Converting *Escherichia coli* into an archaebacterium with a hybrid heterochiral membrane

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Supplemental Information

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1. Supplementary Methods

Operon integration and cloning procedures. *E. coli* MG1655 genomic DNA was used as template for the amplification of the *IDI*, *IspDF* and *DXS* genes encoding for the MEP-DOXP operon. The primers and the plasmids used for the integration of the operon into *E. coli* are listed in **Table S4 and Table S5**. The three genes were cloned into the same plasmid vector. Subsequently, a *lox71-kanR-lox66* selection marker cassette (1) was inserted downstream of the previously constructed MEP-DOXP operon using an extended BsiWI and AflII restriction site. The selection marker cassette along with the MEP-DOXP operon or the single *IDI* gene was amplified by PCR with primers BG4885 and BG4886 to obtain a DNA fragment with extended homologous ends for the integration into *E. coli* JM109(DE3) competent cells harboring pKD46. *E. coli* cells containing the integrated operon were subsequently transformed with a plasmid expressing the Cre recombinase to remove the selection marker. The obtained *E. coli* strains (**Table S1**) containing the integrated *IDI* gene and the MEP-DOXP operon were used as parental strains for the following strain engineering. The primers and plasmids used for expressing the ether lipids genes in the engineered *E. coli* strains are listed in **Table S4** and **S5**.

Bacterial strains and growth conditions. Engineered *E. coli* strains were grown under aerobic conditions at 37°C in 200 ml of LB medium supplemented with the antibiotics ampicillin (100 µg/ml) and kanamycin (50 µg/ml). OPT1 medium (2) was prepared by autoclaving a solution based on glycerol 1% (v/v), KH_2PO_4 2.4% (w/v), $(NH_4)_2HPO_4$ 0.4% (w/v), citric acid 0.17% (w/v) and by adding sterile 1 mM $NiCl_2$, 0.12 mM $MgSO_4$ and 1x MEM vitamin solution (Sigma-Aldrich). When not specified, the cells were induced with 0.1 mM IPTG for 3 hours or overnight, to allow the heterologous protein overexpression. IPTG is required to induce recombinant protein expression. By changing the concentration of IPTG the level of expression of the heterologous protein can be modulated.

Thin Layer Chromatography. An aliquot (3 µl) of lipid extracts from the different *E. coli* strains was spotted on Silica Gel 60 (Merck) plates. A solvent mixture of chloroform, methanol and water (50:10:1) was used as mobile phase for the separation of the different lipid species which were detected by molybdenum blue (3). A solvent system chart from Avanti (http://avantilipids.com/tech-support/analytical-procedures/tlc-solvent-systems/) was used as reference for the lipid identification. The spots were relatively quantified using ImageJ software.

Expression and purification of GGGPS, FadD and PlsB enzymes. The archaeal protein GGGPS from *M. maripaludis* was expressed and purified as previously described (4). The bacterial FadD and PlsB proteins from *E. coli* were overexpressed in *E. coli* BL21 induced with 1 mM IPTG as will be detailed elsewhere. After 2 hours of induction, the cells were harvested (8,754 x*g*) and washed with buffer A containing 50 mM Tris-HCl pH 8.0, 100 mM KCl and 20% Glycerol. After re-suspension, the cells were supplemented with 0.5 mg/ml of DNAse and complete

EDTA free protease inhibitor tablet (Roche). The suspension was subjected to cell disruption at 13,000 psi and the cell lysate was centrifuged for 15 minutes at low spin (12,000 x*g*) to remove unbroken cells.

Purification of the cytoplasmic protein FadD was performed by separation of the cytosolic fraction from membranes by a centrifugation step at 43,667 x*g* for 15 min. The supernatant was incubated with Ni-NTA beads (Sigma Aldrich) in buffer A for 60 min at 4 °C. The beads were washed 5 times with 20 column volumes (CV) of buffer A supplemented with 10 mM Imidazole and eluted 2 times with 2 CV of buffer A supplemented with 300 mM imidazole.
The membrane protein PlsB was purified by a high-speed centrifugation at (235,000 *x g*) for 1 hour to isolate the membrane fraction. Total membrane (pellet) were suspended in buffer A and solubilized at 4 ˚C for 1 hour in 2% of n-dodecyl-β-D-maltopyranoside (DDM) detergent. Insolubilized materials were removed by centrifugation (15,800 *x g*) for 10 minutes and the supernatant was incubated with Ni-NTA beads for 90 min at 4 °C. The beads were washed 5 times with 40 CV of buffer B (0.05% DDM, 50 mM Tris pH 8.0, 100 mM KCl, 20 % glycerol) supplemented with 10 mM imidazole and eluted 3 times with 0.5 CV of buffer B supplemented with 300 mM imidazole. The purity of the proteins was checked by 15% SDS-PAGE, stained with Coomassie Brilliant Blue. Absorbance was measured at 280 nm to determine the concentration of purified protein.

***In vitro* enzyme reactions**. *In vitro* reactions were performed in 100 µl of buffer containing a final concentration of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 60 mM NaCl, 100 mM Imidazole, 0.08% DDM and 4% glycerol. Where specified, 100 µM GGPP, 10 mM G3P, 10 mM G1P and the indicated amount of purified enzymes were added to the reaction mixture. The reactions were incubated at 37°C for 1 hour. Kinetic assays were performed using the same reaction mixture but the reactions were incubated at 37°C for 2 hours. The coupled FadD-PlsB *in vitro* assay was performed in a 100 µl reaction volume containing: 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 2mM DTT, 2.8 mM lipids (DOPG:DOPE, 2:1). Where specified, 160 µM Oleic acid, 40 µM CoA, 1 mM ATP, 0.5 µM purified FadD, 0.5 µM purified PlsB, 10 mM G3P and 10 mM G1P were added to the reaction mixture. Reactions were incubated at 37 °C for 3 hours. The products were extracted two times with 0.3 ml of *n*-butanol. Extracted lipids were evaporated under a stream of nitrogen gas and resuspended in 50 µl of methanol for the LC-MS analysis.

Lipid analysis. *E. coli* strains induced for the archaeal lipids synthesis were grown as described above. The total membrane fractions were isolated and the total lipid content was extracted according to the Bligh and Dyer method (5). Samples were then resuspended in 100 µl of methanol for LC-MS analysis, or total lipid quantitation by a colorimetric assay, based on the formation of a complex between phospholipids and ammonium ferrothiocyanate (6). Samples (20 µl) were evaporated under a nitrogen stream and resuspended in 500 µl of chloroform; 250 µl of ferrothiocyanate reagent was then added to the chloroform layer, mixed for 1 min and allowed to phase separate for 5 minutes. The lower red phase was collected and the

absorbance at 490 nm was measured and calibrated against standards. The obtained values were also used to normalized the LC-MS ion counts for amounts of individual lipids.

LC-MS analysis. The lipid extracts and the sample from *in vitro* reactions were analyzed using an Accela1250 high-performance liquid chromatography system coupled with an electrospray ionization mass spectrometry (ESI-MS) Orbitrap Exactive (Thermo Fisher Scientific). A volume of 5 µl of each sample was used for the analysis. The LC-MS method parameters used in this study to analyzed both type of samples were the same as described previously (5).

NMR and ether phospholipid stereochemical analysis. All chemical reactions were carried out under a nitrogen atmosphere using oven-dried glassware and using standard Schlenk techniques. Reaction temperature refers to the temperature of the oil bath. All reagents and catalysts were purchased from Sigma-Aldrich, Acros, J&K Scientific and TCI Europe and used without further purification unless otherwise mentioned, any purification of reagents was performed following the methods described by Armarego *et al*. (7) TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using either Seebach's reagent (a mixture of phosphomolybdic acid (25 g), cerium (8) sulfate (7.5 g), H_2O (500 mL) and H_2SO_4 (25 mL)), 2,4-DNP stain (2,4-dinitrophenylhydrazine (12 g), conc. sulfuric acid (60 ml), water (80 ml), ethanol (200 ml)) or elemental iodine. Flash chromatography was performed using SiliCycle silica gel type SiliaFlash P60 (230–400 mesh) as obtained from screening. GC-MS measurements were performed with an HP 6890 series gas chromatography system equipped with an HP1 or HP5 column (Agilent Technologies, Palo Alto, CA), and equipped with an HP 5973 mass sensitive detector. High resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL. (ESI+, ESI- and APCI). ^{1}H-, ^{13}C- and ^{19}F-NMR spectra were recorded on a Varian AMX400 (400, 100.6 and 376 MHz, respectively) using $CDCl_3$ as solvent unless stated otherwise. Chemical shift values are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: δ 7.26 for ^{1}H, δ 77.16 for ^{13}C). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, td = triple doublet, t = triplet, q = quartet, b = broad, m = multiplet), coupling constants *J* (Hz), and integration. Enantiomeric excesses were determined by chiral HPLC analysis using a Shimadzu LC- 10ADVP HPLC instrument equipped with a Shimadzu SPD-M10AVP diode-array detector. Optical rotations were measured on a Schmidt+Haensch polarimeter (Polartronic MH8) with a 10 cm cell (c given in 100 g/mL) at ambient temperature (± 20°C).

Scanning Electron Microscopy and Bright field Microscopy. For the scanning electron microscopy analysis, 150 µl of cell suspension (sampled during late exponential phase) was immobilized on poly-L-lysine coated cover slips (Corning art. 354085) for 1 hour. 2.5 % glutaraldehyde in 0.05 M sodium cacodylate buffer pH 7.2 was added to the glass at room temperature for 1 hour. The sample was rinsed three times in the same buffer and fixed for 1 hour in 1% OsO4 (w/v) in the same buffer. Two washes with water were performed, followed by a dehydration in a graded ethanol series (10, 30, 50, 70, 90, 100, 100%) and dried with

carbon dioxide (Leica EM CPD 300). The glasses were attached on a sample holder by carbon adhesive tabs (EMS Washington USA), sputter coated with tungsten (Leica EM SCD 500) and analyzed and digitally imaged with a field emission scanning electron microscope (FEI Magellan 400). Sample preparation, imaging and measurements were performed by the Wageningen Electron Microscopy Centre (WEMC) facility.

The bright field microscopy was performed on cells grown until exponential phase. Aliquots of 1 ml were centrifuged at max speed for 30 s on the top bench centrifuge. The obtained pellet was resuspended in 100 µl of Phosphate Buffered Saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4 and 68 mM NaCl pH 7.3). The FM4-64 and DAPI dyes were added to the solution at the final concentration of 0.8 µM and 36 nM respectively (9). The solution was incubated at room temperature for 10 minutes and centrifuged at max speed for 30 s. The stained cell pellet was suspended in 40 µl of PBS and spotted on agarose pad (1% w/v in PBS). Cells were imaged using a Nikon Ti-E-microscope (Nikon Instruments) equipped with a Hamamatsu Orca Flash 4.0 camera. The image analysis was performed by the software ImageJ (https://imagej.net/ImageJ).

Robustness tests. The engineered *E. coli* strains and the control strains were grown and induced as described above. A dilution into fresh medium was performed to reach the OD_{600} = 1.0 and the obtained culture was diluted again for a dilution factor of 1000x in order to have approximately 10^5- 10^6 cells/ml. For the freeze-thawing survival assay, cell aliquots of 20 µl were frozen in liquid nitrogen and kept at -80°C for 4 days. An untreated cell sample was plated in a 100x dilution to be used as reference. Heat shock treatment was performed by exposing the strains to different temperatures for 2 minutes. The cells were then recovered by adding 980 µl of LB medium and incubated at 37°C for 1 hour and plated for CFU counts. Butanol tolerance was tested by incubating cells in LB supplemented with different butanol concentrations for one hour at 37°C. After all the treatments 100 µl of cells were plated on LB agar plate supplemented with the proper antibiotics (Ampicillin 100 µg/ml and kanamycin 50 µg/ml) and incubated at 37°C overnight. The colony counting was performed using a developed plugin for the software ImageJ.

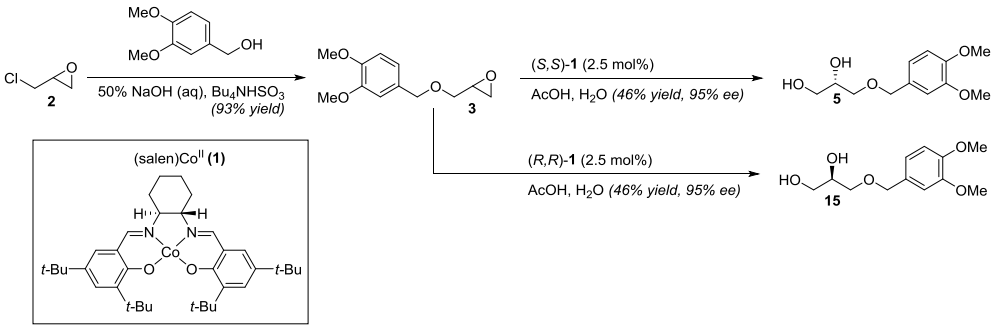
PacBio and Illumina whole genome sequencing

Cell suspension of the *E. coli* MEP/DOXP$^+$EL$^+$ strain grown on LB (control) and OPT medium and induced with IPTG was collected for chromosomal DNA extraction, using the EPICENTRE masterpure DNA purification kit (MGPO4100). Quality and quantity of the isolated DNA was checked by QUBIT quantitation (Thermo Fisher Scientific). Next Generation Sequencing was executed by Baseclear B.V. Leiden. A *de novo* hybrid assembly was performed by means of PacBio sequencing on the both samples by Illumina sequencing. The *de novo* hybrid assembled genome was annotated using the Baseclear annotation pipeline based on Prokka version 1.6 Prokaryotic Genome Annotation System. Illumina generated sequence reads were aligned against the assembled hybrid genome using the “Map reads to reference” options of the CLC Genomics Workbench version 8.5.1. Output of the variant detection was given in single

nucleotide variants, multiple nucleotide variants and short InDels. Identified mutations were interactively analyzed using the BaseClear online annotation browser. Intergenic regions were inspected for possible alterations of promoter regions and transcription factor sequences by means of the online transcriptional regulatory network database (http://regulondb.ccg.unam.mx/).

Chemical synthesis of the standard used for the phospholipid chirality analysis

Scheme 1:



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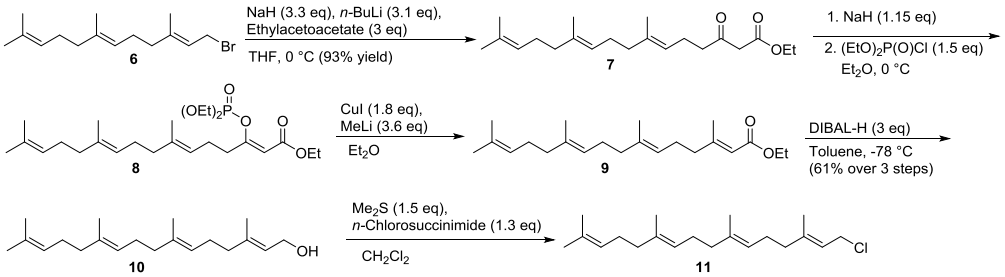

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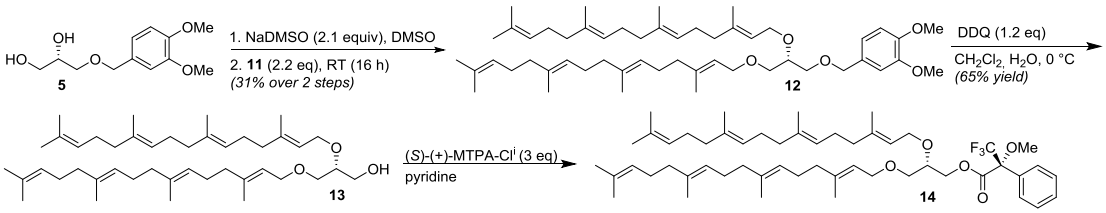

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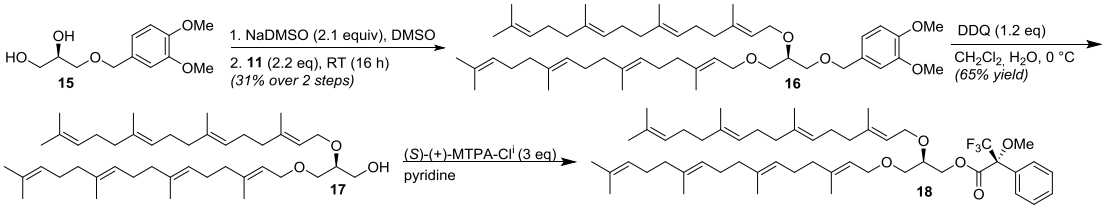
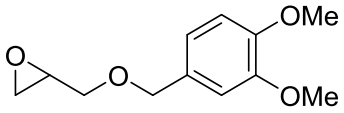
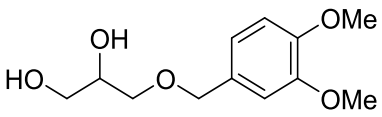




2-(((3,4-dimethoxybenzyl)oxy)methyl)oxirane (10) [3]. To a 100 mL 3-necked flask equipped with magnetic stirrer bar was added 25 mL of a 50% NaOH solution, epichlorohydrin (18.5 g, 15.6 mL, 0.2 mol) and Bu_4NHSO_4 (1.5 mmol, 525 mg, 4 mol%). The resulting solution was cooled to 0˚C (ice/water-bath) after which neat 3,4-dimethoxybenzyl alcohol (37.5 mmol, 5.5 mL, 6.3 g) was added dropwise over 30 min while the solution was stirred vigorously. The resulting turbid mixture was allowed to warm up over a 5 h period, after which complete conversion was observed by TLC. The entire content of the flask was poured into 100 mL of ice water which was subsequently extracted with diethyl ether (3 x 50 mL). The combined organic layers were washed with brine (2 x 50 mL) dried over $MgSO_4$ and concentrated *in vacuo*. The resulting crude was further purified by column chromatography (1:3 EtOAc/pentane) to give 2-(((3,4-dimethoxybenzyl)oxy)methyl)oxirane as a pale yellow oil (94% yield, 7.9 g).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.94 – 6.78 (m, 3H), 4.52 (q, *J* = 11.6 Hz, 2H), 3.89 (s, 3H), 3,87 (s, 3H), 3.75 (dd, *J* = 11.5, 2.9 Hz, 1H), 3.41 (dd, *J* = 11.4, 5.9 Hz, 1H), 3.19 (td, *J* = 6.3, 3.2 Hz, 1H), 2.80 (t, *J* = 4.6 Hz, 1H), 2.61 (dd, *J* = 4.9, 2.7 Hz, 1H).

