

Supplemental Material

β-Lactone Synthetase Found in the Olefin Biosynthesis Pathway

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Materials and Methods

Synthesis and characterization of 3-hydroxy-2-octyldodecanoic acid (3). Synthesis of **3** was prepared from reaction of 1.0 g of decanoic acid with 2.2 equivalents of 1 M LDA in THF at -30° C followed by warming and solvent exchange to remove hexane as described by Mulzer (1). Addition of decanal (2.2 equivalents in THF) produced 95% pure **3** by NMR with the major contaminant identified as decanoic acid. The (+/-) *syn*- β -hydroxy acid diastereomers (2R, 3S and 2S, 3R) were produced in 1:1.3 ratio with the (+/-) *anti*- β -hydroxy acid diastereomers (2R, 3R and 2S, 3S). Recrystallization yielded crystals of an equal molar ratio of all four possible diastereomeric isomers by ^1H NMR and GC/MS analyses, leaving the *anti*-diastereomer highly enriched in the supernatant. **Figure S15** shows the general synthesis and highlights the four diastereomeric products. All ^1H NMR (400 MHz, CDCl_3) peaks match literature values well (2) with the single β -hydrogens of the *syn*- and *anti*- β -hydroxy acids showing shifts of 3.86 and 3.72 ppm, respectively. The single α -hydrogens of the two diastereomeric pairs were not resolved, both falling near 2.48 ppm.

HPLC separation of *syn*- and *anti*-diastereomer pairs. The synthesized mixture of *syn*- and *anti*-3-hydroxy-2-octyldodecanoic acids (**3**) was separated into the *syn*- and *anti* diastereomeric pairs (**3a** and **3b**) by High Pressure Liquid Chromatography (HPLC, Hewlett Packard) using a reverse phase C18 column (Agilent eclipse plus 4.6x250 mm) and flow rates of 1.0 mL/min. The LC column was prewashed with 100% acetonitrile (ACN) with 4 mM HCl and 40% methyl tert-butyl ether (MTBE) in ACN prior to injection of 100 μL samples of 2.0 mg/ml of (**3**) in the acidic ACN. The elution program at room temperature was as follows: 100% ACN hold for 2 min; linearly ramp MTBE to 40% in ACN over 8 min; hold 40% MTBE for 5 min; reverse to 100% ACN in 3 minutes. Total program was 18 min, with a detection wavelength of 220 nm. Fractions were collected manually from 10 runs to accumulate approximately 1.0 mg of each racemic diastereomer. The elution of *syn*- before *anti*-**3** in reverse phase HPLC is consistent with

literature (2). GC/MS and ^1H NMR analyses confirmed both **3a** and **3b** at 90% purity. ^1H NMR (400 MHz, CDCl_3) values for the single α - and β -hydrogens of the (+/-) *syn*- β -hydroxy acids were 2.50 and 3.85 ppm, respectively, while the same hydrogens in the (+/-) *anti*- β -hydroxy acid show shifts of 2.47 and 3.72 ppm, respectively (**Fig S5** and **S6**).

Synthesis and characterization of *cis*- and *trans*-4-nonyl-3-octyloxan-2-one (4a, 4b).

Starting with decanoic acid, the method of Lee (3) produced octylketene dimer in good yield. Only one isomer (presumably *cis*) was observed by NMR. Prior to hydrogenation the ketene dimer was purified by silica gel chromatography. Hydrogenation with 10% Pd/C catalyst in EtOAc/EtOH , as described by Lee (3), produced (+/-) *cis*-4-nonyl-3-octyloxan-2-one (**4a**) and low levels (~3%) of the *trans* isomer, as observed by ^1H NMR/ CDCl_3 . The methine protons of the *cis*-isomer at carbons 4 and 3 are sharp multiplets (d,d,d) at 4.53 and 3.59 ppm, respectively (**Fig S3**). The corresponding multiplets for the *trans* isomer were at 4.21 and 3.16 ppm (**Fig S4**). *trans*-4-nonyl-3-octyloxan-2-one (**4b**) was isolated and purified from a complex mixture of products containing about equal amounts of the *cis* and *trans*-isomers of the oxan-2-one. The complex mixture was prepared by the reaction of methane sulfonyl chloride (2 equivalents Et_3N) in dichloromethane with 3-hydroxy-2-octyldodecanoic acid (**3**) (~1:1 *syn/anti*) according to Crossland (4). Column chromatography (SiO_2 gel, hexane stepwise to dichloromethane) gave fractions of (+/-) *trans*-4-nonyl-3-octyloxan-2-one eluting before the (+/-) *cis*-isomer. This separation was monitored by ^1H NMR, and the final *trans*- β -lactone (**4b**) spectrum is shown in **Figure S4**.

Synthesis and characterization of *cis*- and *trans*-9-nonadecene (5a, 5b).

The alkyne precursor, 9-nonadecyne, was produced in ~80% yield by the reaction of lithiated (n-butyl lithium/THF, -78° C) 1-decyne under reflux with 1-bromononane and catalytic amounts of sodium iodide (5). The product was distilled at 112° C/0.06 mm Hg. 9-Nonadecyne was hydrogenated to *cis*-9-nonadecene under Lindlar conditions (6). A glass-lined, 50 mL, Parr

pressure reactor (50mL) was charged with commercial Lindlar catalyst (5% Pd/CaCO₃ poisoned with Pb), quinoline, and 9-nonadecyne and stirred under 500 psi H₂. The reaction was followed by ¹H NMR and various batches produced ~90% yield of *cis*-9-nonadecene along with low levels (<10%) of the *trans*- isomer and nonadecane overnight. The quinoline, catalyst, and hydroquinolines were removed by filtration through silica gel. Distillation (127-129° C/0.15 mmHg) gave 90% pure *cis*-9-nonadecene (**5a**) (10% trans by ¹H NMR and GC/MS) (**Fig S1**). *trans*-9-Nonadecene (**5b**) was generated by the reduction of 9-nonadecyne by Na/NH₃ using standard conditions (7). Analysis by ¹H NMR showed a mixture of products with 57% starting alkyne, 36% *trans*-9-nonadecene, and 7% *cis*-9-nonadecene. The vinyl proton multiplets for the *trans*- and *cis*-isomers are nearly completely separated, falling at 5.39 and 5.36 ppm, respectively. The multiplet for the four propargylic methylene protons of the starting alkyne at 2.15 ppm is clearly separated from the allylic multiplets of the *trans*- and *cis*-alkenes at 1.97 and 2.01 ppm, respectively. GC/MS also supports the conclusions of the ¹H NMR analysis. To further confirm our assignments of *cis*- and *trans* olefin, photoisomerization of *cis*-9-nonadecene to *trans* was done according to literature (8). The overnight irradiation (254nm) of a solution of 11mg of 90:10 mixture of *cis*- and *trans*-9-nonadecene and 4 mg diphenyl disulfide in hexane (1.65 g) in a quartz UV cell produced a 21:79 mixture of the *cis*- and *trans*- alkenes respectively by ¹H NMR (**Fig S2**).

¹H NMR analyses of the Ole-pathway metabolites. All NMR spectra were acquired on a Varian Inova 400 MHz NMR spectrometer using a 5 mm Auto-X Dual Broadband probe at 20°C. Typically 512 pulses were used with a one second pulse delay. Solvent was always CDCl₃ with TMS as a reference. Enzyme reactions contained 1-bromo-naphthalene as an integration reference. Common known contaminant resonances in our spectra are the CDCl₃ (singlet, 7.26 ppm), dichloromethane (singlet, 5.30 ppm), and the plasticizer bis(2-ethylhexyl) phthalate (dioctyl phthalate) (multiplet 4.22 ppm). The origin of the quartet at 3.98 ppm in several figures is unknown.

