Cooperation between a Coenzyme A-Independent Stand-Alone Initiation Module and an Iterative Type I Polyketide Synthase during Synthesis of Mycobacterial Phenolic Glycolipids

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Supporting Information

A. Additional Results

A.1. Sequence relatedness between mycobacterial Pks15/1 orthologs

Figure S1a

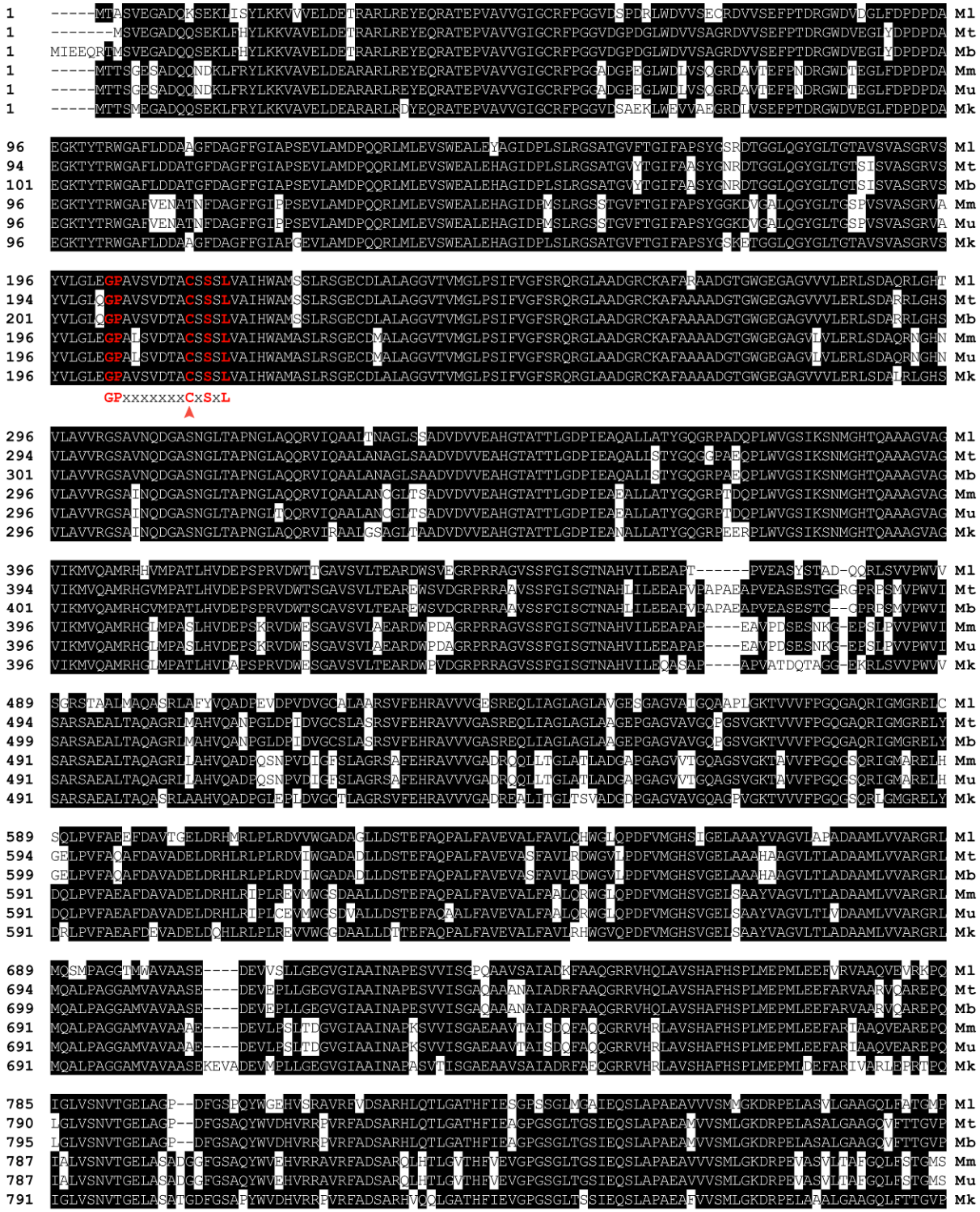


Figure S1a (continuation)

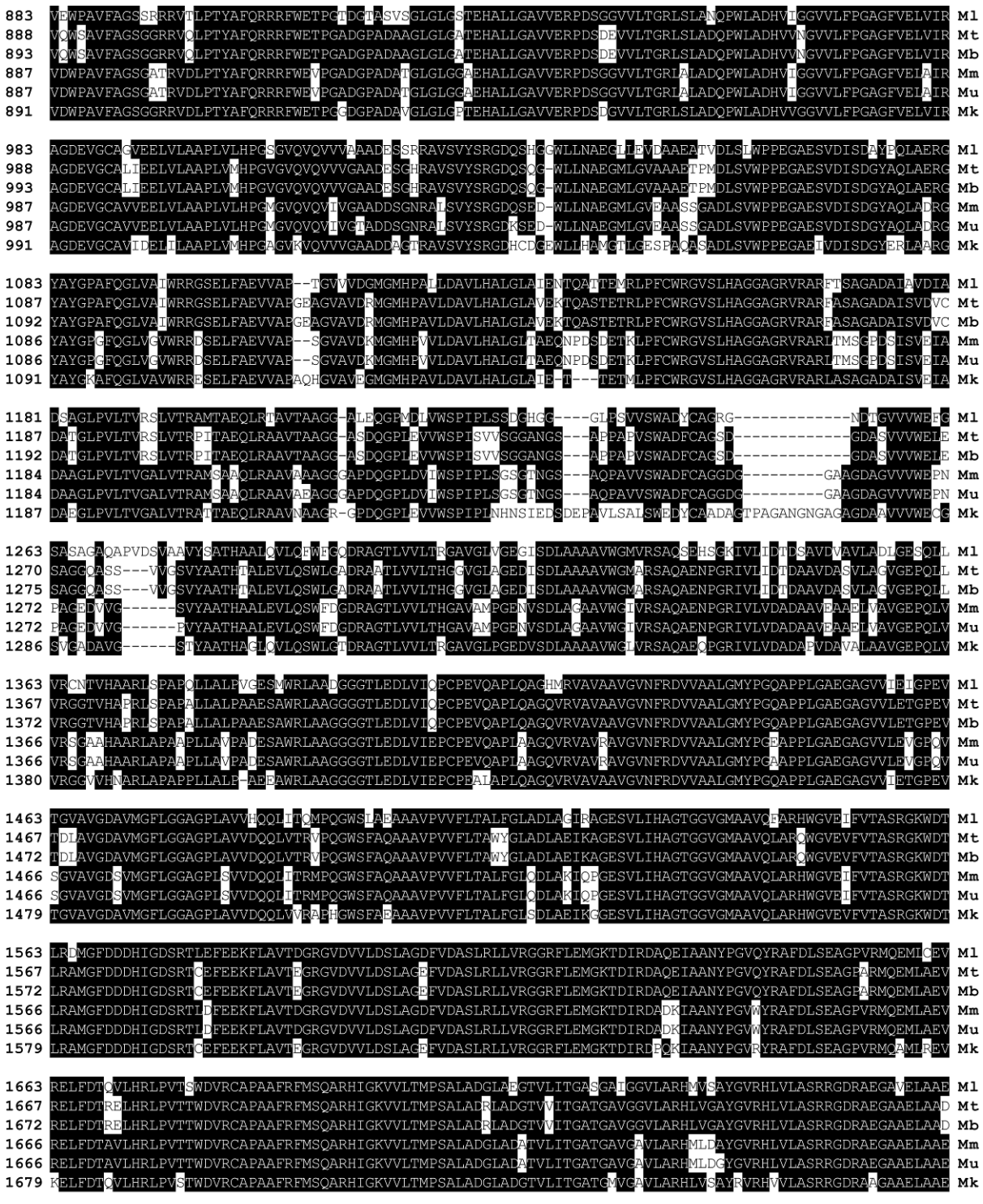


Figure S1a (continuation)

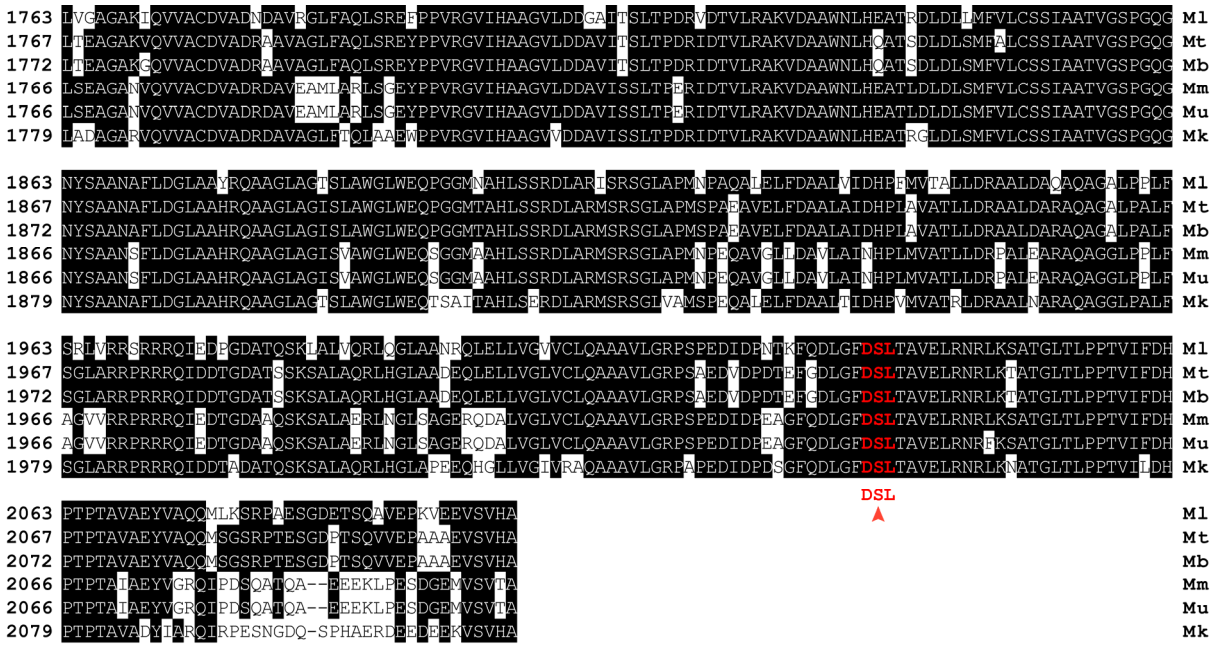


Figure S1b

Percent Identity

	Mt	Mb	Mm	Mu	Mk	
	84.3	84.4	79.2	78.7	80.4	Ml
		99.8	81.2	80.7	83.4	Mt
			81.5	80.9	83.3	Mb
				99.4	81.6	Mm
					81.1	Mu