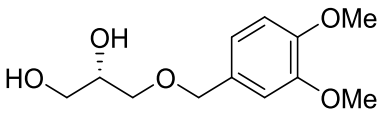
^{13}C NMR (101 MHz, Chloroform-*d*) δ 149.0, 148.7, 130.4, 120.4, 111.1, 110.9, 73.2, 70.6, 55.9, 55.8, 50.8, 44.3.



3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol [4]. Epoxide **3** (200 mg, 0.9 mmol) with 6 mL water was added to a 10 mL round-bottomed flask equipped with magnetic stirrer bar. To this mixture was added 0.2 mL of 10% aqueous sulfuric acid followed by stirring for 5 h at rt. The resulting acidic solution was neutralized with 1 M NaOH and extracted with ethyl acetate (3 x 5 mL). The combined organic layers were washed with brine (2 x 5 mL), dried over $MgSO_4$ and concentrated *in vacuo* which yielded the desired product as a colorless thick oil (98% yield, 210 mg).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.87 – 6.77 (m, 3H), 4.44 (s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.67 – 3.60 (m, 1H), 3.55 (dd, *J* = 11.5, 5.9 Hz), 3.52 – 3.42 (m, 2H), 3.03 (br s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 149.1, 148.8 , 130.3 , 120.5 , 111.2 , 111.0 , 73.5 , 71.5 , 70.8 , 64.1 , 55.6 , 55.9.



(*S*)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol(10) [5]. A 25 mL flask equipped with a magnetic stirrer bar was charged with (*S,S*)-**1** (70 mg, 0.005 equiv). The catalyst was exposed to 2-(((3,4-dimethoxybenzyl)oxy)methyl)oxirane (5 g, 22.3 mmol) and AcOH (25 µL, 0.2 equiv). The resulting red mixture was allowed to stir for 30 min in order to oxidize the catalyst. To the resulting brown mixture

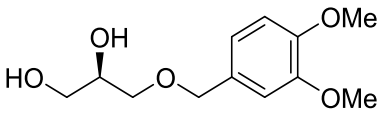
was added H_2O (220 µL, 0.55 equiv) and was stirred rt for 48 h. The final product was isolated as a brown oil by flash column chromatography (100% EtOAc) (45% yield, 2.2 g).

Chiral HPLC analysis on a Lux® 5 µm Cellulose-3 column, *n*-heptane : *i*-PrOH = 90 : 10, 40 ˚C, flow = 1 mL/min, UV detection at 274 nm, t_R(major): 25.29 min, t_R(minor): 29.06 min, 97% ee

^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.87 – 6.77 (m, 3H), 4.44 (s, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.67 – 3.60 (m, 1H), 3.55 (dd, *J* = 11.5, 5.9 Hz, 1H) 3.52 – 3.42 (m, 2H), 3.03 (br s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 149.1, 148.8, 130.3, 120.5, 111.2, 111.0, 73.5, 71.5, 70.8, 64.1, 55.6, 55.9.

$[\alpha]_D^{20}$= -2.4 (c = 0.1 g/mL, $CHCl_3$).



(*R*)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol(10) [15]. This compound was prepared with the same synthetic procedure that was used for **(*S*)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol [5]**, using (*R,R*)-**1** as catalyst (45% yield).

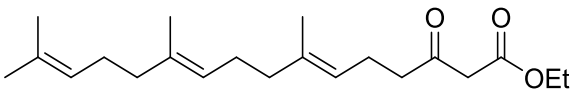
Chiral HPLC analysis on a Lux® 5 µm Cellulose-3 column, *n*-heptane : *i*-PrOH = 90 : 10, 40 ˚C, flow = 1 mL/min, UV detection at 274 nm, t_R(minor): 26.01 min, t_R(major): 29.21 min, 95% ee.

^{1}H NMR (400 MHz, Chloroform-*d*): Same as reported for compound **5**

^{13}C NMR (101 MHz, Chloroform-*d*): Same as reported for compound **5**

$[\alpha]_D^{20}$= +2.4 (c = 0.1 g/mL, $CHCl_3$).

The spectral data correspond to those previously reported(11).

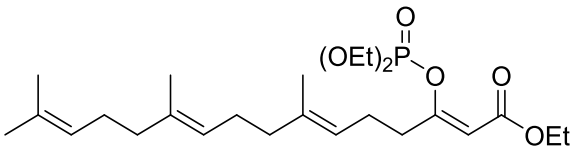


ethyl (6*E*,10*E*)-7,11,15-trimethyl-3-oxohexadeca-6,10,14-trienoate(12) [7]. An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with NaH (60% dispersion, 136 mg, 3.3 equiv). The mineral oil was removed by 3 successive washings with pentane. The remaining white solid was dried in vacuum, suspended in dry THF (2.5 mL) and cooled to 0 °C (ice/water-bath). To the resulting suspension, freshly distilled ethyl acetoacetate (400 mg, 3 equiv) was added dropwise over 15 min after which the solution turned light yellow. After stirring for an additional 15 min at 0 °C, a solution of *n*-BuLi in hexanes (1.6 M, 1.95 mL, 3 equiv) was added over 15 min. The resulting dark yellow solution was allowed to stir further for 15 min at 0 °C. Farnesyl bromide (**6**) (286 mg, 1 mmol) in 0.55 mL of dry THF was added dropwise over 10 min. The resulting orange suspension was quenched by the addition of HCl (1 M, 1.5 mL). The aqueous layer was separated and extracted with Et_2O (3 x 2 mL), the

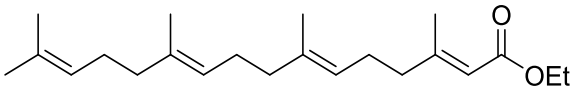
organic layers were combined, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The obtained crude oil was further purified by flash column chromatography after which the pure aceto-ester was obtained as a pale yellow oil (10% Et_2O in pentane) (yield 93%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.06 (s, 0.2H), 5.10 – 4.99 (m, 3H), 4.22 – 4.08 (m, 2H), 3.38 (d, 2H), 2.52 (t, *J* = 7.4 Hz, 2H), 2.24 (q, *J* = 7.4 Hz, 2H), 2.08 – 1.88 (m, 8H), 1.63 (s, 3H), 1.56 (m, 9H), 1.23 (t, *J* = 7.1 Hz, 3H).

The spectral data correspond to those previously reported(13).

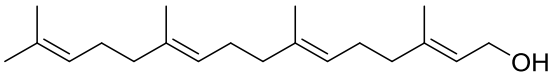


ethyl(2*Z*,6*E*,10*E*)-3-((diethoxyphosphoryl)oxy)-7,11,15-trimethylhexadeca-2,6,10,14-tetraenoate (12) [8]. An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with NaH (60% dispersion, 46 mg, 1.15 mmol, 1.15 equiv). The mineral oil was removed by 3 washings with pentane. The remaining white solid was dried in vacuum and suspended in dry Et_2O (4.5 mL). The suspension was cooled to 0 °C (ice/water-bath) and a solution of ethyl (6*E*,10*E*)-7,11,15-trimethyl-3-oxohexadeca-6,10,14-trienoate (**7**) in dry Et_2O (1.5 mL) was added over 15 min. The resulting yellow homogeneous mixture was stirred for 15 min at 0 °C and for 15 min at rt. The solution was again cooled to 0 °C and neat diethylchlorophosphate was added over 5 min. The resulting mixture was stirred for 15 min at 0 °C after which the reaction was quenched by addition of saturated aqueous NH_4Cl solution (3 mL). The organic layer was separated and the aqueous layer was extracted with Et_2O (3 x 3 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (3 x 3 mL), brine (3 x 3 mL), dried over $MgSO_4$ and the solvent was removed *in vacuo*. The resulting yellow oil (400 mg) was used without further purification in the successive step.



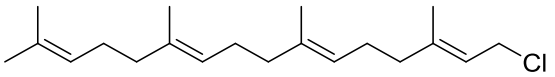
ethyl (2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenoate(12) [9]. An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with CuI (340 mg, 1.8 mmol, 1.8 equiv) and 1.1 mL Et_2O. The resulting suspension was treated with MeLi (1.6 M in Et_2O, 2.25 mL 3.6 equiv) upon which the mixture turned bright yellow. After complete addition of the MeLi the mixture was homogeneous and colorless. The mixture was cooled to -78 °C (cryostat, acetone-bath) and a solution of the phosphate **8** (400 mg) in dry Et_2O (1.4 mL) was added dropwise such that the phosphate solution was cooled by the cold wall of the Schlenk flask, during which the color of the reaction mixture turned to yellow. After complete addition the resulting bright orange solution was stirred at -78 °C for 1 h, after which the mixture was allowed to warm up to -45 °C and was stirred for an additional 2 h. The resulting dark red mixture was quenched by adding 130 µL of MeI and after stirring for 10 minutes the entire content of the Schlenk flask was carefully poured into a saturated aqueous solution of NH_4Cl (5

mL) and NH_4OH (25%, 6 mL) during which gas evolution was observed. The resulting mixture was stirred until it became homogeneous. The layers were separated, the aqueous layer was extracted with Et_2O (3 x 5 mL), the organic layers were combined and washed with an aqueous solution of NH_4OH (25%, 2 x 8 mL), brine (2 x 8 mL), dried and concentrated *in vacuo*. 290 mg of a yellow oil was obtained and used in the successive reaction without further purification.

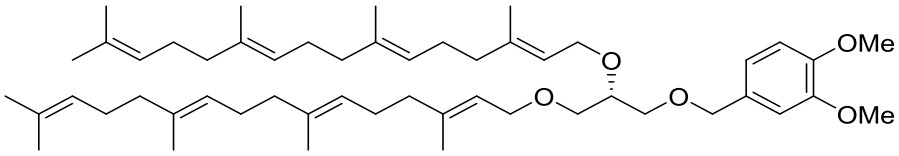


(2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol [10]. To an oven dried Schlenk flask under N_2 atmosphere was added ethyl (2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenoate (**9**), from the previous step in dry toluene (3 mL). The resulting solution was cooled to -78 °C (cryostat, acetone-bath) and a solution of DIBAL (1 M in CH_2Cl_2, 2.6 mL, 3 equiv) was added dropwise over 20 min. The resulting mixture was stirred at -78 °C for 1h after which full consumption was observed by TLC (30% Et_2O in pentane, I_2-stain, R_f ~0.3). The reaction was quenched by dropwise addition of MeOH over 10 min (gas evolution observed) after which the mixture stirred until gas evolution ceased. The solution was allowed to warm up to ambient temperature and was allowed to stir for 10 min. The resulting mixture was poured into a 1:1 saturated aqueous solution of NH_4Cl/1N HCl solution (20 mL) and was stirred until a clear separation of layers was observed. The aqueous layer was extracted with Et_2O (3 x 10 mL) and the combined organic layers were washed with water (2 x 10 mL), brine (3 x 10 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The residual yellow oil was purified by flash column chromatography (30% Et_2O in pentane) which yielded 181 mg of a yellow oil (63% yield over 3 steps)

The spectral data correspond to those previously reported(13).



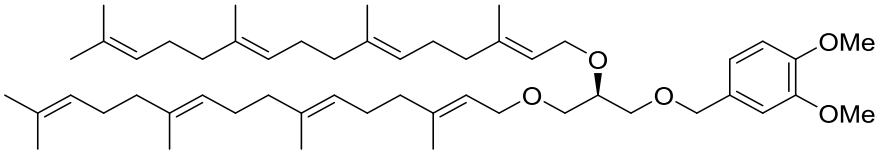
(2*E*,6*E*,10*E*)-1-chloro-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraene [11]. *N*-chlorosuccinimide (145 mg, 1.1 mmol, 1.3 equiv.) was suspended in dry CH_2Cl_2 (2.5 mL) under N_2 atmosphere in a pre-dried Schlenk flask. The turbid mixture was cooled to -30 °C (acetone/liquid N_2 bath) after which dimethyl sulfide (90 µL, 1.25 mmol, 1.5 equiv.) was added dropwise. The reaction mixture was stirred for 10 min at -30 °C after which it was allowed to warm up to 0 °C for 10 min. The resulting solution was cooled to -40 °C and geranylgeraniol (**10**, 240 mg, 0.83 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise over 15 min. The resulting suspension was allowed to warm up over 1 h to -20 °C after which the acetone/liquid N_2 bath was replaced by an ice/water bath and the resulting suspension was stirred for another h at 0 °C after which it was poured into pentane (20 mL). The pentane mixture was decanted from the white precipitate, the white crystals were washed with pentane (20 mL) and decanted again, this process was repeated three times. The pentane extracts were combined and evaporated obtaining geranylgeranyl chloride as a yellow oil quantitatively which was used immediately for the dialkylation of (*S*)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol.



1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-*sn*-glycerol [12]. An oven dried Schlenk flask equipped with magnetic stirrer bar was charged with NaH (60% dispersion in mineral oil, 33 mg, 825 µmol, 2 equiv). The mineral oil was removed by three successive washings with pentane and then dried under vacuum. The resulting white solid was suspended in DMSO (0.6 mL) and stirred at 60 ˚C (oil bath) until a clear solution was observed (45 min). The pale yellow solution was allowed to cool down to rt after which (*S*)-3-((3,4-dimethoxybenzyl)oxy)propane-1,2-diol **5**, (83 mg, 340 µmol) in dry DMSO (freshly distilled from CaH, 0.6 mL) was added in a dropwise manner. After the solution was stirred for 1.5 h at rt, the crude mixture of geranylgeranyl chloride **11**, (250 mg, 2.2 equiv) in dry DMSO (0.2 mL) was added dropwise over 20 min. The resulting reaction mixture was stirred for 16 h after which it was poured into a saturated aqueous solution of NH_4Cl (5 mL). The aqueous layer was extracted with diethyl ether (3 x 5 mL). The combined organic layers were washed with brine, dried and concentrated *in vacuo*. The resulting crude was further purified by flash column chromatography (20 % diethyl ether in pentane) to afford the dialkylated product (31% yield over 2 steps, 41 mg).

HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{52}H_{82}O_5Na$, 809.6055; found, 809.6041.

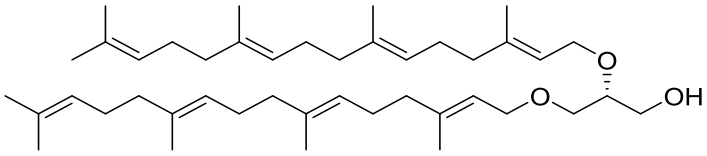
The spectral data correspond to those previously reported(12).