GC/MS analysis of Ole pathway metabolites. Separation and identification of metabolites was accomplished by gas chromatography/mass spectrometry (GC/MS Agilent 7890a & 5975c) equipped with an Agilent J&W bd-ms1 column (30 m, 0.25 mm diameter, 0.25 µm film). Olefin (from either β-lactone breakdown or standards) was detected without derivatization, but the β-hydroxy acid required methylation of the carboxylic acid group by diazomethane. Preparation of ethereal alcoholic solutions of diazomethane from N-methyl-N-nitroso-p-toluenesulfonamide was carried out using a commercial kit from Aldrich (Technical information Bulletin No. AI-113). All samples were extracted with ethyl acetate (EtOAc) and mixed with 100 µL of diazomethane solution when needed. Five microliters of each sample were injected into the injection port (230° C). The 20 min program was as follows: hold 100° C for 3 min; ramp linearly to 280° C for 16 min; hold 280° C for 2 min.

Protein expression and purification. All genes were *Escherichia coli* (*E. coli*) codon optimized and obtained from either IDT or DNA 2.0. Constructs were created using standard cloning techniques with restriction digest, ligation, and transformation reagents all purchased from New England Biolabs (NEB) and used according to manufacturer's protocols. Constructs with pET28b⁺ contained an N-terminal 6X His tag, while pET30b⁺ contained a C-terminal tag. All vector constructs were sequence verified and are listed in **Table S1**.

Table S1

Protein	Organism	Ascension #	Vector	Buffer Additive
OleA	<i>Xanthomonas campestris</i>	WP_011035468.1	pET28b ⁺	5 mM BME
OleC	<i>Xanthomonas campestris</i>	WP_011035474.1	pET30b ⁺	-
OleD	<i>Xanthomonas campestris</i>	WP_011035475.1	pET28b ⁺	0.025% Tween 20
OleC	<i>Stenotrophomonas maltophilia</i>	AFC01244.1	pET30b ⁺	-
OleC	<i>Arenimonas malthae</i>	WP_043804215.1	pET30b ⁺	-
OleC	<i>Lysobacter dokdonensis</i>	WP_036166093.1	pET30b ⁺	-
OleB-C	<i>Micrococcus luteus</i>	WP_010078536.1	pET30b ⁺	-

OleB-C is a natural fusion of OleB and OleC in *Ml*

All vectors were transformed into BL21 (DE3) cells (NEB). Starter cultures (5 mL) were grown in LB media overnight at 37° C with kanamycin selection. Cells were transferred to 2 L flasks

containing 1 L of LB media with kanamycin and grown to an optical density of 0.5 at 37° C. Growth was transitioned to 16° C, and expression was induced by isopropyl β -D-1-thiogalactopyranoside (IPTG, 100 μ M). Cells were pelleted and resuspended in 10 mL of buffer containing 200 mM NaCl, 20 mM NaPO₄, and 10% glycerol at pH 7.4. Protein-specific buffer additives are indicated in **Table S1**. Cells were sonicated, spun at 33,000xg for 45 min, and the supernatant was filtered through 0.45 and 0.22 μ m filters (Millipore), successively. The supernatant was then loaded onto a GE HisTrip HP 1 mL column using an Äkta fast protein liquid chromatography instrument (GE). The column was first washed with 125 mM imidazole buffer before protein elution with 400 mM imidazole buffer. Protein concentration was determined using the Bradford protein assay before samples were aliquoted and frozen at -80° C for future use.

Formation of β -lactones by OleC from β -hydroxy acids. Reactions to generate β -lactones contained 1.0 mg OleC, 20 mg of ATP, and 30 mg of MgCl²•6H₂O in 100 mL of buffer without glycerol (200mM NaCl and 20 mM NaPO₄ at pH 7.4). The appropriate β -hydroxy acids were dissolved in DMSO to 1.0 mg/mL, and 1-bromo-naphthalene at 0.5 mg/mL was included as an internal reference. Replacing DMSO with ethanol for substrate delivery had no effect on the results. Reactions were initiated with 1.5 mL of the DMSO-substrate solution and allowed to run for 24 hours before three successive extractions were performed with 10 mL, 5 mL, and 5 mL of dichloromethane. Samples were allowed to evaporate at room temperature before dissolving in CDCl₃ for NMR. Extraction with ethylacetate instead of dichloromethane produced the same results. **Figures S8-S14** show the ¹H NMR results of OleC experiments.

Computational analyses of OleC homologues. A BLAST search with the non-redundant protein sequence database from the National Center for Biotechnology Information (NCBI) was used to identify homologs of OleC (WP_011035474.1) from *Xanthomonas campestris* pv. *campestris* ATCC 33913. BLAST parameters were as follows: matrix = BLOSUM62; gap cost =

Existence:11 Extentension:1; word size = 6; max matches in a query range = 0; expect threshold = 10. The most distantly related sequences that maintained 25% identity had E values of 2e-14 or better.

Supplemental Figures

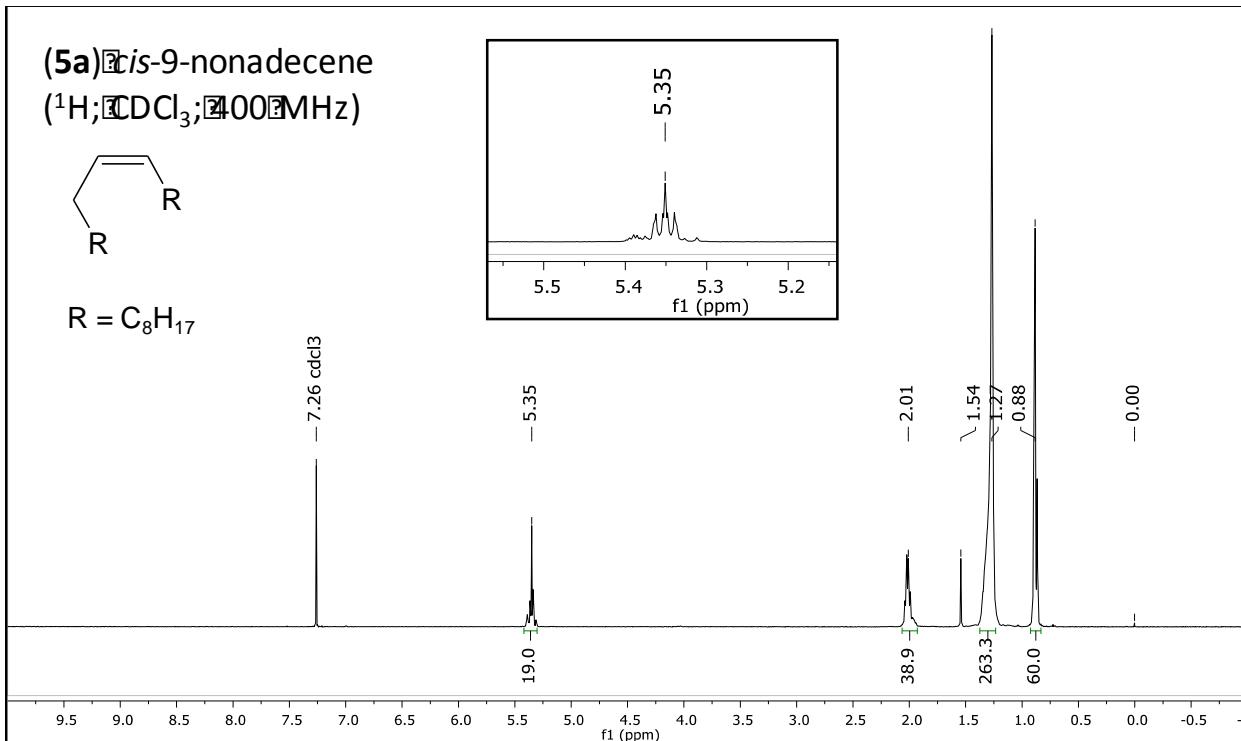


Figure S1. *cis*-Olefin standard.

