Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information

Assembly Line Termination in Cylindroclcyophane Biosynthesis: Discovery of an Editing Type II Thioesterase Domain in a Type I Polyketide Synthase

Hitomi Nakamura[a], Jennifer X. Wang[b], Emily P. Balskus[a]*

[a]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, USA

[b]Small Molecule Mass Spectrometry Facility, FAS Division of Science, Cambridge, Massachusetts 02138, USA

*Correspondence to: balskus@chemistry.harvard.edu

Supplementary Materials

1. General Materials and Methods

All chemicals were obtained from Sigma-Aldrich except for EDC•HCl (Creosalus, Advanced Chemtech), glycerol (Avantor Performance Materials, Macron Fine Chemicals), and [Acetyl-1-^{14}C]-Coenzyme A (Perkin Elmer). Solvents were obtained from Sigma-Aldrich except ethanol (KOPTEC) and diethyl ether (EMD Millipore). Luria-Bertani Lennox (LB) medium was purchased from EMD Millipore and IPTG was purchased from Teknova. All NMR solvents were purchased from Cambridge Isotope Laboratories.

Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Recombinant plasmid DNA was purified with a QIAprep Kit from Qiagen. Gel extraction of DNA fragments and restriction endonuclease clean up were performed using an Illustra GFX PCR DNA and Gel Band Purification kit from GE Healthcare. DNA sequencing was performed by Beckman Coulter Genomics (Danvers, MA). Nickel-nitrilotriacetic acid-agarose (Ni-NTA) resin was purchased from Qiagen and Thermo Scientific. SDS-PAGE gels were purchased from Bio-Rad.

Gel filtration FPLC was performed on a BioLogic DuoFlow Chromatography System (Bio-Rad) equipped with a Superdex column, 200 10/300 GL or 200 26/600 PG (GE Healthcare). Bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1350 Da) were used as molecular weight markers (Bio-Rad, #151-190). The molecular weights of the proteins analyzed by gel filtration were calculated from their elution volume, using a linear relationship between log(molecular weight) and ve/vo (elution volume/void volume). Protein concentrations were determined according to the method of Bradford using bovine serum albumin (BSA) as a standard,[1] or using a NanoDrop 2000 UV-Vis Spectrophotomter (Thermo Scientific) for $CylH_{PKS}$ (ε = 169,820 $M^{-1}cm^{-1}$) and $CylH_{TE}$ (ε = 30,035 $M^{-1}cm^{-1}$). The extinction coefficients for $CylH_{PKS}$ and $CylH_{TE}$ were calculated using ExPASy ProtParam tool.[2] Optical densities of *E. coli* cultures were determined with a DU 730 Life Sciences UV/Vis spectrophotometer (Beckman Coulter) by measuring absorbance at 600 nm.

Analytical HPLC was performed on a Dionex Ultimate 3000 instrument (Thermo Scientific) using a Chromolith RP-18e endcapped monolithic silica column (4.6 x 100 mm, EMD Millipore). High-resolution mass spectral data and the spectrophotometric assay data were obtained in the Small Molecule Mass Spectrometry Facility, FAS Division of Science. Mass spectral data were obtained on an Agilent 1290 Infinity UHPLC system (Agilent Technologies) and a maXis impact UHR time-of-flight mass spectrometer system (Bruker Daltonics Inc) equipped with an electrospray ionization (ESI) source or a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) source with Agilent Jet Stream technology. Spectrophotometric assay was performed on Spectramax i3 Plate Reader (Molecular Devices).

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were obtained on a Varian Inova-500 (500 MHz, 125 MHz), Varian-Mercury 400 (400 MHz, 100 MHz) or Varian-Mercury 300 (300 MHz, 75 MHz) NMR spectrometers in the Magnetic Resonance Laboratory in the Harvard University Department of Chemistry and Chemical Biology. Chemical shifts are reported in parts per million downfield from tetramethylsilane using the solvent resonance as internal standard for ^{1}H ($CDCl_3$, δ_H = 7.26 ppm) and ^{13}C ($CDCl_3$, δ_C = 77.16 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration, and coupling constant. NMR spectra were visualized using iNMR Version 5.3.6 (Mestrelab Research).

High-resolution mass spectral (HRMS) data for the synthetic compounds were obtained in the Magnetic Resonance Laboratory in the Harvard University Department of Chemistry and Chemical Biology on a Bruker Micro qTOF-QII fitted with a dual-spray electrospray ionization (ESI) source or in the Small Molecule Mass Spectrometry Facility, FAS Division of Science on an Agilent 6210 TOF. The capillary voltage was set to 4.5 kV and the end plate offset to –500 C, the drying gas temperature was maintained at 190 ºC with a flow rate of 8 L/min and a nebulizer pressure of 21.8 psi. The liquid chromatography (LC) was performed using an Agilent Technologies 1100 series LC with 50% H_2O and 50% acetonitrile as solvent.

2. Cloning, overexpression and purification of CylI, CylH tetra-domain, and standalone TE domain.

N-His_6-tagged CylI was cloned, expressed and purified according to a previously published procedure.[3] pET30a-TycF was obtained from the Walsh group, and TycF was expressed and purified following a previously reported procedure.[4]

Table S1. Oligonucleotides used for cloning (restriction sites underlined).

Oligo	Sequence	Target
CylH-KS-F-NdeI	5' CCGCCCATATGAATACTGTTATTTCCAAGC 3'	$CylH_{PKS}$ (KS-AT-T-TE)
CylH-RC-XhoI-nostop	5' GCCCTCGAGATTGATTGTACAGAAAATAG 3'	
TE-20aa-F-NdeI	5' GCACGACATATGAAGGTTGTAGATGAAGCC 3'	$CylH_{TE}$
TE-RC-XhoI-nostop	5' GTAGGGCTCGAGGAAAATAGTCTCTTCAAT 3'	
CylH-TE-S74A	5' CATTTTTCGGTCATGATATGGGAGCACTACTC 3'	TE active site serine to alanine mutants $CylH_{PKS}$-S1201A and $CylH_{TE}$-S95A
CylH-TE-S74A-RC	5' GAGTAGTGCTCCCATATCATGACCGAAAAATG 3'	

Cloning of *cylH* tetra-domain and TE domain

CylH$_{PKS}$ (KS-AT-T-TE) and *cylH*$_{TE}$ (TE domain only) were PCR amplified from *Cylindrospermum licheniforme* ATCC 29412 genomic DNA using the primers shown in **Table S1**: *cylH*$_{PKS}$ (forward primer **CylH-KS-F-NdeI** + reverse primer **CylH-RC-XhoI-nostop**), and *cylH*$_{TE}$ (forward primer **TE-20aa-F-NdeI** + reverse primer **TE-RC-XhoI-nostop**). All forward primers have *Nde*I restriction sites and all reverse primers have *Xho*I restriction sites. All PCR reactions contained Phusion High-Fidelity PCR Master Mix (New England BioLabs), 50-100 ng DNA template, and 25 pmoles of each primer in a total volume of 50 µL. Thermocycling was carried out in a C1000 Gradient Cycler (Bio-Rad) using the following parameters: denaturation for 30 sec at 98 ºC, followed by 30 (*cylH*$_{PKS}$) or 35 cycles (*cylH*$_{TE}$) of 10 sec at 98 ºC, 30 sec at 56 ºC, 5 min (*cylH*$_{PKS}$) or 45 sec (*cylH*$_{TE}$) at 72 ºC, and a final extension time of 10 min at 72 ºC. PCR reactions were analyzed by agarose gel electrophoresis with ethidium bromide staining, pooled, and purified.

Amplified fragments were digested with *Nde*I and *Xho*I (New England BioLabs) for 2.5 h at 37 ºC. Digests contained 1 µL water, 3 µL NEB buffer 4 (10x), 3 µL of BSA (10x), 20 µL of PCR product, 1.5 µL of *Nde*I (20 U/µL), and 1.5 µL of *Xho*I (20 U/µL). Restriction digests were purified directly using agarose gel electrophoresis; gel fragments were further purified using the Illustra GFX kit. The digests were ligated into linearized expression vector using T4 DNA ligase (New England BioLabs). Both $cylH_{PKS}$ and $cylH_{TE}$ digested inserts were ligated into the pET-29b vector to encode a C-terminal His_6-tagged construct. Ligations were incubated at 16 ºC overnight and contained 3 µL water, 1 µL T4 Ligase Buffer (10x), 1 µL digested vector, 3 µL digested insert DNA, and 2 µL T4 DNA Ligase (400 U/µL). 5 µL of each ligation was used to transfer a single tube of chemically competent *E. coli* TOP10 cells (Invitrogen). The identities of the resulting pET29b-$cylH_{PKS}$ and pET29b-$cylH_{TE}$ constructs were confirmed by sequencing of purified plasmid DNA. The pET29b-$cylH_{PKS}$ and pGro7 (Takara) were co-transformed into chemically competent *E. coli* BL21 (DE3) cells (Invitrogen), while the pET29b-$cylH_{TE}$ was transformed into *E. coli* BL21 (DE3) cells by itself. The resulting transformants were stored at –80 ºC as frozen 1:1 LB/glycerol stocks.

Site-directed mutagenesis of the CylH TE domain

Site-directed mutagenesis of the TE domains of $CylH_{PKS}$ and $CylH_{TE}$ were performed using the corresponding oligonucleotides (**CylH-TE-S74A** and **CylH-TE-S74A-RC**) listed in **Table S1**. The residue mutated was the catalytic Ser74 residue on the CylH TE domain, which corresponded to Ser1201 on $CylH_{PKS}$ and Ser95 on $CylH_{TE}$. The catalytic serine residue (S74) for mutagenesis was identified through multiple sequence alignment of the CylH TE domain with 8 other previously characterized TE domains by ClustalW in Geneious Pro Version 7.1.6 software (**Fig. S1**).[5]

PCR reactions of 50 µL contained 25 µL of Phusion High Fidelity PCR Master Mix (New England BioLabs), 10 ng of pET29b-$cylH_{PKS}$ or pET29b-$cylH_{TE}$ template, and 15 pmoles of each primer. Thermocycling was carried out in a C1000 Gradient Cycler (Bio-Rad) using the following parameters: denaturation for 1 min at 95 ºC, followed by 18 cycles of 30 sec at 95 ºC, 1 min at 57 ºC, and 6 min ($cylH_{TE}$) or 9.5 min ($cylH_{PKS}$) at 70 ºC. Digestion of the methylated template plasmid was performed with DpnI (New England BioLabs), and 5 µL of each digestion

was used to transform a single tube of chemically competent *E. coli* TOP10 cells. The identities of the resulting pET29b-*cylH*$_{PKS}$-S1201A and pET29b-*cylH*$_{TE}$-S95A constructs were confirmed by sequencing purified plasmid DNA. pET29b-*cylH*$_{PKS}$-S1201A and pGro7 (Takara) plasmids were co-transformed into chemically competent *E. coli* BL21 (DE3) cells (Invitrogen), while the pET29b-*cylH*$_{TE}$-S95A plasmid was transformed into *E. coli* BL21 (DE3) cells by itself. The resulting transformants were stored at –80 ºC as frozen 1:1 LB/glycerol stocks.

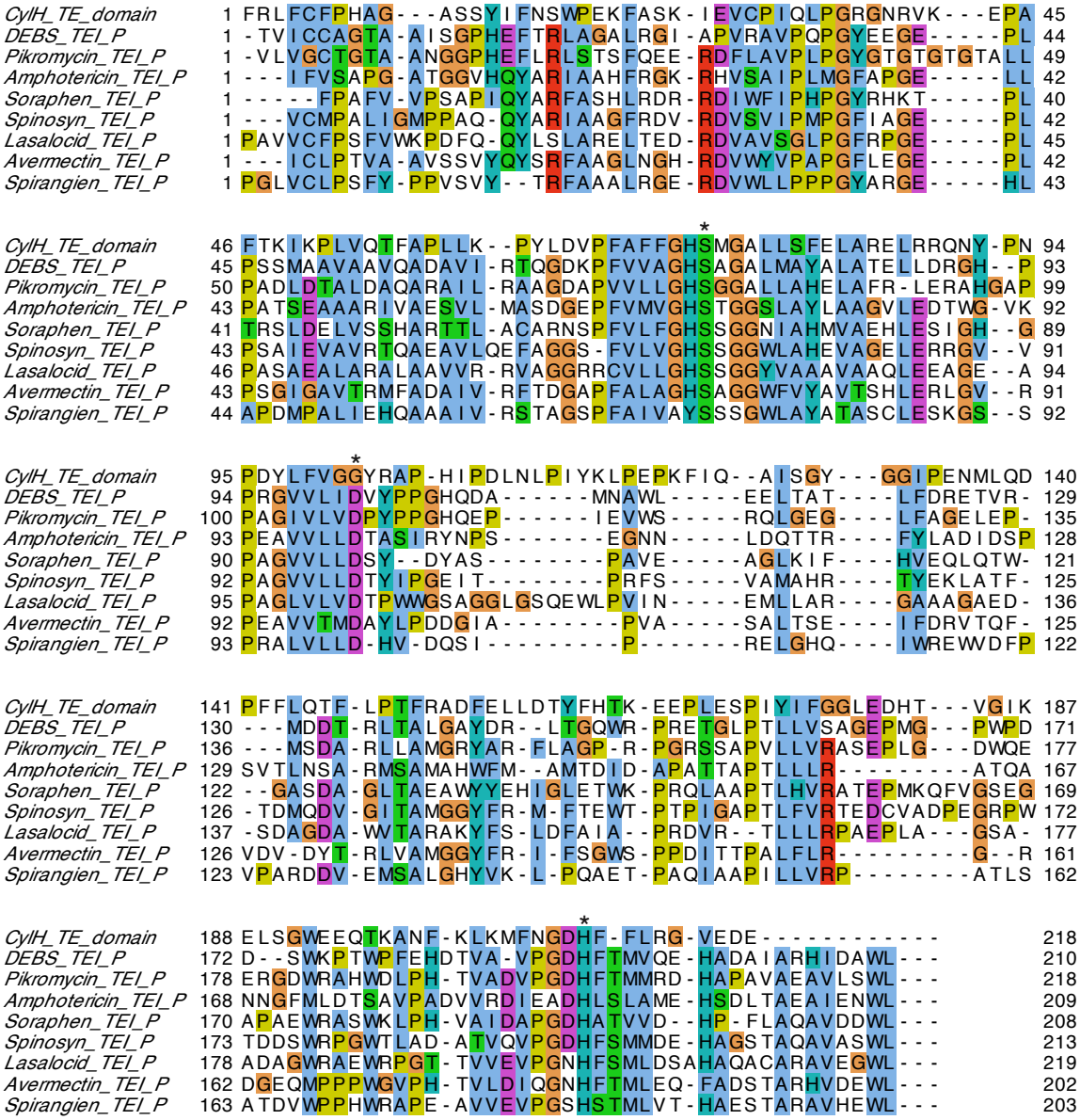


Figure S1. Multiple sequence alignment of the CylH TE domain with other previously characterized C terminal TE domains of type I modular PKS enzymes by ClustalW. The catalytic serine, aspartate and histidine residues are marked with asterisk (*).

Large scale overexpression and purification of CylH$_{PKS}$, CylH$_{TE}$, and the TE domain mutants

CylH$_{PKS}$ and CylH$_{PKS}$-S1201A mutant: A 50 mL starter culture of pET29b-*cylH*$_{PKS}$ + pGro7 BL21 *E. coli* was inoculated with frozen stock and grown overnight at 37 ºC in LB medium supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Overnight cultures were diluted 1:100 into 2 L LB medium containing kanamycin and chloramphenicol. Expressions of GroES and GroEL were induced by addition of 0.5 mg/mL of L-arabinose. The cultures were incubated at 37 ºC with 175 rpm shaking until OD_{600} = 0.3–0.45. The cultures were then transferred to 15 ºC, protein expression was induced with 100 µM of IPTG when OD_{600} = 0.65–0.75, and the cultures were incubated at 15 ºC for ~20 h.

CylH$_{TE}$ and CylH$_{TE}$-S95A mutant: A 50 mL starter culture of pET29b-*cylH*$_{TE}$ BL21 *E. coli* was inoculated with frozen stock and grown overnight at 37 ºC in LB medium supplemented with 50 µg/mL kanamycin. Overnight cultures were diluted 1:100 into 2 L LB medium containing kanamycin. The cultures were incubated at 37 ºC with 175 rpm shaking until OD_{600} = 0.3–0.45, and the cultures were transferred to 15 ºC incubator. The protein expression was induced with 500 µM of IPTG when OD_{600} = 0.65–0.75, and incubated at 15 ºC for ~18 h.