1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-*sn*3-glycerol [16]. This compound was prepared with the same synthetic procedure that was used for **(1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-*sn*-glycerol**, **(12).**

^{1}H NMR (400 MHz, Chloroform-*d*): Same as reported for **12**

^{13}C NMR (101 MHz Chloroform-*d*): Same as reported for **12**

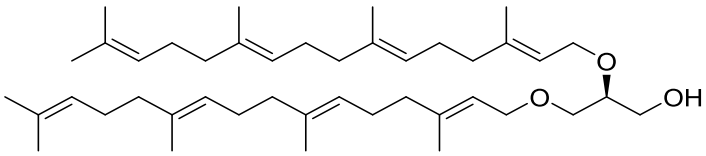


2,3-bisgeranylgeranyl-*sn*-glycerol [13]. 1-(3,4-dimethoxybenzyl)-2,3-bisgeranylgeranyl-*sn*-glycerol (**12**) (35 mg, 45 µmol) was dissolved in CH_2Cl_2 (0.9 mL) to which water (0.1 mL) was added in order to form a biphasic system. The solution was cooled to 0˚C (ice/water bath) and DDQ (12.5 mg, 55 µmol, 1.2 equiv) was added in portions. The resulting green mixture was stirred for 2 h at 0˚C during which it turned light brown. The entire mixture was filtered over a small silica pad with CH_2Cl_2 and purified

by flash column chromatography (20% diethyl ether in pentane, I_2 and 2,4-DNP stain) which afforded the desired product as a viscous yellow oil (70% yield, 20 mg).

HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{43}H_{72}O_3Na$, 659.5374; found, 659.5369.

The spectral data correspond to those previously reported(12).



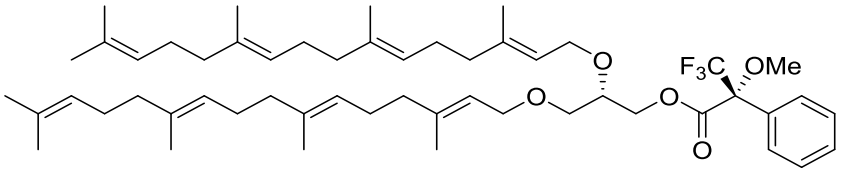
2,3-bisgeranylgeranyl-*sn*3-glycerol (17). This compound was prepared with the same synthetic procedure that was used for **2,3-bisgeranylgeranyl-*sn*-glycerol 13.**

^{1}H NMR (400 MHz, Chloroform-*d*): Same as reported for **13**

HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{43}H_{72}O_3Na$, 659.5374; found, 659.5371.

Saponification of ester-lipids from natural lipid extract mixture. 480 mg NaOH was added to a mixture of MeOH (0.6 mL) and CH_2Cl_2 (4.4 mL) and stirred until all NaOH was dissolved. To this resulting stirred solution was added the natural lipid extract (40 mg) from the modified *E. coli* cultures in CH_2Cl_2 (1 mL) such that the resulting mixture had a 1:9 MeOH/CH_2Cl_2 ratio and a 2 N NaOH concentration. The solution was allowed to stir for 80 h at rt during which it became turbid. The mixture was filtered over celite and the residue was washed with CH_2Cl_2. The filtrate was neutralized with 1 M aqueous HCl, transferred to a separatory funnel and separated. The aqueous layer was extracted with CH_2Cl_2 (3 x 5 mL) and the combined organic layers were washed with brine (2 x 5mL), dried over $MgSO_4$ and concentrated *in vacuo*. The obtained residue was further purified by column chromatography (20% Et_2O in pentane) in order to yield 7 mg of the natural di-ether glycerol lipid (**20**) as a yellow oil with some co-eluted impurities.

HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{43}H_{72}O_3Na$, 659.5374; found, 659.5371.

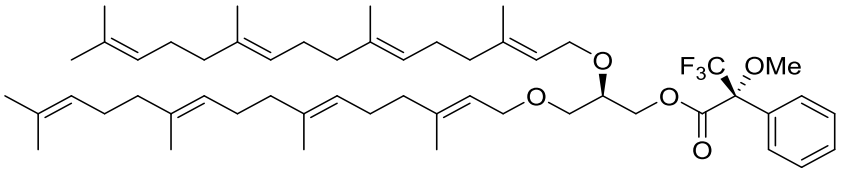


2,3-bisgeranylgeranyl-*sn*-glycerol (+)-Mosher's ester derivative [14]. Alcohol **13** (5 mg, 7.8 µmol) was dissolved in pyridine (0.2 mL) under N_2 atmosphere. (*S*)-(+)-MTPA-Cl (6 mg, 5 µL, 23.4 µmol, 3 equiv, Mosher's chloride) was added dropwise and the reaction mixture was stirred for 2 h at rt before it was quenched with 0.2 mL saturated $NaHCO_3$ solution. A small quantity of water (1 mL) was added and the aqueous mixture was extracted with ethyl acetate (3 x 1 mL), the organic layers were combined, washed with brine (2 x 1 mL) dried over $MgSO_4$ and concentrated *in vacuo*. The residue was filtered

over silica with pentane and the residue was washed with a large amount of pentane. The filtrate was concentrated *in vacuo* in order to yield a yellow crude mixture containing the desired Mosher's ester (6 mg).

^{1}H NMR (599 MHz, Chloroform-*d*) δ 7.57 – 7.50 (m, 2H), 7.42 – 7.37 (m, 3H), 5.42 – 5.23 (m, 1H), 5.16 – 5.05 (m, 6H), 4.69 (s, 1H), 4.51 (dd, J = 11.5, 3.5 Hz, 1H), 4.37 – 4.32 (m, 1H), 4.09 – 4.05 (m, 2H), 4.00 – 3.96 (m, 2H), 3.74 (m, 1H), 3.55 (s, 3H), 3.49 (m, J = 10 Hz, 1H), 3.42 (m, J = 10.0 Hz, 1H), 2.11 – 1.94 (m, 24H), 1.68 (s, 6H), 1.60 (s, 18H), 1.55 (s, 6H).

HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{53}H_{79}F_3O_5Na$, 875.5772; found, 875.5772.



2,3-bisgeranylgeranyl-*sn*3-glycerol (+)-Mosher's ester derivative [18]. This compound was prepared with the synthetic route, identical to that used for **2,3-bisgeranylgeranyl-*sn*-glycerol Mosher's ester derivative** (**14**)

^{1}H NMR (599 MHz, Chloroform-*d*) δ 7.57 – 7.51 (m, 2H), 7.41 – 7.36 (m, 3H), 5.29 (m, 1H), 5.18 – 5.07 (m, 6H), 4.71 – 4.65 (m, 1H), 4.56 (dd, J = 11.5, 3.5 Hz, 1H), 4.37 – 4.32 (m, 1H), 4.07 (d, J = 6.5 Hz, 2H), 3.96 (d, J = 6.5 Hz, 2H), 3.75 – 3.71 (m, 1H), 3.56 (s, 3H), 3.49 (m, J = 10 Hz, 1H), 3.42 (m, J = 10 Hz, 1H), 2.12 – 1.94 (m, 24H), 1.64 (s, 6H), 1.59 (s, 18H), 1.56 (s, 6H).

HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{53}H_{79}F_3O_5Na$, 875.5772; found, 875.5773.

Naturally derived di-ether lipid (+)-Mosher's ester derivative [19]. This compound was prepared with the synthetic route, identical to that used for **2,3-bisgeranylgeranyl-*sn*-glycerol Mosher's ester derivative** (**14**)

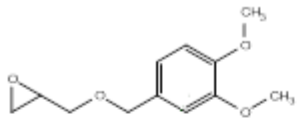
^{1}H NMR (599 MHz, Chloroform-*d*): Same as reported for **14**

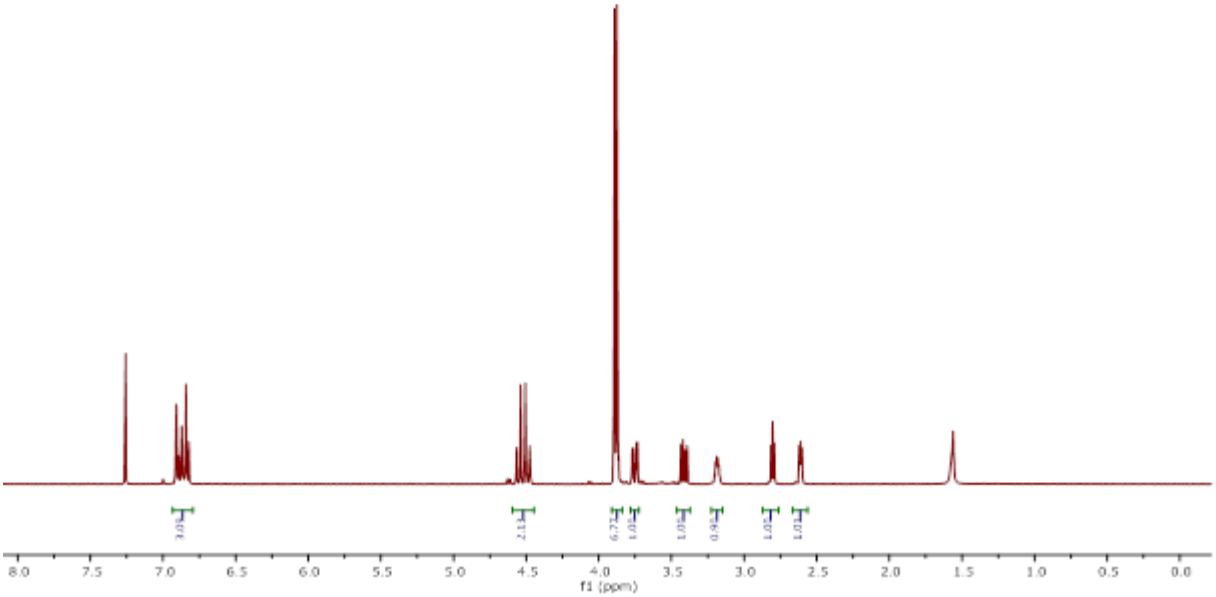
HRMS-ESI+ (*m/z*): $[M + Na]^+$ calculated for $C_{53}H_{79}F_3O_5Na$, 875.5772; found, 875.5773.

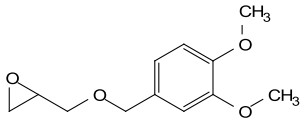
Naturally derived di-ether lipid (+)-Mosher's ester derivative from G1P deficient strain [20]. This compound was prepared with the synthetic route, identical to that used for **2,3-bisgeranylgeranyl-*sn*-glycerol Mosher's ester derivative** [**14**]

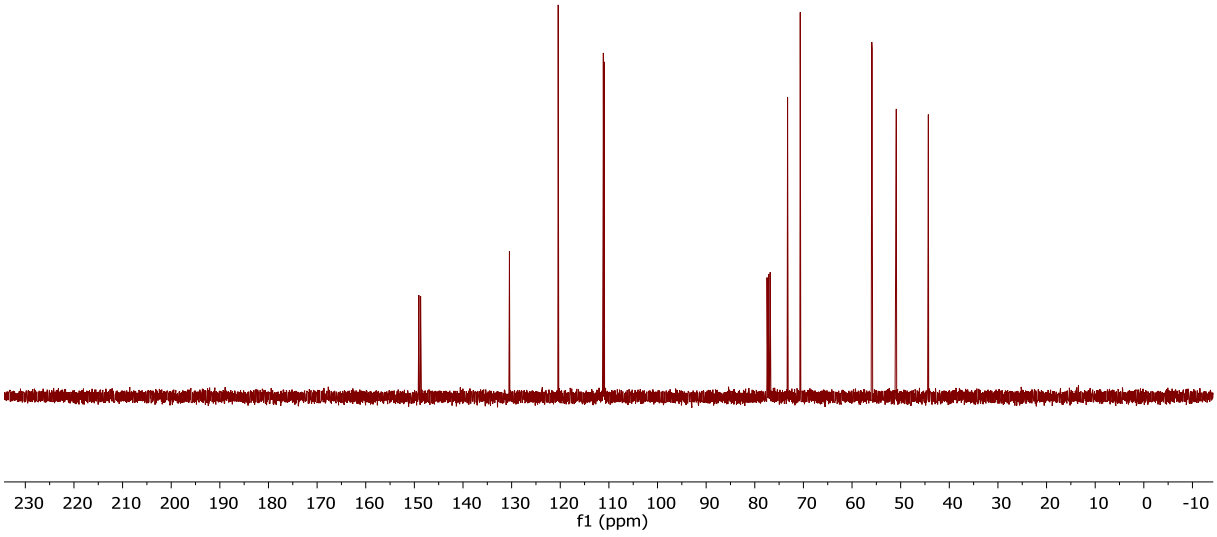
^{1}H NMR (599 MHz, Chloroform-*d*): Same as reported for **14**

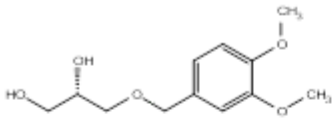
HRMS-ESI + (*m/z*): $[M + Na]^+$ calculated for $C_{53}H_{79}F_3O_5Na$, 875.5772; found, 875.5773.

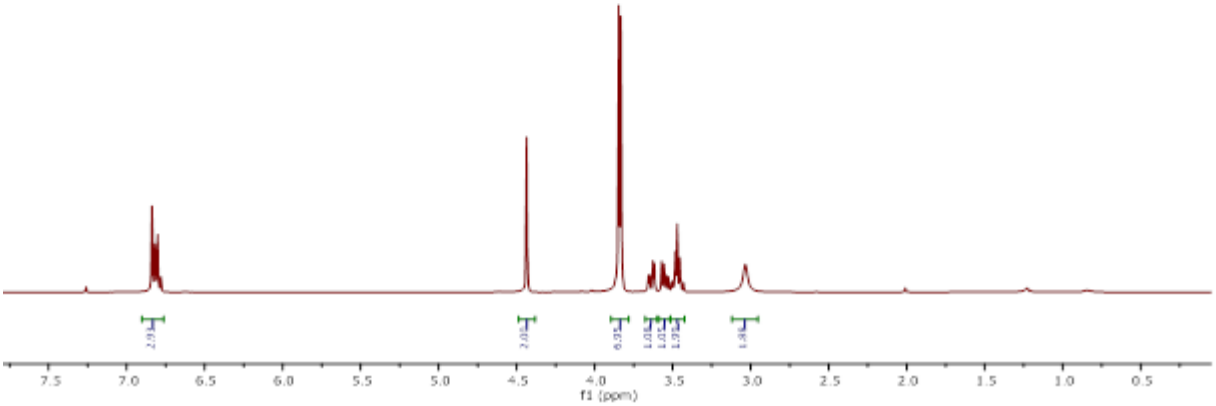


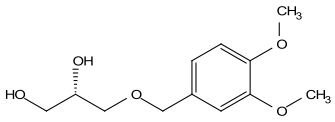

3.00
2.72
6.72
1.03
1.01
0.96
1.00
1.03
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

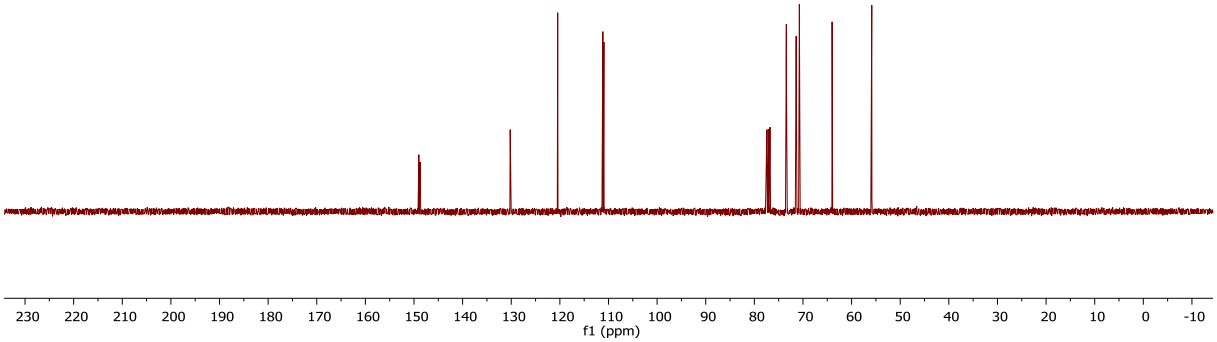



230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)