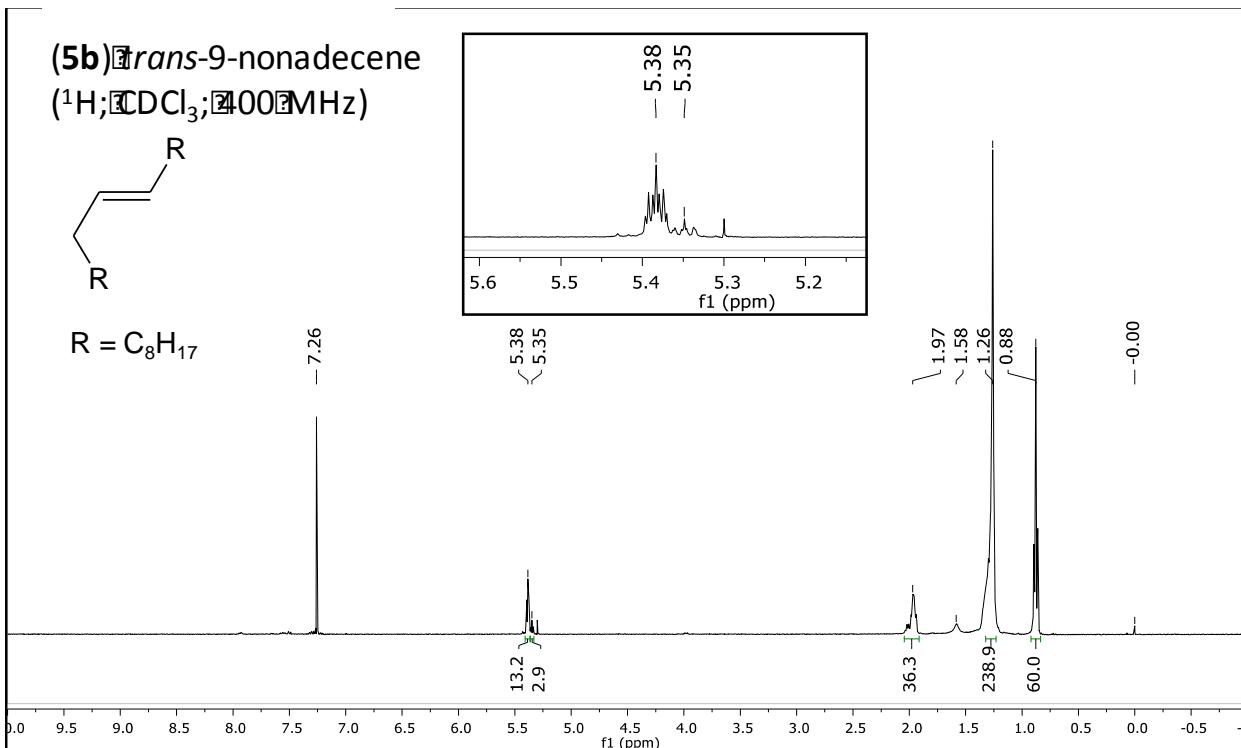


Figure S2. *trans*-Olefin standard. Approximately 20% *cis*-olefin can be seen.

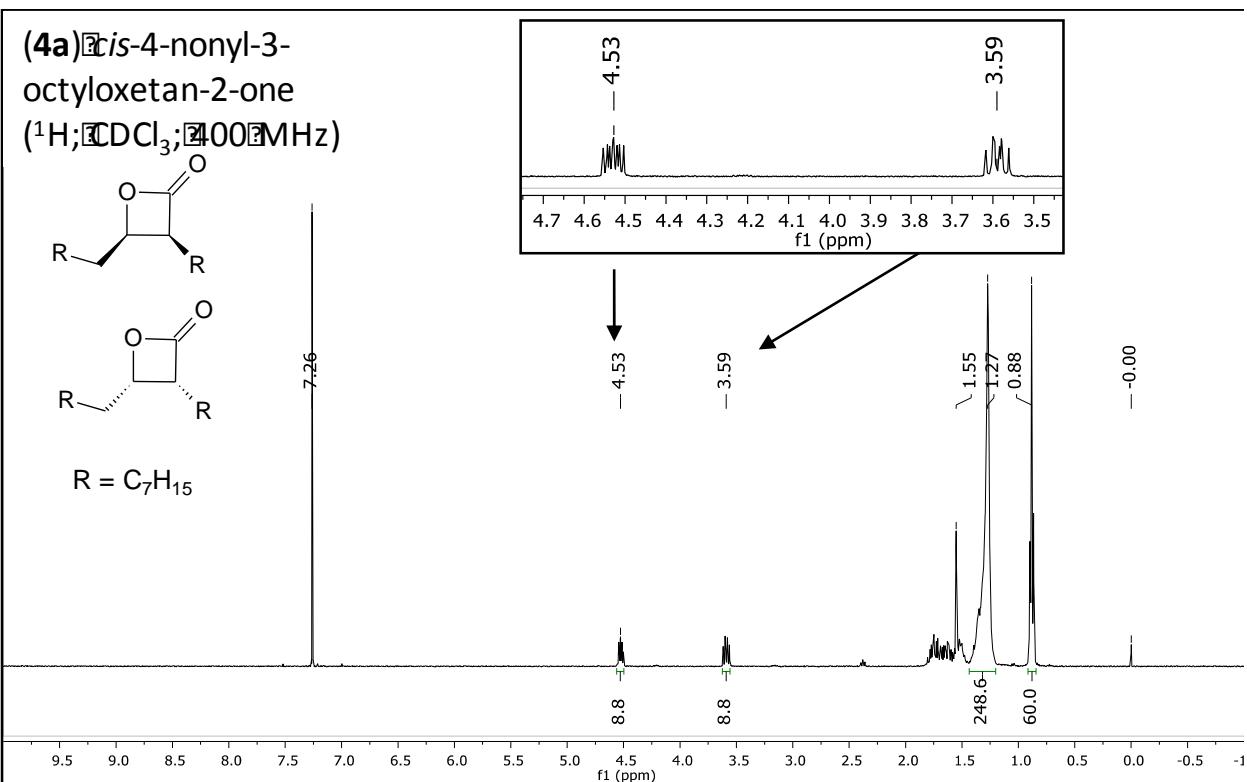


Figure S3. Synthetic *cis*- β -lactone.