Cells from 2 L of culture were pelleted by centrifugation (6,000 rpm x 15 min) and resuspended in 40 mL of lysis buffer (CylH$_{PKS}$ and CylH$_{PKS}$-S1201A mutant = 100 mM potassium phosphate, 300 mM NaCl, pH 8.0; CylH$_{TE}$ and CylH$_{TE}$-S95A = 25 mM Tris•HCl, 500 mM NaCl, 10 mM $MgCl_2$, pH 8.5) supplemented with EDTA-free Pierce Protease Inhibitor Tablets (Thermo Fisher). The cells were lysed by passage through a cell disruptor (Avestin EmulsiFlex-C3) twice at 8,000–10,000 psi, and the lysate was clarified by centrifugation (13,000 rpm x 30 min). The supernatant was incubated with 2 mL of Ni-NTA resin and 5 mM imidazole for 2 h at 4 ºC. The mixture was centrifuged (4,000 rpm x 5 min) and the unbound fraction was discarded. The Ni-NTA was resuspended in 10 mL of wash buffer 1 (lysis buffer containing 20 mM imidazole), loaded into a glass column, and washed with 50 mL of wash buffer 1. CylH$_{PKS}$ and CylH$_{PKS}$-S1201A mutant was washed further with 50 mL of wash buffer 2 (lysis buffer containing 40 mM imidazole). Protein was eluted from the column using a stepwise imidazole gradient in elution buffer (50 mM, 100 mM, 200 mM) and collecting 5 mL fractions. SDS-PAGE analysis (4–15% Mini-PROTEAN TGX precast gel) was employed to determine the presence and purity of

protein in each fraction. Fractions containing the desired protein were combined and dialyzed twice against 2 L of dialysis buffer ($CylH_{PKS}$ and $CylH_{PKS}$-S1201A mutant = 50 mM potassium phosphate, 100 mM NaCl, pH 8.0; $CylH_{TE}$ and $CylH_{TE}$-S95A mutant = 25 mM Tris•HCl, 50 mM NaCl, pH 8.5). The collected proteins were further purified by size-exclusion gel filtration using FPLC. Fractions containing protein were combined and dialyzed against storage buffer ($CylH_{PKS}$ and $CylH_{PKS}$-S1201A mutant = 50 mM potassium phosphate, 100 mM NaCl, pH 8.0; $CylH_{TE}$ and $CylH_{TE}$-S95A mutant = 25 mM Tris•HCl, 50 mM NaCl, 10% glycerol, pH 8.5). The solutions were concentrated using a Spin-X® UF 20 mL centrifugal concentrator with a 100,000 ($CylH_{PKS}$) or 10,000 ($CylH_{TE}$) MWCO membrane (Corning®), and the concentrated protein solutions were frozen with liquid N_2 for storage at –80 ºC. This procedure afforded yields of 1.5–1.7 mg/L for the C-His_6-tagged $CylH_{PKS}$, 1.7–2.5 mg/L of the C-His_6-tagged $CylH_{PKS}$-S1201A mutant, 8.3 mg/L for the C-His_6 tagged $CylH_{TE}$, and 6.0 mg/L for the C-His_6 tagged $CylH_{TE}$-S95A mutant (**Fig. S2**).

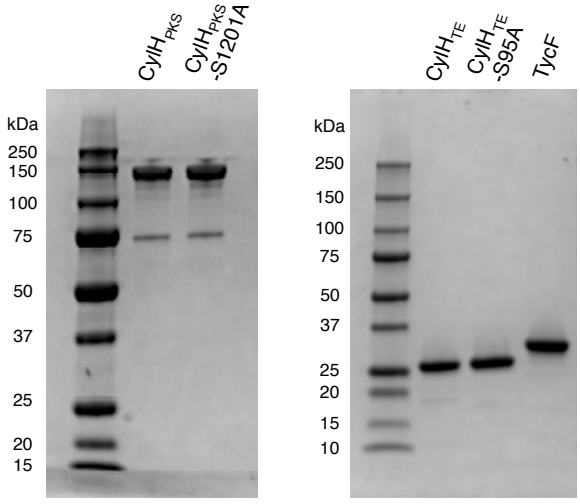


Fig. S2 SDS-PAGE of purified His_6-tagged CylI, $CylH_{PKS}$, and $CylH_{PKS}$ S1201A mutant run on 10% Mini-PROTEAN TGX precast gel (Bio-Rad) (*left*); $CylH_{TE}$, $CylH_{TE}$-S95A mutant, and TycF run on 4-15% Mini-PROTEAN TGX precast gel (Bio-Rad) (*right*). (MW=Precision Plus Protein™ All Blue Standards (Bio-Rad)).

Determination of the molecular mass of the $CylH_{PKS}$, $CylH_{TE}$, and the TE domain mutants

A ~50 μM solution of purified protein was analyzed by gel filtration during large-scale purification as described in 'general materials and methods'. The $CylH_{PKS}$ was eluted over 72 min with 50 mM potassium phosphate buffer pH 8, 100 mM NaCl at 0.5 mL/min using Superdex 200 30/100 GL column (**Fig. S3b**). $CylH_{PKS}$-S1201A mutant was eluted over 450 min with the same buffer as the wild type $CylH_{PKS}$ at 0.8 mL/min using a Superdex 200 26/600 PG column (**Fig. S3d**). $CylH_{TE}$ and $CylH_{TE}$-S95A mutant were eluted over 450 min with 25 mM Tris•HCl pH 8.5, 50 mM NaCl at 0.8 mL/min using a Superdex 200 26/600 PG column (**Fig. S3 e and f)**. A solution of molecular weight markers was analyzed under the same conditions (**Fig. S3 a and c)**.

Molecular weight analysis by gel filtration shows that $CylH_{PKS}$ exists as mixture of oligomers and dimers in solution (**Table S2**). Fractions that contained dimeric $CylH_{PKS}$ were collected separately and used for subsequent biochemical assays. $CylH_{PKS}$-S1201A mutant mainly exists as dimers. $CylH_{TE}$ and $CylH_{TE}$-S95A both exist as monomers. Interestingly, known structures of type I TEs on type I modular PKS assembly lines are dimers while type II TEs are monomers.[6]

Table S2. Molecular weight analysis of $CylH_{PKS}$, $CylH_{TE}$ and their mutants by gel filtration

Protein	Calculated MW (kDa)	Observed MW (kDa)
$CylH_{PKS}$	152.7	739.8 411.5
$CylH_{PKS}$-S1201A mutant	152.7	402.1
$CylH_{TE}$	29.8	21.0
$CylH_{TE}$-S95A mutant	29.8	22.0

[a] Molecular weights were calculated for the C-terminal His_6-tagged constructs using ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/).

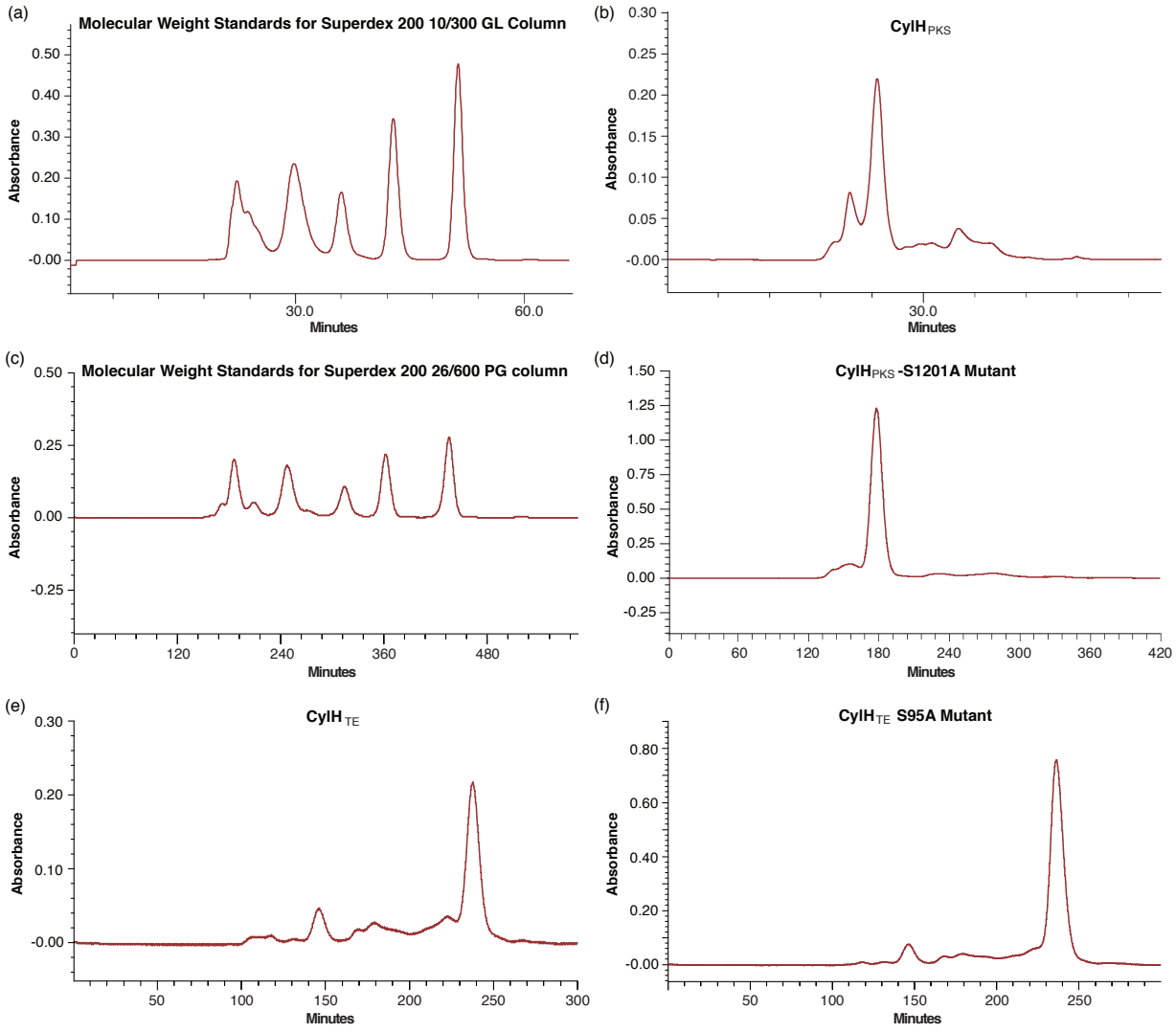


Fig. S3 Determination of CylH molecular mass via size exclusion chromatography. FPLC chromatogram using the Superdex 200 10/300 GL column of the (a) molecular weight standrards and (b) $CylH_{PKS}$; FPLC chromatogram using the Superdex 200 26/600 PG column of the (c) molecular weight standards, (d) $CylH_{PKS}$-S1201A mutant, (e) $CylH_{TE}$, and (f) $CylH_{TE}$-S95A mutant.

3. Biochemical characterization of $CylH_{PKS}$ and $CylH_{PKS}$-S1201A mutant

BODIPY-CoA fluorescent phosphopantetheinylation assay

BODIPY-CoA[7] and Sfp[8] were prepared using previously reported procedures. Reaction mixtures (25 μL) contained 2 μM $CylH_{PKS}$ (wild type or S1201A mutant), 10 μM Sfp, 25 μM BODIPY-CoA, 2 mM $MgCl_2$, 1 mM DTT and 50 mM potassium phosphate pH 8.0. Reactions were incubated in the dark at room temperature for 1 h. Reaction mixtures were diluted 1:1 in 2x Laemmli sample buffer (Bio-Rad), boiled for 10 min, and then separated by SDS-PAGE on a 4-15% Mini-PROTEAN TGX precast gel. The gel was first imaged at λ = 365 nm, then stained with Coomassie and imaged again (**Fig. S4**). The relative amount of BODIPY-CoA loading for $CylH_{PKS}$ and $CylH_{PKS}$-S1201A was quantified by taking the ratios of the band intensities of Coomassie stain and BODIPY absorption at 365 nm. The analysis shows that 80% of $CylH_{PKS}$ is loaded with BODIPY relative to $CylH_{PKS}$-S1201A (**Table S3**).

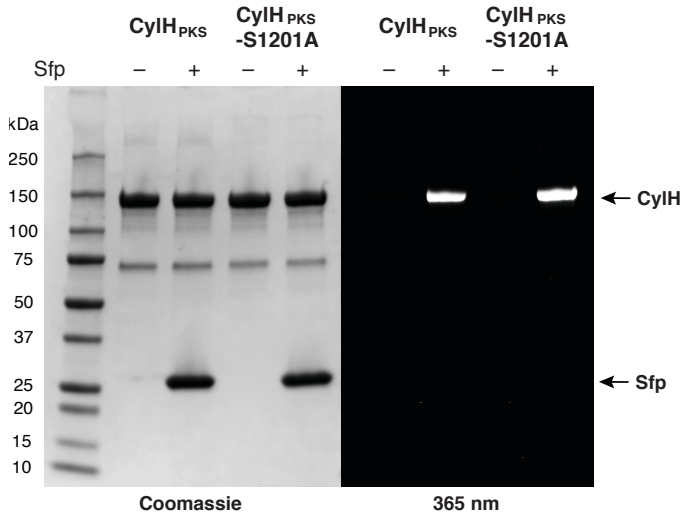


Fig. S4 Phosphopantetheinyl transferase Sfp can recognize and load the ACP domain of *apo* $CylH_{PKS}$ with BODIPY-CoA. MW = Precision Plus Protein™ All Blue Standards (Bio-Rad).

Table S3. Relative quantitation of BODIPY loading assay.

Sample	Coomassie signal intensity	BODIPY signal intensity (365 nm)	Signal ratio (BODIPY/Comassie)	Relative loading
$CylH_{PKS}$	1893474	1916200	1.012	80%
$CylH_{PKS}$-S1201A	2097282	2645045	1.261	100%

LC-MS assay for $CylH_{PKS}$ elongation reaction

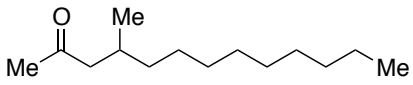
A stock solution of *holo* $CylH_{PKS}$ and *holo* $CylH_{PKS}$-S1201A mutant were prepared prior to the assay by incubating 30 μM *apo* $CylH_{PKS}$ (wild type or S1201A mutant), 5 μM Sfp, 250 μM CoA, and 1 mM $MgCl_2$ in 50 mM potassium phosphate buffer pH 8.0 in 100 μL volume for 1 h at room temperature. The elongation assay contained 100 μM acyl-SNAC substrate **10**, 24 μM *holo* $CylH_{PKS}$ (wild type or S1201A mutant), and 200 μM malonyl-CoA in 100 mM potassium phosphate buffer pH 8.0 in 100 μL volume. Reactions were incubated at room temperature for 2 h, and the 50 μL of each sample was quenched with 10 μL of 1 M KOH. Quenched samples were heated to 65 ºC for 20 min and then acidified with 10 μL of 1.2 M HCl. 50 μL of cold acetonitrile was added to each sample and the samples were incubated overnight at –20 ºC. Samples were thawed on ice and then centrifuged (13,200 rpm x 10 min at 4 ºC) before LC-MS analyses using a high accuracy Bruker qTOF mass spectrometer in the Small Molecule Mass Spectrometry Facility, Harvard Faculty of Arts and Sciences (FAS) Division of Science.

The UHPLC equipped with a G4220A binary pump, a built-in vacuum degasser and a thermostatted G4226A high performance autosampler was used for the analysis. 8 μL Samples were analyzed by LC-MS (8 μL injection volume) on an XTerra MS C18 analytical column (2.1 x 50 mm, 3.5 μm) equipped with a guard column (2.1 x 10 mm, 3.5 μm, Waters Corporation) at a flow rate of 0.35 mL/min with the column temperature maintained at room temperature. The following elution conditions were applied: a gradient increasing from 20% to 70% solvent B in solvent A over 5 min, a gradient increasing to 100% solvent B over 0.1 min, 100% solvent B for 5 min, a gradient decreasing to 20% solvent B in solvent A over 0.1 min, and 20% solvent B in solvent A for 5 min. (solvent A = 10 mM ammonium formate and 0.03% ammonium hydroxide in water; solvent B = 0.03% ammonium hydroxide in acetonitrile).

