMEPNTNYDMIOMLFREISVRDLOANAYDIADFIKIEGHMKEALCVANNRHLVQDVPPTLQKEQHQLLVHELSDITEYPDTHQLFTEQVEKTPHEAVVFEDEKTVYRELHERSNQLAFLREKYGKESIIGIMMERSVMIGILGKAGGasFVPIDPEPKERINARYLMVLRVTVTQAFKSTTIEPDPESELTSSLEEDFYIIYTSGGTPQKAVGVMLEKNVNLHFTFEKTNINSKDVLVQYTTSCFVQYFISFLGSGGQLYLRICKORQVDOLDLVKRENIEVLSFPAVLFKIFNEREFINNFRCTVHIITAGEQVEVNLFENKRYLHENVVHLHNYGSEPETHVVTITAINEAPEIELPFPKGPISNTW1YDQEQLOQFQGIGVGRQSISANGVGRGYNLQETEAKFPPDFRNPENRGLNRDLGPNGIEFLGRADQHVRGHRILGEAELLNNCGKVEAVVIDADDKGGYLCAYAVMEVENSDRELRYLGALPDIQPFPFPVLQPLTNGKDRKSLPNLIEGNTNAYQVPVTNELEKAKLWEVEVGLQIGISIQGDNFSSLGHSKAIITLSRMKCEVNDIPLRLLEEFAPITEQINSHEYANLEGKESYXIAPQVPEEQYVYYPSSVQKRFMPILNEFDRSTAYNLP6VMFGLDKNRYQLEAAVKKLVVERHALRTSESHINGEPVCRQVHVNLQAYSETEDQVEIIAEFMQFQALEVAPLRLVRVLLKALEHERLFIIMDDHMQGVDSGMIQEMIAEDLYKEKELPTLIGQYKDFTWVHRNRLIQGDSVEQIEAYWLNVFAIEFVNLNPTDYRPTIQSFDGKRTFTPSTGQKLMDDLYKVTETTGTLVYLLAVANNVLQSYQQDDIVTGYTFPTIAGRSHADVNLGLMFTNLAIRSLNNDTDFLANKQNTALHAYENPDYPDFDLKELQIRDDLRMSPLMDFMLDNTRDSEVEQITITTPYPNRSRHSDKFL
Table S4. R Script for Michaelis-Menten Plots.

#install missing packages (remove the hashtag and run the next lines manually once)
#install.packages("dplyr")
#install.packages("stringr")
#install.packages("plotmath")

library(dplyr)
library(stringr)
#read csv file named "data.csv"
dat <- read.csv(file="data.csv", sep=" ", header=FALSE, dec=".")
S <- dat$V1
v0 <- dat$V2
v1 <- dat$V3
#fit to model
m <- nls(v0~kcat*S/(Km+S), start = list(kcat = 100, Km = 1))
#extract coefficients
r <- signif(cor(v0,predict(m)), 4)
coeffs <- summary(m)$coefficients
summary(m)
kcat <- signif(coeffs[1,1], 3)
Km <- signif(coeffs[2,1], 3)
kcat_err <- signif(coeffs[1,2], 1)
Km_err <- signif(coeffs[2,2], 1)
kcatKm <- signif(kcat/Km, 3)
#predicted curve
S_pred <- seq(0,max(dat$V1),max(dat$V1)/100)
v0_pred <- predict(m,list(S = S_pred))
#plot
dev.new(width=6, height=4, noRStudioGD = TRUE)
par(mfcol=c(1, 1), mar=c(4,4,1,1), mgp=c(2,0.4,0), tcl=-0.2)
x_label<expression(paste("substrate concentration \( (M) \))
y_label<expression(italic(v)

S, v0, xlab=x_label, ylab=y_label, ylim=c(0,1.1*max(dat$V2)))
lines(S_pred,v0_pred,lty=1,col="red",lwd=3)
pow< expression(italic(v)[0]/E[0]~ (s^{(-1)}))
plot(S, v0, xlab=x_label, ylab=y_label, ylim=c(0,1.1*max(dat$V2)))
points(S, v0, pch=19, cex=1.4)
arrows(x0=S, y0=v0-v1, x1=S, y1=v0+v1, code=3, angle=90, length=0.1)
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