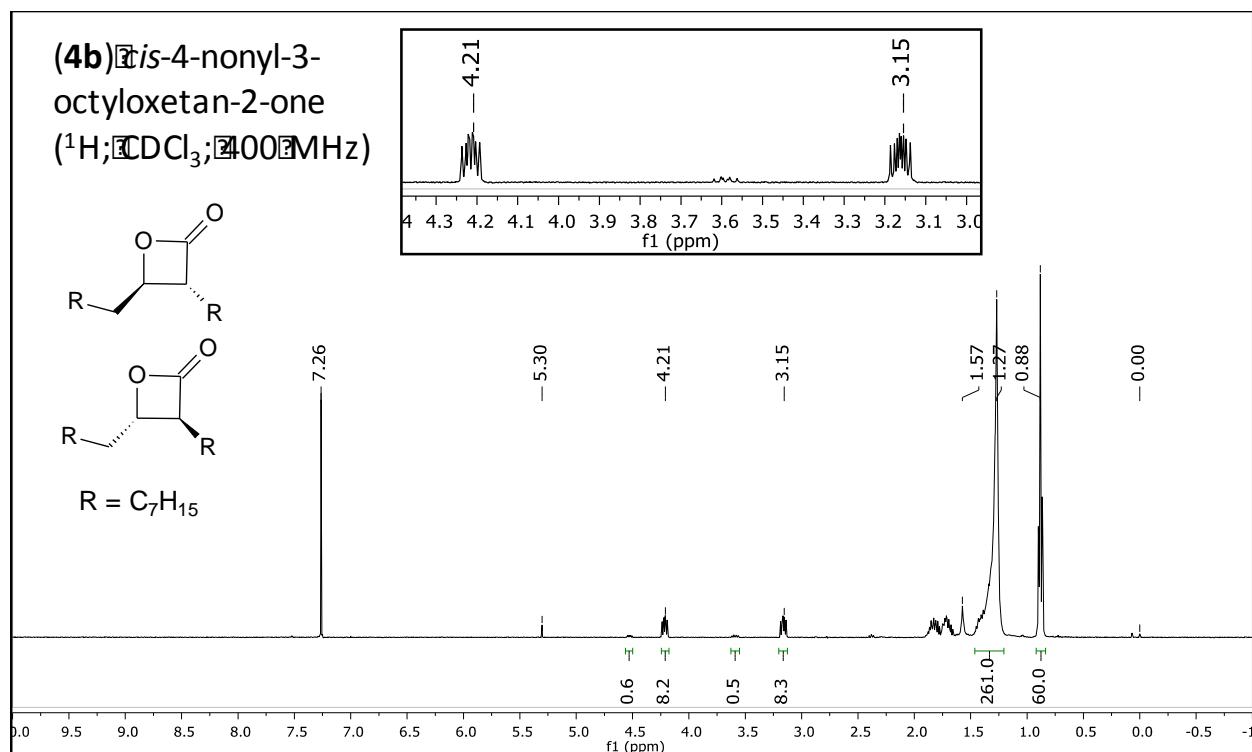


Figure S4. Synthetic *trans*- β -lactone. Singlet at 5.30 ppm is dichloromethane. A very small amount *cis*- β -lactone (<10%) can be seen at 3.59 ppm

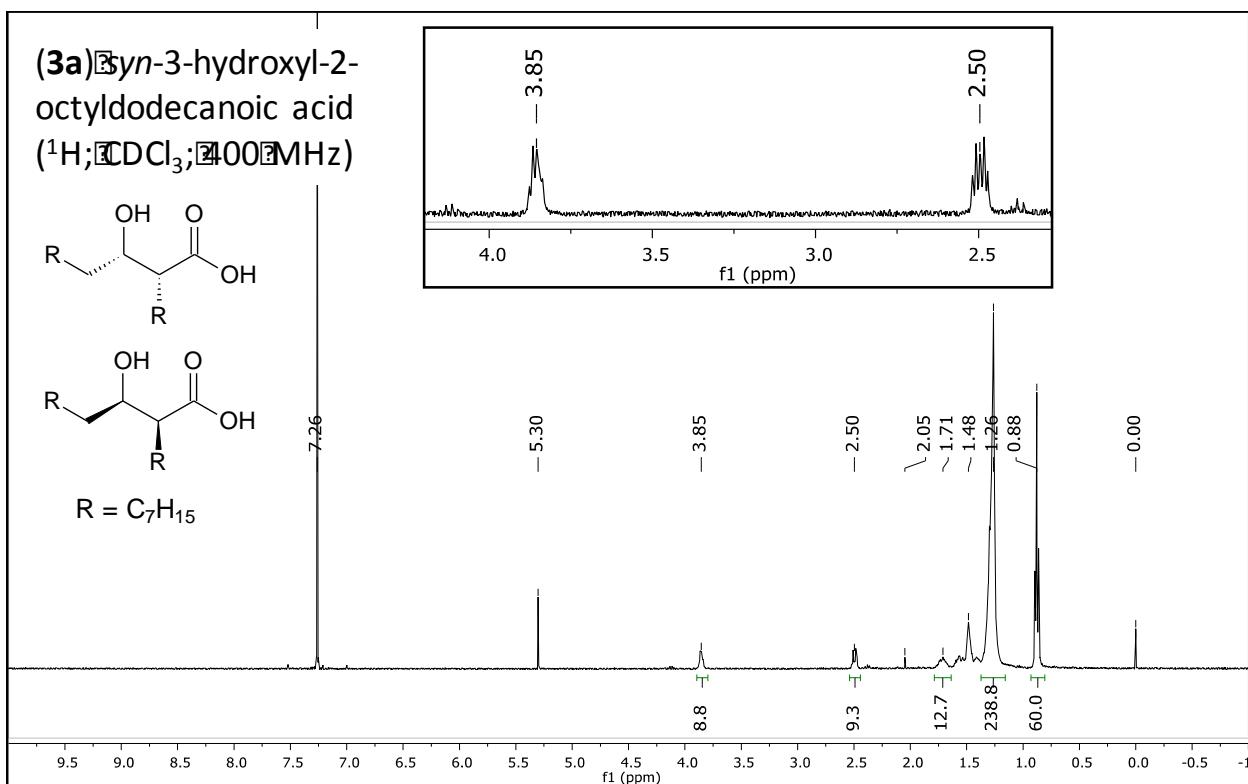


Figure S5. HPLC separated (+/-) *syn*-diasteromeric pairs (2R,3S and 2S, 3R). Singlet at 5.30 is dichloromethane.