For the MS detection, the ESI mass spectra data were recorded on a negative ionization mode for a mass range of m/z 50 to 1200; calibration mode, HPC; spectra rate, 1.00 Hz; capillary voltage, 3400 V; nebulizer pressure, 30.0 psi; drying gas (N_2) flow, 10.0 L/min; drying gas (N_2) temperature, 220 ºC. A mass window of ± 0.005 Da was used to extract the ion of $[M-H]^-$. Hydrolysis product **11** was detected only in the full assay with mass accuracy within 5 ppm, a

match between the observed and theoretical isotopic patterns, and a match between the actual samples and the synthetic standard (**Table S4** and **Fig. S6**).

We initially monitored for masses of hydrolysis product **11** as well as ketone **12**, which could form through decarboxylation of **11** (**Fig. S5**). The amount of **12** detected in the assays, however, was very small, and the mass was not reproducibly detected in all the assays. As a consequence, we only monitored hydrolysis product **11** for the LC-MS assays.



12

Figure S5. Structure of ketone product resulting from decarboxylation of hydrolysis product **11**

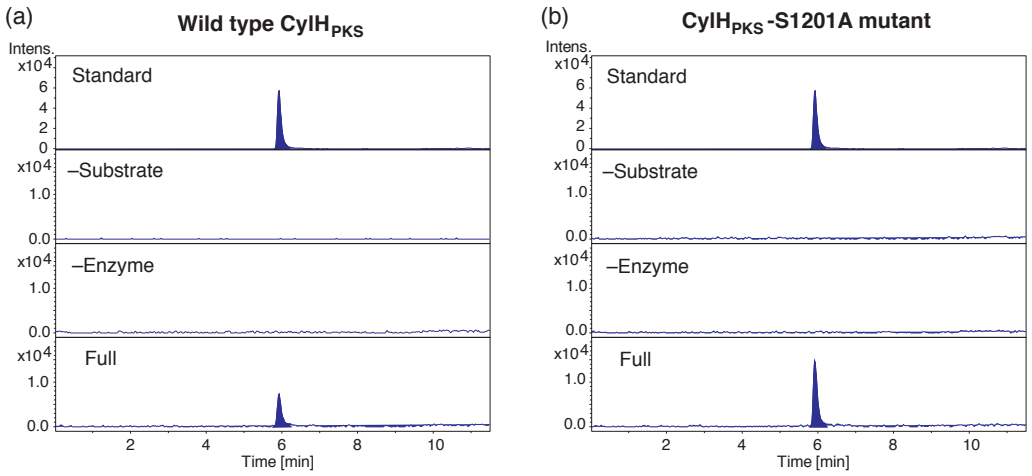


Figure S6. LC-MS assay shows that CylH$_{PKS}$ can catalyze the elongation reaction. Hydrolysis product **11** is only observed in the full assay and not in the negative controls for both CylH$_{PKS}$ and CylH$_{PKS}$-S1201A mutant. (a) Extracted ion chromatogram (EIC) of hydrolysis product **11** in assay with wild type CylH$_{PKS}$, (b) CylH$_{PKS}$-S1201A mutant.

Table S4. LC-MS analysis of standards for CylH$_{PKS}$ elongation assays.

Sample	Formula	Charge state	Calculated Mass	Observed Mass	Error (ppm)
Acyl-SNAC substrate **10**	$C_{17}H_{33}NO_2S$	$[M+H]^+$	316.2305	316.2306	0.3
Hydrolysis product **11**	$C_{15}H_{28}O_3$	$[M–H]^-$	255.1966	255.1963	-1.2

HPLC assay for CylI resorcinol formation with CylH acyl-ACP substrate

Stock solutions of *holo* $CylH_{PKS}$ and *holo* $CylH_{PKS}$-S1201A mutant were prepared prior to the assay by incubating 50 μM *apo* $CylH_{PKS}$, 5 μM Sfp, 250 μM CoA, and 1 mM $MgCl_2$ in 50 mM potassium phosphate buffer pH 8.0 in 100 μL volume for 1 h at room temperature. A typical CylI assay contained 100 μM substrate **5**, 20 μM *holo* $CylH_{PKS}$ (wild type or S1201A mutant), 1.2 μM CylI, 500 μM malonyl-CoA, and 1 mM EDTA in 100 mM potassium phosphate buffer pH 8.0 in a final assay volume of 100 μL. The reaction was incubated at room temperature and 30 μL of the reaction was quenched with 60 μL of cold acetonitrile after 15 min, 30 min, 1 h, and 2 h. The quenched sample was incubated on ice for 10 min and then centrifuged (13,200 rpm x 10 min at 4 ºC). The supernatant was analyzed by HPLC (80 μL injection volume) on a Chromolith RP-18e column (4.6 x 100 mm, EMD Millipore) at a flow rate of 1 mL/min and monitoring at 210 nm. The following elution conditions were applied: 20% solvent B in solvent A for 2 min, a gradient increasing to 100% solvent B in solvent A over 15 min, 100% solvent B for 5 min, a gradient decreasing to 20% solvent B in solvent A over 1 min, 20% solvent B in solvent A for 6 min (solvent A = water; solvent B = acetonitrile).

Resorcinol formation is not observed in the absence of CylI, MalonylCoA or substrate **10** (**Fig. S7**). A small amount of resorcinol **8** is formed in the absence of $CylH_{PKS}$ due to promiscuous activity of CylI toward substrate **10**. Increasing the ratio of $CylH_{PKS}$ to CylI did not change the qualitative rate of resorcinol formation, which implies that the CylI-catalysed resorcinol formation is the rate-limiting step in these assays (data not shown).

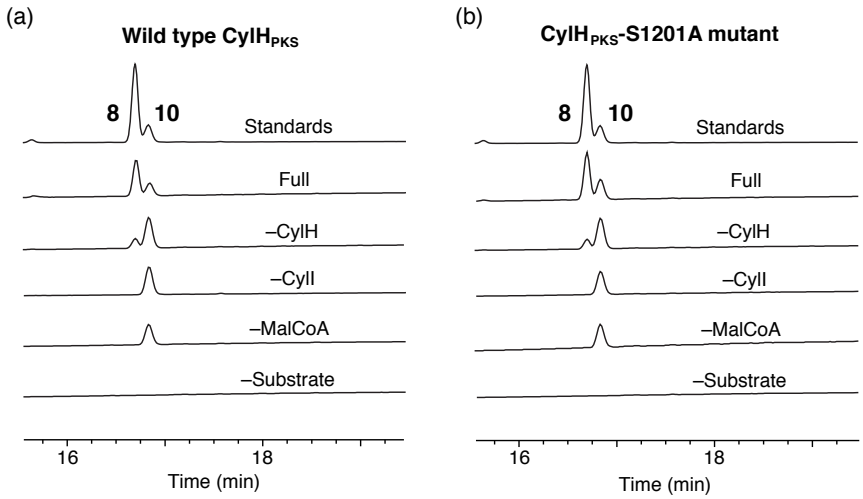


Figure S7. HPLC analysis of the full assay and the negative controls of the CylI catalyzed resorcinol **8** formation using CylH acyl-ACP substrate at 2 h time point. (A) Assays performed with wild type CylH$_{PKS}$, and (B) assays performed with CylH$_{PKS}$-S1201A mutant (210 nm).

Quantitative LC-MS assay for CylI resorcinol formation with CylH acyl-ACP substrate

Stock solutions of *holo* CylH$_{PKS}$ and *holo* CylH$_{PKS}$-S1201A mutant were prepared prior to the assay by incubating 50 μM *apo* CylH$_{PKS}$, 5 μM Sfp, 250 μM CoA, and 1 mM $MgCl_2$ in 50 mM potassium phosphate buffer pH 8.0 in 100 μL volume for 1 h at room temperature. A typical quantitation assay contained 100 μM substrate **10**, 20 μM *holo* CylH$_{PKS}$ (wild type or S1201A mutant), 1.2 μM CylI, 500 μM malonyl-CoA, and 1 mM EDTA in 100 mM potassium phosphate buffer pH 8.0 in a final assay volume of 50 μL. The reaction was incubated at room temperature for 2 h, and then 10 μL of an internal standard stock solution containing 120 μM hydrolysis product internal standard **13** and 120 μM d_3-resorcinol **14** was added to each sample (**Fig. S8**). The reaction was quenched with 50 μL of cold acetonitrile, and then the quenched sample was incubated overnight at –20 ºC. The sample was thawed on ice, centrifuged (13,200 rpm x 10 min at 4 ºC), and analyzed using an Agilent QQQ mass spectrometer in the Small Molecule Mass Spectrometry Facility, Harvard Faculty of Arts and Sciences (FAS) Division of Science.

The UHPLC equipped with a G4220A binary pump, a built-in vacuum degasser and a thermostatted G4226A high performance autosampler was used for the analysis. Calibration standards and samples (4 μL injection volume) were analyzed using an ACQUITY^{TM} UPLC BEH C_{18} analytical column (2.1 x 50 mm, 1.7 μm) equipped with a VanGuard BEH C_{18} Pre-

column (2.1 x 5 mm, 1.7 µm, Waters Corporation) at the flow rate of 0.6 mL/min. The column temperature was maintained at room temperature. The following elution conditions were applied: a gradient increasing from 20 to 65% solvent B in solvent A for 2.91 min, a gradient increasing from 65 to 100% solvent B in solvent A over 1.01 min, 100% solvent B for 3.5 min, a gradient decreasing to 20% solvent B in solvent A over 0.1 min, 20% solvent B for 2.35 min. (solvent A = 5 mM ammonium hydroxide in water; solvent B = 5 mM ammonium hydroxide in acetonitrile).

For the MS detection, the ESI mass spectra data were recorded on a negative ionization mode by MRM. The precursor → product ions MRM transition used for hydrolysis product **11** were m/z 255.19 $[M-H]^-$ → 211.1 $[C_{14}H_{27}O]^-$ (fragmentor voltage 115 V, collision energy (CE) = 12 V) as the quantifier and m/z 255.19 $[M-H]^-$ → 57 $[C_3H_5O]^-$ (fragmentor voltage 115 V, CE = 24 V) as the qualifier (**Fig. S9a**); for the hydrolysis product internal standard **13**, they were m/z 213.15 $[M-H]^-$ → 169 $[C_{11}H_{21}O]^-$ (fragmentor voltage 110 V, CE = 8 V) as the quantifier and m/z 213.15 $[M-H]^-$ → 153 $[C_{10}H_{17}O]^-$ (fragmentor voltage 110 V, CE = 16 V) as the qualifier (**Fig. S9b**); for resorcinol **8** they were m/z 277.22 $[M-H]^-$ → 235.2 $[C_{16}H_{27}O]^-$ (fragmentor voltage 155 V, CE = 20 V) as the quantifier and m/z 277.2 $[M-H]^-$ → 121.9 $[C_7H_6O_2]^-$ (fragmentor voltage 155 V, CE = 24 V) as the qualifier (**Fig. S9c**); for d_3-resorcinol **14** they were m/z 280.24 $[M-H]^-$ → 238.2 $[C_{16}H_{24}D_3O]^-$ (fragmentor voltage 155 V, CE = 20 V) as the quantifier and m/z 277.2 $[M-H]^-$ → 121.9 $[C_7H_6O_2]^-$ (fragmentor voltage 155 V, CE = 24 V) as the qualifier (**Fig. S9d**). Nitrogen was used as the drying, sheath, and collision gas. All the source and analyzer parameters were optimized using Agilent MassHunter Source and iFunnel Optimizer and Optimizer software, respectively. The source parameters are as follows: drying gas temperature 320 ºC, drying gas flow 13 L/min, nebulizer pressure 45 psi, sheath gas temperature 300 ºC, sheath gas flow 11 L/min, capillary voltage 3000 V, and nozzle voltage 0 V. The UHPLC eluant before 0.5 min was diverted to waste.

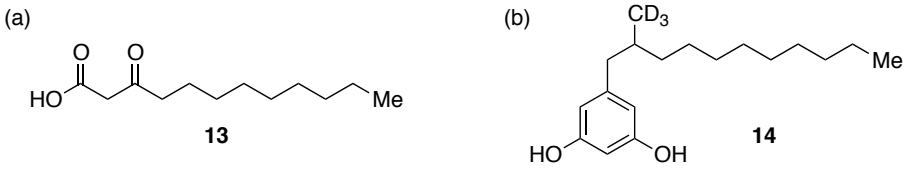


Figure S8. Structures of internal standards used for the LC-MS quantitation assay. (a) Structure of hydrolysis product internal standard **13** and (b) d_3-resorcinol **14**.

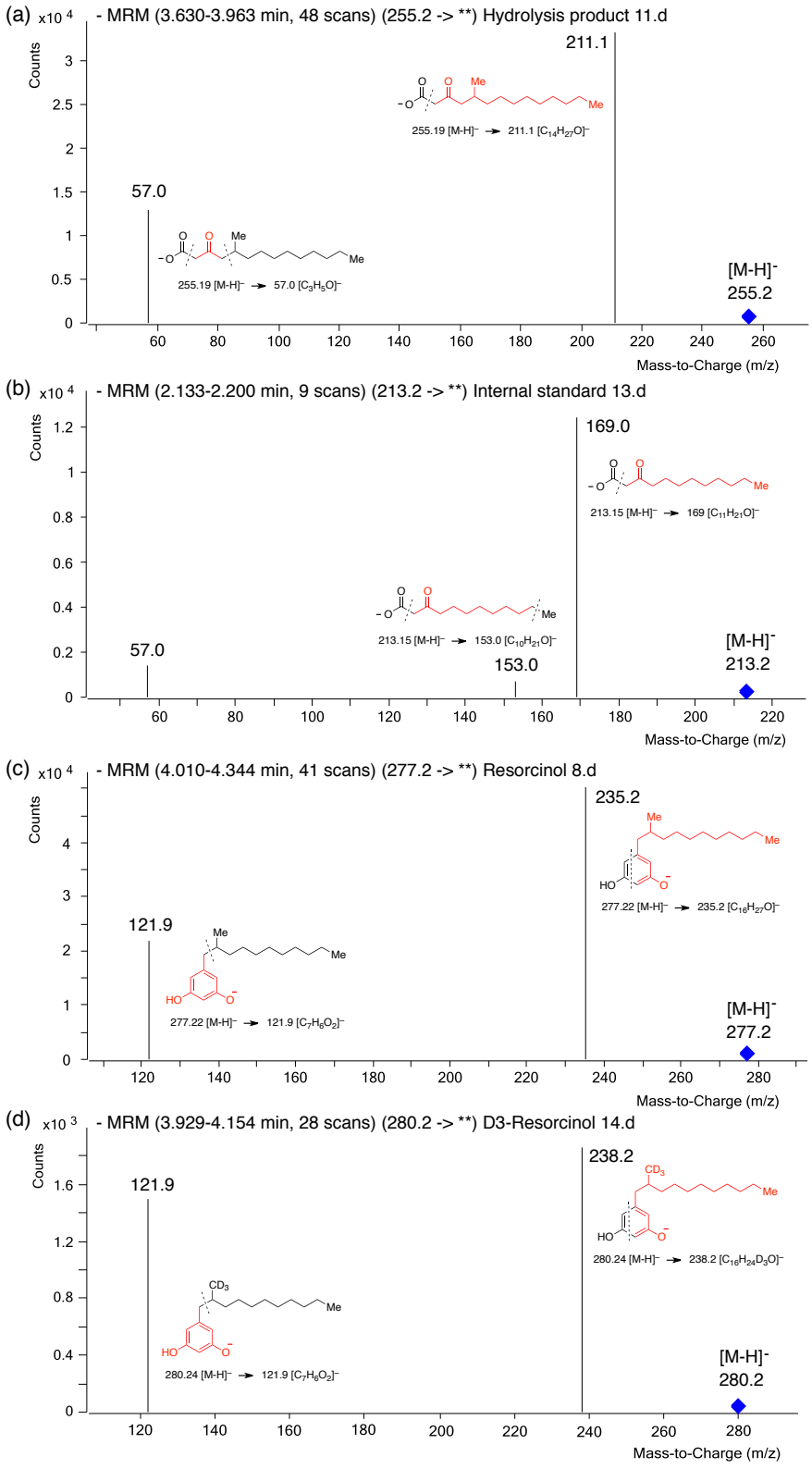


Figure S9. MRM transitions monitored in quantitative LC-MS. (a) MRM transitions of hydrolysis product **10**, (b) internal standard **13**, (c) resorcinol **8**, and (d) d_3-resorcinol **14**.