Figure S1. Sequence relatedness between mycobacterial Pks15/1 orthologs and suspected catalytic Cys residue in the ketosynthase domain and Ser residue site of phosphopantetheinylation in the acyl carrier protein domain of Pks15/1. **(a)** Alignment of Pks15/1 orthologs. Conserved amino acids are highlighted in white font over black background. The motif GPxxxxxxxCxSxL, which is the active site consensus for PKS and FAS ketosynthase domains, is marked.[11] The predicted catalytic Cys residue in this motif is indicated by the red arrowhead. The 4′-phosphopantetheine attachment site DSL conserved motif (NCBI's Conserved Domain Database Pfam family identifier: PF00550P) in the acyl carrier protein domain with the Ser residue (red arrowhead) that is phosphopantetheinylated is marked. **(b)** Percentage of amino acid identity among Pks15/1 orthologs. Sequence alignments were carried out using the MegAlign module of DNASTAR Lasergene software package. Mb, *M. bovis*; Mk, *M. kansasii*; Ml, *M. leprae*; Mm, *M. marinum*; Mt, *M. tuberculosis*; and Mu, *M. ulcerans*. Only confirmed PGL-producing strains of species for which *pks15/1* is available via http://www.ncbi.nlm.nih.gov, or via The J. Craig Venter Institute in the case of Mt strain 210 Pks15/1, are included in the alignment. Sequence Accession Numbers at http://www.ncbi.nlm.nih.gov/sites/entrez: Mb strain AF2122/97 Pks15/1 (Pks1), NP_856616.1; Mk ATCC 12478 Pks15/1, NZ_ACBV01000072.1; Ml strain TN Pks15/1, NP_301229.1; Mm strain M Pks15/1, YP_001850066.1; and Mu strain Agy99 Pks15/1, YP_905915.1.

A.2. Phosphopantetheinylation of Pks15/1 and Pks15/1(S2039A)

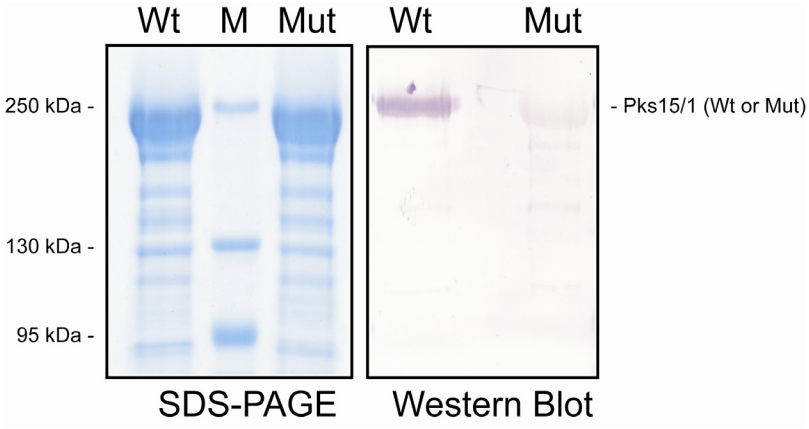


Figure S2. Sfp-dependent phosphopantetheinylation of Pks15/1 and Pks15/1(S2039A) *in vitro*. Phosphopantetheinylation was assessed as incorporation of the biotinylated phosphopantetheinyl group derived from the CoA analog biotinyl-CoA (Avanti Polar Lipids) onto the proteins. The GelCode Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 5%) and its parallel Western blot for analysis of biotinylated proteins are shown. Lanes: Wt, wild-type; Mut, mutant; M, molecular weight marker. Phosphopantetheinylation reactions, SDS-PAGE, and Western blot were carried out using standard protocols described in the Experimental Section of the manuscript. Briefly, phosphopantetheinyl transferase Sfp was utilized for protein phosphopantetheinylation. Phosphopantetheinylation reactions were quenched by addition of SDS-PAGE loading buffer and analyzed by SDS-PAGE and by Western blot to detect biotinylated proteins. Proteins were electrophoretically transferred from the polyacrylamide gel to a Hybond-P membrane (GE Healthcare). Biotinylated-phosphopantetheinyl group covalently incorporated onto the proteins was detected using a commercial mouse monoclonal anti-biotin–alkaline phosphatase conjugated antibody (Sigma-Aldrich). Colorimetric detection of biotinylated proteins was achieved using alkaline phosphatase BCIP/NBT liquid substrate system (Sigma-Aldrich).

A.3. Mass spectrometry data

Figure S3 1a to 10a (*in vitro* system): MS spectra of compounds 1 to 10, respectively, shown in Figure 3a and Table 1