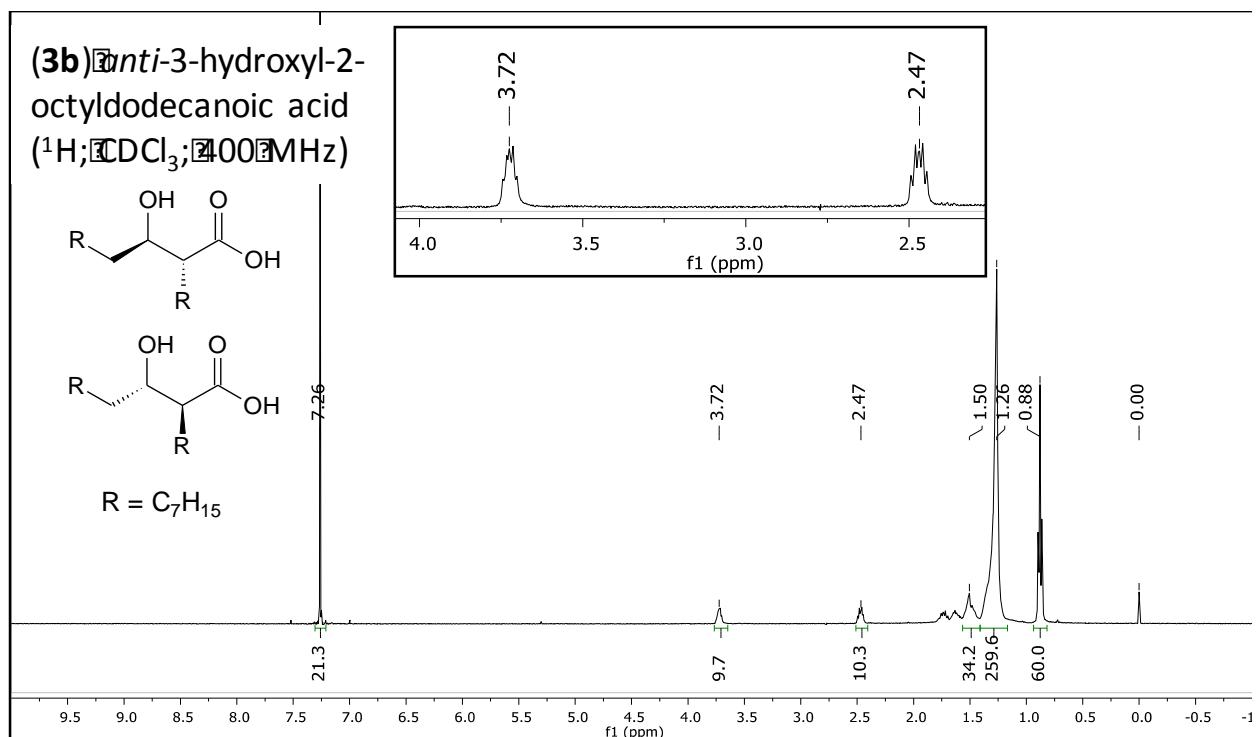


Figure S6. HPLC separated (+/-) *anti*-diasteromeric pairs (2R,3R and 2S, 3S).

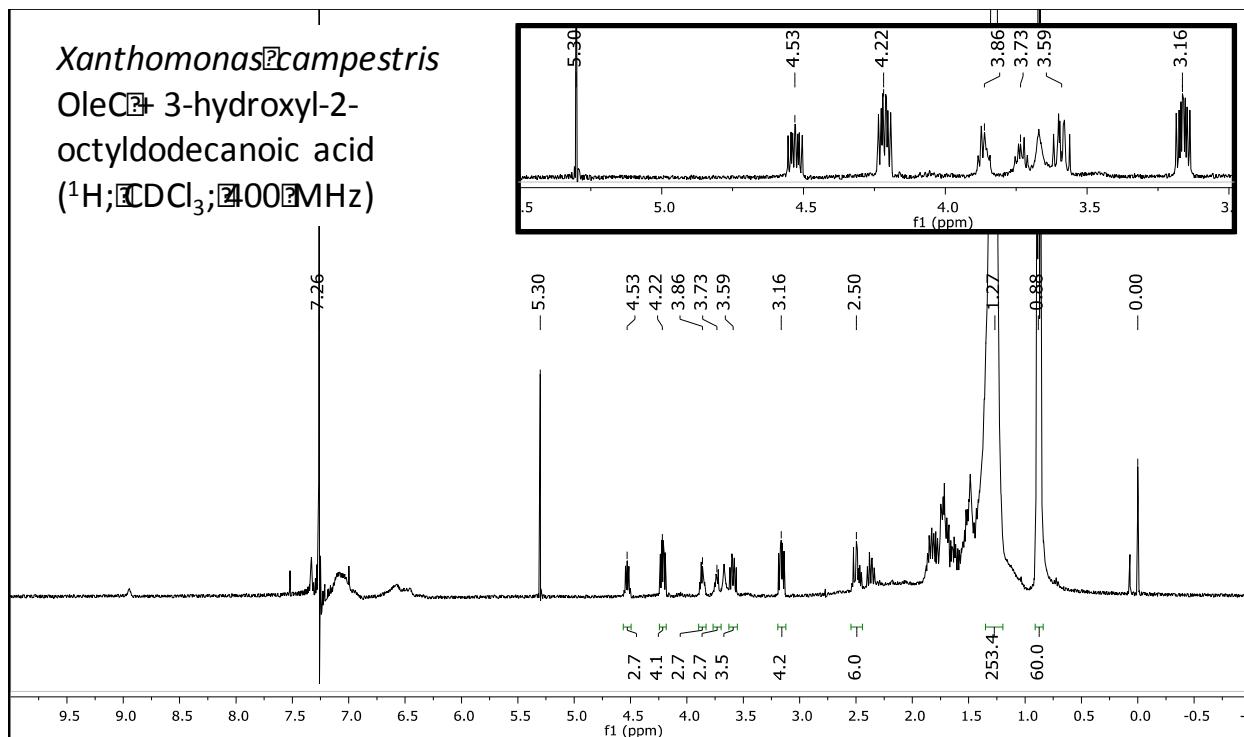


Figure S7. All four isomers of **3** with *Xc* OleC overnight. The singlet of dichloromethane is visible at 5.30 ppm.

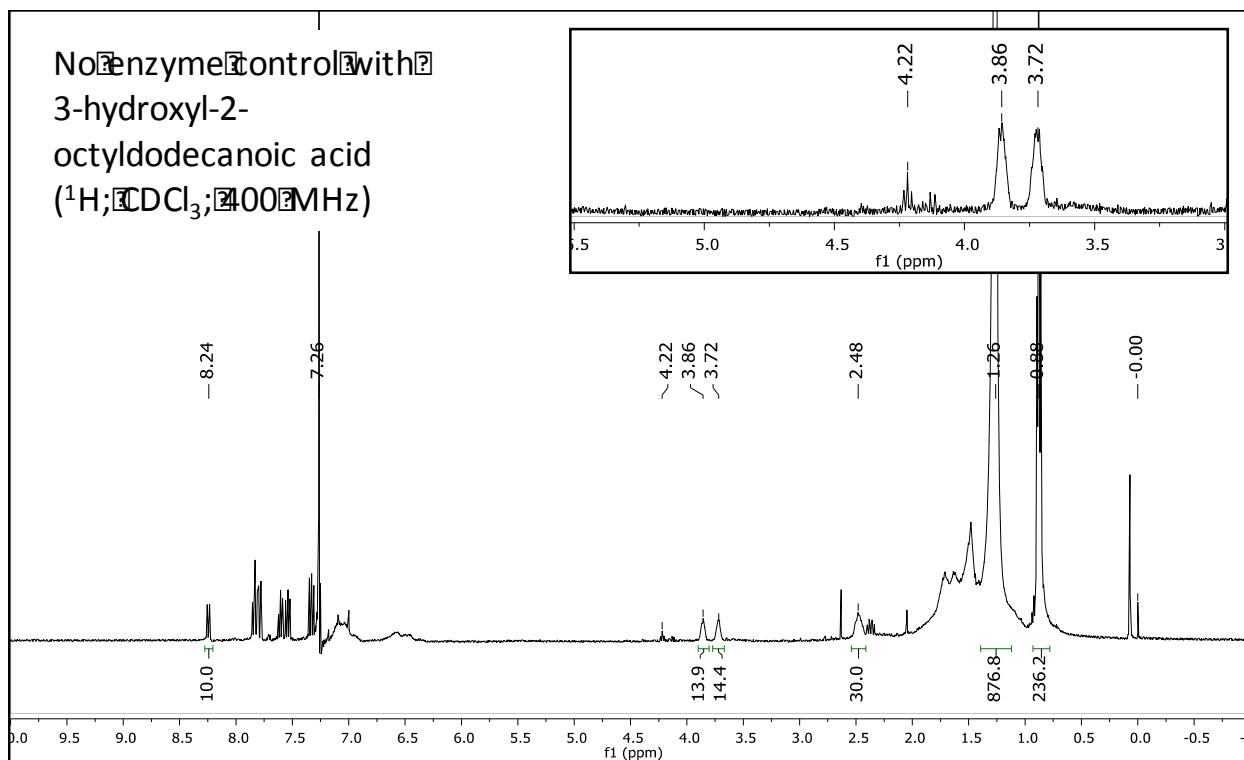


Figure S8. All four isomers of **3** with no enzyme. Some diethyl phthalate contamination is visible at 4.22 ppm.