4. Biochemical characterization of $CylH_{TE}$ and $CylH_{TE}$-S95A mutant

HPLC assay for CylI resorcinol formation in presence of $CylH_{TE}$

A typical assay contained 100 µM substrate **9**, 10 µM of $CylH_{TE}$ (wild type or S95A mutant), 1.2 µM CylI, 250 µM malonyl-CoA, and 1 mM EDTA in 100 mM potassium phosphate buffer pH 8.0 in a final assay volume of 100 µL. The reaction was incubated at room temperature and a 30 µL reaction aliquot was quenched with 60 µL of cold acetonitrile after 5 min, 10 min, 15 min, 30 min, 1 h, and 2 h. The quenched sample was incubated on ice for 10 min and centrifuged (13,200 rpm x 10 min at 4 ºC). Supernatant was analyzed by HPLC (80 µL injection volume) on a Chromolith RP-18e column (4.6 x 100 mm, EMD Millipore) at a flow rate of 1 mL/min. The following elution conditions were applied: 20% solvent B in solvent A for 2 min, a gradient increasing to 100% solvent B in solvent A over 15 min, 100% solvent B for 5 min, a gradient decreasing to 20% solvent B in solvent A over 1 min, 20% solvent B in solvent A for 6 min (solvent A = water; solvent B = acetonitrile).

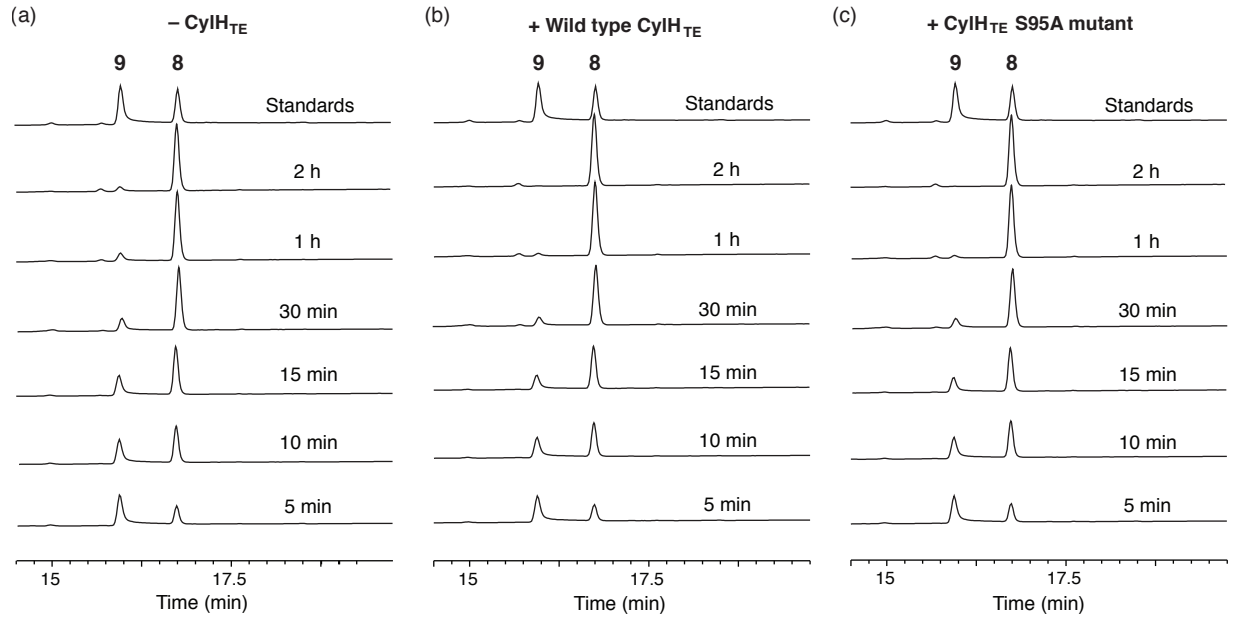


Figure S10. HPLC analysis of CylI-catalyzed resorcinol formation with or without $CylH_{TE}$ shows that the rate of reaction does not appear to be impacted by the presence of the $CylH_{TE}$ (monitored at 230 nm). (a) Time course of resorcinol formation in the absence of $CylH_{TE}$, (b) in the presence of wild type $CylH_{TE}$ and (c) in the presence of $CylH_{TE}$ S95A mutant.

Spectrophotometric assay for the hydrolysis activity of $CylH_{TE}$

A continuous spectrophotometric assay using dithionitrobenzoic acid (DTNB) reduction to thionitrobenzoic acid was employed to monitor generation of free thiol after thioester hydrolysis. Each assay contained 50 mM tris•HCl buffer pH 8, 50 mM NaCl, 2 mM DTNB, 50 µM EDTA, 2 µM enzyme ($CylH_{TE}$, $CylH_{TE}$-S95A mutant, or TycF), and acetyl-SNAC (0.1 mM, 0.5 mM, 1 mM, 2.5 mM, 5 mM, or 10 mM) in a final volume of 100 µL. The reaction was initiated by adding acetyl-SNAC and monitored at 412 nm by Spectramax i3 Plate Reader. Michaelis-Menten kinetics were calculated employing the value ε = 14,140 $M^{-1}cm^{-1}$ and plotted (**Fig. S11**). The mean ± SEM values of the kinetic parameters are shown in **Table S5**. The signal due to background hydrolysis of acetyl-SNAC was taken into consideration by subtracting the averaged signal of the negative controls lacking an enzyme performed in triplicate.

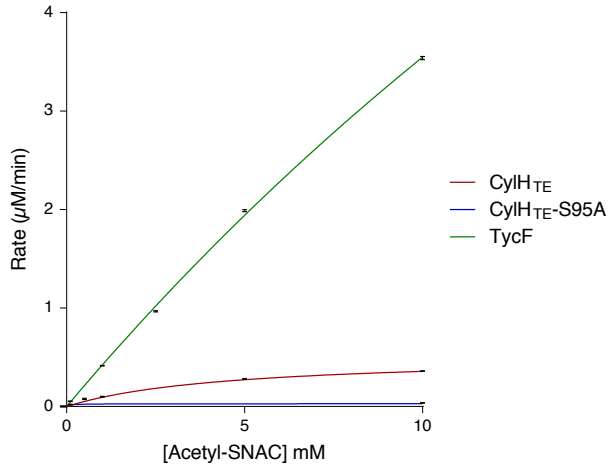


Figure S11. Michaelis-Menten analysis of the spectrophotometric assay shows that wild type $CylH_{TE}$ hydrolyses acetyl-SNAC, while $CylH_{TE}$-S95A mutant is inactive. Data points represent the average of results from triplicate reactions with standard error bars.

Table S5. Kinetic parameters of $CylH_{TE}$ and TycF thioesterase activity on acetyl-SNAC.

Enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
$CylH_{TE}$	4.5 ± 1.7	0.26 ± 0.04	1.0 ± 0.4
$CylH_{TE}$-S95A	N/A	N/A	N/A
TycF	47.9 ± 9.7	10.28 ± 1.78	3.6 ± 1.0

Assessment of the substrate scope of $CylH_{TE}$

A continuous spectrophotometric assay using dithionitrobenzoic acid (DTNB) reduction to thionitrobenzoic acid was employed to monitor generation of free thiol after thioester hydrolysis. Each assay contained 50 mM tris•HCl buffer pH 8, 50 mM NaCl, 2 mM DTNB, 50 µM EDTA, 2 µM $CylH_{TE}$ and acyl-SNAC (0.1 mM, 0.5 mM, 1 mM, 2.5 mM, 5 mM, or 10 mM) in a final volume of 100 µL. The reaction was initiated by adding acyl-SNAC and monitored at 412 nm by Spectramax i3 Plate Reader. Michaelis-Menten kinetics were calculated employing the value ε = 14,140 $M^{-1}cm^{-1}$ and plotted (**Fig. S12**). For the hexanoyl-SNAC assay, which did not follow Michaelis-Menten kinetics, the k_{cat}/K_M value was calculated from the linear slope of the plot at low substrate concentration. The mean ± SEM values of the kinetic parameters are shown in **Table S6**. The signal due to background hydrolysis of acyl-SNAC was taken into consideration by subtracting the averaged signal of the negative controls with $CylH_{TE}$-S95A mutant performed in duplicate.

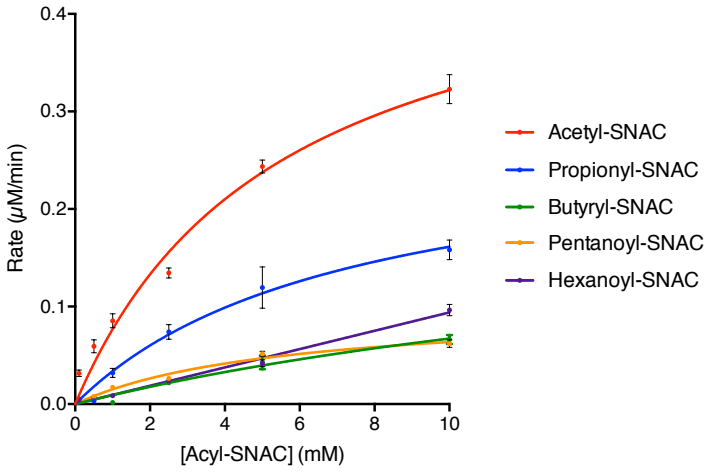


Figure S12. Michaelis-Menten analysis of the spectrophotometric assay shows that wild type $CylH_{TE}$ has greater activity for acyl-SNAC substrates with shorter chain length (acetyl- and propionyl-SNACs). Data points represent the average of results from duplicate reactions with standard error bars.

Table S6. Kinetic parameters of $CylH_{TE}$ activity on other short chain acyl-SNAC.

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Propionyl-SNAC	7.3 ± 2.5	0.14 ± 0.03	0.32 ± 0.12
Butyryl-SNAC	23.1 ± 17.9	0.11 ± 0.06	0.08 ± 0.08
Pentanoyl-SNAC	5.5 ± 1.8	0.05 ± 0.01	0.15 ± 0.05
Hexanoyl-SNAC	N/A	N/A	0.08 ± 0.01

5. Gel autoradiography assay of wild type $CylH_{PKS}$ and $CylH_{PKS}$-S1201A mutant using [Acetyl-1-^{14}C]-Coenzyme A

^{14}C-Acetyl-ACP was generated by activation with [acetyl-1-^{14}C]-CoA using the phosphopantetheinyl transferase Sfp. A typical assay contained 10 μM $CylH_{PKS}$ (wild type or S1201A mutant), 8 μM Sfp, 50 μM ^{14}C-acetyl-CoA (6.25 nCi), 1 mM $MgCl_2$, and 1 mM TCEP in 50 mM potassium phosphate buffer pH 8. The assay was initiated by addition of ^{14}C-acetyl-CoA and incubated at room temperature. After 15 min, 30 min, 1 h, and 2 h, a 10 μL reaction aliquot was removed and quenched by dilution into 10 μL of 2x SDS-loading buffer (no DTT or β-mercaptoethanol added). The quenched samples were heated at ~90 ºC for 10 min and then 10 μL of each sample was run on a SDS-PAGE gel (4-15% Mini-PROTEAN TGX precast gel). The gel was washed with water, stained with Coomassie, destained, dried using a gel dryer (Labconco), and then visualized by phosphorimaging (GE Typhoon Imager, FAS Center for Systems Biology). Reactions with $CylH_{PKS}$-S1201A mutant or no Sfp were used as negative controls. DTT and β-mercaptoethanol reducing agents were excluded from the loading dye since they are known to cleave acyl-ACP species.[9]

6. Bioinformatics of the CylH TE domain and other thioesterases

Multiple sequence alignment of the CylH TE domain with other thioesterases

52 amino acid sequences of type I and type II thioesterases from various PKS, NRPS, and hybrid PKS/NRPS biosynthetic systems were obtained from the NCBI public database (**Table S7**),[10] and the domain boundaries were determined using PKS/NRPS Analysis Web Server.[11] Multiple sequence alignment for the phylogenetic tree was performed by MUSCLE alignment using Geneious Pro Version 7.1.6.[5]

Table S7. Amino acid sequences of the TEs used in the MSA and the phylogenetic tree.

Thioesterase	Accession number
C-1027_TEII_P	4I4J_A
Calicheamycin_TEII_P	AAM94793
Bacitracin_TEI_N	O68008
Lichenysin_TEI_N	CAA06325
Surfactin_TEI_N	1JMK_C
Tyrocidine_TEI_N	O30409
Fengycin_TEI_N	AAB00093
Fusaricidin_TEI_N	ABQ96384
Mycosubtilin_TEI_N	Q9R9I9
Bacillorin_TEI_N	WP_007410142
Iturin_TEI_N	ABY89500
B. pseudomallei_TEI-1_PN B. pseudomallei_TEI-2_PN	WP_009967664
FmoA5_TEI-1_N FmoA5_TEI-2_N	BAP16699
Tyrocidine_TEII_N	WP_0158910190
Bacitracin_TEII_N	WP_020452080
DEBS_TEII_P	CAA42028
Megalomicin_TEII_P	AAG13923
Pikromycin_TEII_P	AAC69333
ScoT_TEII_P	WP_003972638
Borrelidin_TEII_P	WP_019330222
Tylosin_TEII_P	KDS84464
Kendomycin_TEII_P	CAQ52621
Rifamycin_TEII_P	AAG52991
Jerangolid_TEI_P	ABK32291
Ajudazol_TEI_PN	CAQ18835

Thioesterase	Accession number
Legioliulin_TEI_P	AIU36104
G. violaceus_TEI_P	WP_011141939
C. acetobutylicum_TEII_uk	WP_010964340
Gramicidin_TEII_N_ B.pseudomycoides	WP_006096422
Cylindrocyclophane_TEI_P	AFV96142
M. aeruginosa_TEI_P	WP_024970096
Gramicidin_TEII_N_ A. migulans	P14686
Microcystein_TEII_N	ACA83967
A. variabilis_TEII_uk	WP_011321392
Soraphen_TEI_P	AAA79984
Amphotericin_TEI_P	AAK73503
Lasalocid_TEI_P	BAG85032
Spinosyn_TEI_P	AAG23262
Spirangien_TEI_P	CAD43451
Avermectin_TEI_P	WP_010982381
Pikromycin_TEI_P	AAC69332
DEBS_TEI_P	WP_011873139
Disorazol_TEI_PN	CAI43932
Melithiazol_TEI_PN	CAD89778
Myxothiazol_TEI_PN	AAF19815
DKxanthene_TEI_PN	WP_011554299
Tubulysin_TEI_PN	CAF05651
Chondramid_TEI_PN	Q0VZ70
Cryptophycin_TEI_PN	ABM21572
Hectochlorin_TEI_PN	AAY42398

Abbreviations: TEI = type I TE, TEII = type II TE, P = from PKS assembly lines, N = from NRPS assembly lines, PN = from PKS-NRPS hybrid assembly lines, uk = unknown.

Generation of homology model using HHpred

CylH TE domain was used as the query to search for structural homologs using HHpred.[12] The two highest hits were known type II TEs from rifamycin and prodiginine biosynthetic pathways (RifR[13] and RedJ,[14] respectively). A homology model of the CylH TE domain was generated by Modeller[15] using the crystal structure of RifR as a template and the resulting PDB file was aligned with the RifR PDB file (3FLA) using MacPyMOL version 1.7.[16]

Construction of phylogenetic tree

52 amino acid sequences were aligned by MUSCLE, and phylogenetic trees were created using MrBayes[17] provided by Geneious Pro Version 7.1.6[5] following a method described in the study of the TE domain in the ajudazole biosynthetic pathway.[18] The Bayesian inference method used the Poisson process with an outgroup set as a type II thioesterase C-1027 from the enediyne polyketide biosynthetic pathway, which has a hotdog fold unlike other TEIIs and TEIs.[19] Markov chain Monte Carlo analysis (MCMC) was performed with 1.1 million generations and four independent chains, and the Markov chain was sampled every 200 generations.

BLAST searches for other TE domains with sequence homology to type II TEs

The CylH TE domain amino acid sequence was used as the query for a BLASTp search on NCBI.[10] Any protein hits with lengths shorter than 300 amino acids were removed to eliminate freestanding type II thioesterases from the search results. The search results were manually curated to remove hits that appeared to be identical from the organisms that have multiple protein sequences in the NCBI database. All the hits that did not appear to be type I PKSs or NRPSs were removed from the search results to give 84 unique hits (**Table S8**). The domain organization of each protein hit was determined using the Conserved Domain on NCBI and the PKS/NRPS Analysis tool.[10] Protein hits with an E value greater than 1 E-10 were removed from the list, and the TE domain hits were confirmed to have higher homology to type II TEs by phylogenetic analysis using the Mr. Bayes tool and multiple sequence alignment using the MUSCLE alignment tool on Geneious.[11]

Table S8. BLASTp search result for type II TE-like domains on type I PKS and NRPS enzymes.