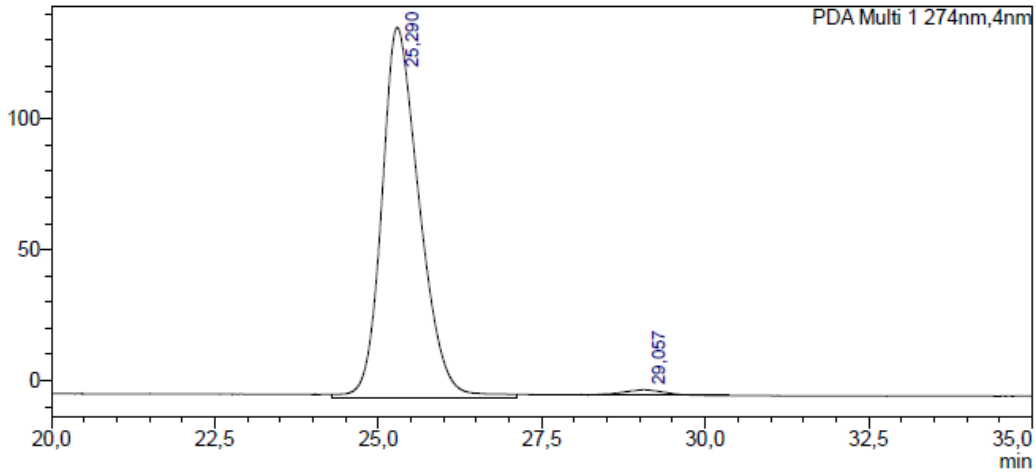




(*S*)-enantiomer:

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 274nm

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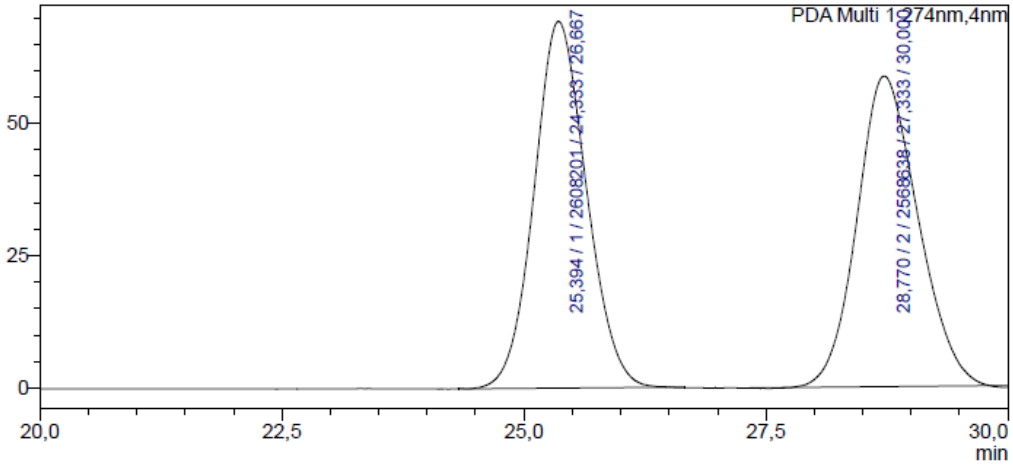
2	29,057	77410	1956	1,346		M	
Total		5752217	143244				

Peak Start	Peak End	Area%
24,280	27,112	98,654
28,264	30,368	1,346
		100,000

racemic mixture:

<Chromatogram>

mAU

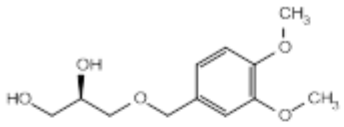


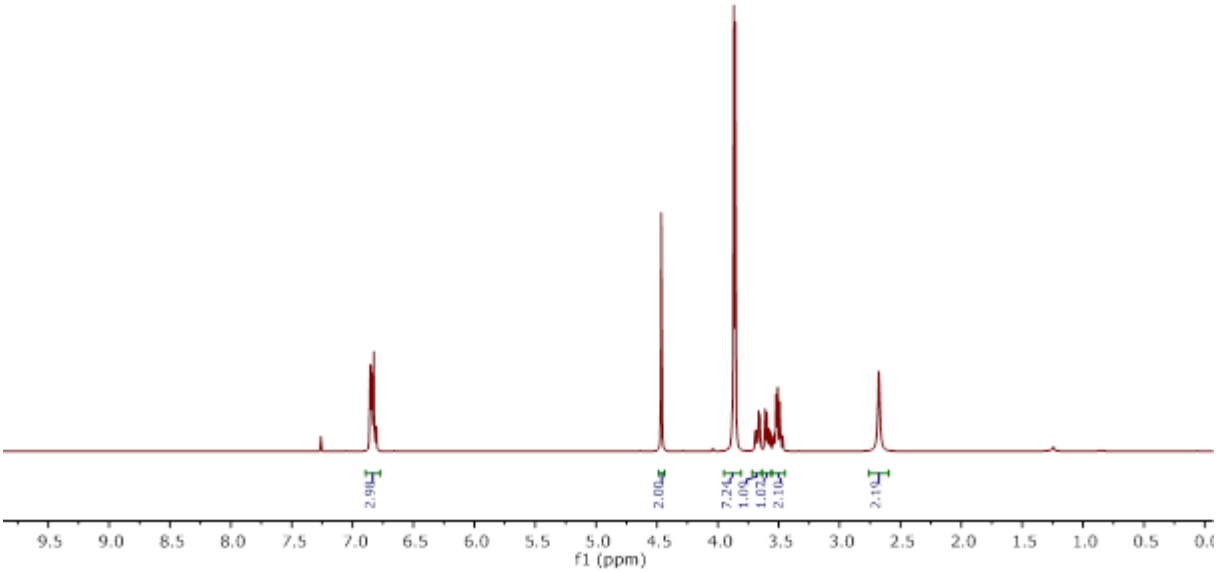
<Peak Table>

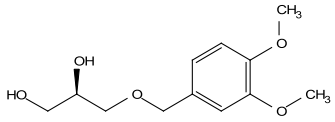
PDA Ch1 274nm

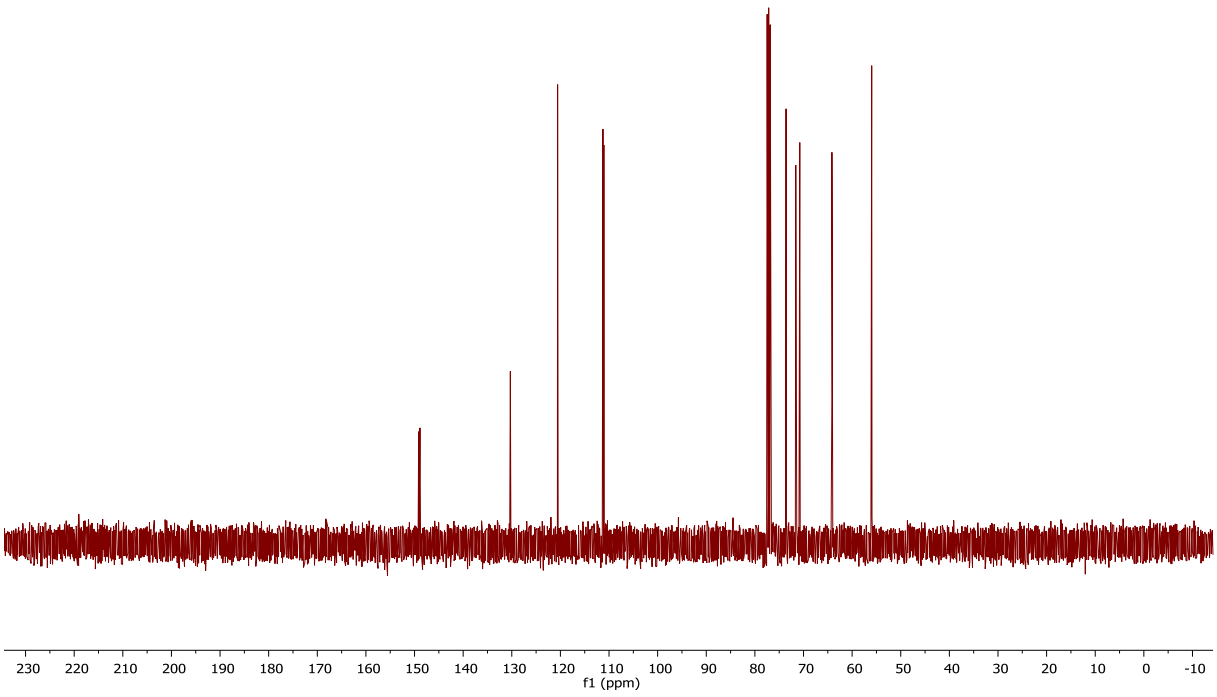
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	25,394	2608201	56409	50,382		M	
2	28,770	2568638	52337	49,618		M	
Total		5176839	108746				

Area%	Peak Start	Peak End
50,382	24,333	26,667
49,618	27,333	30,000
100,000		


CH3
O
OH
HO
O
O
CH3


9.5 9.0 8.5 8.0 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
f1 (ppm)

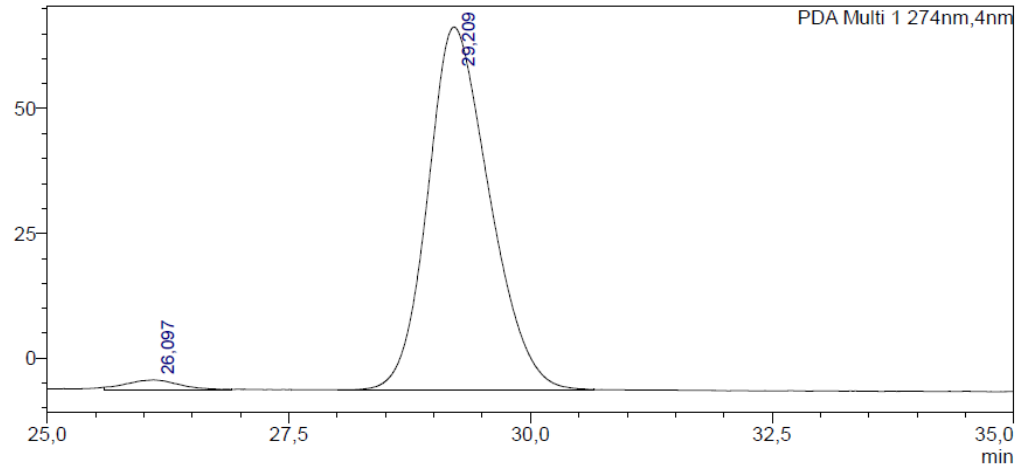

CH3
O
OH
HO
O
O
CH3


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
f1 (ppm)

(*R*)-enantiomer:

<Chromatogram>

mAU



<Peak Table>

PDA Ch1 274nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
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2	29,209	3240443	72698	97,555		M	
Total		3321665	74750				

Area%	Peak Start	Peak End
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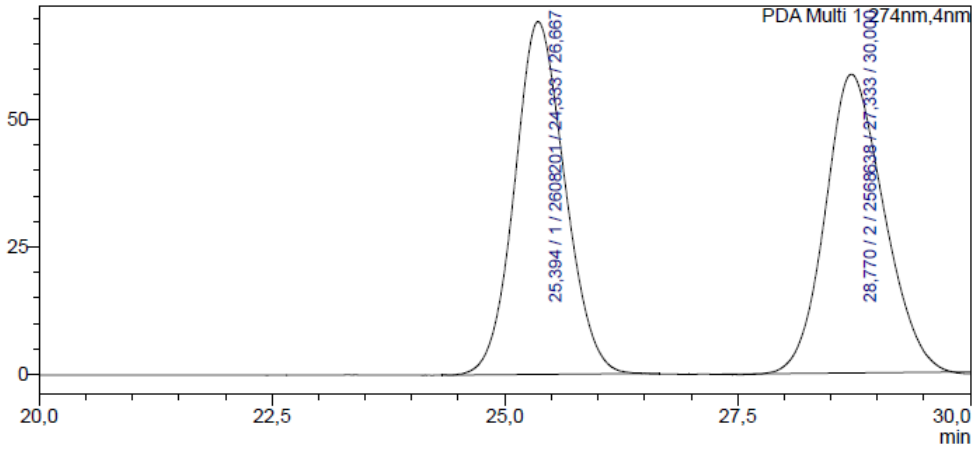
97,555	28,008	30,656
100,000		

Racemic mixture:

<Chromatogram>

mAU

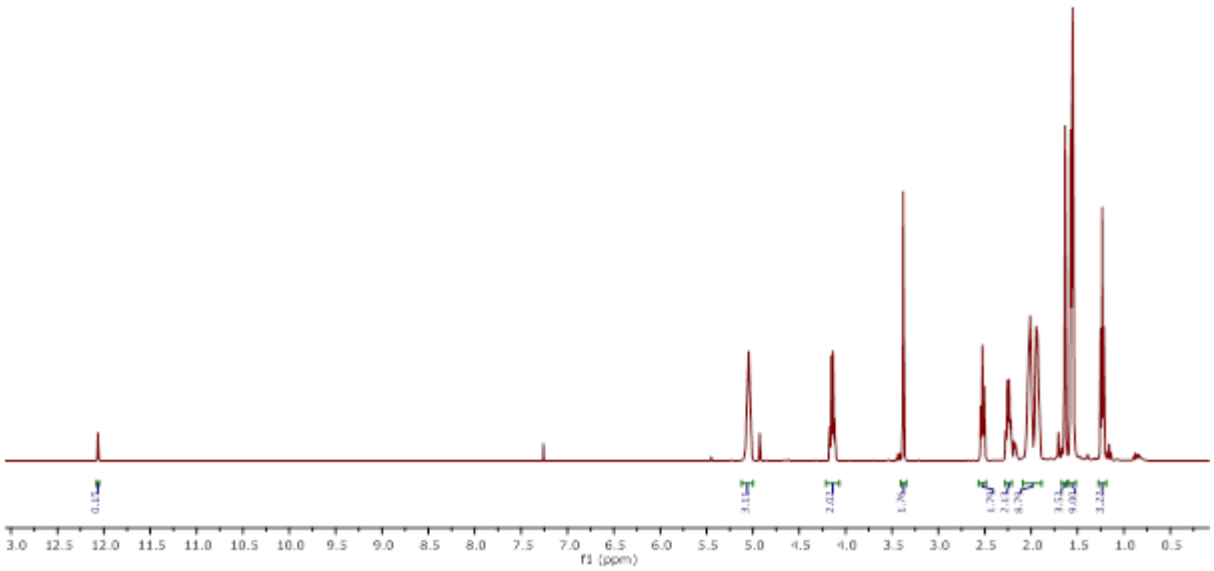


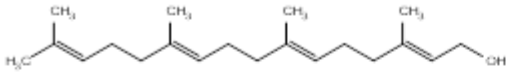
<Peak Table>

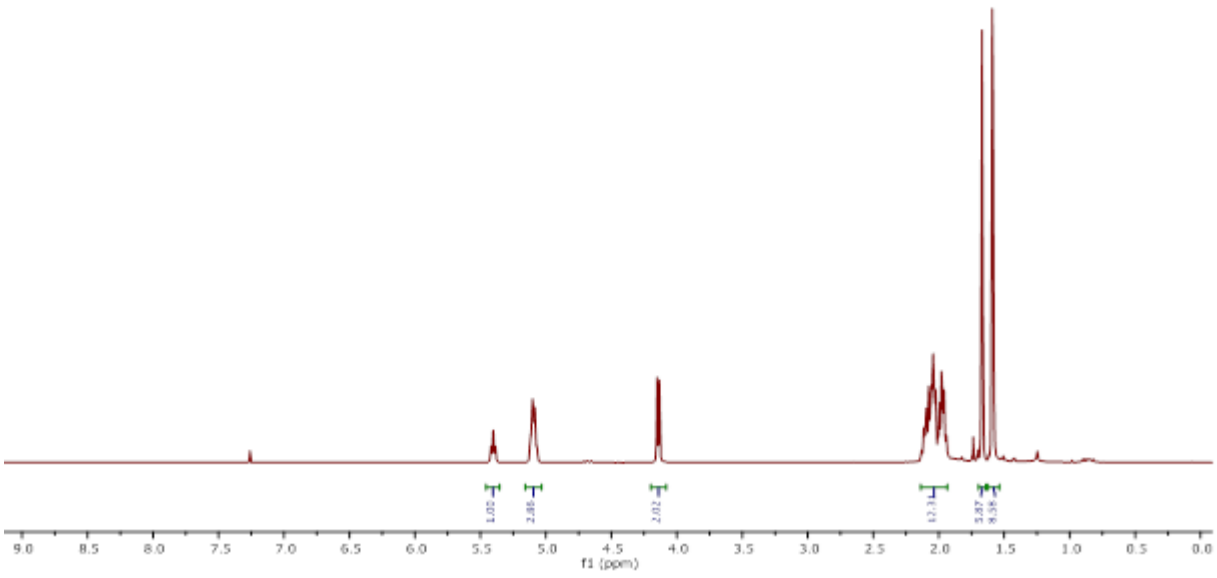
PDA Ch1 274nm

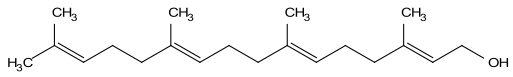
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	25,394	2608201	56409	50,382		M	
2	28,770	2568638	52337	49,618		M	
Total		5176839	108746				