Figure S3, 1a

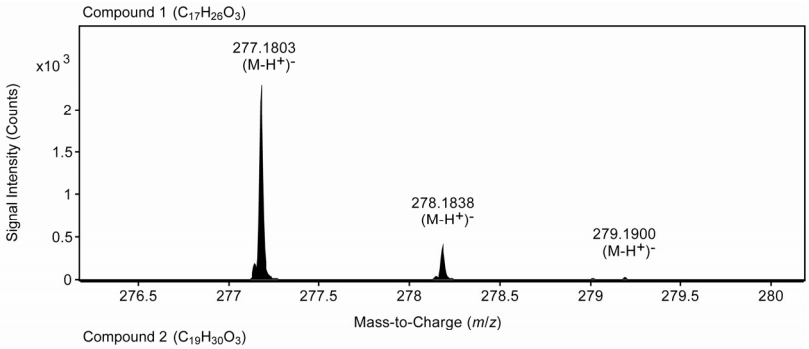


Figure S3, 2a

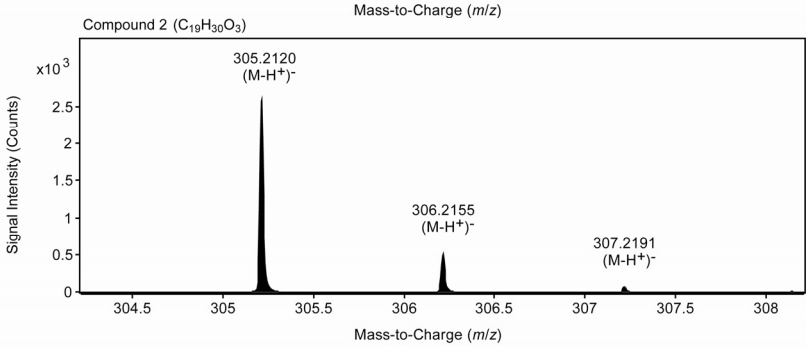


Figure S3, 3a

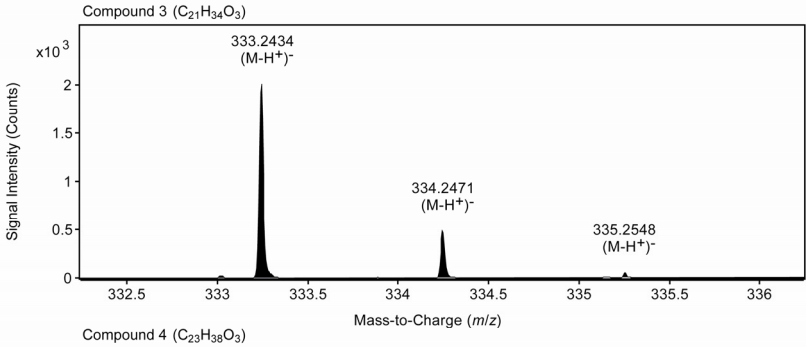


Figure S3, 4a

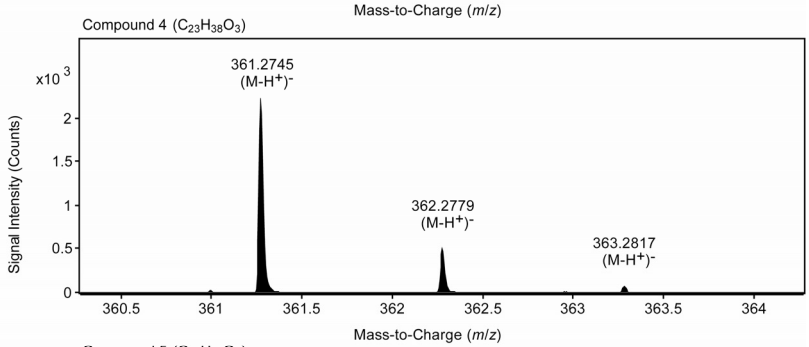


Figure S3, 5a

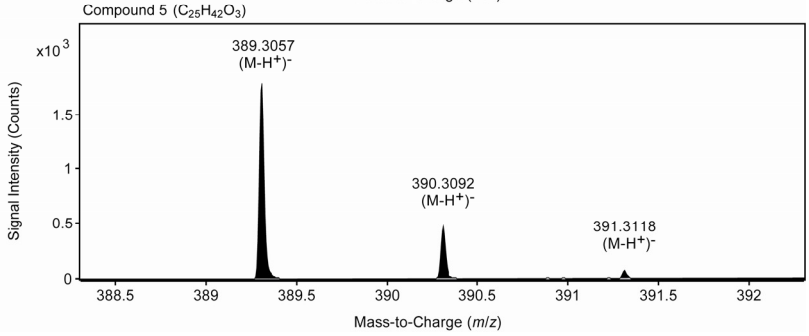


Figure S3, 6a

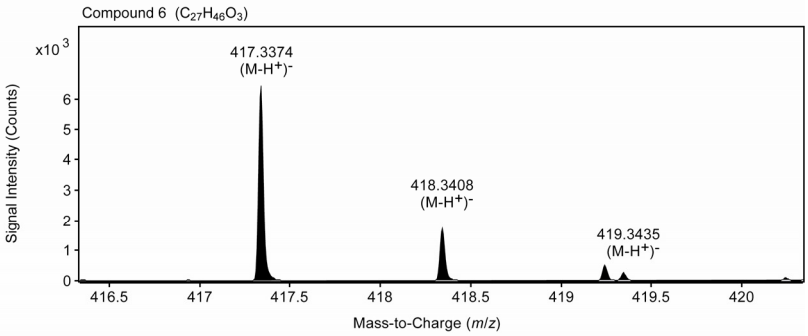


Figure S3, 7a

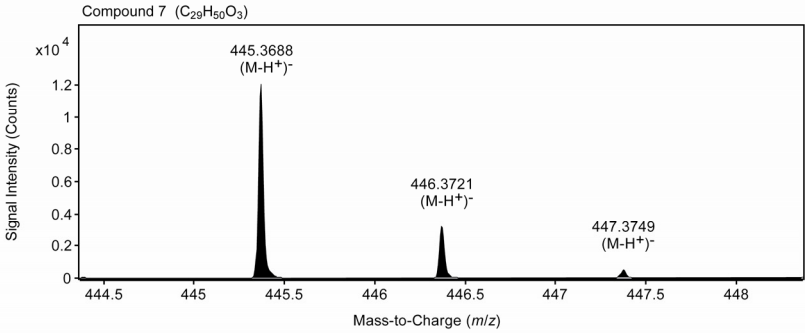


Figure S3, 8a

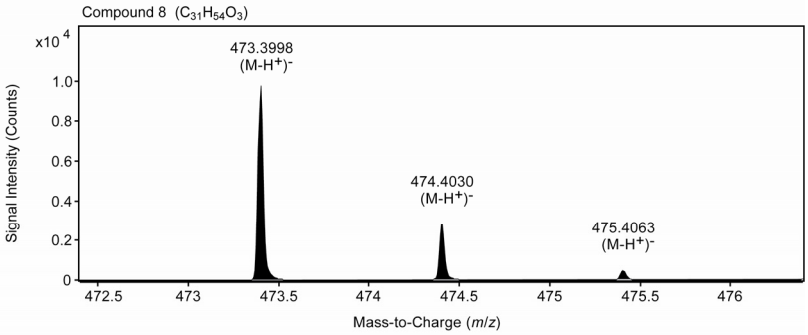


Figure S3, 9a

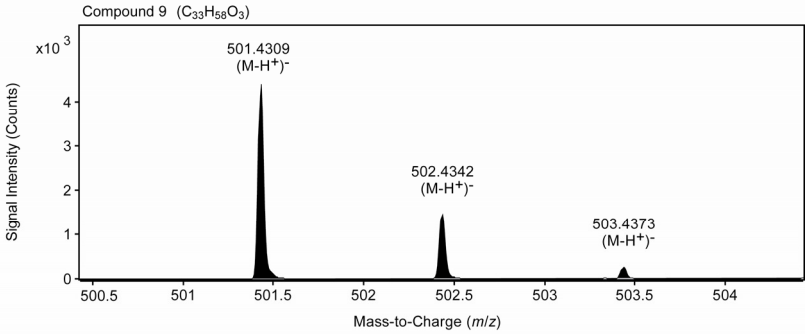


Figure S3, 10a

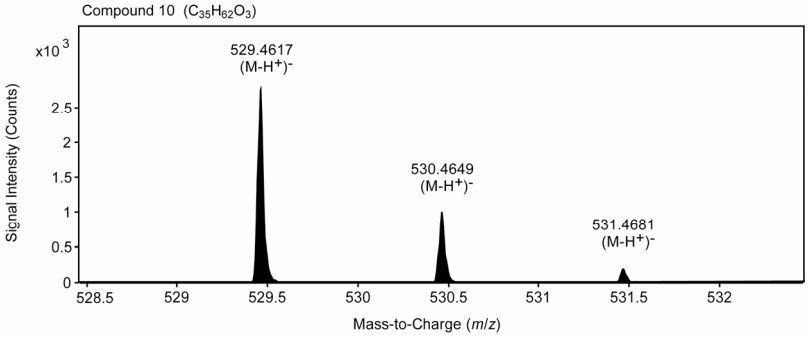


Figure S3 1b to 10b (*in vivo* system): MS spectra of compounds 1 to 10, respectively, shown in Figure 3b and Table 1.