#	Organism	Accession number	Length (aa)	Annotated function	Domain organization	E value
1	*Cylindrospermum stagnale*	WP_015207397.1	2241	PKS	ECH-ER-KS-AT-T-TE	1.40E-163
2	*Pleurocapsa* sp. PCC 7319	WP_019504305.1	1593	PKS	KS-AT-DH-T-TE	1.87E-77
3	*Cyanothece* sp. PCC 7424	WP_015954747.1	1337	PKS	KS-AT-T-TE	4.42E-76
4	*Gloeocapsa* sp. PCC 73106	WP_006530113.1	1316	PKS	KS-AT-T-TE	1.14E-74
5	*Chlorogloeopsis fritschii*	WP_016872856.1	1281	PKS	KS-AT-T-TE	4.46E-73
6	*Microcystis aeruginosa*	WP_002776857.1	1320	PKS	KS-AT-T-TE	9.33E-61
7	*Cylindrospermum stagnale*	WP_015328279.1	2172	PKS	KS-AT-MT-KR-T-TE	1.47E-51
8	*Kamptonema* (multispecies)	WP_007358014.1	1312	PKS	KS-AT-T-TE	1.79E-49
9	*Burkholderia glumae*	WP_012732747.1	2381	PKS	KS-AT-DH-EH-KR-T-TE	6.15E-48
10	*Paenibacillus alvei*	WP_005550795.1	1815	NRPS	C-A-T-E-TE	9.76E-48
11	*Stigmatella aurantiaca*	WP_013375988.1	2092	PKS	KS-AT-DH-KR-T-TE	4.43E-44
12	*Chondromyces crocatus*	CAQ18835.1	1620	AjuH (PKS)[18]	KS-AT-DH-T-TE	3.81E-43
13	*Mesorhizobium amorphae*	WP_006199852.1	1488	PKS	TE-A-T-KS-AT	1.00E-42
14	*Gloeobacter violaceus*	WP_011141939.1	963	PKS	KS-T-TE	1.61E-41
15	*Sorangium cellulosum*	ABK32291.1	2869	JerE (PKS)[18]	KS-AT-KR-T-KS-AT-T-TE	2.92E-41
16	*Trichodesmium erythraeum*	WP_011613196.1	1354	PKS	KS-AT-T-TE	3.85E-41
17	*Mesorhizobium* sp. L48C026A00	WP_023803946.1	1920	PKS	TE-A-T-KS-AT-T	6.61E-41
18	*Sorangium cellulosum*	CCE88378.1	2453	PKS	KS-AT-DH-ER-KR-T-TE	3.49E-40
19	*Candidatus Magnetoglobus multicellularis str. Araruama*	ETR71534.1	1348	PKS	KS-DH-T-TE	7.53E-38
20	*Clostridium pasteurianum*	WP_023973746.1	1502	PKS	KS-AT-DH-T-TE	3.31E-37
21	*Rhizobium sullae*	WP_027513632.1	1773	PKS	KS-AT-KR-T-TE	1.06E-36
22	*Pseudomonas corrugata*	WP_024779985.1	1787	PKS	KS-AT-KR-T-TE	5.00E-36
23	*Chondromyces apiculatus* DSM 436	EYF05476.1	4240	PKS	KS-AT-T-KS-AT-KR-T-KS-AT-DH-T-TE	2.88E-35
24	*Sinorhizobium arboris*	WP_028001517.1	1768	PKS	KS-AT-KR-T-TE	3.20E-35

25	*Cyanothece* sp. ATCC 51142	WP_012362254.1	1285	PKS	KS-AT-T-TE	1.42E-34
26	*Bacillus cereus*	WP_002107075.1	1531	PKS	KS-AT-DH-T-TE	3.19E-34
27	*Clostridium* sp. CAG:411	WP_022438322.1	2203	PKS-NRPS	TE-A-KR-T-SDR	2.83E-33
28	*Legionella cherrii*	WP_028380815.1	1293	PKS	KS-DH-T-TE	5.58E-33
29	*Streptomyces longisporoflavus*	ACR50795.1	1502	PKS	KS-AT-DH-T-TE	1.23E-31
30	*Mesorhizobium ciceri*	WP_027038666.1	1758	PKS	KS-AT-KR-T-TE	9.68E-31
31	*Streptomyces mobaraensis*	WP_004946356.1	2674	PKS/NRPS	TE-A-T-KS-AT-TE-KR-T	1.60E-30
32	*Magnetospirillum* sp. SO-1	WP_008618542.1	2056	PKS	KS-AT-DH-KR-T-TE	3.58E-30
33	*Corallococcus coralloides*	WP_014397901.1	1359	PKS	DH-ER-KR-T-TE	6.46E-30
34	*Burkholderia* sp. UYPR1.413	WP_028371022.1	2062	PKS	KS-AT-DH-KR-T-TE	6.93E-30
35	*Nocardia* sp. BMG51109	WP_024805020.1	2730	PKS	KS-AT-DH-MT-ER-KR-T-TE	1.02E-29
36	*Bradyrhizobium japonicum*	WP_028153765.1	2060	PKS	KS-AT-DH-KR-T-TE	1.49E-29
37	*Streptomyces* sp. CNQ329	WP_027770067.1	1499	PKS	KS-DH-T-TE	1.76E-28
38	*Mesorhizobium loti*	WP_010915895.1	1780	PKS	KS-AT-KR-T-TE	3.99E-28
39	*Branchiostoma floridae*	XP_002598380.1	3473	PKS/NRPS	KS-AT-DH-ER-KR-T-TE-C-A	6.23E-28
40	*Pseudomonas aeruginosa*	WP_023124984.1	1392	NRPS	A-MT-T-C-T-TE	1.02E-27
41	*Streptomyces* sp. NRRL 11266	BAE93739.1	1508	PKS	KS-DH-T-TE	1.43E-27
42	*Burkholderia pseudomallei*	WP_024430902.1	2832	PKS/NRPS	TE-A-T-KS-AT-TE-KR-T	2.75E-27
43	*Burkholderia mallei*	WP_004195624.1	2366	PKS/NRPS	TE-A-T-KS-AT-TE	2.77E-27
44	*Paenibacillus* sp. OSY-SE	WP_019419387.1	1660	PKS	KS-AT-DH-T-TE	6.47E-27
45	*Azospirillum lipoferum*	WP_012978027.1	2157	PKS	KS-AT-DH-KS-T-TE	9.99E-27
46	*Fulvivirga imtechensis*	WP_009578358.1	1986	PKS	KS-AT-DH-KR-T-TE	2.23E-26
47	*Ornithinibacillus scapharcae*	WP_010094403.1	1319	PKS	KS-AT-DH-T-TE	3.19E-26
48	*Yersinia rohdei*	WP_004716672.1	1966	PKS	KS-AT-DH-ER-KR-T-TE	4.03E-26
49	*Bradyrhizobium japonicum* SEMIA 5079	AHY53341.1	2299	PKS	KS-AT-DH-KR-T-T-TE	4.31E-26
50	*Mesorhizobium* sp. WSM3224	WP_027170255.1	1843	PKS/NRPS	TE-A-T-KS-AT-T	5.53E-26
51	*Bacillus amyloliquefaciens* SQR9	AHZ14674.1	1632	PKS	KS-AT-DH-T-TE	1.07E-25
52	*Burkholderia thailandensis*	WP_011401503.1	2792	PKS/NRPS	TE-A-T-KS-AT-TE-KR-T	1.30E-25

53	*Nocardiopsis chromatogenes*	WP_026123546.1	2674	PKS	TE-A-T-KS-AT-TE-KR-T	1.47E-25
54	Uncultured bacterium psy1	ADA82585.1	12645	PKS/NRPS	KS-MT-T-C-A-T-KS-KR-T-KS-KR-MT-T-KS-DH-KR-T-KS-KR-MT-T-KS-KR-T-KS-MT-T-KS-T-KS-DH-T-TE	1.75E-25
55	*Branchiostoma floridae*	XP_002610053.1	3311	PKS	KS-AT-DH-KR-T-TE-C	2.62E-25
56	*Burkholderia oklahomensis*	WP_010117951.1	1596	PKS/NRPS	TE-A-T-KS-AT	4.63E-25
57	*Branchiostoma floridae*	XP_002613499.1	1705	PKS/NRPS	KR-T-TE-C-A	6.50E-25
58	*Branchiostoma floridae*	XP_002598386.1	3458	PKS/NRPS	KS-AT-DH-ER-KR-T-TE-C-A	9.03E-25
59	*Chondromyces apiculatus* DSM 436	EYF04558.1	1153	PKS	KS-DH-T-TE	5.47E-24
60	*Burkholderia thailandensis*	WP_009908075.1	1559	PKS/NRPS	TE-A-T-KS-AT	9.36E-23
61	*Hahella ganghwensis*	WP_020406515.1	2382	PKS	FAAL-KS-AT-KR-T-TE	9.67E-23
62	*Coccomyxa subellipsoidea* C-169	XP_005650993.1	15797	PKS	KS-MT-MT-MT-MT-T-T-T-T-KS-KR-T-T-KS-DH-ER-KR-T-T-KS-KR-T-KS-KR-T-T-T-KS-KR-T-T-KS-KR-T-KS-DH-KR-T-T-KS-DH-KR-T-T-KS-KR-T-TE-T-TE	1.73E-22 2.03E-22
63	*Propionibacterium propionicum*	WP_014846098.1	1536	PKS	KS-AT-DH-T-TE	1.67E-21
64	*Vibrio caribbenthicus*	WP_009601858.1	1072	NRPS	A-T-TE	2.16E-21
65	*Yersinia kristensenii*	WP_004388876.1	2392	PKS	KS-AT-DH-ER-KR-T-TE	3.35E-21
66	*Streptomyces* sp. A7248	AFS33452.1	1641	SiaI (PKS)[20]	KS-AT-DH-T-TE	1.06E-20
67	*Trichophyton interdigitale* MR816	KDB25511.1	2403	PKS	KS-AT-DH-ER-KR-T-TE	3.86E-20
68	*Mycobacterium tuberculosis* TKK_03_0022	KCD30187.1	881	NRPS	A-T-TE	6.35E-20
69	*Mycobacterium tuberculosis* MAL010108	KBG75468.1	1414	Phenyloxazoline synthase MbtB (NRPS)	T-Cy-A-T-TE	7.03E-20
70	*Mycobacterium bovis*	WP_024458669.1	991	NRPS	A-T-TE	1.06E-19
71	*Mycobacterium tuberculosis*	WP_003901388.1	1280	NRPS	C-A-T-TE	1.53E-19
72	*Mycobacterium bovis*	WP_019283915.1	1414	NRPS	C-A-T-TE	2.02E-19
73	*Arthroderma gypseum* CBS 118893	XP_003175627.1	2406	PKS	KS-AT-DH-ER-KR-T-TE	2.33E-19
74	*Mycobacterium abscessus*	WP_025239395.1	1257	PKS	TE-KS-AT-T	1.07E-18

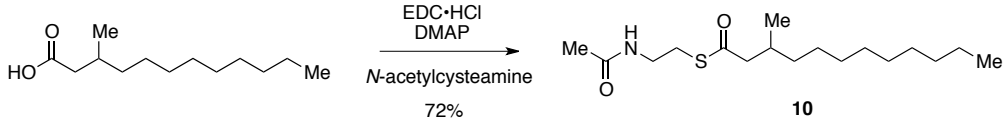
75	*Streptomyces fungicidicus*	ABD65958.1	8986	Enduracidin biosynthesis EndC (NRPS)[21]	C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-TE-TE	3.45E-18
76	*Saccoglossus kowalevskii*	XP_006823210.1	2198	PKS	AT-DH-ER-KR-T-TE	7.60E-18
77	*Segniliparus rotundus*	WP_013139326.1	1274	PKS	TE-KS-AT-T	2.48E-17
78	*Emiliania huxleyi* CCMP1516	XP_005790240.1	2677	PKS/NRPS	TE-A-T-KS-AT-TE-KR-T	1.88E-14
79	*Fusarium oxysporum* FOSC 3-a	EWY85877.1	2372	PKS	KS-AT-DH-ER-KR-T-TE	2.34E-14
80	*Strongylocentrotus purpuratus*	NP_001239013.1	2606	PKS	KS-AT-DH-ER-KR-T-TE	4.20E-14
81	*Phytophthora parasitica*	ETL32105.1	1311	NRPS	TE-A-T-Re	6.40E-14
82	*Nannochloropsis gaditana*	EWM26301.1	2887	PKS	KS-AT-DH-ER-KR-T-TE	6.02E-13
83	*Phytophthora infestans* T30-4	XP_002905400.1	1311	NRPS	TE-A-T-Re	6.73E-12
84	*Sorangium cellulosum*	ABK32263.1	1773	AmbH (PKS)[22]	KS-AT-KR-T-TE	1.28E-10

The results are ordered by E-value. Protein sequences from previously reported biosynthetic pathways are highlighted in green.

7. Chemical synthesis procedures and characterization data

The acetyl-SNAC used in the spectrophotometric assay for thioesterase activity was prepared following the previously reported synthesis.[23]

Synthesis of CylH acyl-SNAC substrate (10)



3-Methyldodecanoic acid was prepared following the previously reported synthesis.[3]

S-(2-acetamidoethyl)-3-methyldodecanethioate (**10**)

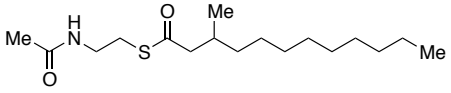
EDC•HCl (89 mg, 0.467 mmol) was added to a solution of 3-methyldodecanoic acid (50 mg, 0.233 mmol) in dichloromethane (2.5 mL) under argon cooled to 0 ºC. The mixture was stirred at 0 ºC for 20 min, and then *N*-acetylcysteamine (0.030 mL, 0.280 mmol) and 4-dimethylaminopyridine (2 mg, 0.016 mmol) were added. The reaction was warmed to room temperature. After 3 hours, water (10 mL) was added, and then the reaction mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The crude extract was purified with flash chromatography, eluting with 75–100% ethyl acetate in hexanes to afford *S*-(2-acetamidoethyl)-3-methyldodecanethioate **10** (53 mg, 0.17 mmol, 72%).

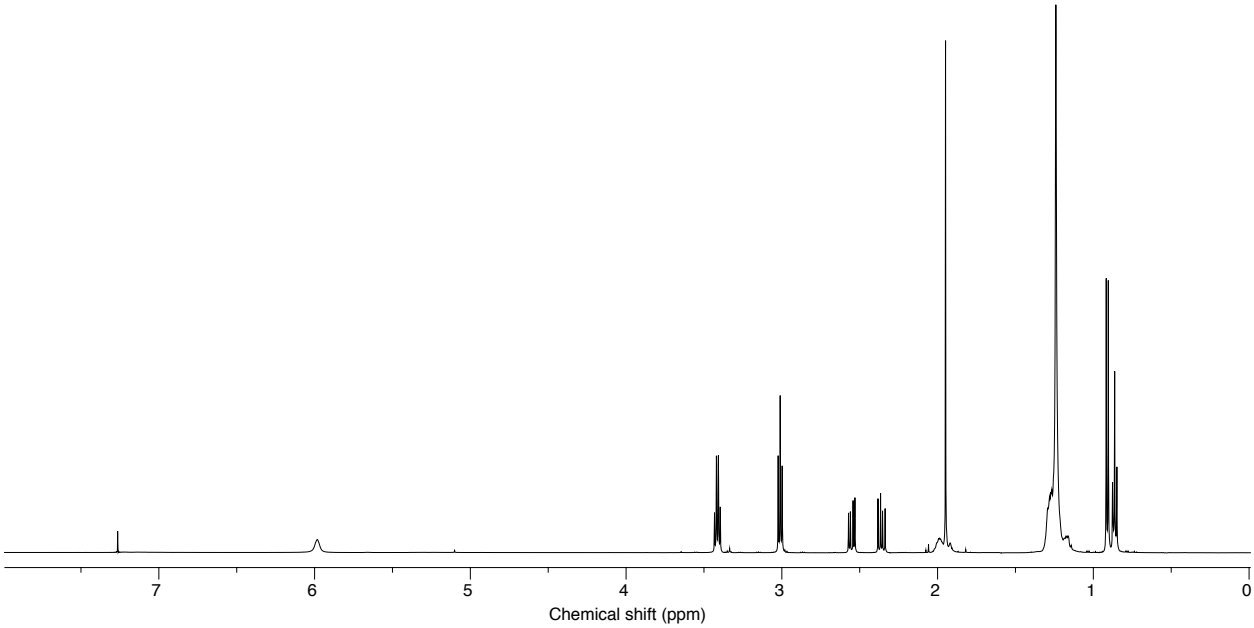
TLC: R_f = 0.24 (silica gel, 1:1 hexanes:ethyl acetate).