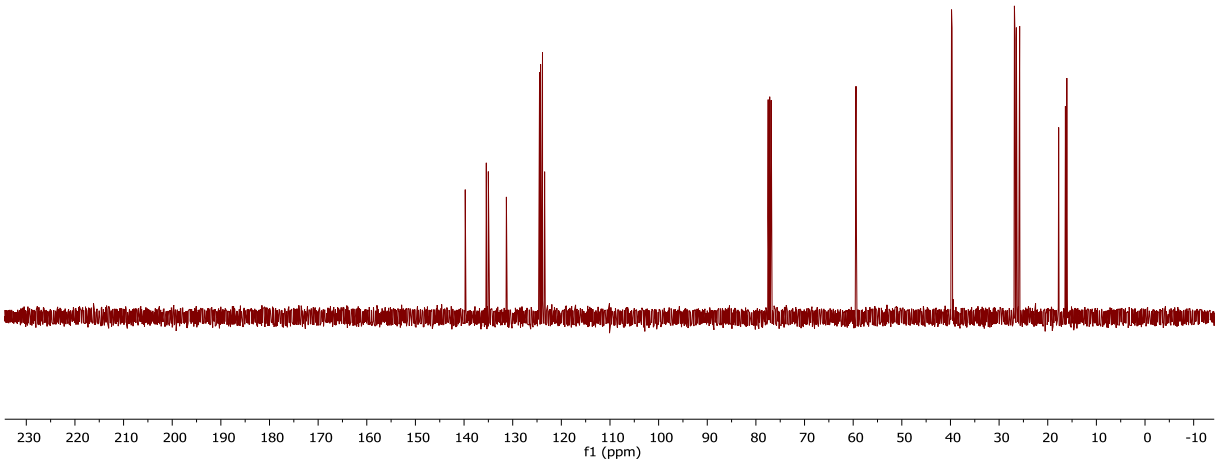
Area%	Peak Start	Peak End
50,382	24,333	26,667
49,618	27,333	30,000
100,000		


f1 (ppm)




f1 (ppm)


CH3
CH3
CH3
CH3
H3C
OH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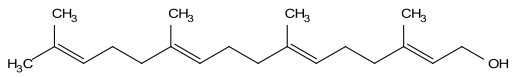
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CH3
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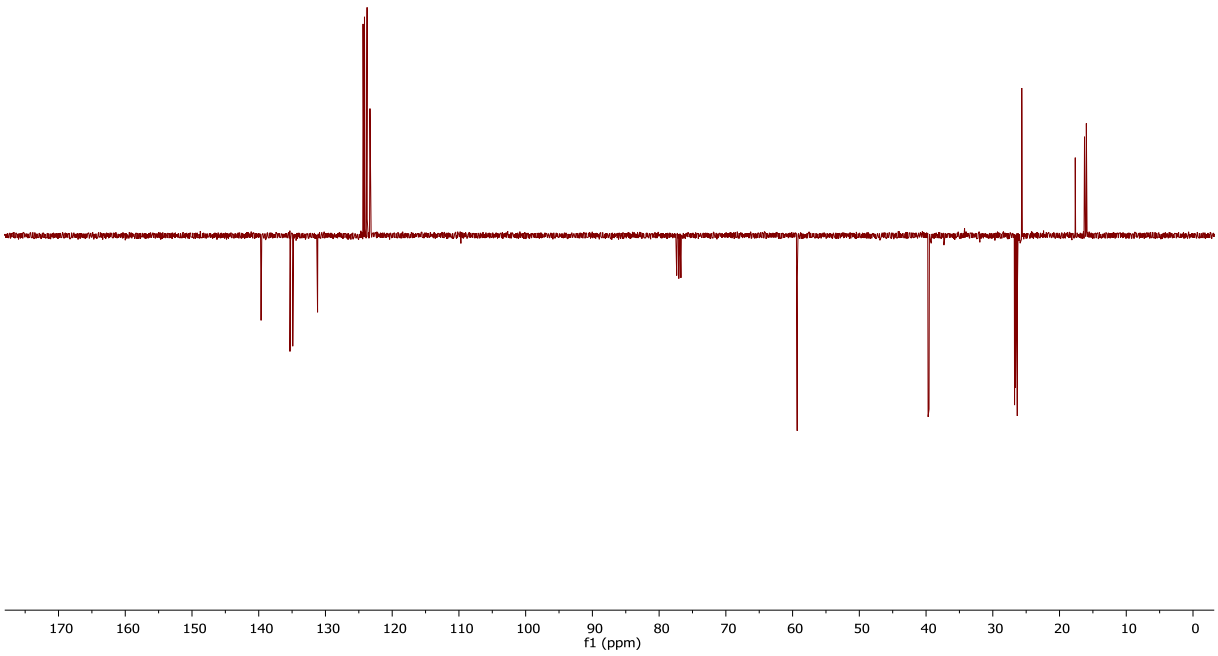
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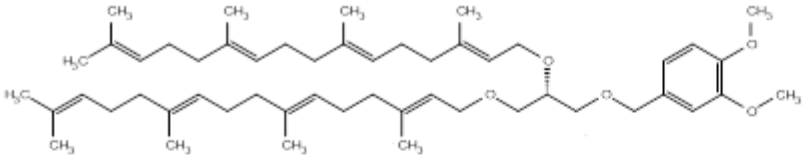

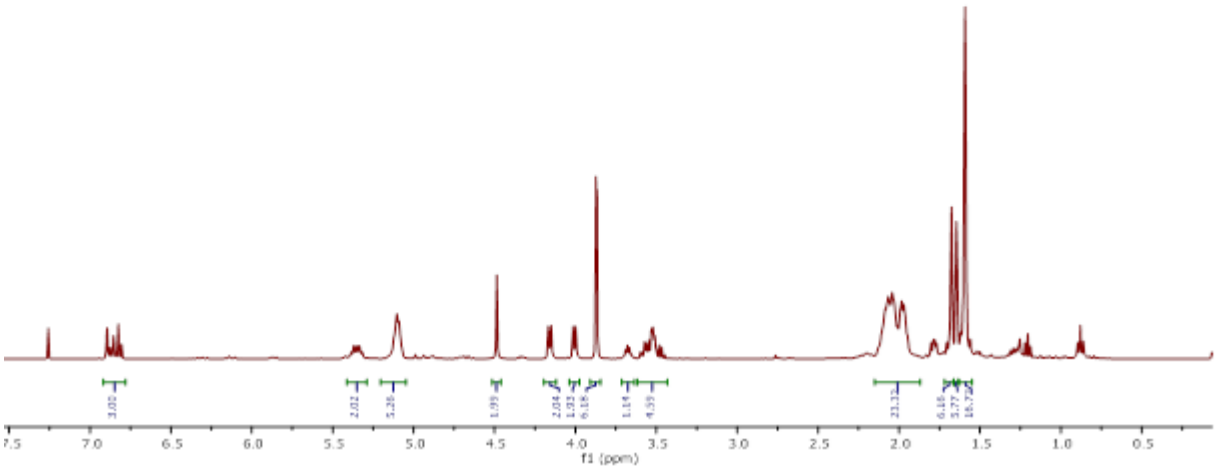
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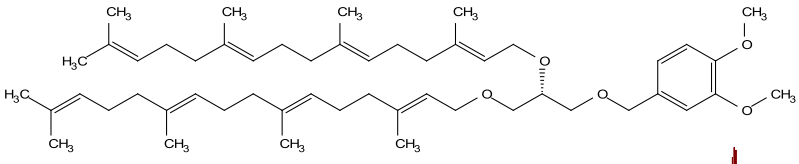
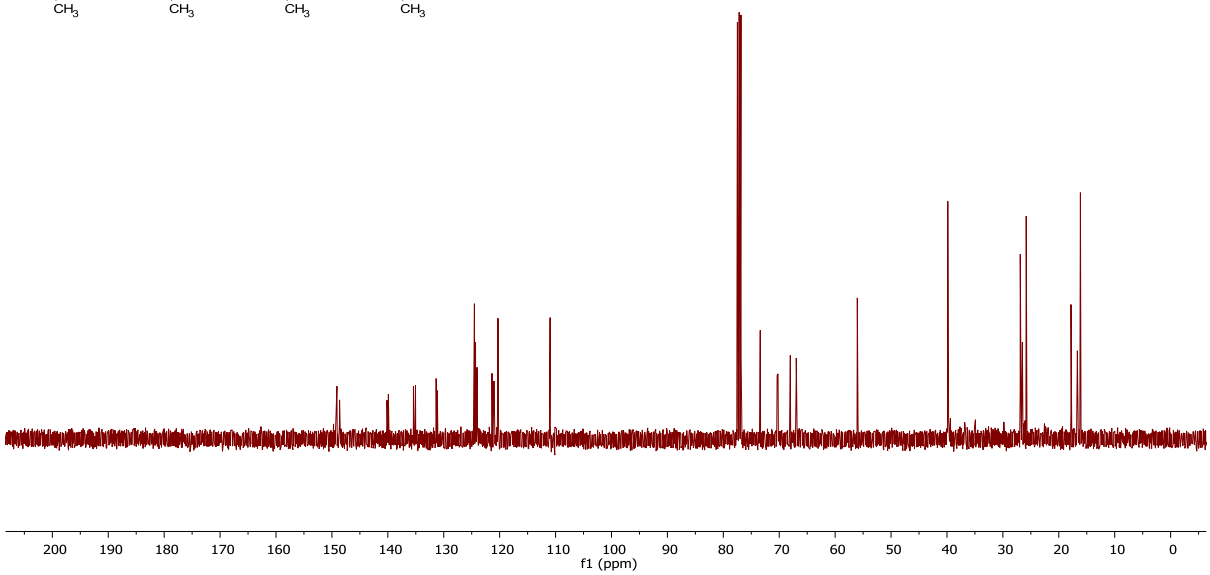
f1 (ppm)

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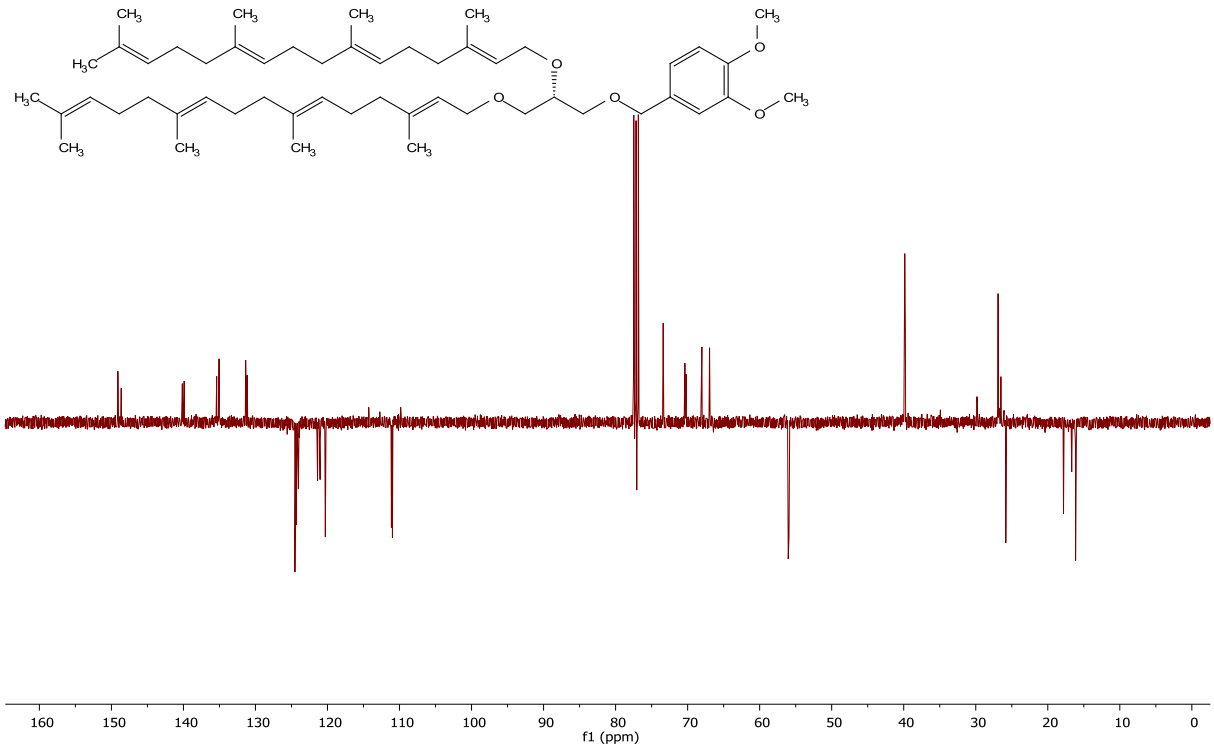
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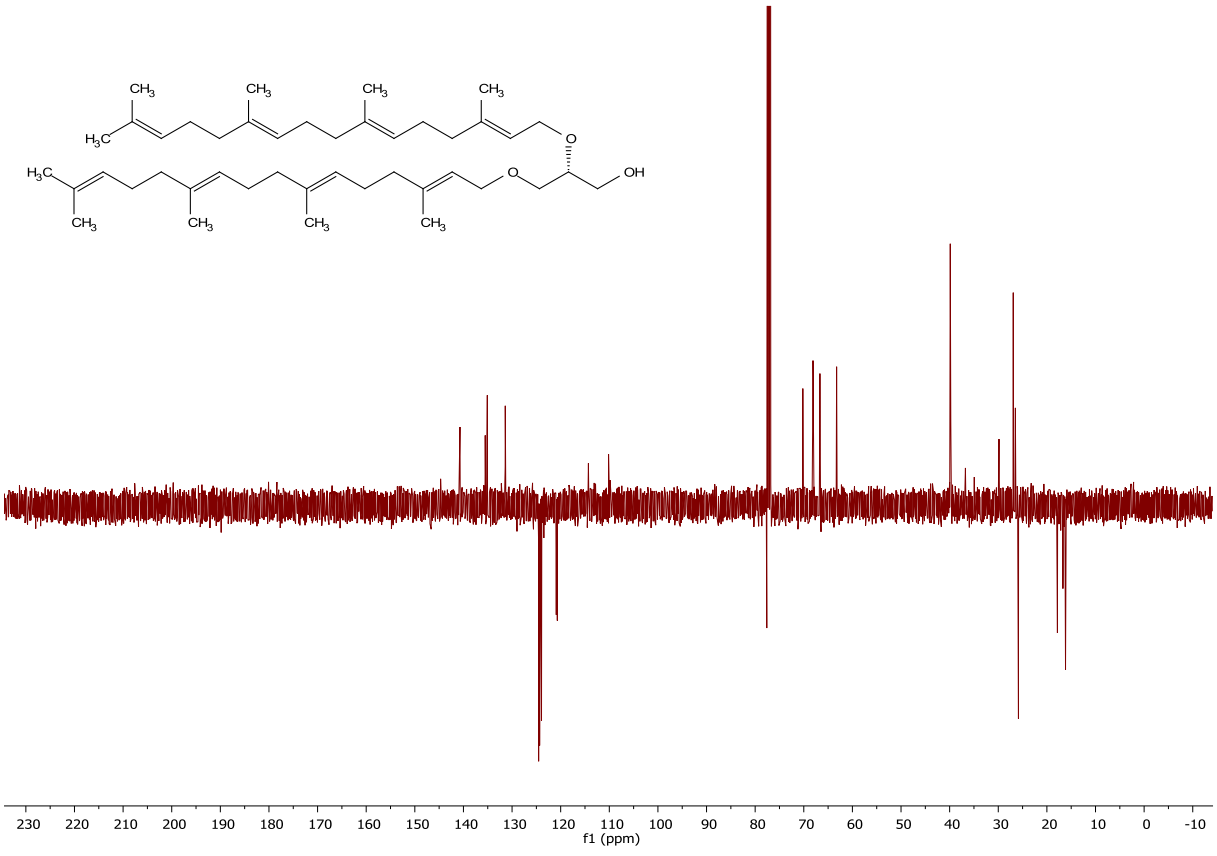
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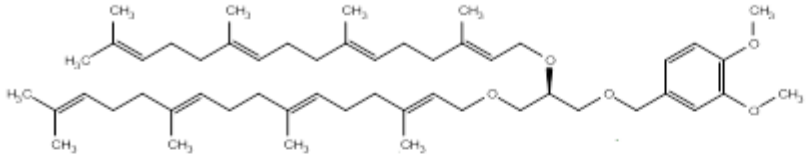