Figure S3, 1b

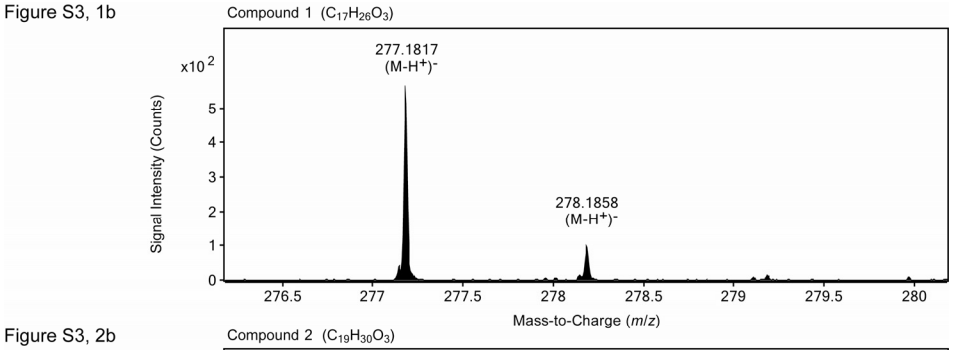


Figure S3, 2b

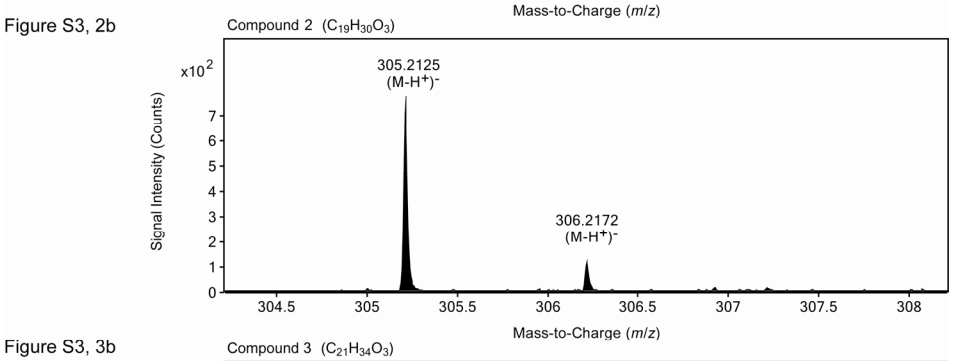


Figure S3, 3b

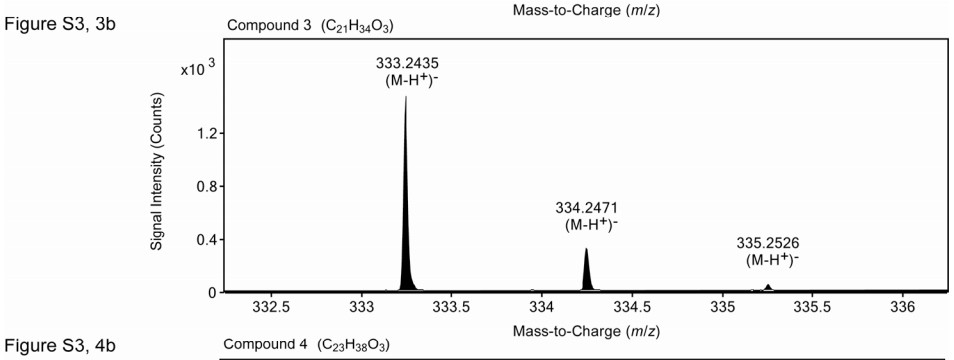


Figure S3, 4b

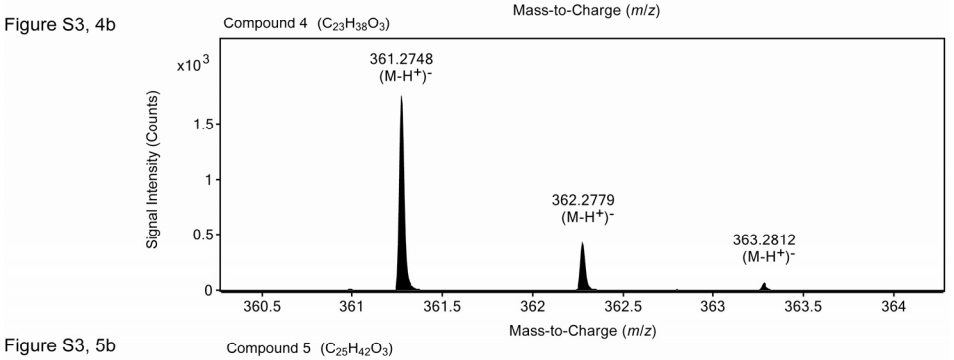


Figure S3, 5b

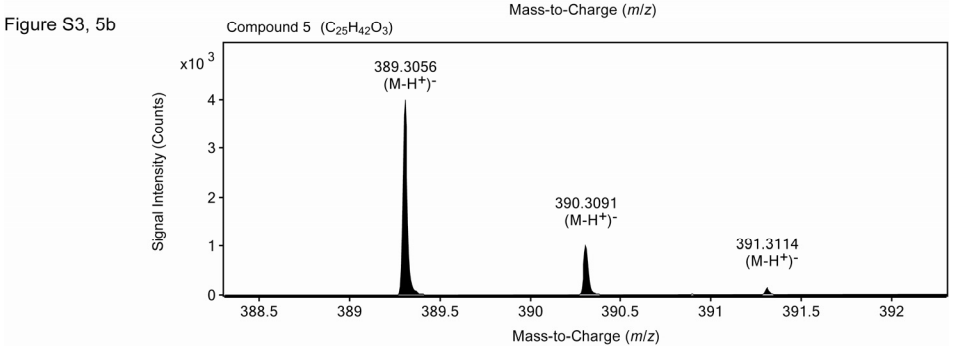


Figure S3, 6b

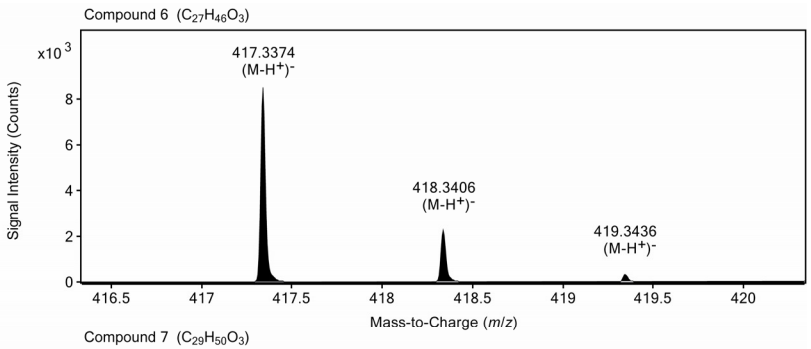


Figure S3, 7b

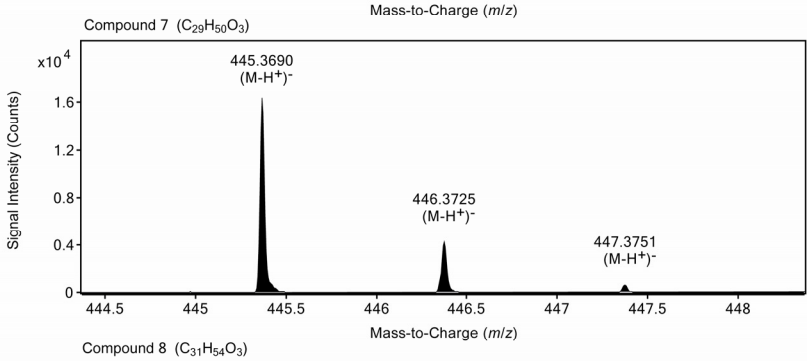


Figure S3, 8b

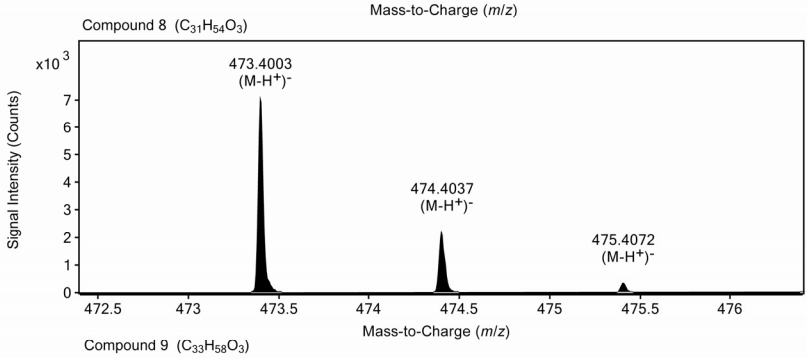


Figure S3, 9b

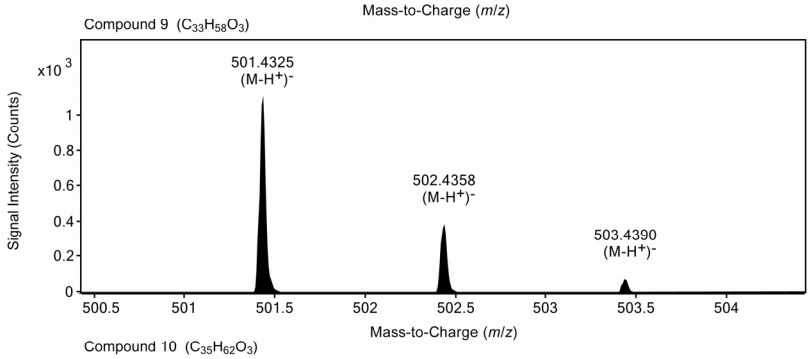


Figure S3, 10b

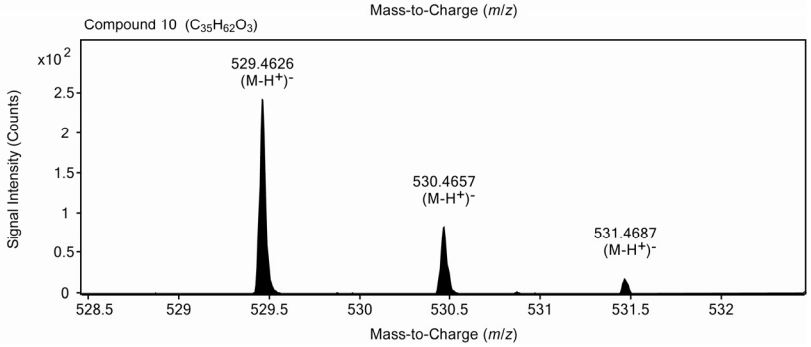


Figure S3 1c to 10c (*in vitro* system): MS/MS spectra of compounds 1 to 10, respectively, shown in Figure 3a and Table 1. The parent ion is marked (♦) in the MS/MS spectra.

Figure S3, 1c

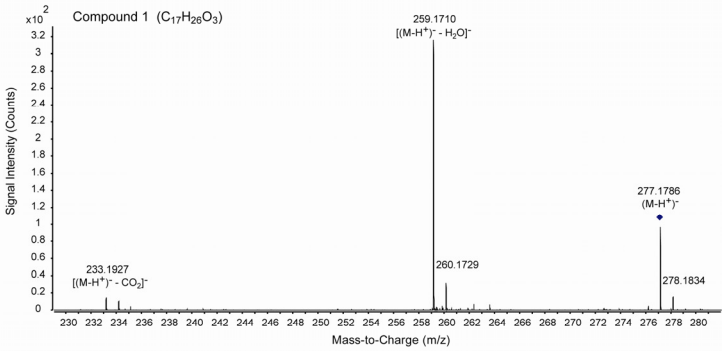


Figure S3, 2c

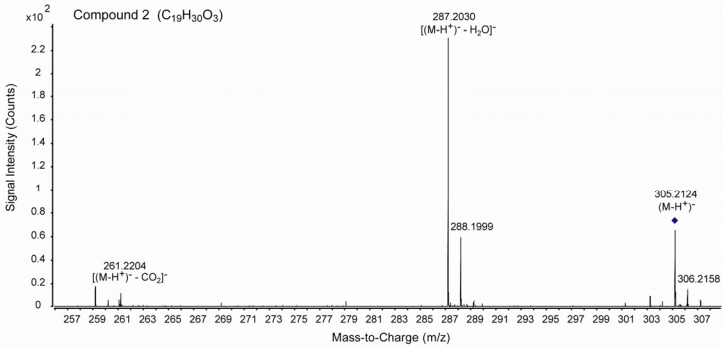


Figure S3, 3c

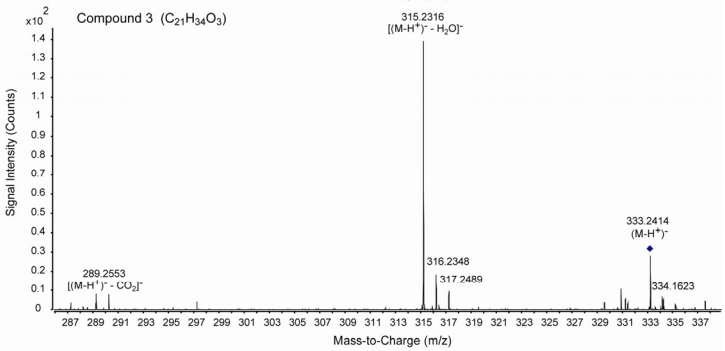


Figure S3, 4c

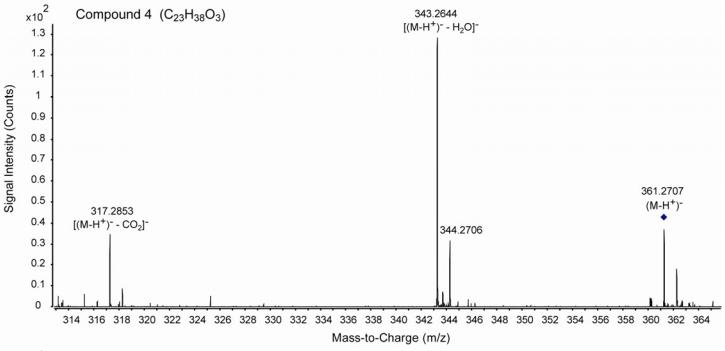


Figure S3, 5c

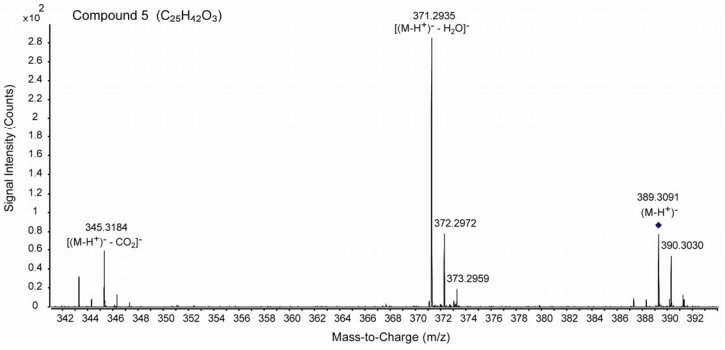


Figure S3, 6c

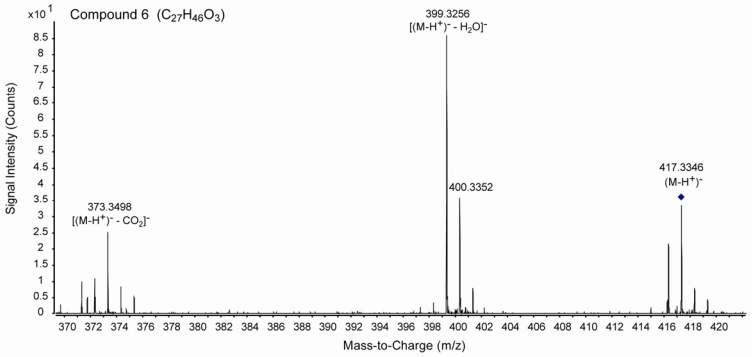
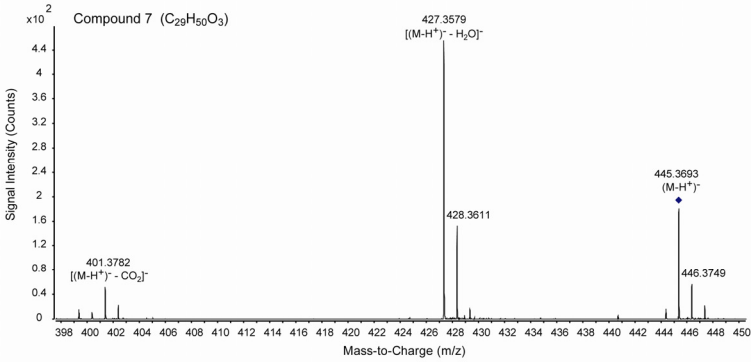


Figure S3, 7c



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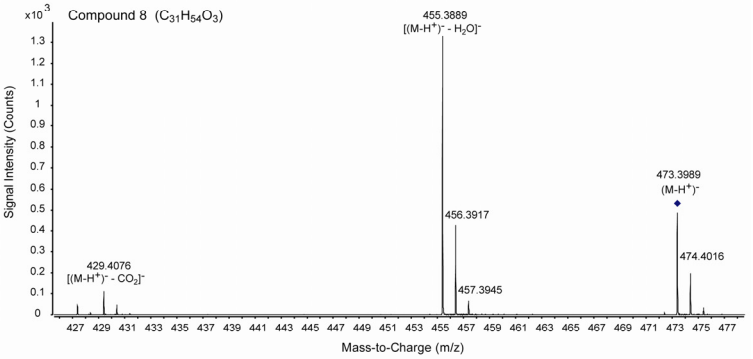

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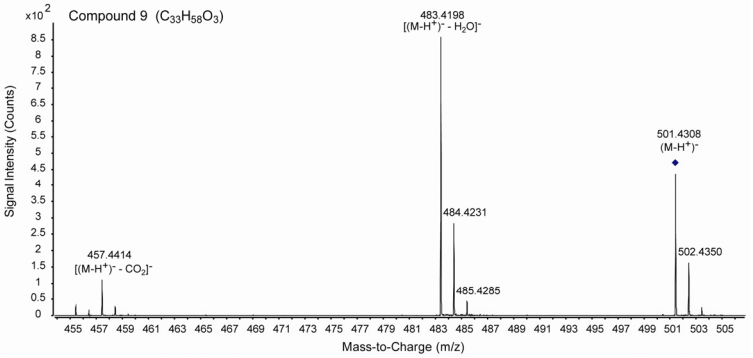


Figure S3, 10c

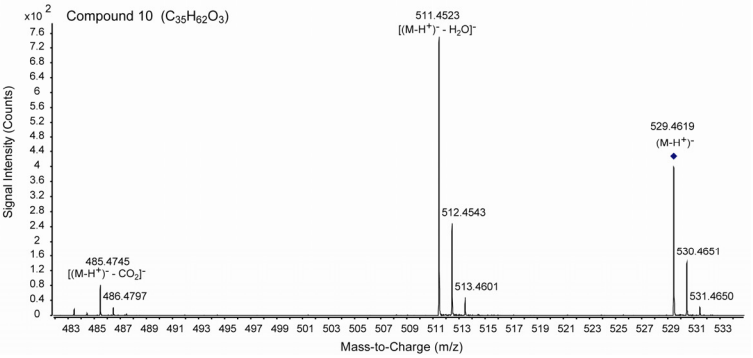


Figure S3 1d to 10d (*in vivo* system): MS/MS spectra of compounds 1 to 10, respectively, shown in Figure 3b and Table 1. The parent ion is marked (♦) in the MS/MS spectra.