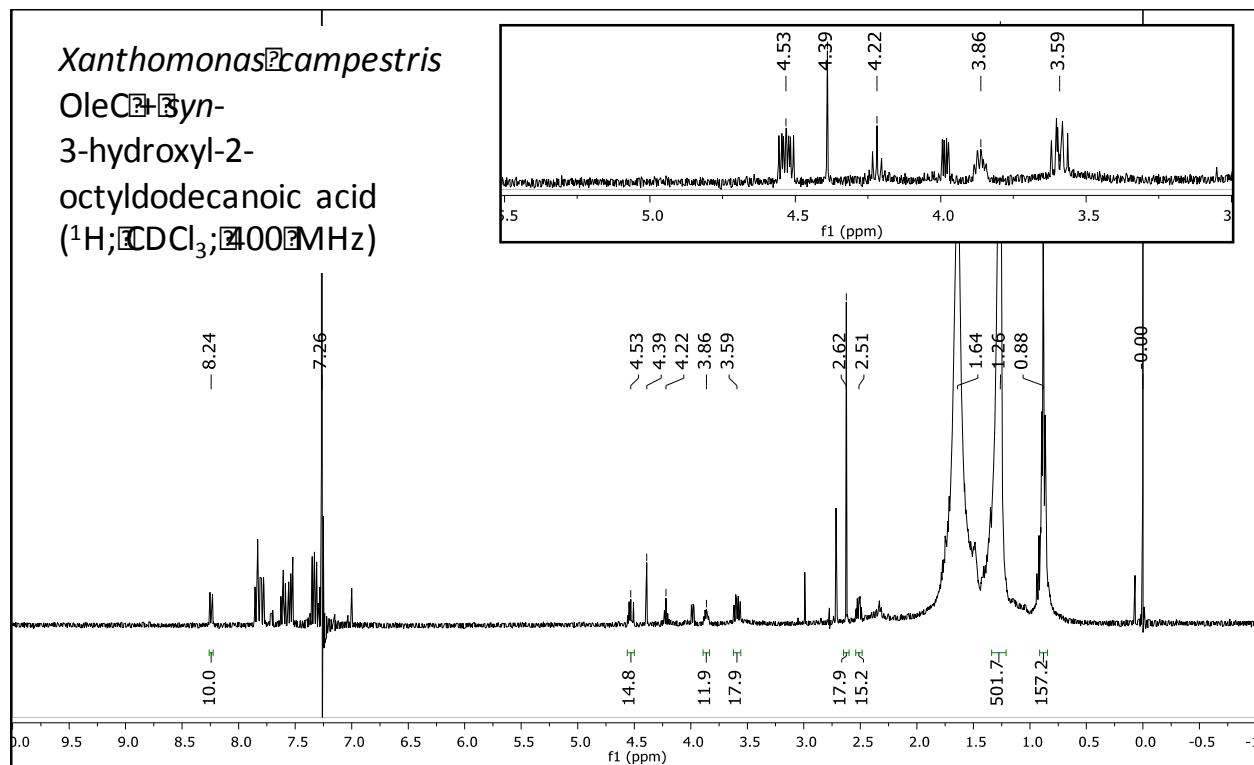


Figure S9. Xc OleC with **3a** overnight. A multiplet from the plasticizer diethyl phthalate is visible at 4.22 ppm. Only (+/-) *cis*- β -lactone is observed.

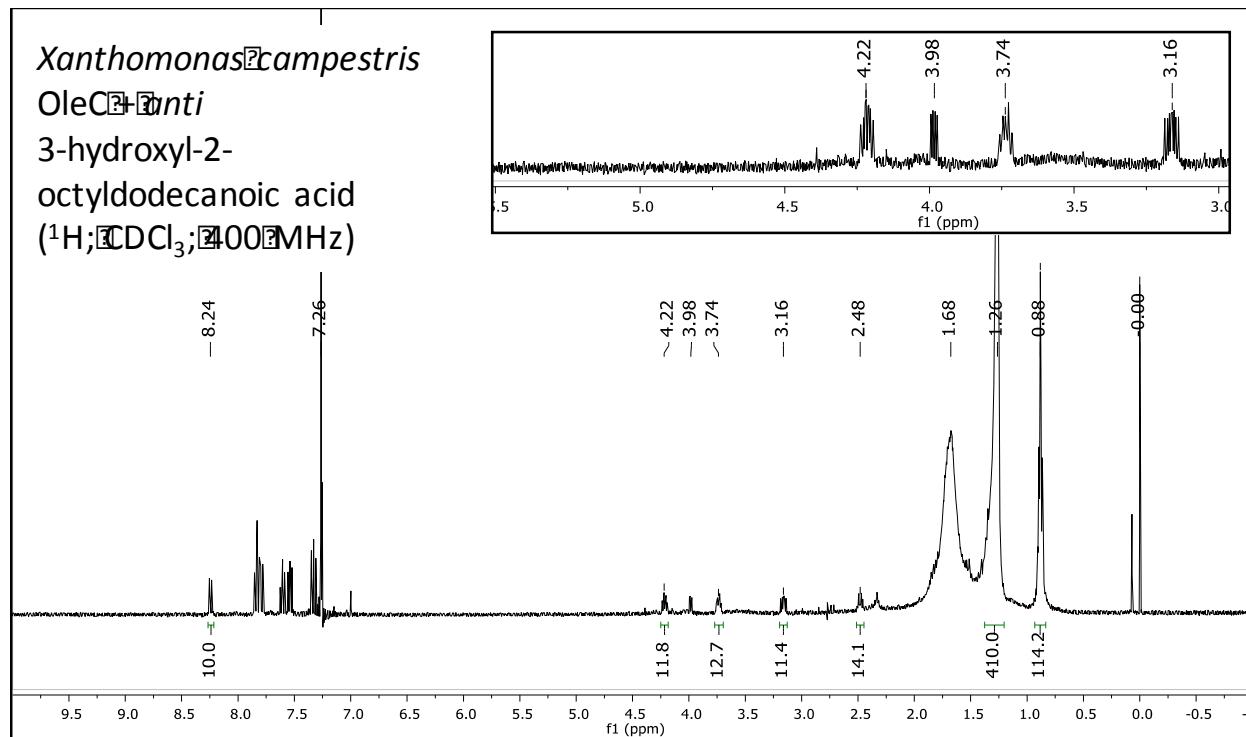


Figure S10. Xc OleC with **3b** overnight. Only (+/-) *trans*- β -lactone is observed.