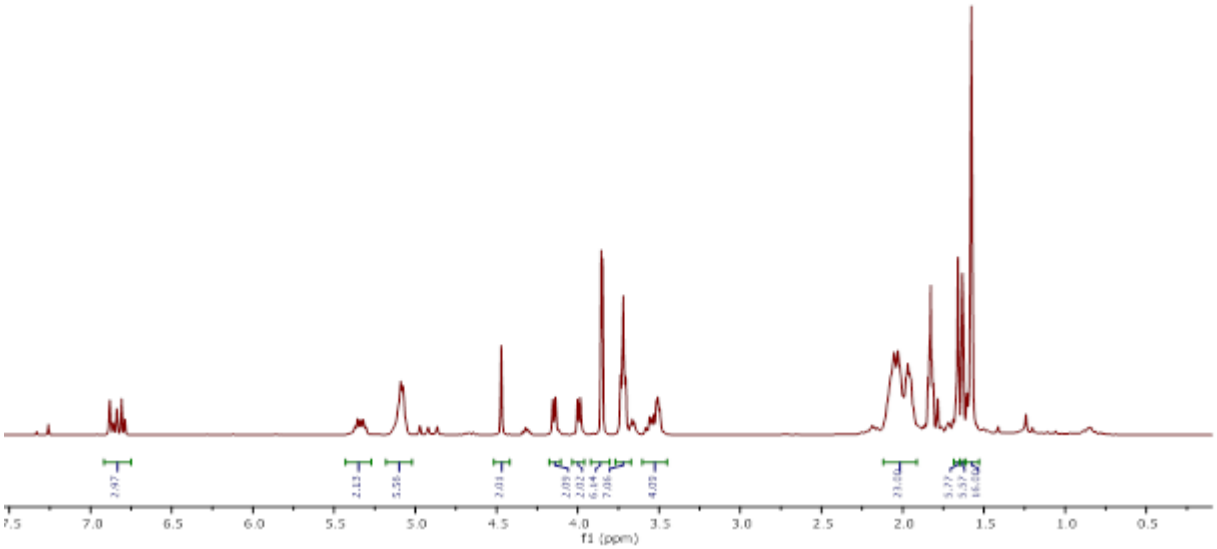
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2.0
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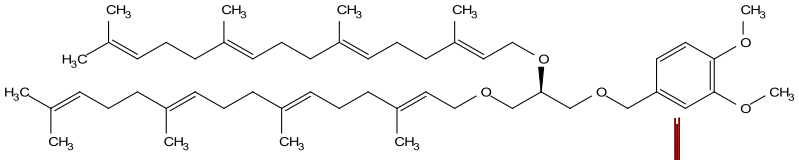
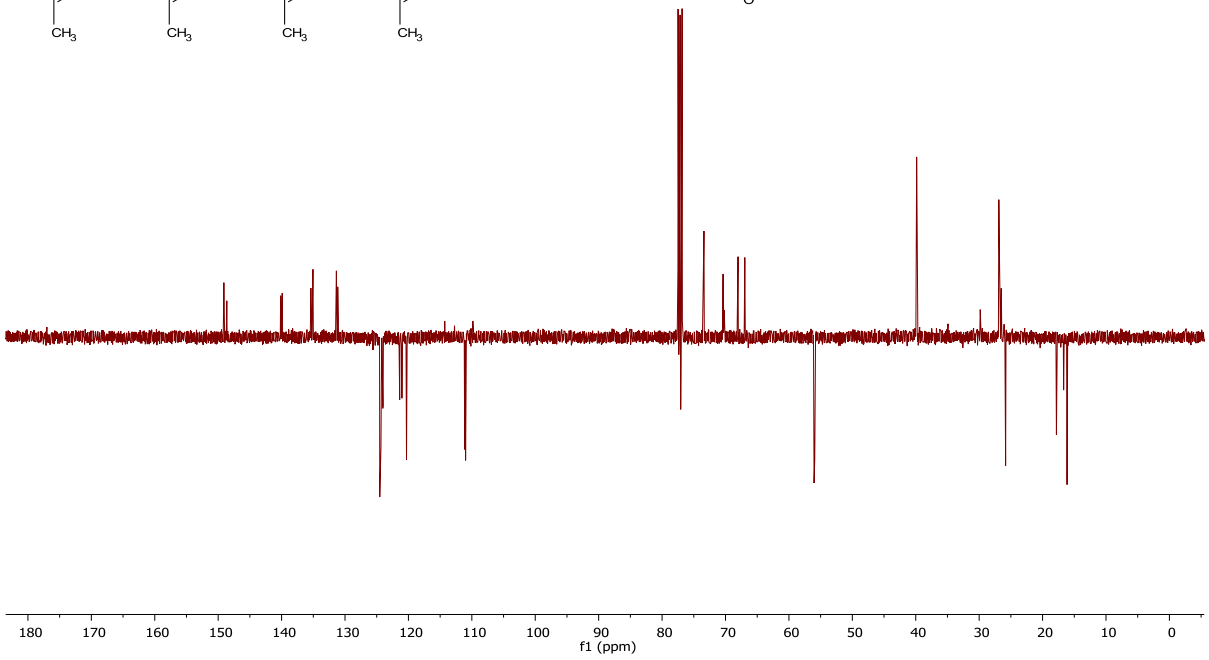
0.5
f1 (ppm)

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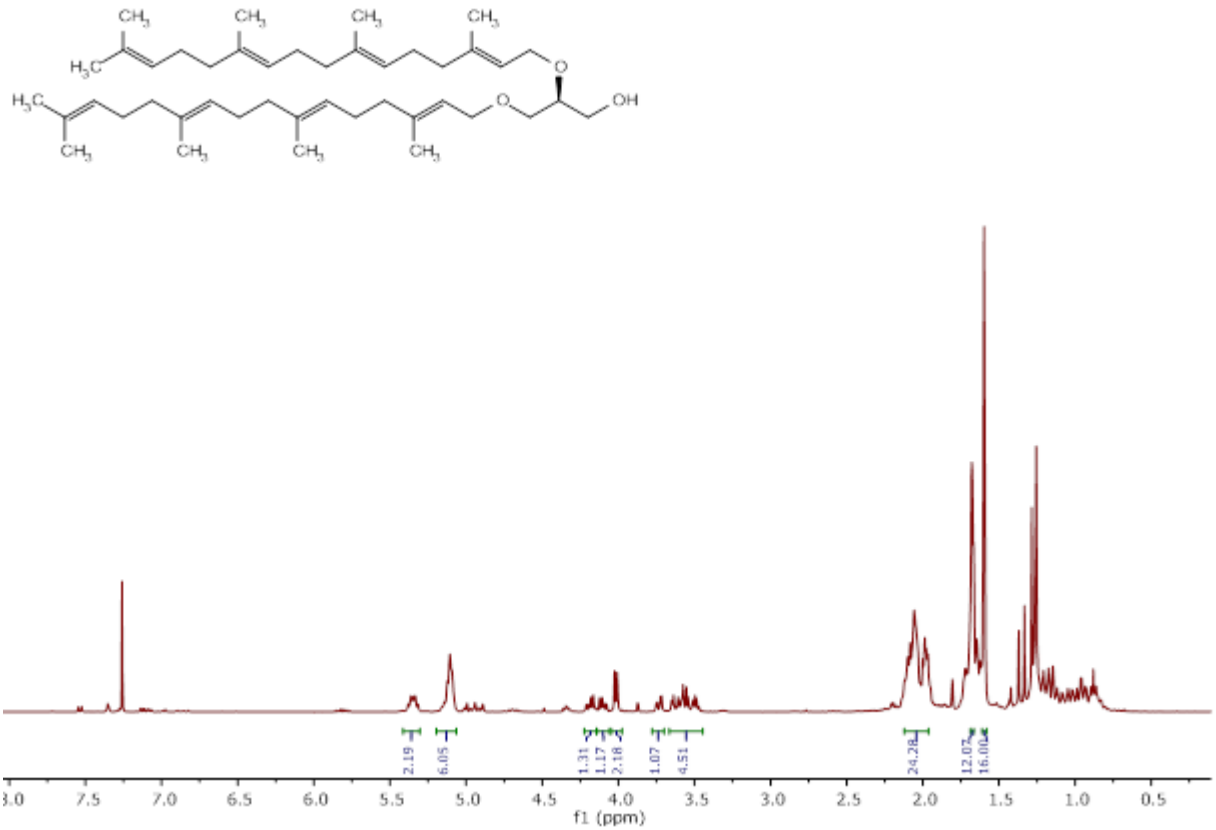
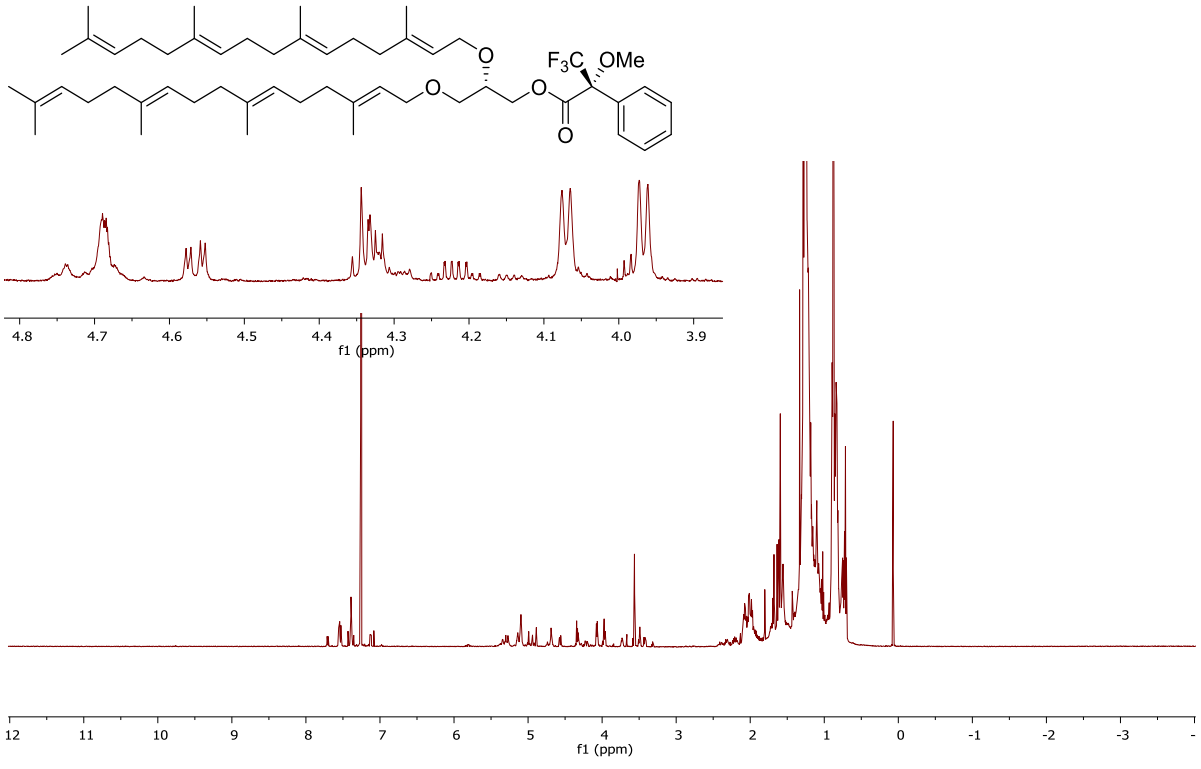
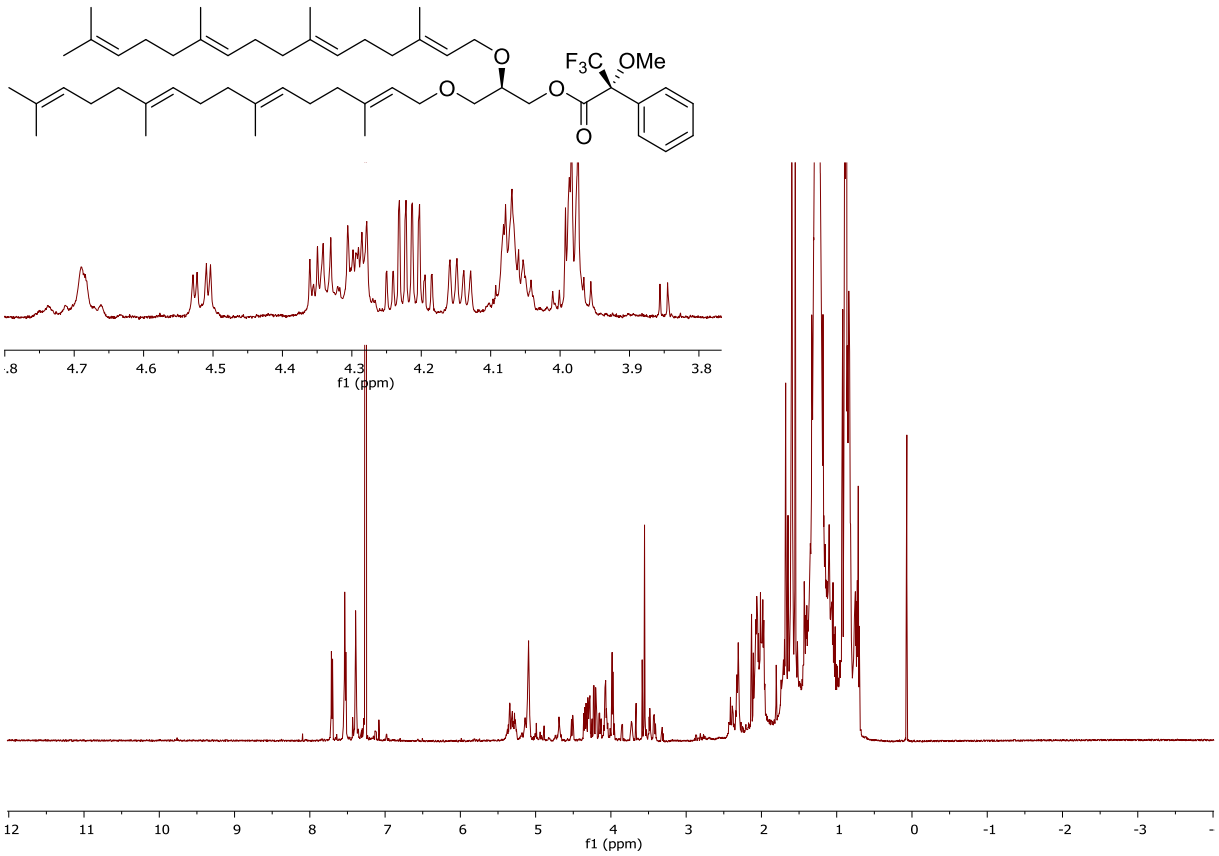


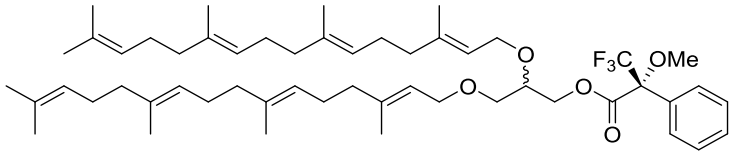
Synthetic *sn*-1 Mosher's ester lipid (**14**)