Figure S3, 1d

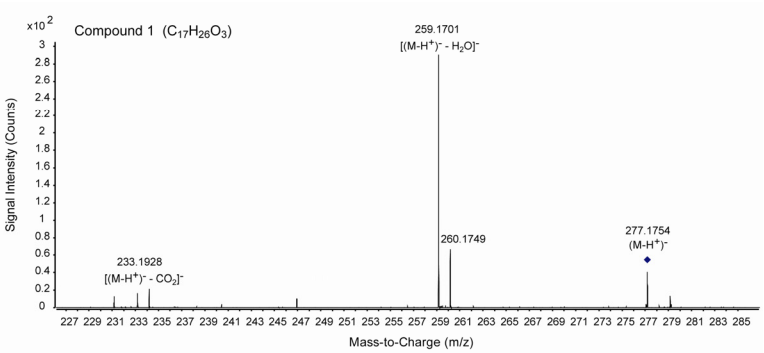


Figure S3, 2d

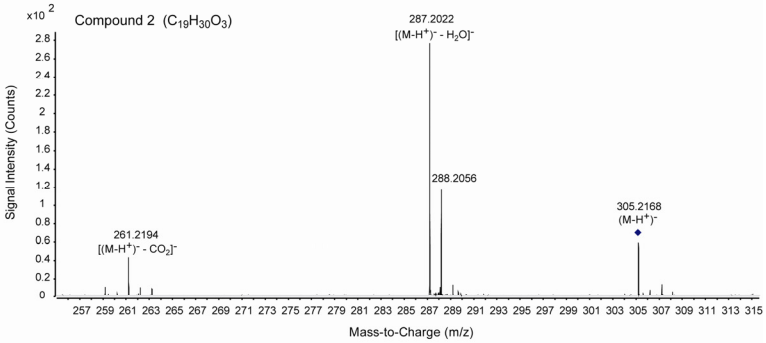
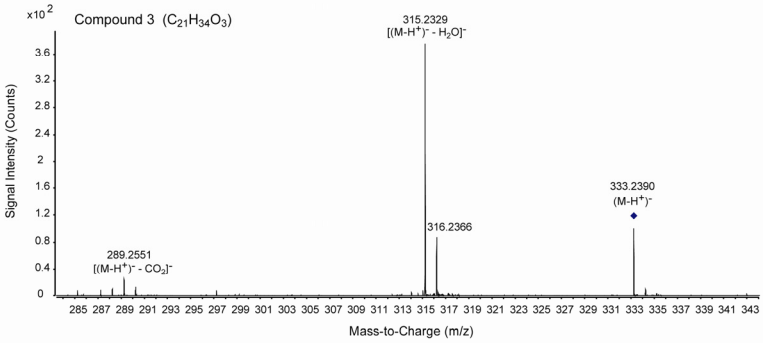


Figure S3, 3d



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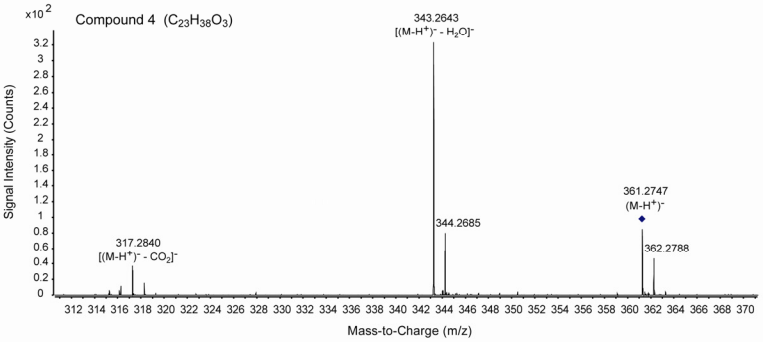


Figure S3, 5d

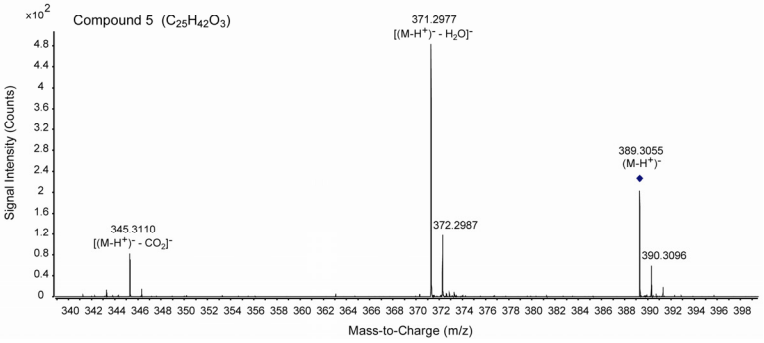
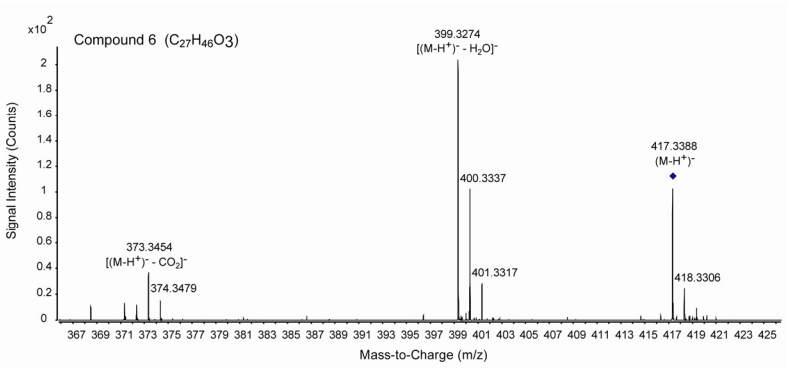


Figure S3, 6d



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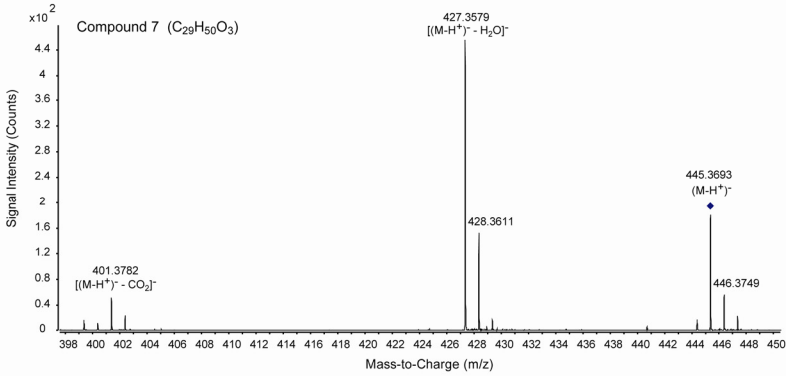

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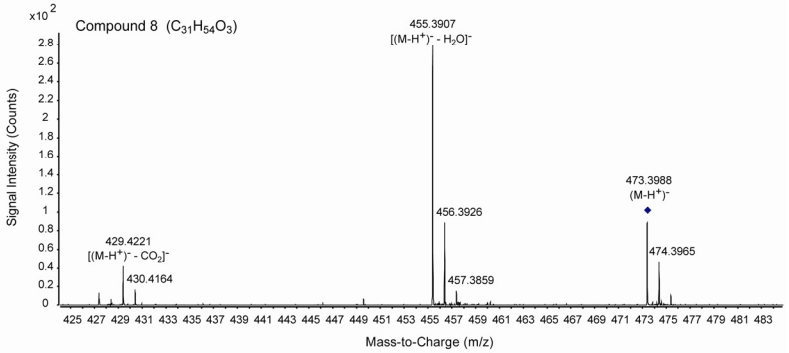


Figure S3, 9d

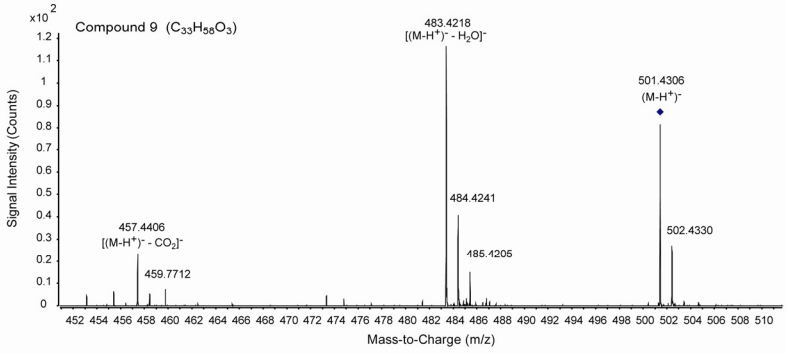


Figure S3, 10d

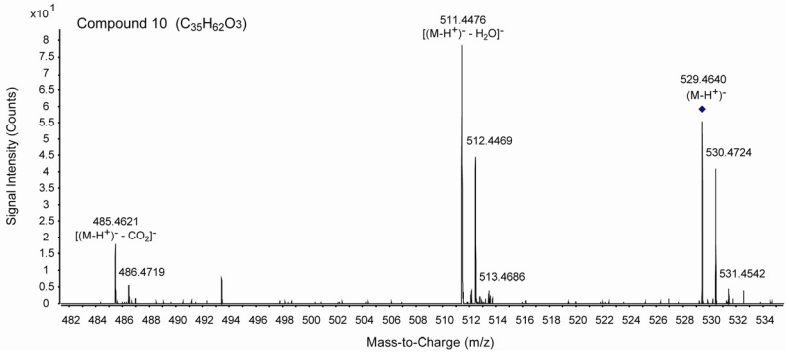


Figure S3 11 and 12: MS and MS/MS of the synthetic PHPA standard, 21-(4-hydroxyphenyl)henicosanoic acid ($C_{27}H_{46}O_3$). Ions corresponding to the isotopic distribution of the $[M\text{-}H^+]^-$ ion are shown. The parent ion is marked (♦) in the MS/MS spectrum.