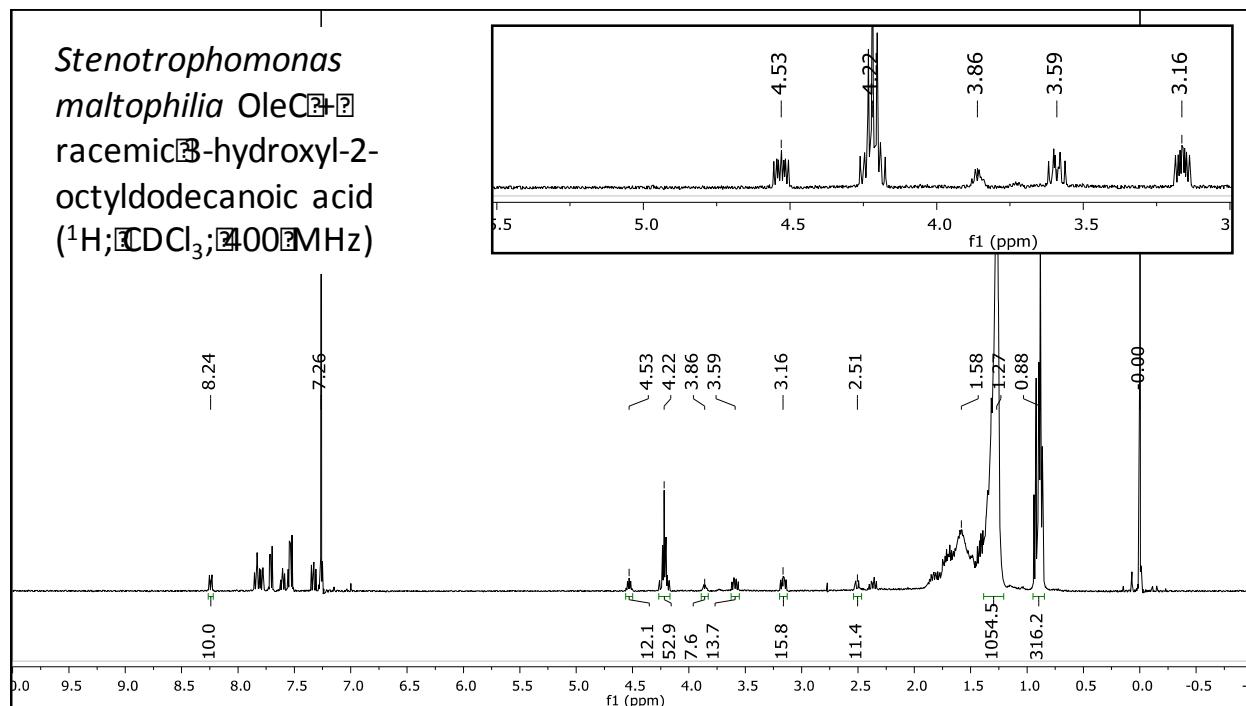


Figure S11. *Sm OleC* with **3** overnight. Some dioctyl phthalate contamination masks the *trans*- β -lactone at 4.22 ppm.

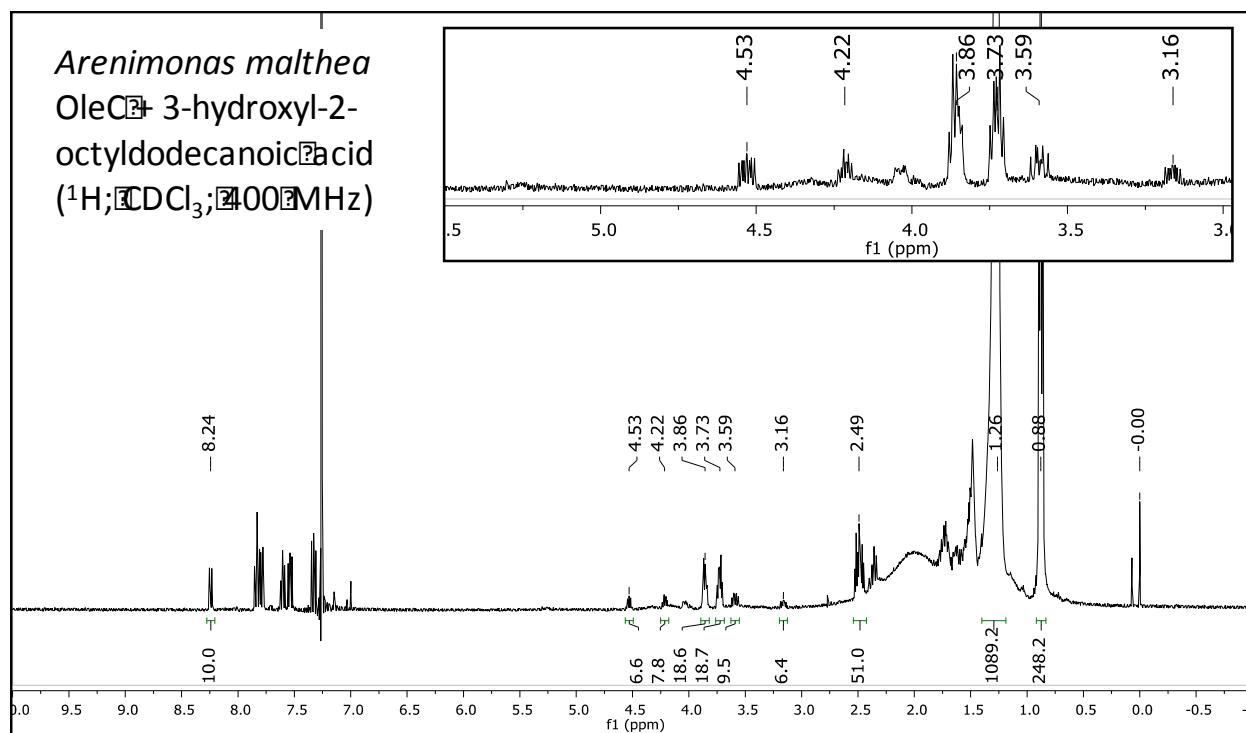


Figure S12. Am OleC with 3 overnight.