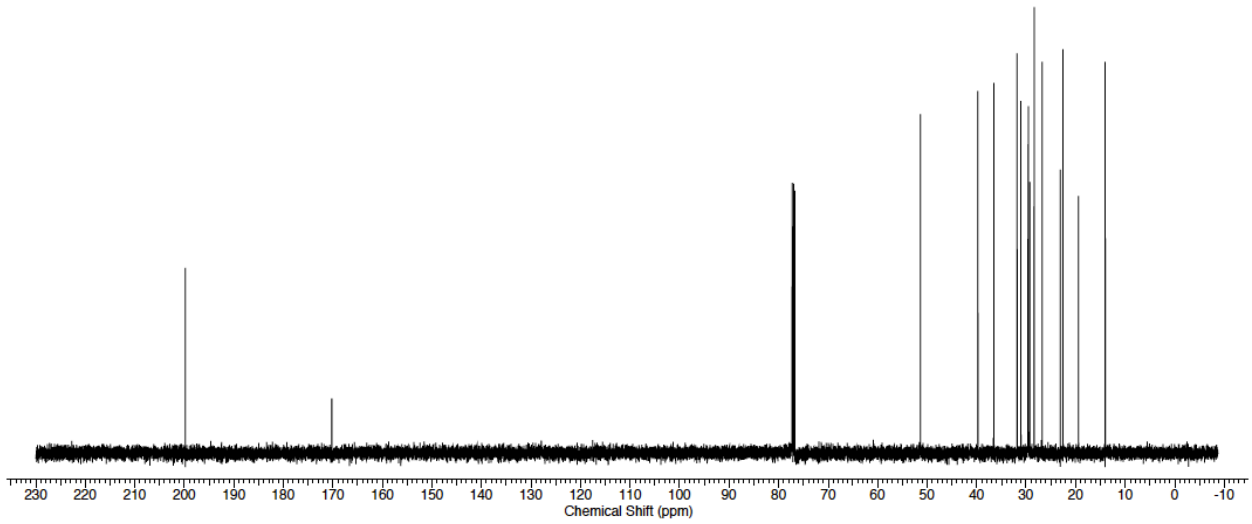
HRMS (ESI): calc'd for $C_{17}H_{34}NO_2S^+$ $[M+H]^+$, 316.2305; found, 316.2306.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.98 (s, 1H, N**H**), 3.41 (q, 2H, J = 6.2 Hz, NHC**H**$_2$), 3.01 (t, 2H, J = 6.5 Hz, CH_2C**H**$_2$S), 2.55 (dd, 1H, J = 14.5 Hz, 5.9, SCOC**H**$_2$), 2.36 (dd, 1H, J = 14.5, 8.2 Hz, SCOC**H**$_2$), 1.98 (m, 1H, C**H**), 1.95 (s, 3H, NHCOC**H**$_3$), 1.16-1.29 (m, 16H, C**H**$_2$), 0.91 (d, 3H, J = 6.7 Hz, CHC**H**$_3$), 0.86 (t, 3H, J = 6.9 Hz, CH_2C**H**$_3$).

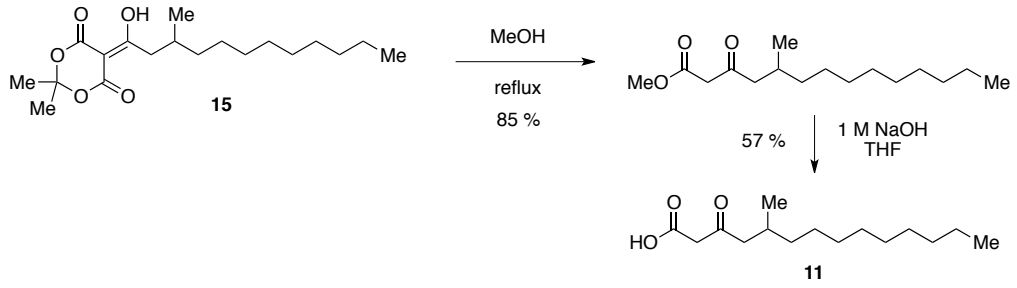
^{13}C NMR (125 MHz, CDCl3) δ: 199.84, 170.23, 51.34, 39.77, 36.56, 31.84, 31.09, 29.65, 29.56, 29.53, 29.26, 28.40, 26.79, 23.14, 22.62, 19.44, 14.06.







Synthesis of standard for hydrolysis product (11)



5-(1-Hydroxy-3-methyldodecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (**15**) was prepared following the previously reported synthesis.[3]

Methyl 5-methyl-3-oxotetradecanoate

15 (556 mg, 1.63 mmol) was dissolved in anhydrous methanol (10 mL), and the solution was refluxed overnight under a nitrogen atmosphere. The solvent was removed *in vacuo*, and the crude reaction mixture was purified by flash chromatography eluting with 0-20% ethyl acetate in hexanes to afford methyl 5-methyl-3-oxotetradecanoate as mixture of keto and enol tautomers (375 mg, 1.39 mmol, 85%).

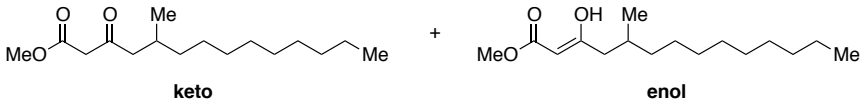
TLC: R_f = 0.92 (silica gel, 1:1 hexanes:ethyl acetate).

HRMS (ESI): calc'd for $C_{16}H_{31}O_3^+$ $[M+H]^+$, 271.2268; found 271.2257: calc'd for $C_{16}H_{30}NaO_3^+$ $[M+Na]^+$, 293.2087; found 293.2081: calc'd for $C_{16}H_{30}KO_3^+$ $[M+K]^+$, 309.1827; found 309.1816.

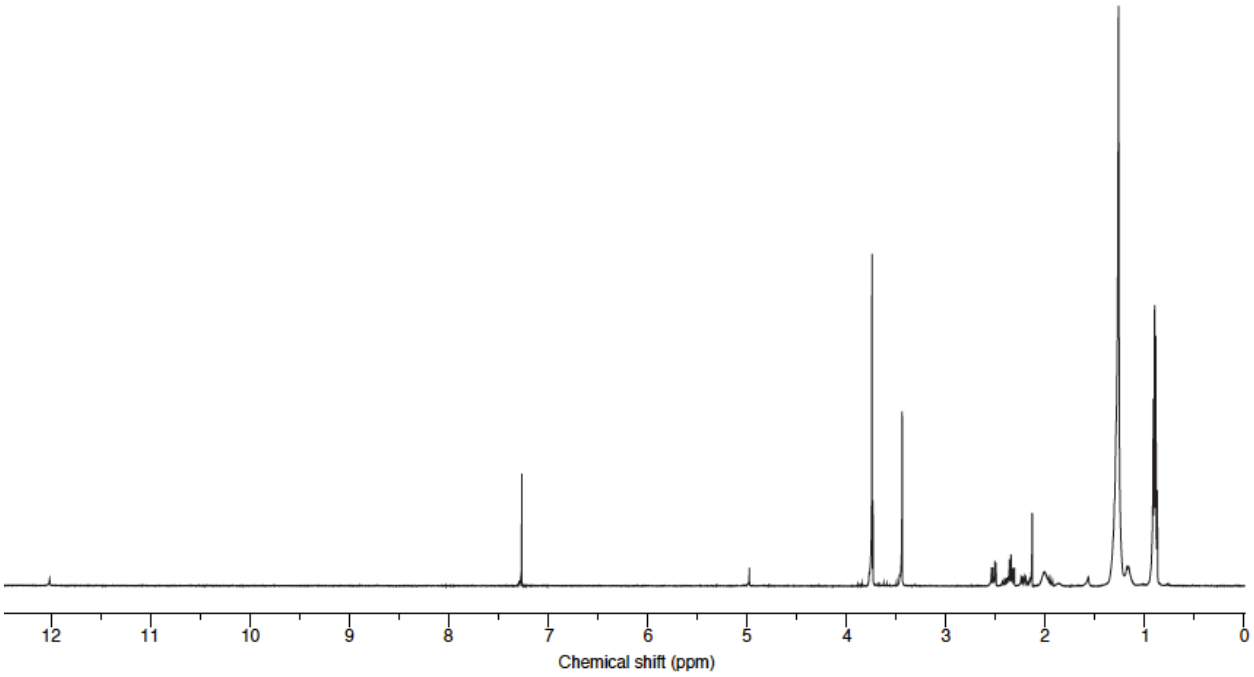
^{1}H-NMR (500 MHz; $CDCl_3$): δ 12.02 (s, 1H, enol-CHCO**H**), 4.98 (s, 1H, enol-C**H**COH), 3.78–3.74 (s, 3H, COOC**H**$_3$), 3.45 (s, 2H, keto-CH_3OCOC**H**$_2$), 2.52 (dd, 1H, *J* = 16.5, 5.6 Hz, keto-COC**H**$_2$), 2.41 (dd, 1H, *J* = 15.8, 5.7 Hz, enol-COC**H**$_2$), 2.34 (dd, 1H, *J* = 16.5, 8.0 Hz, keto-COC**H**$_2$), 2.25–2.19 (m, 1H, enol-COC**H**$_2$), 2.05–1.95 (m, 1H, C**H**), 1.21–1.35 (m, 16H, C**H**$_2$), 0.96–0.86 (m, 6H, C**H**$_3$).

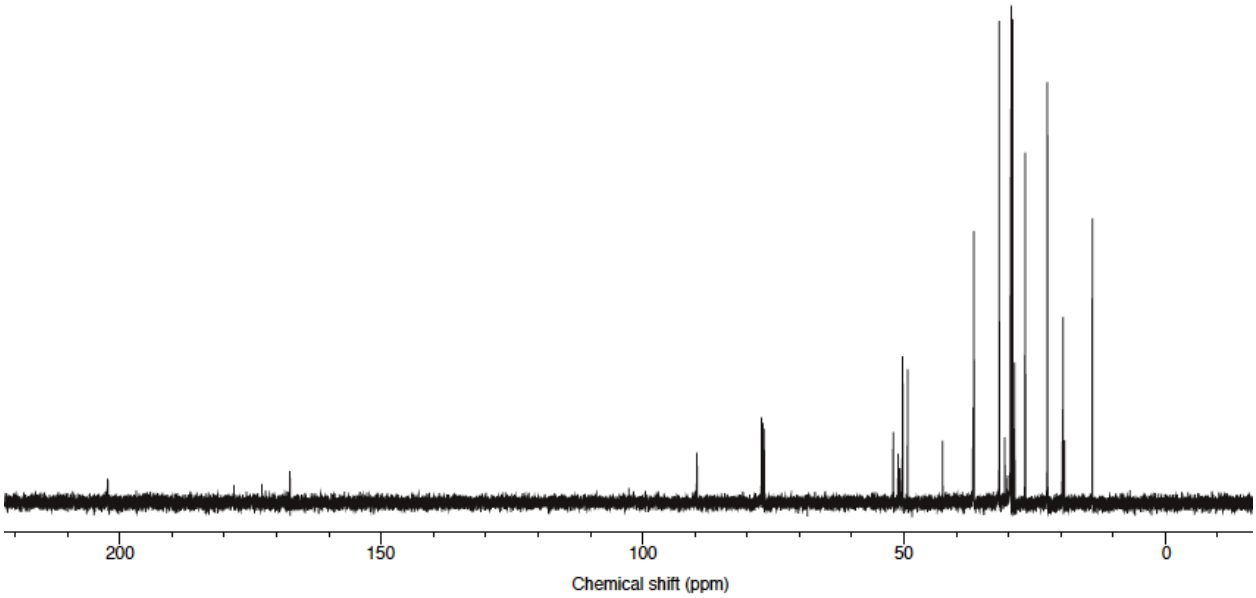
^{13}C-NMR (125 MHz; $CDCl_3$): δ 202.47, 178.34, 172.98, 167.63, 89.77, 52.20, 51.28, 50.41,

49.45, 42.76, 36.96, 36.80, 31.95, 30.89, 29.82, 29.78, 29.67, 29.65, 29.38, 29.00, 26.97, 22.72, 19.82, 19.72, 14.11.



keto **enol**





5-Methyl-3-oxotetradecanoic acid (Hydrolysis product **11**)

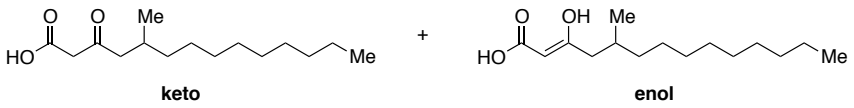
Methyl 5-methyl-3-oxotetradecanoate (50 mg, 0.185 mmol) was dissolved in isopropanol (2 mL), and 1 M NaOH aqueous solution (2 mL) was added. The reaction was stirred at room temperature for 3 h. The reaction was quenched with 1 M HCl (5 mL), and the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The residue was purified by flash chromatography, eluting with 40–60% ethyl acetate in hexanes to give **11** as mixture of keto and enol tautomers (27 mg, 0.105 mmol, 57%).

TLC: R_f = 0.12 (silica gel, 1:1 hexanes:ethyl acetate).

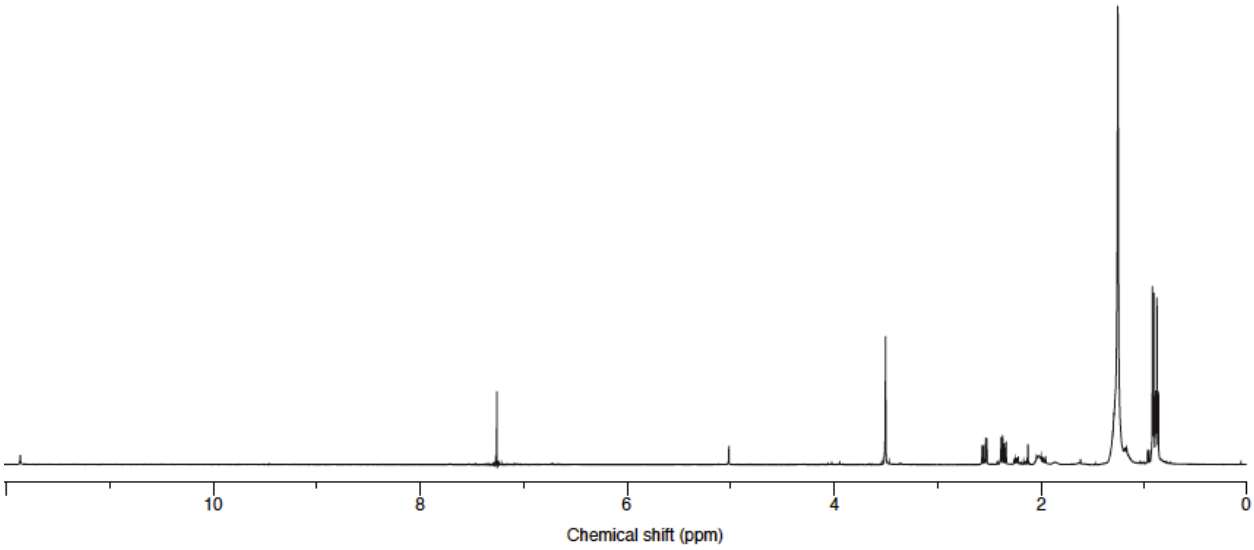
HRMS (ESI): calc'd for $C_{15}H_{27}O_3^-$ $[M-H]^-$, 255.1966; found, 255.1963.