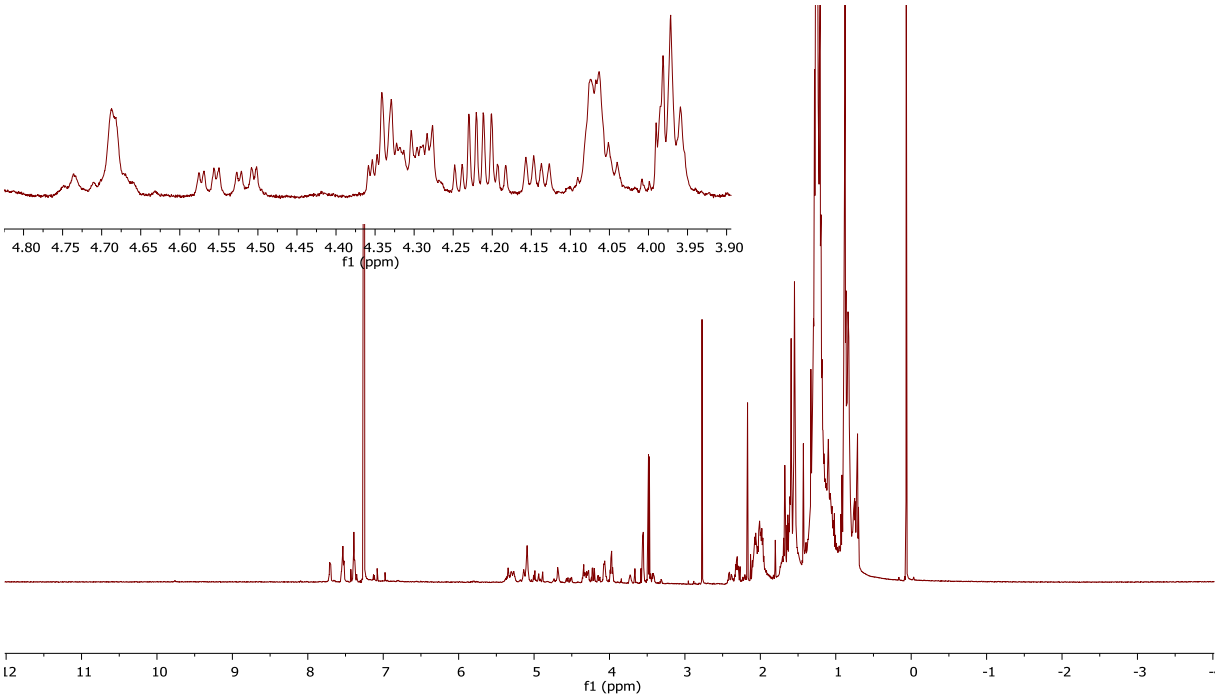


Synthetic *sn*-3 Mosher's ester lipid (**18**)

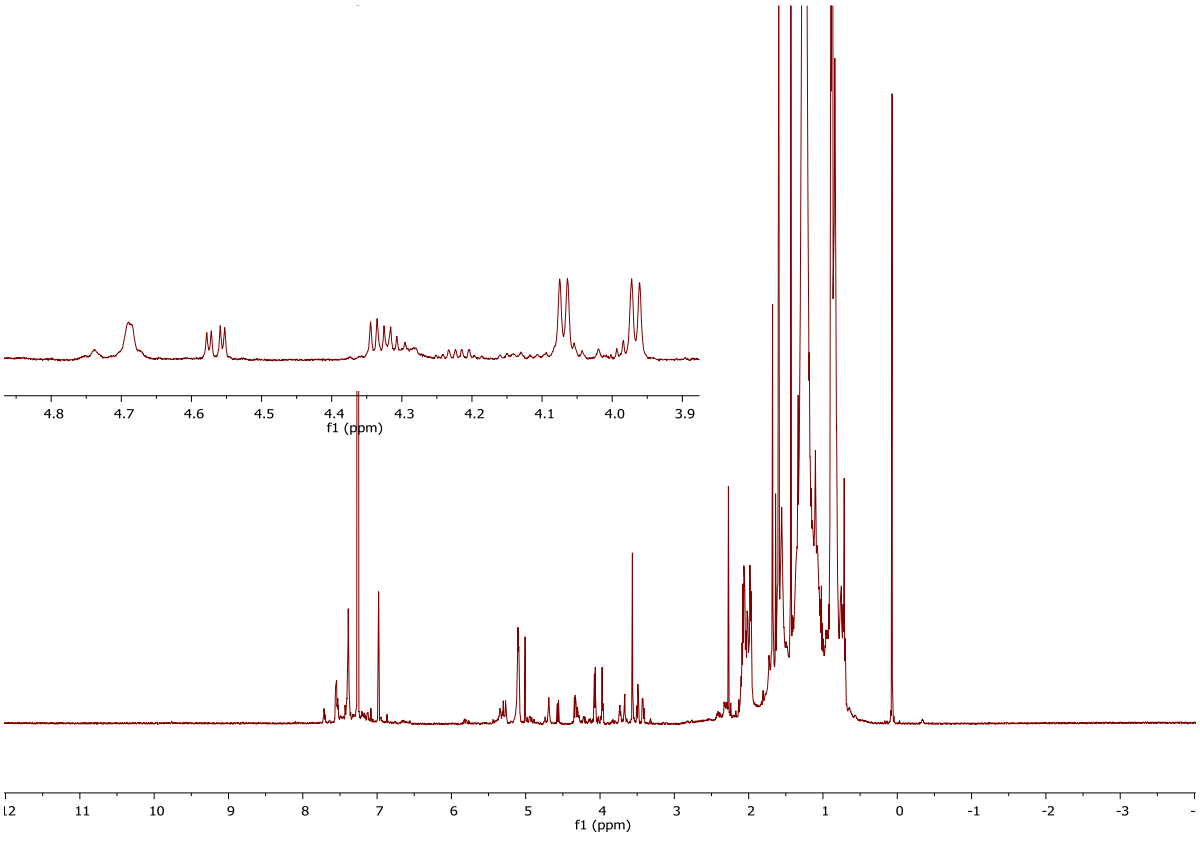


Mixture of *sn*-1 and *sn*-3 Mosher's ester lipids

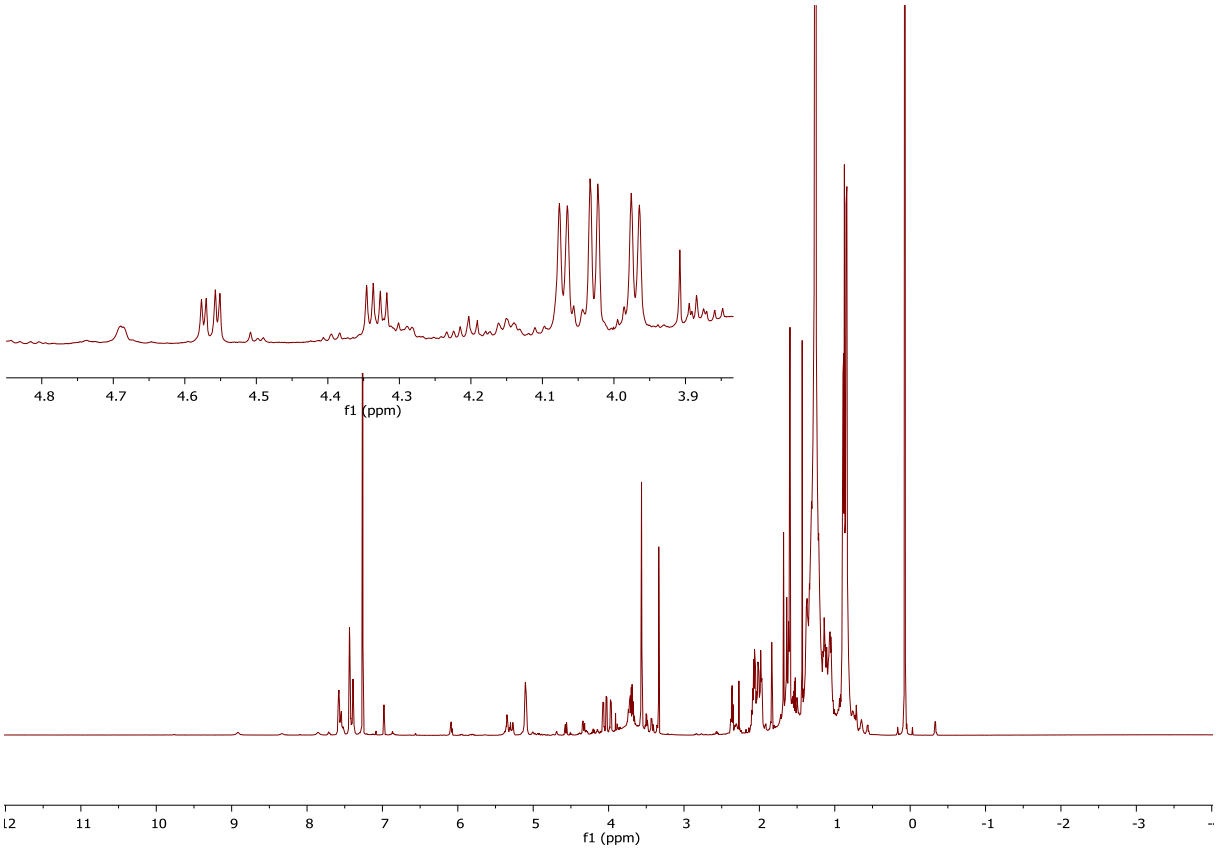




Naturally derived Mosher's ester lipid (**19**)



Naturally derived Mosher's ester lipid (G1P-deficient strain) (**20**)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4.3
4.2
4.1
4.0
3.9
f1 (ppm)
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
f1 (ppm)

2. Supplementary Figures

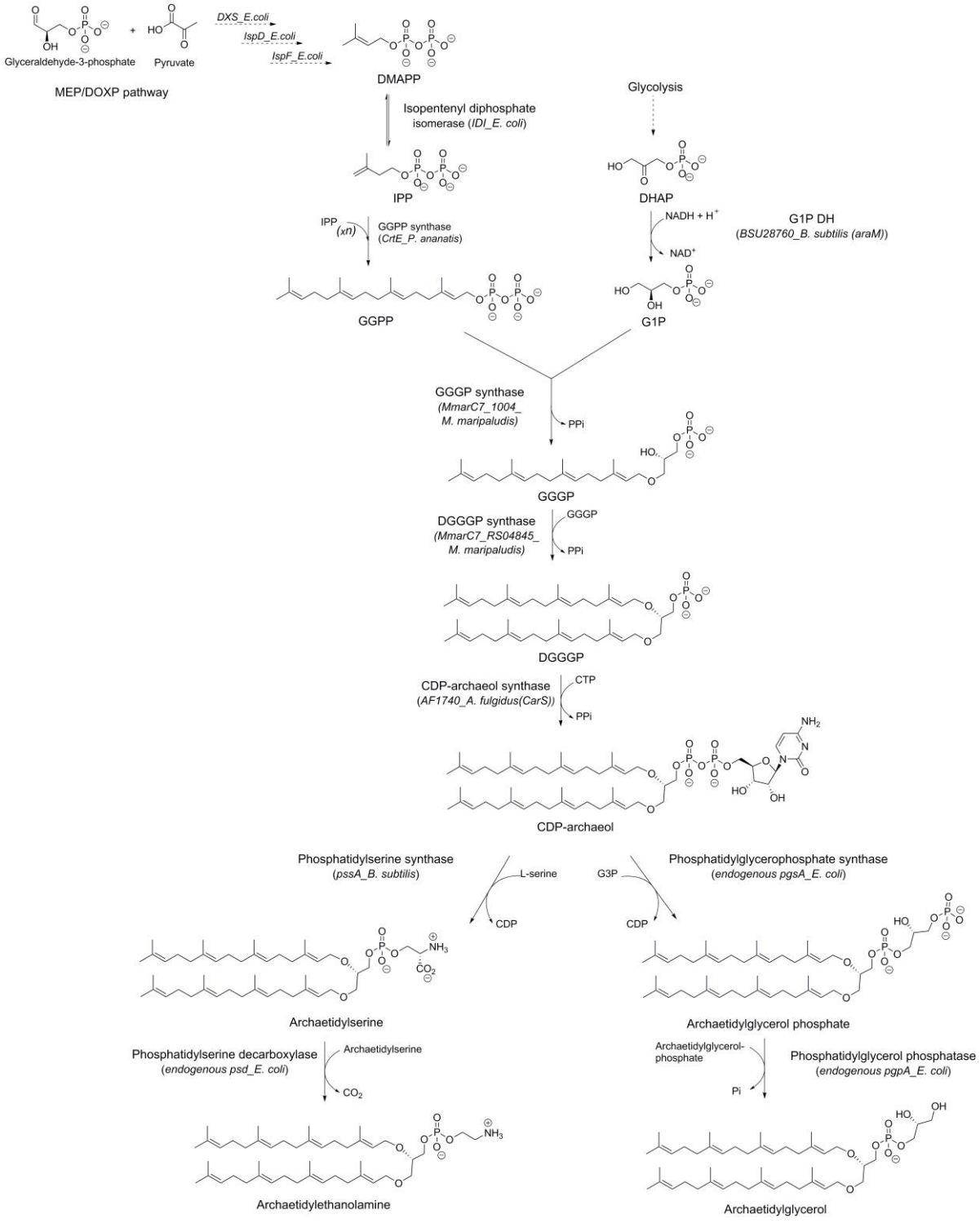

Figure S1 related to Figure 1. Schematic representation of the biosynthetic pathway introduced into the bacterium *E. coli* for archaeal lipids synthesis. The bacterial MEP/DOXP pathway enzymes used to overproduce the isoprenoid building blocks IPP and DMAPP and the genes encoding the enzymes for ether phospholipid biosynthesis. The scheme indicates

all the biosynthetic steps introduced in the bacterium *E. coli* for the production of a heterochiral mixed membrane.

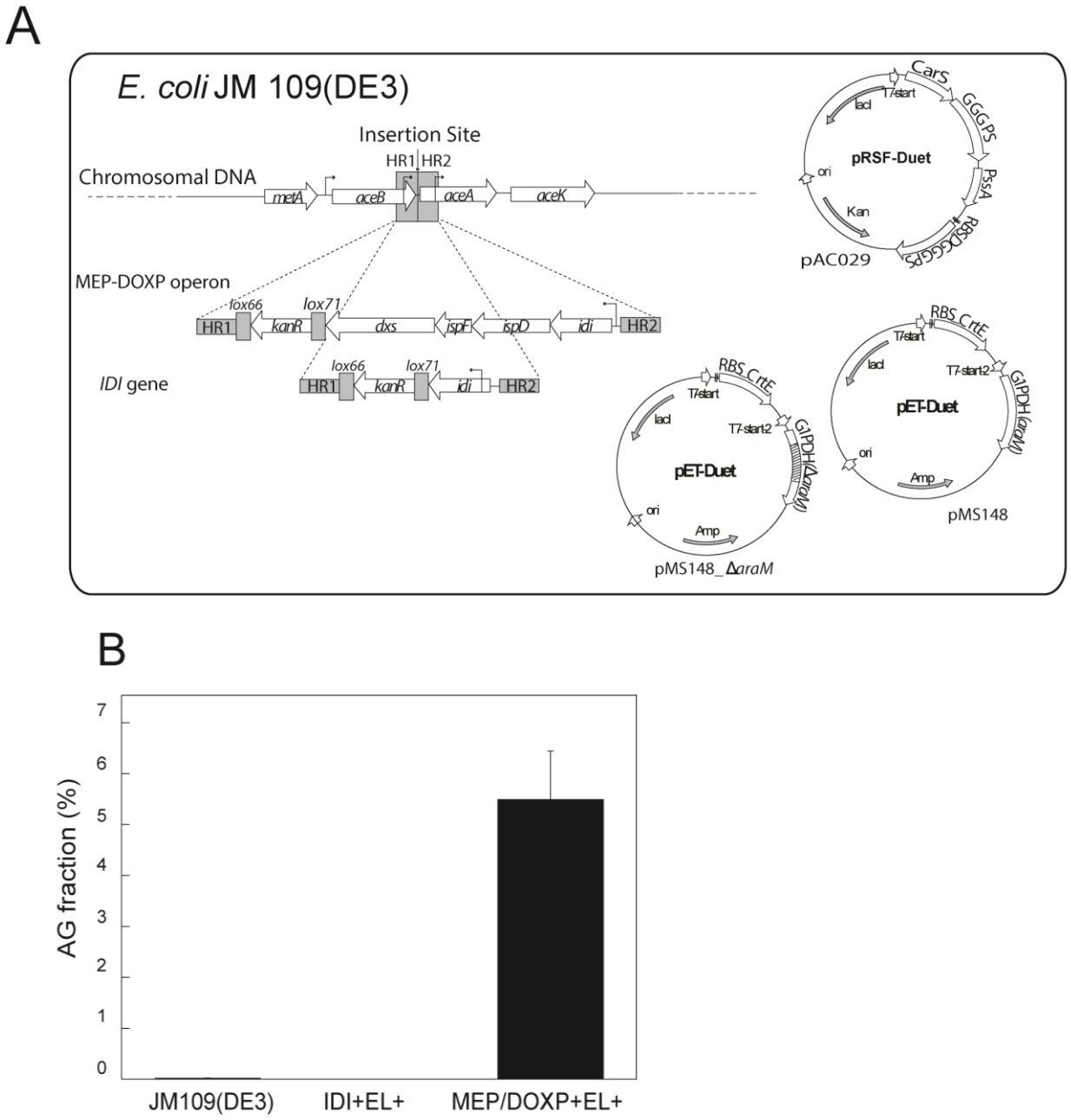

Figure S2 related to Figure 1. *E. coli* metabolic engineering. (**A**) Schematic representation ofthe engineering of *E. coli* JM109DE3 showing the integration of the MEP-DOXP operon or the *IDI* gene into the chromosome and the three vectors harbouring the ether lipids enzymes. (**B**) *In vivo* production of AG by engineered *E. coli* strains (JM109(DE3), IDI^{+}/EL^{+} and MEP/DOXP^{+}/EL^{+}) with improved IPP and DMAPP synthesis. Total ion count from LC-MS werenormalized for the total amount of phospholipids present in each sample. The data are the averages of three biological replicates ± S.E.M. (AG: archaetidylglycerol).