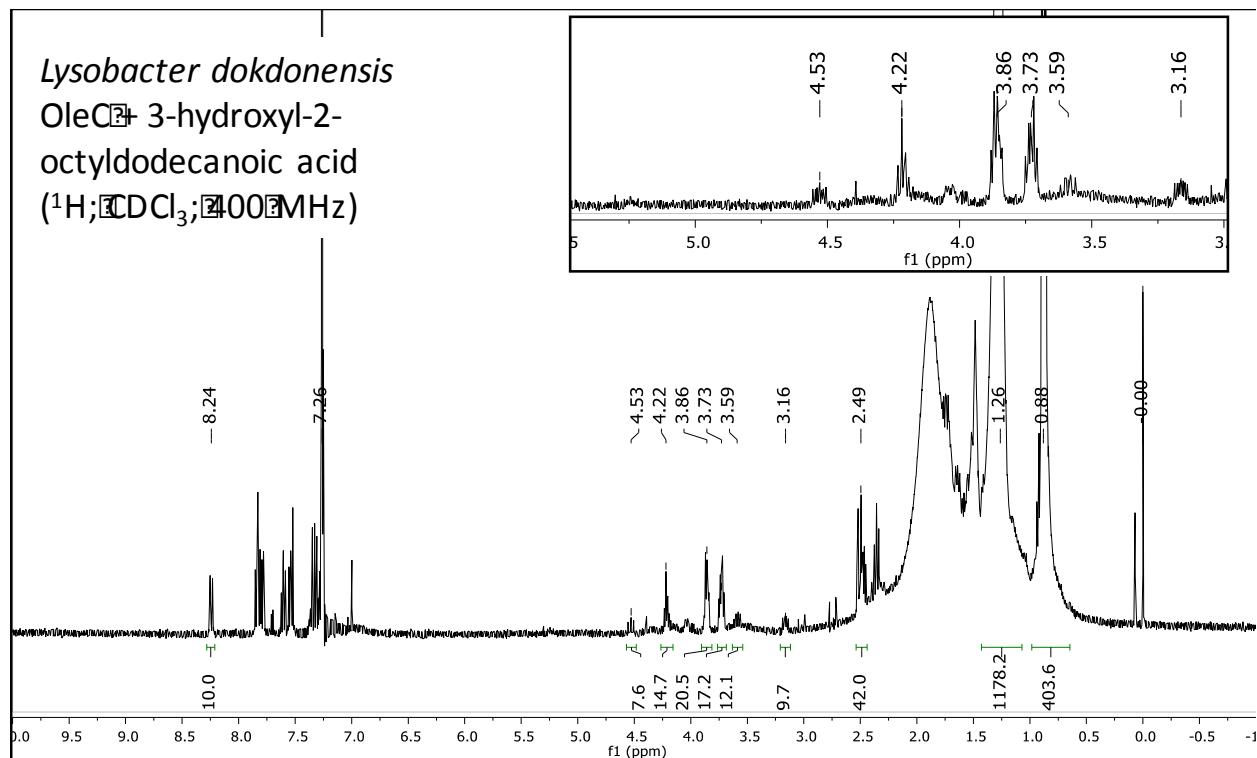


Figure S13. *Ld* OleC with 3 overnight. Some diethyl phthalate contamination masks the *trans*- β -lactone at 4.22 ppm.

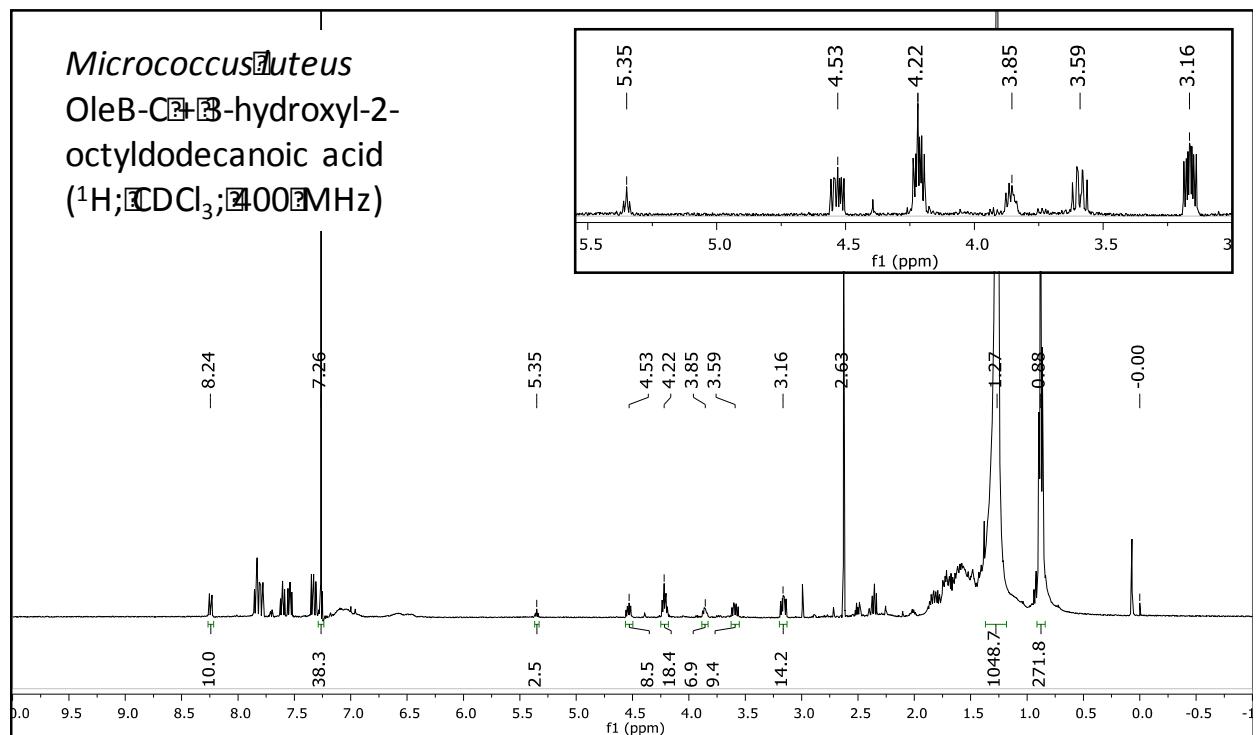


Figure S14. *M/* OleB-C fusion with 3 overnight. Note the presence of only *cis*-olefin at 3.35 ppm.

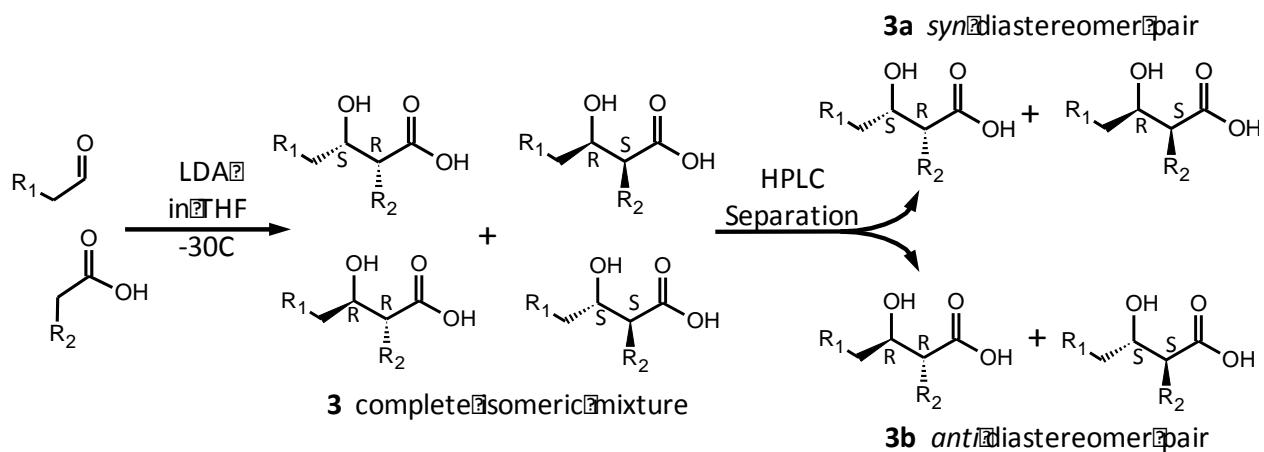


Figure S15. Preparation of -3-hydroxy-2-octyldodecanoic acid as substrates for OleC

Supplemental References

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