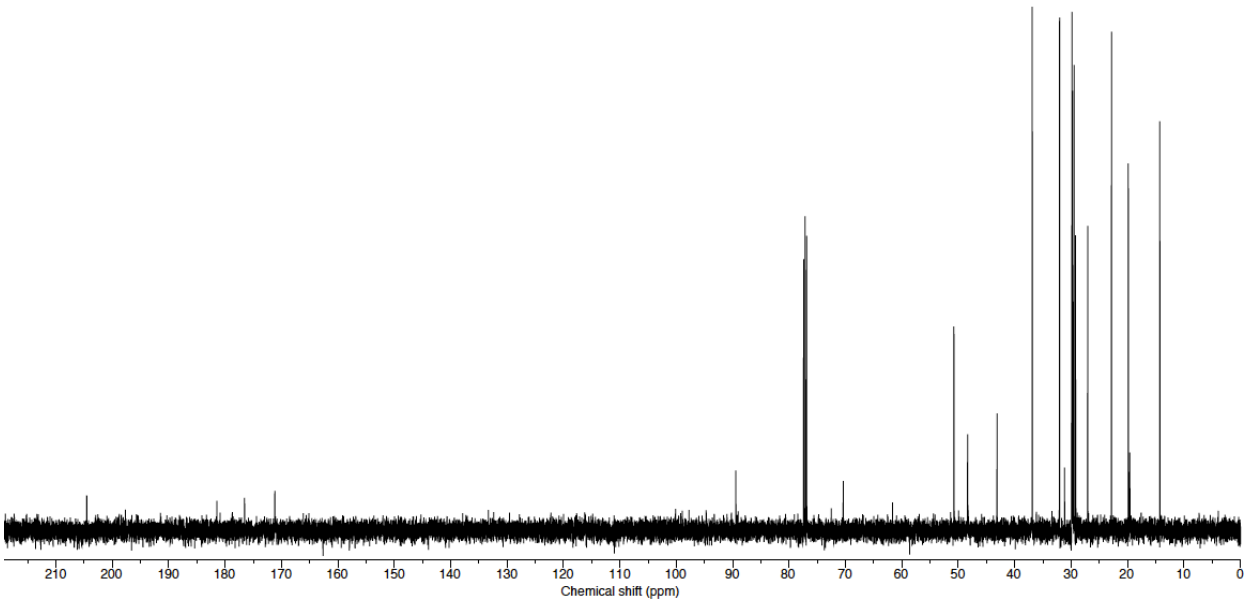
^{1}H-NMR (500 MHz; $CDCl_3$): δ 11.87 (s, 1H, enol-CHCO**H**), 5.02 (s, 1H, enol-C**H**COH), 3.50 (s, 2H, keto-$CH_3OCOC\mathbf{H}_2$), 2.55 (dd, 1H, *J* = 16.5, 5.6 Hz, keto-$COC\mathbf{H}_2$), 2.36 (dd, 1H, *J* = 16.5, 8.0 Hz, keto-$COC\mathbf{H}_2$), 2.24 (dd, 1H, *J* = 13.6, 5.9 Hz, enol-$COC\mathbf{H}_2$), 2.05–2.01 (m, 1H, C**H**), 1.98 (dd, 1H, *J* = 13.7, 8.3 Hz, enol-$COC\mathbf{H}_2$), 1.31-1.26 (m, 19H, $C\mathbf{H_2}$), 0.92 (d, 3H, *J* = 6.6 Hz, $CHC\mathbf{H}_3$), 0.88 (t, 3H, *J* = 6.9 Hz, $C\mathbf{H}_3$).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 204.52, 181.46, 176.55, 171.18, 89.44, 70.40, 61.66, 50.77, 48.35, 43.12, 36.89, 32.04, 31.15, 29.85, 29.76, 29.47, 29.21, 27.06, 22.83, 19.86, 19.59, 14.26.

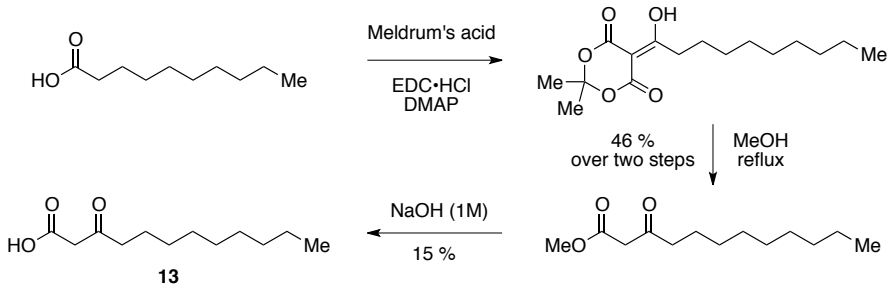

O
O
Me
HO
Me
+
O
OH
Me
HO
Me

keto **enol**


10
8
6
4
2
0
Chemical shift (ppm)


210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical shift (ppm)

Synthesis of internal standards for LC-MS quantitation assay



5-(1-Hydroxydecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione

To an ice-cooled (0 ºC) solution of decanoic acid (200 mg, 1.16 mmol) in dichloromethane (9 mL) under argon was added 2,2-dimethyl-1,3-dioxane-4,6-dione (167 mg, 1.16 mmol) and DMAP (426 mg, 3.48 mmol), followed by the dropwise addition of a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (245 mg, 1.28 mmol) in dichloromethane (3 mL). The reaction was allowed to slowly warm up to room temperature overnight. The reaction mixture was diluted with dichloromethane (30 mL), and the organic layer was washed with 1 M aqueous HCl (3 x 30 mL) and brine (30 mL). The organic layer was dried over sodium sulfate, filtered and concentrated *in vacuo* to give 5-(1-hydroxydecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione as a yellow oil. The crude product was used directly in the next reaction without further purification.

TLC: R_f = 0.35 (silica gel, 1:1 hexanes:ethyl acetate).

HRMS (ESI): calc'd for $C_{16}H_{25}O_5^-$ $[M-H]^-$, 297.1707; found, 297.1704.

^1H and ^{13}C NMR data matched previously reported values.[24]

Methyl 3-oxododecanoate

5-(1-Hydroxydecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (100 mg, 0.335 mmol) was dissolved in anhydrous methanol (5 mL), and the solution was refluxed overnight under an argon atmosphere. The solvent was removed *in vacuo* and the crude reaction mixture was purified by

flash chromatography, eluting with 0–20% ethyl acetate in hexanes to afford methyl 3-oxododecanoate as mixture of keto and enol tautomers (36 mg, 0.16 mmol, 46%).

TLC: R_f = 0.71 (silica gel, 1:1 hexanes:ethyl acetate).

HRMS (ESI): calc'd for $C_{13}H_{25}O_3^+$ $[M+H]^+$, 229.1798; found, 229.18117: calc'd for $C_{13}H_{24}NaO_3^+$ $[M+Na]^+$, 251.1618; found 251.1631: calc'd for $C_{13}H_{24}KO_3^+$ $[M+K]^+$, 267.1357; found, 267.1345.

^1H and ^{13}C NMR data matched previously reported values.[25]

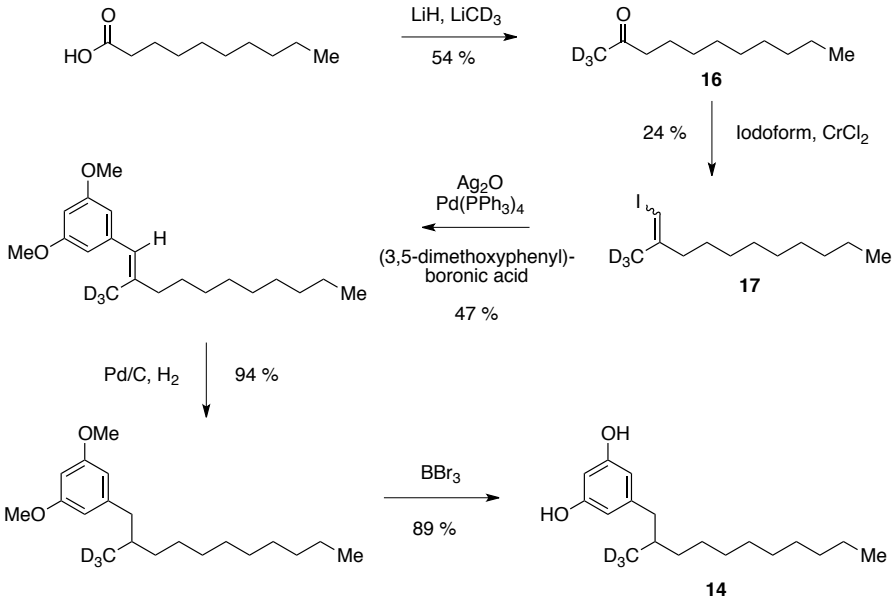
3-Oxododecanoic acid (**13**)

Methyl 3-oxotetradecanoate (35 mg, 0.15 mmol) was dissolved in THF (2 mL), and 1 M NaOH solution (2 mL) was added. The reaction mixture was stirred at room temperature for 3 h. and then the THF was removed *in vacuo*. The resulting mixture was diluted with ethyl acetate (5 mL) and acidified with 1 M aqueous HCl (5 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The residue was purified by flash chromatography, eluting with 50–100% ethyl acetate in hexanes to give **13** as mixture of keto and enol tautomers (5.0 mg, 0.023 mmol, 15%).

TLC: R_f = 0.28 (silica gel, 1:1 hexanes:ethyl acetate).

HRMS (ESI): calc'd for $C_{12}H_{21}O_3^-$ $[M-H]^-$, 213.1496; found 213.1498.

^1H NMR data matched previously reported values.[25]



[1,1,1-$^2H_3$]Undecan-2-one (**16**)

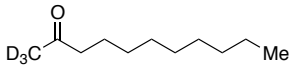
To a suspension of lithium hydride (73 mg, 8.7 mmol) in dimethoxyethane (30 mL), a solution of decanoic acid (1.0 g, 5.8 mmol) in dimethoxyethane (40 mL) was added dropwise. The reaction mixture was refluxed for 2.5 h under an argon atmosphere, and then cooled to room temperature. 0.5 M *d*$_3$-methyllithium solution (17.4 ml, 8.71 mmol) was added dropwise, and the mixture was stirred overnight. Excess *d*$_3$-methyllithium was quenched by addition of 1 M aqueous HCl (30 mL), the layers were separated, and the aqueous layer was extracted with ether (3 x 30 mL). The combined organic layers were washed with brine (40 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified with flash chromatography, eluting with 5-10% ether in hexanes to give [1,1,1-$^2H_3$]undecan-2-one (**16**) as clear oil (540 mg, 3.12 mmol, 54%).

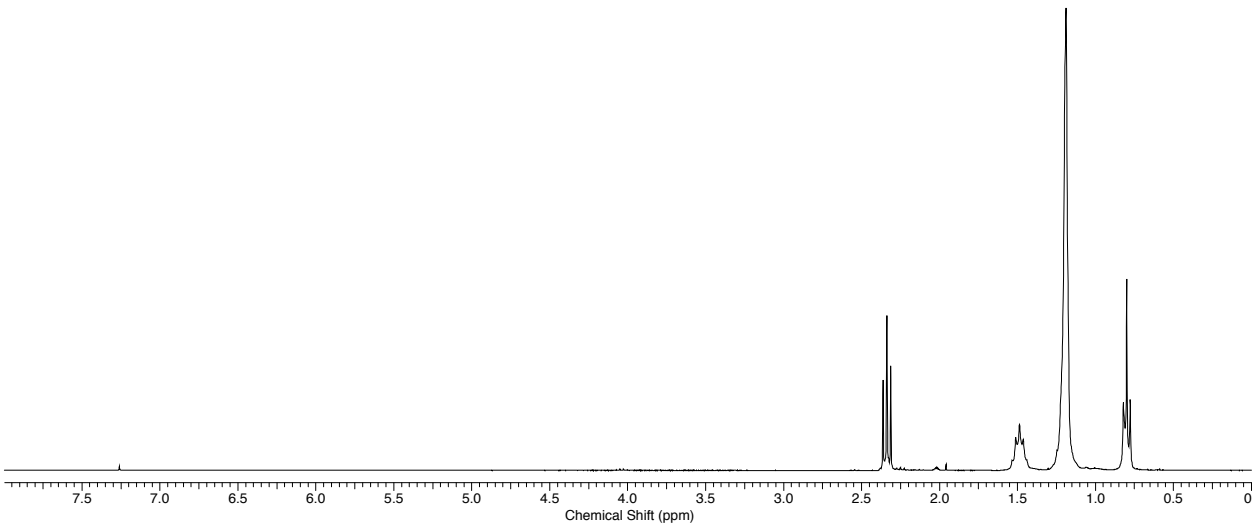
TLC: R_f = 0.25 (silica gel, 1:9 ether:hexanes).

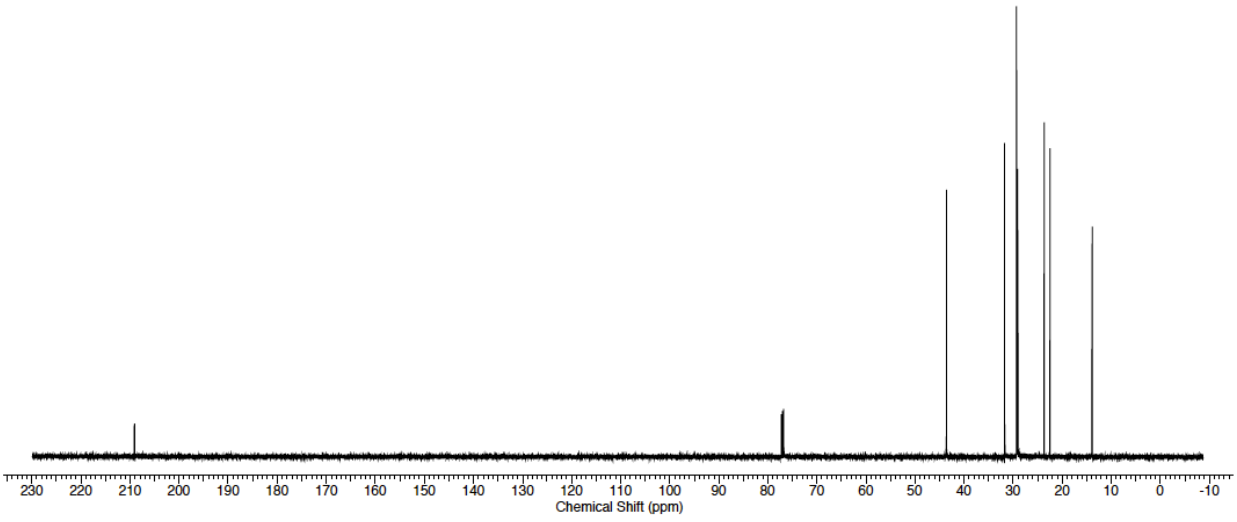
HRMS (ESI): calc'd for $C_{11}H_{20}D_3O^+$ $[M+H]^+$, 174.1932; found 174.1932: cal'cd for $C_{11}H_{19}NaD_3O^+$ $[M+Na]^+$, 196.1751; found 196.1749.

^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.34 (t, 2H, *J* = 7.6 Hz, COC**H**$_2$), 1.49 (m, 2H, C**H**$_2$), 1.29–1.12 (m, 12H, C**H**$_2$), 0.80 (t, 3H, *J* = 6.6 Hz, CH_2C**H**$_3$).

^{13}C NMR (125 MHz, CDCl3) δ: 209.09, 43.60, 31.72, 29.29, 29.27, 29.12, 29.04, 23.68, 22.51, 13.91.







1-Iodo-2-[$^2H_3$]methylundec-1-ene (**17**)

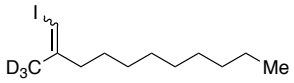
To a solution of **16** (535 mg, 3.09 mmol) in THF (20 mL) under argon, chromium chloride (1.52 g, 12.4 mmol) was added. Then, a solution of iodoform (2.43 g, 6.17 mmol) in THF (10 mL) was added dropwise and the reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with 1 M aqueous HCl (30 mL) and then extracted with ether (3 x 30 mL). The combined organic layers were washed with a 20% w/v solution of sodium thiosulfate (40 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified with flash chromatography, eluting with 100% hexanes to give 1-iodo-2-[$^2H_3$]methylundec-1-ene (**17**) as 5:3 mixture of *E* and *Z* stereoisomers (220 mg, 0.740 mmol, 24%). Approximately half of the starting material **16** was also recovered.