Figure S3, 11

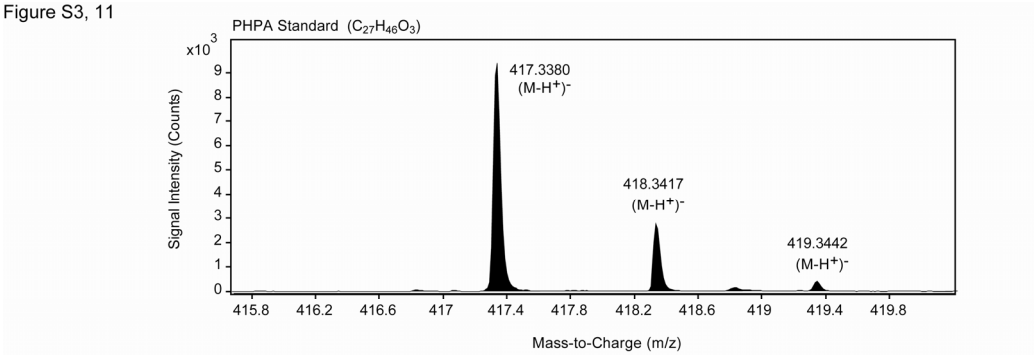
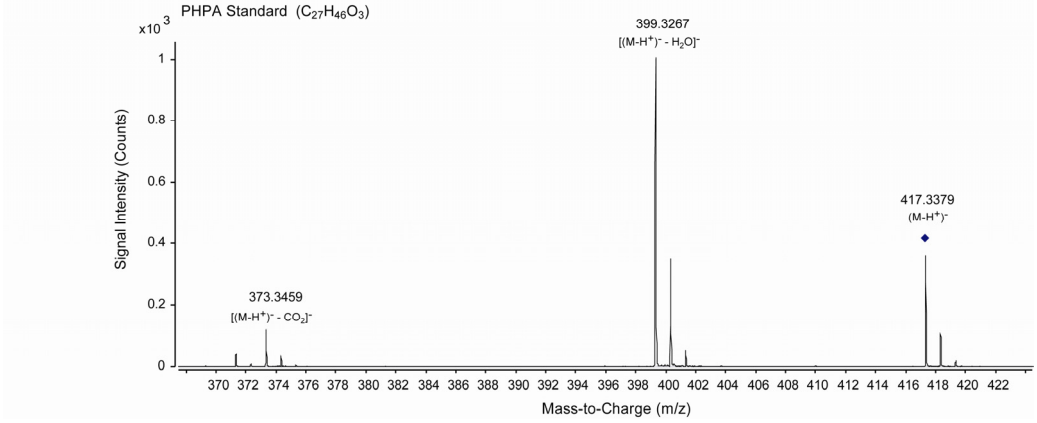


Figure S3, 12



B. Additional Materials and Methods

B.1. Synthesis of 21-(4-hydroxyphenyl)henicosanoic acid standard (PHPA standard)

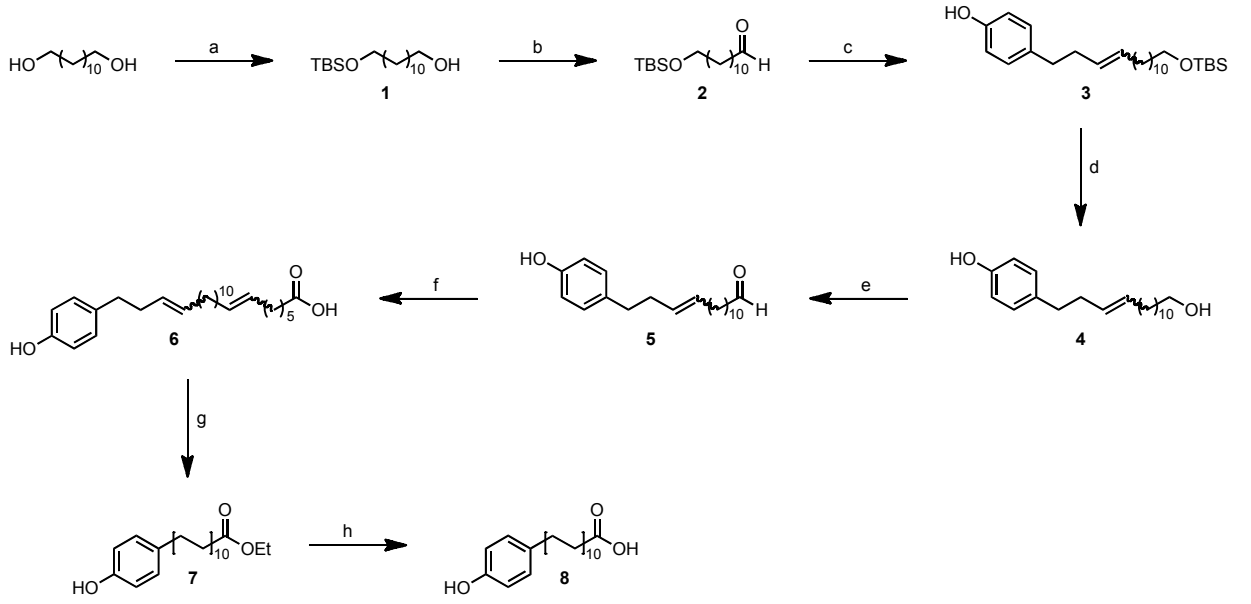


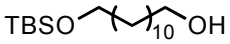
Figure S4. Synthesis of 21-(4-hydroxyphenyl)henicosanoic acid standard. Conditions: **(a)** TBSCl (1.01 equiv), imidazole (1.6 equiv), DMAP (0.1 equiv), CH_2Cl_2, 85 ˚C, microwave, 1 h (45%); **(b)** Dess-Martin periodinane (1.05 equiv), AcOH (0.1 equiv), CH_2Cl_2, rt, 40 min (80%); **(c)** 3-(4-(hydroxyphenyl)propyltriphenylphosphonium bromide (1.5 equiv), KO*t*Bu (1.6 equiv), THF, -10 ˚C, 2.5 h (57%); **(d)** TBAF (1.0 M in THF, 1 equiv), THF, rt, 16 h, (98%); **(e)** oxalyl chloride (7.3 equiv), DMSO, CH_2Cl_2, TEA (21.8 equiv), -78 ˚C, 4 h (35%); **(f)** 5-carboxypentyltriphenylphosphonium bromide (1.5 equiv), KO*t*Bu (1.6 equiv), THF, -10 ˚C, 2.5 h (69%); **(g)** Pd/C (10 mol %), H_2 (1 atm), EtOH, rt, 94 h (42%); (h) LiOH (1.0 M in water), THF/H_2O (4/1), 55 ˚C, 16 hr (55%).

General experimental procedures. All non-aqueous reactions were carried out in oven-dried glassware under an atmosphere of argon. Flash column chromatography was conducted following the protocol reported by Still,[7] using ICN Silted 32-63 D 60 Å silica gel. Automatic chromatography was performed using a Büchi Sepacore system equipped with a C-660 fraction collector. Analytical thin layer chromatography (TLC) was performed employing Merck 250 micron 60F-254 silica plates. The plates were visualized either by exposure to UV light, staining with iodine impregnated silica gel, or by staining with ceric ammonium molybdate (CAM). LC-

MS analysis was conducted as described in the Experimental Section of the manuscript. Microwave-based reactions were performed on a CEM-Discover Microwave irradiator, using the dynamic heating mode with a power limit of 300 W and pressure limit of 17 psi. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker DRX-500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are expressed in parts per million downfield from tetramethylsilane (TMS), using either TMS or the solvent resonance as an internal standard (TMS, ^{1}H: 0 ppm; chloroform, ^{13}C: 77.0 ppm; methanol-d_4, ^{1}H: 3.31 ppm; ^{13}C: 49.0 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), integration, and coupling constant.

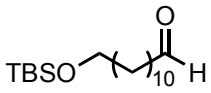
Experimental procedures

12-(*tert*-butyldimethylsilyloxy)dodecan-1-ol (1)



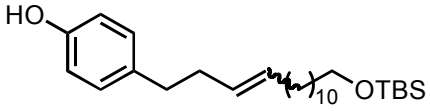
To a 35 mL microwave reaction vessel was added 1,12-dodecanediol (2.02 g, 9.9 mmol), TBSCl (1.51 g, 10.0 mmol), DMAP (130 mg, 1.1 mmol), imidazole (1.09 g, 16.0 mmol), and dichloromethane (15 mL). The mixture was heated to 85 ˚C under microwave irradiation for 1 h, then cooled to room temperature, diluted with diethylether and filtered. The organic layer was washed sequentially with H_2O, saturated NH_4Cl (aq.), and brine, then dried over Na_2SO_4 and concentrated. Purification by flash chromatography (4:1 petroleum ether in diethylether, R_f = 0.28) provided the title compound as a colorless oil (1.40 g, 45%). ^{1}H NMR ($CDCl_3$): δ 3.66-3.61 (m, 2H), 3.59 (t, *J* = 6.7 Hz, 2H), 1.60-1.53 (m, 2H), 1.52-1.47 (m, 2H), 1.30-1.23 (brm, 16H), 0.89 (s, 9H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 63.5, 63.2, 33.1, 33.0, 29.8, 29.8, 29.7, 29.6, 26.1, 26.0, 25.9, 18.5, -5.1.

12-(*tert*-butyldimethylsilyloxy)dodecanal (**2**)



Monoprotected diol **1** (527 mg, 1.7 mmol) was dissolved in 10 mL of dry dichloromethane. To this solution was added a solution of Dess-Martin periodinane (741 mg, 1.8 mmol) and acetic acid (10.2 mg, 0.17 mmol) in dichloromethane (2 mL).[8] The reaction was stirred at room temperature for 40 min, at which point the starting material completely disappeared, as indicated by TLC. The reaction was then diluted with diethylether (25 mL), followed by 1.3 M NaOH (5 mL). The resulting suspension was stirred at room temperature for 15 min, and then separated. The ether layer was washed with 1.3 M NaOH, H_2O, and brine, then dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography (20:1 petroleum ether in diethylether, R_f = 0.23). The pure product appeared as a colorless oil (420 mg, 80 %). ^{1}H NMR ($CDCl_3$): δ 9.76 (t, *J* = 1.9 Hz, 1H), 3.59 (t, *J* = 6.6, 2H), 2.41 (dt, *J* = 7.4, 1.9 Hz, 2H), 1.68-1.59 (m, 2H), 1.55-1.45 (m, 2H), 1.36-1.23 (brm, 14H), 0.89 (s, 9H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 203.1, 63.5, 44.0, 33.0, 29.7, 29.7, 29.6, 29.5, 29.3, 26.1, 26.1, 25.9, 22.2, 18.5, -5.1.