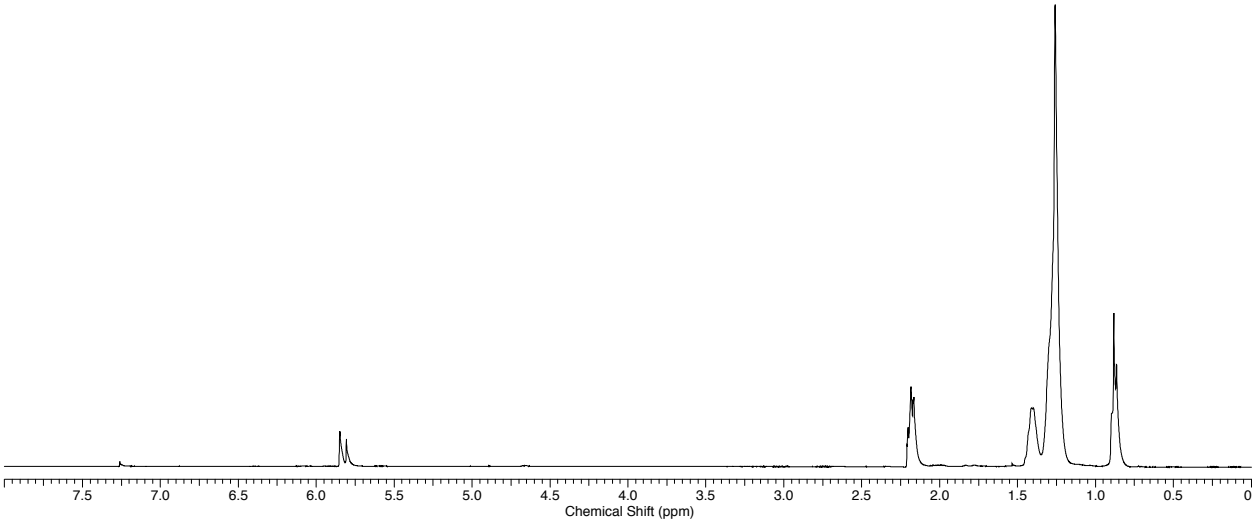
TLC: R_f = 0.88 (silica gel, 1:9 ether:hexanes).

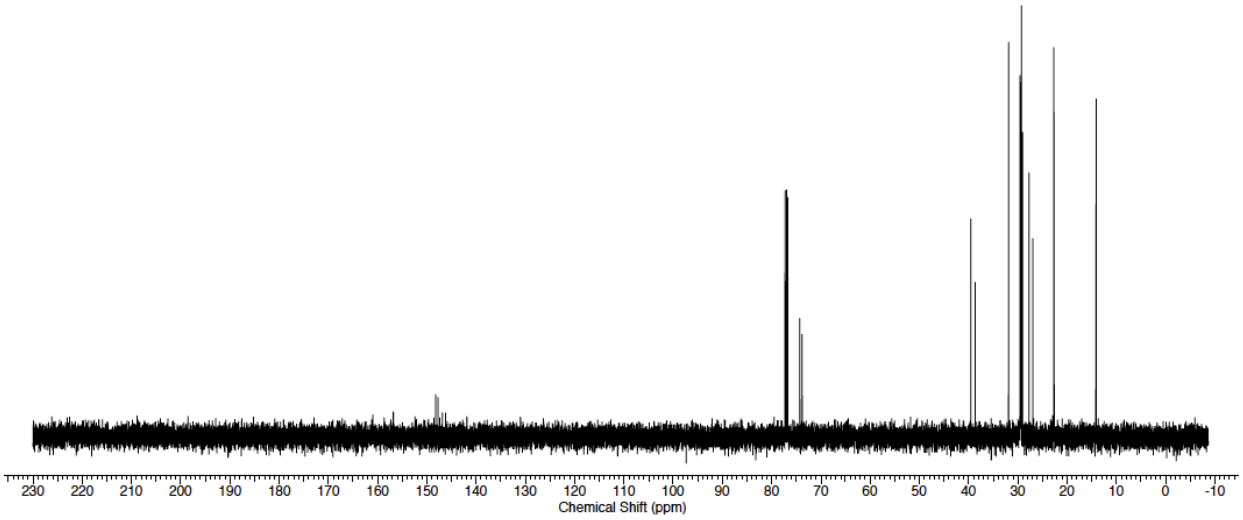
^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.85 (s, 1H, *E*-alkene C**H**I), 5.81 (s, 1H, *Z*-alkene C**H**I), 2.18 (t, 2H, *J* = 7.2 Hz, C$\mathbf{H}_2$) 1.41 (m, 2H, C$\mathbf{H}_2$), 1.35–1.15 (m, 12H, C$\mathbf{H}_2$), 0.88 (t, 3H, *J* = 6.0 Hz, $CH_2C\mathbf{H}_3$).

^{13}C NMR (125 MHz, CDCl3) *E*-stereoisomer δ: 148.21, 74.28, 39.56, 31.87, 29.53, 29.39, 29.30, 29.05, 27.70, 22.67, 14.11; *Z*-stereoisomer δ: 147.76, 73.80, 38.63, 31.87, 29.51, 29.39, 29.30, 29.05, 26.95, 22.67, 14.11.

*^{13}C NMR peaks of the two isomers were assigned by comparing with the previously reported ^{13}C NMR peaks of the pure *E*-stereoisomer.[3]


I
D3C
Me


7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0
Chemical Shift (ppm)


230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
Chemical Shift (ppm)

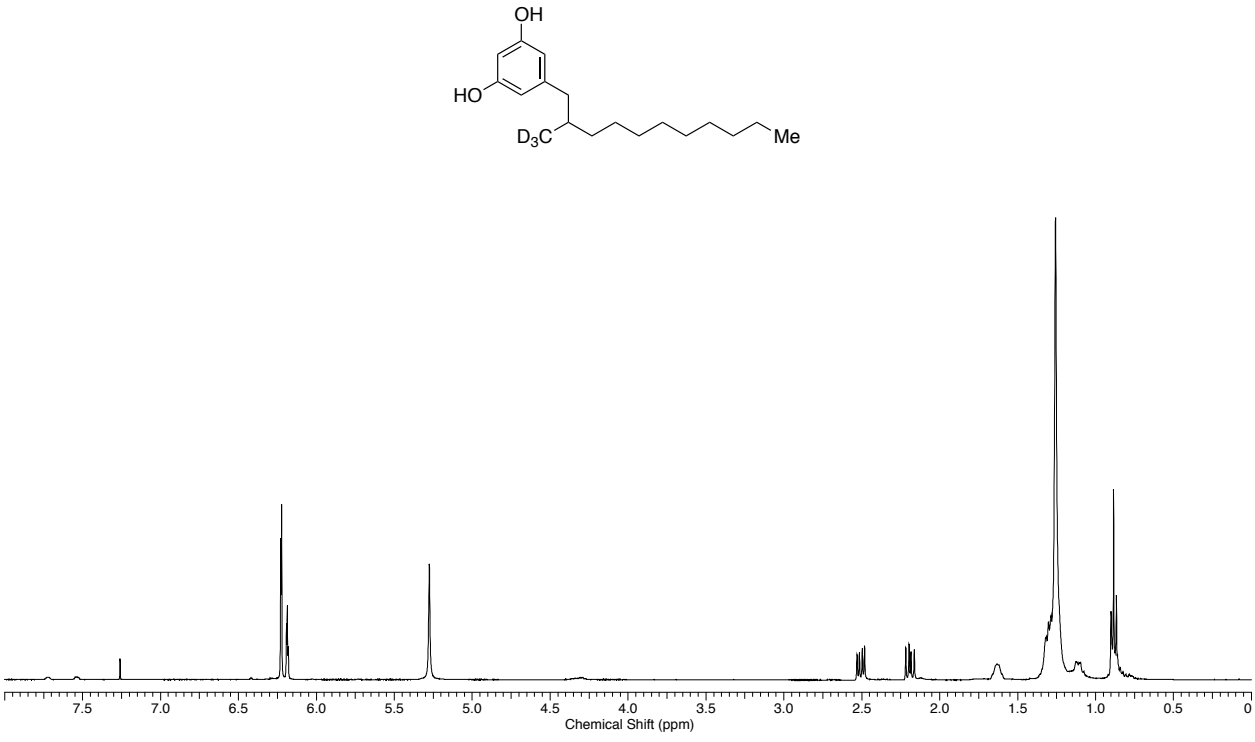
d$_3$-Resorcinol (**14**)

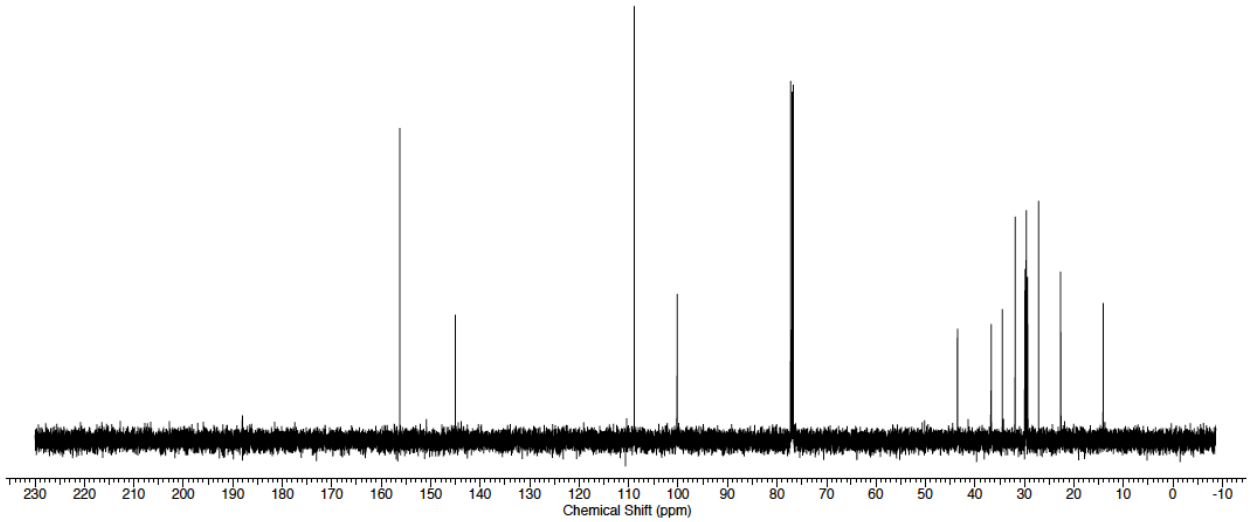
d$_3$-Resorcinol (**14**) was prepared from **17** following the previously reported synthesis.[3]

HRMS (ESI): calc'd for $C_{18}H_{26}D_3O_2^-$ $[M–H]^-$, 280.2361; found 280.2359.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.22 (d, 2H, *J* = 2.2 Hz, aromatic C**H**), 6.18 (d, 1H, *J* = 2.2 Hz, aromatic C**H**), 5.28 (s, 2H, O**H**), 2.51 (dd, 1H, *J* = 13.4, 6.1 Hz, benzylic C**H**$_2$), 2.19 (dd, 1H, *J* = 13.6, 8.5 Hz, benzylic C**H**$_2$), 1.63 (m, 1H, C**H**), 1.35–1.23 (m, 15H, C**H**$_2$), 1.11 (m, 1H, C**H**$_2$), 0.88 (t, 3H, *J* = 6.8 Hz, $CH_2C\mathbf{H}_3$).

^{13}C NMR (125 MHz, CDCl3) δ: 156.25, 145.05, 108.88, 100.22, 43.53, 36.74, 34.46, 31.91, 29.91, 29.72, 29.64, 29.35, 27.12, 22.68, 14.10.





General method for preparation of acyl-SNAC substrates for spectrophotometric assay

The following procedure was used for preparing propionyl-, butyryl-, pentanoyl- and hexanoyl-SNAC substrates. To free acid (1.03 mmol) in a flask cooled to 0 ºC, a solution of EDC•HCl (360 mg, 1.88 mmol) in dry dichloromethane (3 ml) dissolved in dry dichloromethane (3 ml) was added. The mixture was stirred at 0 ºC for 20 min, and then *N*-acetylcysteamine (100 µL, 0.94 mmol) and DMAP (11.5 mg, 0.094 mmol) were added. The reaction was warmed to room temperature and stirred for three more hours. The reaction was quenched with water (10 mL) and extracted with ethyl acetate (3 x 15 mL). The combined organic layer was washed with 1 M HCl (30 mL), saturated sodium bicarbonate solution (30 mL) and then brine (30 mL). The organic fraction was then dried over anhydrous sodium sulfate and concentrated *in vacuo* to afford desired acyl-SNAC products. The isolated yields were as follows: propionyl-SNAC (97 mg, 0.55 mmol, 59%), butyryl-SNAC (135 mg, 0.71 mmol, 76%), pentanoyl-SNAC (138 mg, 0.68 mmol, 72%), and hexanoyl-SNAC (159 mg, 0.73 mmol, 78%).

^{1}H and ^{13}C NMR data matched previously reported values.[26]

References

1. C. M. Stoscheck, *Method. Enzymol,* 1980, **182**, 50.
2. ExPASy ProtParam tool, http://www.expasy.org/tools/protparam.html (accessed October 2013)
3. H. Nakamura, H. A. Hamer, G. Sirasani, E. P. Balskus, *J. Am. Chem. Soc.*, 2012, **134**, 18518.
4. E. Yeh, R. M. Kohli, S. D. Bruner, C. T. Walsh, *ChemBioChem*, 2004, **5**, 1290.
5. (a) Geneious, version 7.1.6, Biomatters, Auckland, New Zealand, 2014; (b) M. Kearse, R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Meintjes, A. Drummond, *Bioinformatics*, 2012, **28**, 1647.
6. J. J. Gehret, L. Gu, W. H. Gerwick, P. Wipf, D. H., Sherman, J. L. Smith, *J. Biol. Chem.*, 2011, **286**, 14445.
7. J. J. La Clair, T. L. Foley, T. R. Schegg, C. M. Regan, M. D. Burkart, *Chem. Biol.*, 2004, **11**, 195.
8. J. Yin, A. J. Lin, D. E. Golan, C. T. Walsh, *Nat. Protoc.*, 2006, **1**, 280.
9. P. Beltran-Alvarez, R. J. Cox, J. Crosby, T. J. Simpson, *Biochemistry*, 2007, **46**, 14672.
10. NCBI public database, http://www.ncbi.nlm.nih.gov/, (accessed October 2013 through January 2014).
11. PKS/NRPS Analysis Web Server, http://nrps.igs.umaryland.edu/nrps/, (accessed January 2014).
12. HHpred, Bioinformatics Toolkit, Max-Planck Institute for Developmental Biology, http://toolkit.tuebingen.mpg.de/hhpred, (accessed January 2014).
13. H. B. Claxton, D. L. Akey, M. K. Silver, S. J. Admiraal, J. L. Smith, *J. Biol. Chem.*, 2009, **284**, 5021.
14. J. R. Whicher, G. Florova, P. K. Sydor, R. Singh, M. Alhamadsheh, G. L. Challis, K. A. Reynolds, J. L. Smith, *J. Biol. Chem.*, 2011, **286**, 22558.
15. Modeller, Bioinformatics Toolkit, Max-Planck Institute for Developmental Biology, http://toolkit.tuebingen.mpg.de/modeller, (accessed January 2014).
16. MacPyMOL, version 1.7, Schrödinger, Inc., New York City, NY, 2014.
17. J. P. Huelsenbeck, F. Ronquist, *Bioinformatics*, 2001, **17**, 754.
18. K. Buntin, K. J. Weissman, R. Müller, *ChemBioChem,* 2010, **11,** 1137.

19. M. Kotaka, R. Kong, I. Qureshi, Q. S. Ho, H. Sun, C. W. Liew, L. P. Goh, P. Cheung, Y. Mu, J. Lescar, Z.-X. Liang, *J. Biol. Chem.*, 2009, **284**, 15739.
20. Y. Zou, H. Yin, D. Kong, Z. Deng, S. Lin, *ChemBioChem,* 2013, **14,** 679.
21. X. Yin, T. M. Zabriskie, *Microbiology*, 2006, **152,** 2969.
22. B. Julien, Z.-Q. Tian, R. Reid, C. D. Reeves, *Chem. Biol.,* 2006, **13,** 1277.
23. *US pat.*, 65900083 B1, 2003.
24. *WO pat.*, 2009050575 A1, 2009.
25. E. E. Kwan, J. R. Scheerer, D. A. Evans, *J. Org. Chem.*, 2013, **78**, 175.
26. G. Prasad, L. S. Borketey, T.-Y. Lin, N. A. Schnarr, *Org. Biol. Chem.,* 2012, **10,** 6717.