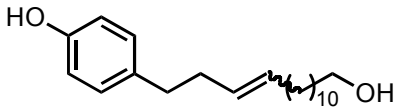
4-(15-(*tert*-butyldimethylsilyloxy)pentadec-3-enyl)phenol (**3**)



To a -10 ˚C solution of 3-(4-(hydroxyphenyl)propyltriphenylphosphonium bromide[9] (601 mg, 1.26 mmol) in anhydrous THF (5 mL) was added potassium *tert*-butoxide (1 M in THF, 1.32 mL, 1.32 mmol) dropwise over 5 min. The color of the solution changed from colorless to orange. The mixture was stirred at -10 ˚C for 20 min before aliphatic aldehyde **2 (**264 mg, 0.84

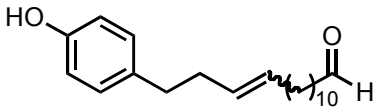
mmol) was added. The reaction continued to stir at -10 ˚C for 2.5 h when it was quenched with 1 M HCl (aq.). The mixture was extracted with ethyl acetate and the organic layer was washed with brine, dried over Na_2SO_4, and then concentrated. Purification by flash chromatography (4:1 petroleum ether in diethylether, R_f = 0.38) yielded the target molecule as a pale yellow oil (210 mg, 57 %). ^{1}H NMR ($CDCl_3$): δ 7.02 (d, *J* = 8.5, 2H), 6.72 (d, *J* = 8.5, 2H), 5.37-5.30 (m, 2H), 3.60 (t, *J* = 6.7, 2H), 2.62-2.49 (m, 2H), 2.31-2.25 (m, 2H), 1.98-1.91 (m, 2H), 1.55-1.43 (m, 2H), 1.32-1.19 (brm, 16H), 0.88 (s, 9H), 0.04 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 153.9, 134.4, 130.8, 129.6, 129.6, 128.9, 115.3, 115.2, 63.7, 35.3, 32.9, 29.7, 29.6, 29.5, 29.4, 27.3, 26.2, 25.9, 18.6, -5.1.

4-(15-hydroxypentadec-3-enyl)phenol (**4**)



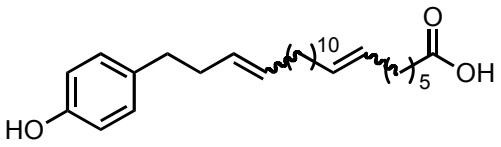
To a solution of silyl ether **3** (90 mg, 0.21 mmol) in anhydrous THF (2 mL) was slowly added tetrabutylammonium fluoride (1.0 M in THF, 210 μL, 0.21 mmol). The mixture was stirred vigorously at room temperature overnight and then concentrated *in vacuo*. Purification by flash chromatography (1:1 hexane in ethyl acetate, R_f = 0.52) yielded the title compound as a yellow oil (65 mg, 98%). ^{1}H NMR ($CDCl_3$): δ 7.05 (d, *J* = 8.5, 2H), 6.75 (d, *J* = 8.5, 2H), 5.58 (brs, 1H), 5.44-5.31 (m, 2H), 3.67 (t, *J* = 6.6, 2H), 2.61-2.56 (m, 2H), 2.36-2.25 (m, 2H), 2.01-1.90 (m, 2H), 1.61-1.54 (m, 2H), 1.39-1.21 (brm, 16H); ^{13}C NMR ($CDCl_3$): δ 154.0, 134.3, 130.8, 129.6, 128.9, 115.2, 77.4, 77.2, 76.9, 76.9, 63.3, 35.3, 32.8, 29.7, 29.6, 29.6, 29.6, 29.5, 29.3, 27.3, 25.8.

15-(4-hydroxyphenyl)pentadec-12-enal (**5**)



A 50 mL round bottom flask was charged with oxalyl chloride (260 mg, 2.05 mmol), anhydrous DMSO (300 μL), and dichloromethane (5 mL). The mixture was cooled to -78 ˚C and stirred for 15 min at which point a solution of alcohol **4** (87 mg, 0.28 mmol) in dichloromethane (2 mL) was added dropwise. The resulting solution was stirred at -78 ˚C for 25 min, and then triethylamine (852 μL, 6.12 mmol) was added to the mixture. This mixture was allowed to warm to room temperature and stirred for 4 h. The mixture appeared bright orange. The reaction was quenched with water and extracted with chloroform. The organic layer was washed with $NaHCO_3$ (sat. aq.) and dilute H_2SO_4, dried over Na_2SO_4, and concentrated. Purification by flash chromatography (4:1 hexane in ethyl acetate, $R_f = 0.39$) yielded the title compound as an orange oil (30 mg, 35%). ^{1}H NMR ($CDCl_3$): δ 9.76 (dd, *J* = 1.8, 1.4 Hz, 1H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.75 (d, *J* = 8.5 Hz, 2H), 5.44-5.32 (m, 2H), 2.61-2.55 (m, 2H), 2.44-2.40 (m, 2H), 2.34-2.27 (m, 2H), 2.01-1.91 (m, 2H), 1.67-1.54 (m, 2H), 1.46-1.37 (m, 2H), 1.35-1.11 (brm, 12H); ^{13}C NMR ($CDCl_3$): δ 205.2, 147.8, 133.1, 131.2, 129.6, 128.2, 111.7, 44.1, 37.3, 32.2, 29.7, 29.6, 29.4, 29.3.

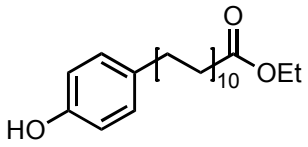
21-(4-hydroxyphenyl)henicosa-6,18-dienoic acid (**6**)



To a -10 ˚C solution of 5-carboxypentyltriphenylphosphonium bromide[10] (65.2 mg, 14 μmol) in THF (2 mL) was added potassium *tert*-butoxide (1.0 M in THF, 150 μL, 15 μmol). After stirring

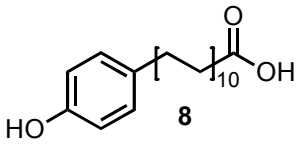
for 15 min, a solution of aldehyde **5** (30 mg, 9.5 μmol) in THF (2.5 mL) was slowly added and the resulting mixture was stirred at -10 ˚C for 2.5 h. At that point, the reaction was quenched with ice cold 1.0 M HCl (aq.) and extracted with ethyl acetate. After drying (with Na_2SO_4) and concentrating, the residue was purified by flash chromatography (4:1 ratio hexane in ethyl acetate, R_f = 0.12). The pure product was a white crystalline solid (27 mg, 69%). ^{1}H NMR ($CDCl_3$): δ 7.05 (d, *J* = 8.5, 2H), 6.75 (d, *J* = 8.5, 2H), 5.42-5.30 (m, 4H), 2.61-2.56 (m, 2H), 2.39-2.34 (m, 2H), 2.33-2.28 (m, 2H), 2.10-1.87 (m, 6H), 1.69-1.61 (m, 2H), 1.40 (s, 2H), 1.35-1.17 (brm, 16H); ^{13}C NMR ($CDCl_3$): δ 185.2, 153.7, 134.5, 130.9, 130.7, 129.7, 129.1, 128.8, 115.2, 35.3, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.2, 27.4, 27.4, 26.9, 24.5.

Ethyl 21-(4-hydroxyphenyl)henicosanoate (**7**)



To a solution of diene **6** (27 mg, 64 μmol) in absolute ethanol (1 mL) was added palladium on activated carbon (10% wt, 8.2 mg), and the mixture was vigorously stirred at room temperature for 94 h under 1 atm of hydrogen. At that point, the solvent was evaporated and the crude material directly purified by flash chromatography (4:1 hexane in ethyl acetate, R_f = 0.45). The pure product appeared as a white crystalline solid (11.0 mg, 42%). ^{1}H NMR ($CDCl_3$): δ 7.04 (d, *J* = 8.5, 2H), 6.74 (d, *J* = 8.5, 2H), 4.13 (q, *J* = 7.2, 2H), 2.52 (dd, *J* = 7.6, 7.9 Hz, 2H), 2.29 (t, *J* = 7.6, 2H), 1.61 (t, *J* = 7.2 Hz, 2H), 1.35-1.19 (brm, 37H); ^{13}C NMR ($CDCl_3$) δ: 189.1, 153.6, 132.5, 129.6, 115.2, 60.3, 35.2, 34.6, 31.9, 29.9, 29.8, 29.8, 29.8, 29.7, 29.7, 29.6, 29.4, 29.4, 29.3, 25.1, 14.4.

21-(4-hydroxyphenyl)henicosanoic acid (PHPA standard, 8)



8

To a solution of ester **7** (5.7 mg, 13 μmol) in anhydrous THF (400 μL) was added lithium hydroxide (1M in H_2O, 100 μL), making a 4:1 ratio of THF to H_2O. The mixture was refluxed at 55 ˚C overnight, at which point TLC confirmed the reaction was complete. The organic content was removed *in vacuo* and the residual material was dissolved in H_2O. The solution was adjusted to pH 14 with 1.0 M NaOH (aq.) and the aqueous layer was washed with diethylether. The pH was then carefully adjusted to 1 with 1.0 M HCl, and the solution was extracted by diethylether. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo* to provide the title compound as a white solid (2.9 mg, 55%). ^{1}H NMR (MeOD): δ 6.97 (d, J = 8.4, 2H), 6.67 (d, J = 8.4, 2H), 2.53-2.47 (m, 2H), 2.27 (t, J = 7.4, 2H), 1.65-1.49 (m, 4H), 1.42-1.24 (brm, 32H). MS: $C_{27}H_{46}O_3$, calculated exact mass = 418.3447; experimental neutral mass = 418.3453, where the experimental neutral mass is the observed ion $[M\text{-}H^+]^-$ m/z plus H^+, 1.00728 Da (Table 1 and Figure S3).

B.2. Protein purification

Pks15/1 and its mutant variants Pks15/1(C211A), Pks15/1(S2039A), and Pks15/1(C211A-S2039A) were expressed from plasmids pET28b-Pks15/1, pET28b-Pks15/1(C211A), pET28b-Pks15/1(S2039A), and pET28b-Pks15/1(C211A-S2039A), respectively. The construction of these and other plasmids used in this study is described below (Section B.3). The proteins were overproduced in *E. coli* BL21(DE3) (Stratagene) as isopropyl β-D-1-thiogalactopyranoside

(IPTG)-inducible, *N*-terminally hexahistidine-tagged fusions. To obtain Pks15/1 and Pks15/1(C211A) in their phosphopantetheinylated (holo) form, the proteins were coexpressed with Sfp using plasmid pSU20-Sfp as reported.[2,6] For protein overproduction, the corresponding expression strains were cultured in Luria-Bertani (LB) broth (Becton, Dickinson and Company) containing kanamycin (30 μg/mL) [or kanamycin (30 μg/mL) and chloramphenicol (20 μg/mL) for strains carrying pSU20-Sfp] under orbital shaking (220 rpm) at 37 °C. When the cultures reached an $OD_{600\ nm}$ of 0.6, the incubation temperature was reduced to 18 °C and recombinant protein production was induced by addition of IPTG (0.025-0.1 mM). After 20 h of additional incubation (18 °C, 220 rpm), cells were harvested by centrifugation (6,000 *g*, 20 min) and resuspended in lysis buffer (75 mM sodium phosphate, pH 7.6; 300 mM NaCl; 10 mM imidazole). Cells were disrupted using a French pressure cell and cellular debris was removed from the lysates by ultracentrifugation at 40,000 rpm (Ti 50.2 rotor from Beckman Coulter, Inc.) for 40 min. The tagged proteins were purified from the clarified supernatant by Ni^{2+}-column chromatography using Ni–NTA Superflow resin according to the manufacturer's instructions (QIAGEN). Fractions containing desired protein with best purity were identified by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[1] pooled, concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore), and buffer-exchanged with 100 mM sodium phosphate buffer (pH 7.2) using PD-10 desalting columns (GE Healthcare) (Figure S5). Additional purification steps (*e.g.*, size exclusion chromatography) were not possible due to poor protein solubility and/or loss of enzymatic activity of Pks15/1 and its mutant variants. FadD22 and FadD22(S576A) were expressed in *E. coli* BL21(DE3) as IPTG-inducible, *C*-terminally hexahistidine-tagged proteins and purified by Ni^{2+}-column chromatography using Ni–NTA Superflow resin as previously reported.[2] Holo-FadD22 was obtained by coexpression with Sfp using plasmid pSU20-Sfp as described earlier.[2] Purified proteins were flash-frozen in a dry ice-ethanol bath and stored at -80 °C.

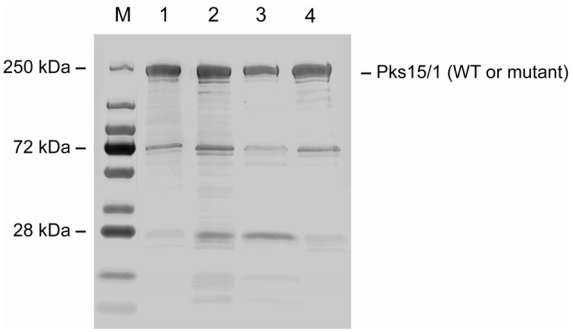


Figure S5. Purity of recombinant holo-Pks15/1 (lane 1), holo-Pks15/1(C221A) (lane 2), Pks15/1(S2039A) (lane 3), and Pks15/1(C211A/S2039A) (lane 4). The purities of apo-Pks15/1 and apo- Pks15/1(S2039A) (not shown) preparations were comparable to that of the proteins shown. Protein samples were resolved by SDS-PAGE (10%) and stained with GelCode Blue Stain (Pierce). The positions of molecular weight markers are indicated (lane M).

B.3. Construction of plasmids

Recombinant DNA manipulations were carried out using reported standard methods[1] and *E. coli* DH5α (Invitrogen) as a cloning host. *pks15/1* mutant alleles were created using QuikChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were sequenced to verify fidelity. DNA sequencing was conducted at Cornell University's Life Sciences Core Laboratories Center. Plasmid constructions are outlined in Figure S6.

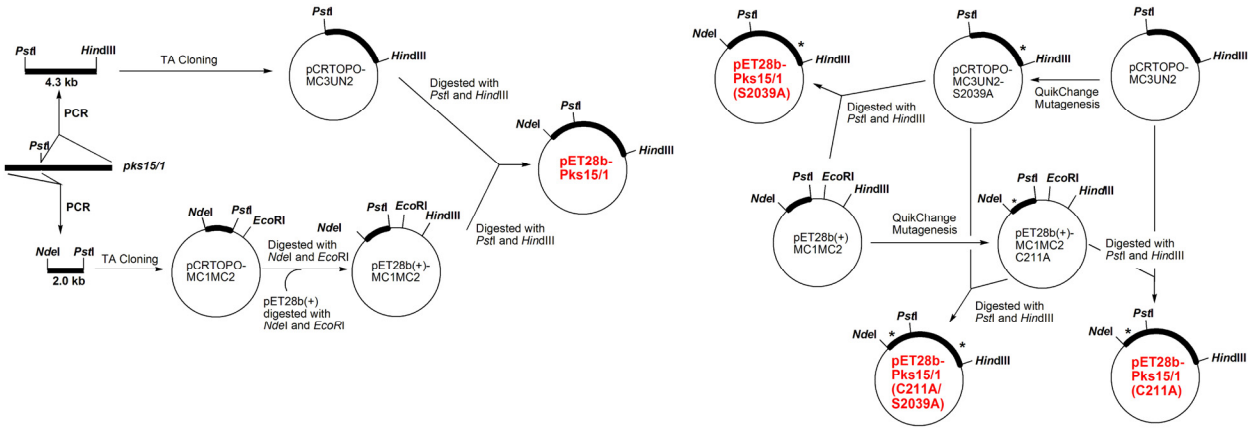


Figure S6. Strategy for construction of plasmids pET28b-Pks15/1, pET28b-Pks15/1(C221A), pET28b-Pks15/1(S2039A), and pET28b-Pks15/1(C211A/S2039A). *, represents introduced mutations.

pET28b-Pks15/1. The 2.0-kb 5'-end and the 4.3-kb 3'-end segments of gene *pks15/1* (genome locus tag: MMAR_1762) from *Mycobacterium marinum* strain M (American Type Culture Collection Number BAA-535) were PCR-amplified with primer pairs MC1 (5'-GCAACTGCATATGACCACCAGCGGTGAAAGCGCCG-3'; introduces an *Nde*I site, underlined) and MC2 (5'-ATCACGAAGTCGGGCTGCAGTCCCCAGCGTTG-3'; a native *Pst*I site is underlined) and MC3 (5'-AACGCTGGG GACTGCAGCCCGACTTCGTGATG-3'; a native *Pst*I site is underlined) and UN2 (5'-GCTCGAAGCTTCTACGCAGTTACCG AAACCATCTCGCCGT-3'; introduces a *Hin*dIII site, underlined), respectively. Genomic DNA from *M. marinum* was used as PCR template. *M. marinum* was cultured as reported previously[2] and its genomic DNA was isolated as described elsewhere.[3] Each 5'-end and 3'-end PCR-generated gene segment was independently cloned into *E. coli* cloning vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen) to create plasmids pCRTOPO-MC1MC2 and pCRTOPO-MC2UN2, respectively. The 5′-end segment was recovered from pCRTOPO-MC1MC2 as an *Nde*I-*Eco*RI fragment and cloned into *E. coli* expression vector pET28b(+) (Novagen) digested with *Nde*I and *Eco*RI, resulting in plasmid pET28b(+)-MC1MC2. The 3′-end segment was recovered from pCRTOPO-MC2UN2 as a *Pst*I-*Hin*dIII fragment and cloned into the pET28b(+)-MC1MC2 digested with *Pst*I and *Hin*dIII. The resulting plasmid, pET28b-Pks15/1, expressed IPTG-inducible, *N*-terminally hexahistidine-tagged Pks15/1 (Figure S6).

pET28b-Pks15/1(C221A). pET28b(+)-MC1MC2 was mutagenized using primer pair C211A (5'-CGGTGGATACGGCGGCCTCGTCGTCGTTGG-3') and C211A-antisense (5'-CCAACGACGACGAGGCCGCCGTATCCACCG-3') to change Cys-221 codon to Ala codon GCC and create plasmid pET28b(+)-MC1MC2-C211A. Then, the wild-type *Pst*I-*Hin*dIII 3′-end *pks15/1* segment from pCRTOPO-MC2UN2 was cloned into pET28b(+)-MC1MC2-C211A

digested with *Pst*I and *Hin*dIII. The resulting plasmid, pET28b-Pks15/1(C221A), expressed IPTG-inducible, N-terminally hexahistidine-tagged Pks15/1(C221A) (Figure S6).

pET28b-Pks15/1(S2039A). pCRTOPO-MC2UN2 was mutagenized using primer pair S2039A (5'-CTTGGCTTCGACGCGCTGACCGCGG-3') and S2039A-antisense (5'-CCGCGGTCAGCGCGTCGAAGCCAAG-3') to change Ser2039 codon to Ala codon GCG and create plasmid pCRTOPO-MC2UN2-S2039A. Then, the mutagenized *Pst*I-*Hin*dIII 3′-end segment was subcloned into pET28b(+)-MC1MC2 digested with *Pst*I and *Hin*dIII. The resulting pET28b-Pks15/1(S2039A) plasmid expressed IPTG-inducible, *N*-terminally hexahistidine-tagged Pks15/1(S2039A) (Figure S6).

pET28b-Pks15/1(C211A/S2039A). The mutagenized *Pst*I-*Hind*III fragment of pCRTOPO-MC2UN2-S2039A was cloned into pET28b(+)-MC1MC2-C211A digested with *Pst*I and *Hin*dIII. The resulting pET28b-Pks15/1(C211A-S2039A) plasmid expressed IPTG-inducible, *N*-terminally hexahistidine-tagged Pks15/1(C211A-S2039A) (Figure S6).

pETDuet-Pks15/1. The *Xba*I-*Hin*dIII fragment from pET28b-Pks15/1, encompassing the segment coding for the *N*-terminally hexahistidine-tagged Pks15/1 and its upstream ribosome binding site, was cloned into the expression vector pETDuet-1 (Novagen) digested with *Xba*I and *Hin*dIII. The resulting pETDuet-Pks15/1 plasmid expressed IPTG-inducible, *N*-terminally hexahistidine-tagged Pks15/1.

pCDF-FadD22. The *Nco*I-*Xho*I fragment from pET28b-FadD22mm^2 encoding *M. marinum* FadD22 was cloned into plasmid pCDFDuet-1 (Novagen) digested with *Nco*I and *Xho*I. The resulting pCDF-FadD22 plasmid expressed IPTG-inducible, *C*-terminally S-peptide-tagged FadD22.

pSU20-Sfp and pRSF-ACC. pSU20-Sfp, constitutively expressing *Bacillus subtilis* phosphopantetheinyl transferase Sfp,[4] was kindly provided by Prof. Christopher T. Walsh

(Harvard Medical School, USA). pRSF-ACC[5] expresses *Corynebacterium glutamicum* acetyl-CoA carboxylase (ACC), and it was included in *E. coli* to increase the intracellular pool of malonyl-CoA available for synthesis of PHPAs. pRSF-ACC was kindly provided by Prof. Nobutaka Funa (University of Tokyo) with the permission of Prof. Hisashi Kawasaki (Tokyo Denki University).

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