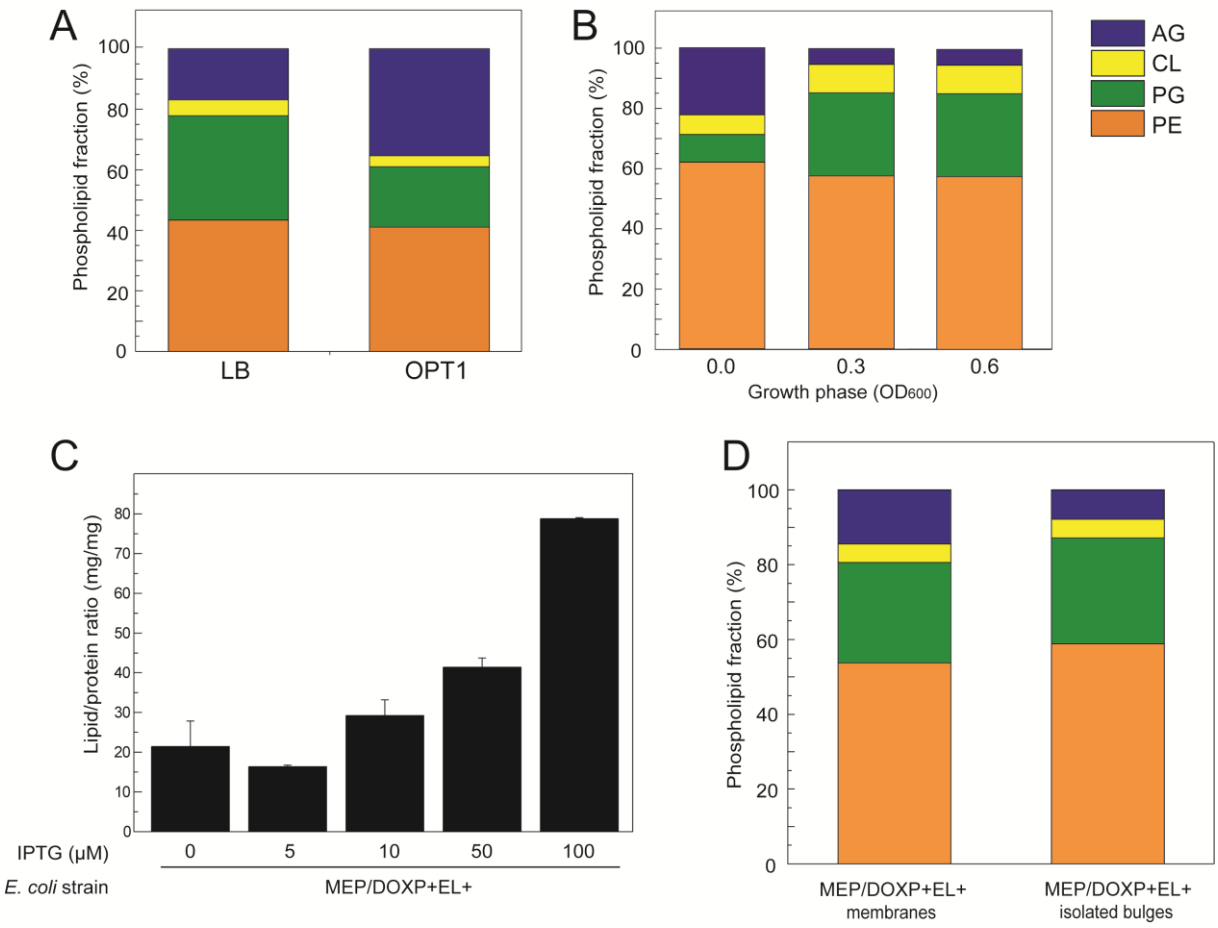

Figure S3 related to Figure 1. Optimization of archaeal lipid production in *E. coli*. Total lipid analysis of the *E. coli* MEP/DOXP^{+}/EL^{+} strain harbouring the entire ether lipid biosynthetic pathway grown and induced under different conditions. (**A**) Comparison between rich LB medium and a defined minimal medium (OPT1) optimized for the isoprenoid production. (**B**) Induction tested at different growth phases and growth on OPT1. Sampling was all done when stationary phase was reached. (**C**) Relative lipid/protein ratio of *E. coli* MEP/DOXP^{+}/EL^{+} induced with different IPTG concentration, showing the increased lipid production in higher induced conditions. Total ion counts were used to represent the amount of phospholipids present in each sample for which also the protein content was determined to yield a lipid to protein ratio that was normalized for the wild-type cells. The data are the averages of three biological replicates ± S.E.M. (AG: archaetidylglycerol, CL: cardiolipin, PG: phosphatidylglycerol and PE: phosphatidylethanolamine). (**D**) Total lipid analysis of the heterochiral mixed membrane *E. coli* strain and the isolated bulges. The lobular appendages were separated from the bacterial cells by a centrifugation step at low speed (5403 *xg*) and high speed (235,000*xg*). The obtained pellet was resuspended and the lipid analysis was performed. Content was compared with the total lipidome of the isolated membranes from the same *E. coli* strain. The total ion counts from LC-MS were normalized using eicosane as internal standard.

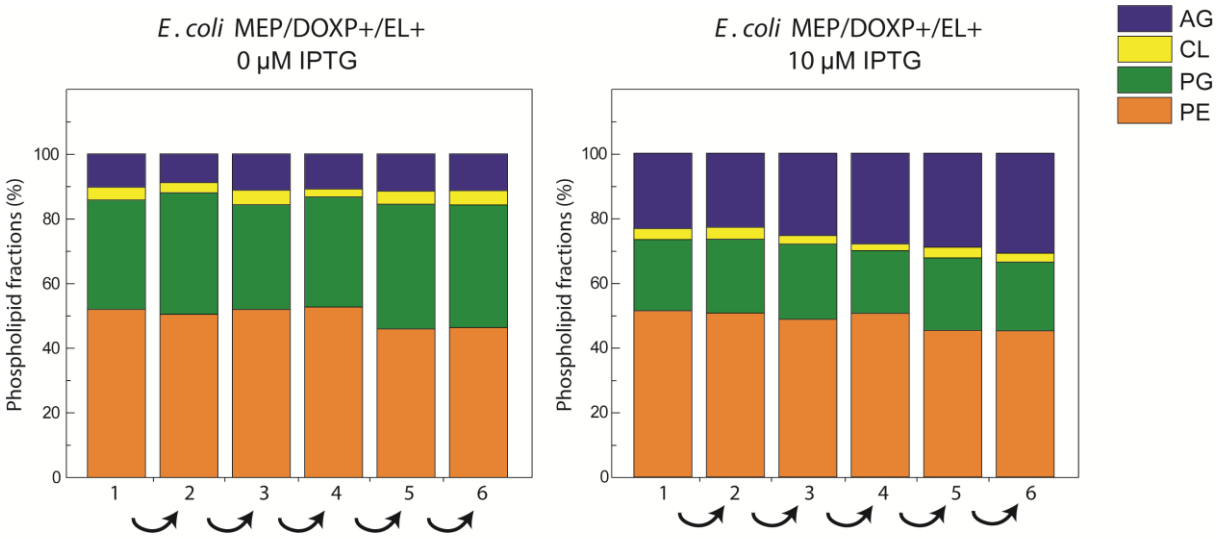

Figure S4 related to Figure 1. Continuous batch growth of the hybrid heterochiral membrane strain. Total phospholipid analysis of the *E. coli* MEP/DOXP^{+}/EL^{+} strain harbouring the entire ether lipid biosynthetic pathway that was grown under non-inducing (0 µM IPTG) and inducing (10 µM IPTG) conditions. The strain was grown for 24 hours and an aliquot of cell culture was transferred to fresh media for six times. The total ion counts from LC-MS were normalized using eicosane as internal standard. The data are the averages of two biological replicates ± S.E.M. (AG: archaetidylglycerol, CL: cardiolipin, PG: phosphatidylglycerol and PE: phosphatidylethanolamine).

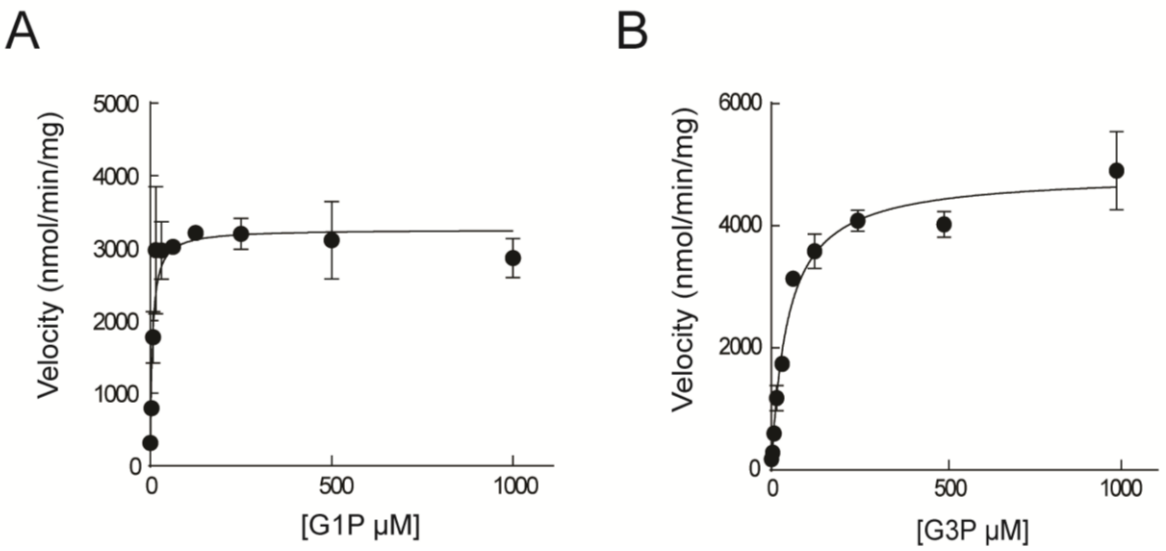

Figure S5. Stereoselective enzyme kinetics of *M. maripaludis* GGGPS. Kinetic analysis of M. Maripaludis GGGPS using different concentrations of G1P (A) or G3P (B). Results are the averages of two experiments ± S.E.M.

3. Supplementary Tables

Table S1. *E. coli* engineered strains used in this study.

Strain name	Genome integration	Plasmids
JM109(DE3)	none	pET-Duet and pRSF-Duet
E. coli IDI^{+}	*idi*	pET-Duet and pRSF-Duet
E. coli MEP/DOXP^{+}	*idi-ispDF-dsx*	pET-Duet and pRSF-Duet
E. coli MEP/DOXP^{+}EL^{+} (Ether Lipid)	*idi-ispDF-dsx*	pMS148 and pAC029
E. coli MEP/DOXP^{+}EL^{+}AraM^{-}	*idi-ispDF-dsx*	pMS148Δ and pAC029
E. coli IDI^{+}EL^{+}	*idi*	pMS148 and pAC029

Table S2. Combination of bacterial and enzymes used in this study for the *in vivo* ether lipids production.

Locus (14)	Source	Protein expressed	Function	Reference
CrtE	*P. ananatis*	GGPP synthase	IPP+DMAPP→GGPP	Albermann *et al.*(15)
BSU28760 (araM)	*B. subtilis*	G1P dehydrogenase –His_6	DHAP+NADH→G1P	Guldan *et al*.(16)
MmarC7_1004	*M. maripaludis*	His_8-GGGP synthase (codon optimized)	G1P+GGPP→ GGGP	Peterhoff *et al*.(17)
MmarC7_RS04845	*M. maripaludis*	His_8-DGGGP synthase (codon optimized)	GGPP+GGGP→ DGGGP	Lai D. *et al.*(18)
AF1740	*A. fulgidus*	CarS-His_8 (codon optimized)	DGGGP+CTP→ CDP-archaeol	Jain *et al*.(4)
pssA	*B. subtilis*	PssA-His_8	CDP-archaeol→ AS	Caforio *et al*.(5)
psd	*E. coli*	Psd-His_8	AS→AE	Caforio *et al.*(5)
pgsA	*E. coli*	PgsA-His_8	CDP-archaeol→ AGP	Caforio *et al*.(5)
pgpA	*E. coli*	PgpA-His_8	AGP→AG	Caforio *et al*.(5)

Table S3 Single nucleotide variants detected by Illumina sequencing. Comparative analysis of the genome of the hybrid heterochiral mixed membrane genome grown in LB and not induced (reference) and the hybrid heterochiral mixed membrane genome grown in OPT medium and induced with IPTG.

ref. allele	allele	gene/intergenic region	effect	predicted function
C	T	*mhpE - mhpT*	none	4-hydroxy-2-oxovalerate aldolase; 3-hydroxyphenylpropionic acid transporter
A	T	*lysZ – lysQ*	none	(tRNA-lys) ttt
G	A	*nu1_2* (possible prophage)	none	possibly nohB
G	C	*rsxC*	E614Q	reducer of SoxR, SoxR iron-sulfur cluster reduction factor component
G	C	*lptB*	V210L	LPS transport; LPS export ABC transporter ATPase
A	T	*yhhI*	A292A	H-repeat-associated protein, RhsE-linked, (predicted transposase)
A	G	*yhhI*	E293E	
G	A	*yhhI*	K294K	
C	deletion	*rop – lacI*	none	non-coding C-terminal region of *lacI*
G	deletion	*rop - lacI*	none	

Table S4. Cloning and expression vectors used in this study.

Plasmid	Description	Reference
pGFPuv	Cloning vector expressing *Aequorea Victoria* GFP	Clontech
pCR2.1 TOPO	Cloning vector with *lox71-kanR-lox66* gene cassette	ThermoFisher
pKD46	Lamba Red recombinase expressing vector	Datsenko *et al.* (19)
pRSF-Duet	Cloning and expression vector (Kan^R), T7 promoter	Novagen
pET-Duet	Cloning and expression vector (Amp^R), T7 promoter	Novagen
pACYC-Duet	Cloning and expression vector (Cm^R), T7 promoter	Novagen
pMS003	*IDI* gene from *E. coli* MG1655 cloned into pGFPuv vector using primers BG3606 and BG3599	This study
pMS008	*IspDF* genes from *E. coli* MG1655 cloned into pMS003 vector using primers BG3600 and BG3601	This study
pMS011	*DXS* gene from *E. coli* MG1655 cloned into pMS008 vector using primers BG3602 and BG3603	This study
pMS051	*lox71-kanR-lox66* gene cassette from pCR2.1 TOPO vector cloned into pMS003 vector using primers BG4429 and BG4430	This study
pMS053	*lox71-kanR-lox66* gene cassette from pCR2.1 TOPO vector cloned into pMS011 vector using primers BG4429 and BG4430	This study
pMS016	*crtE* gene from *Pantoea ananatis* cloned into the pACYC-duet vector using primers BG3899 and BG3900	This study
pMS017	*crtB* and *crtI* genes from *Pantoea ananatis* cloned into the pMS016 vector using primers BG3901 and BG3902	This study
pSJ130	*araM* gene from *Bacillus subtilis* cloned into pET-duet vector using primers 70 and 71	Jain *et al*.(4)
pMS148	c*rtE* gene from *Pantoea ananatis* digested with EcoRI and cloned into the pSJ130 vector	
pMS148Δ	pMS148 vector containing a deleted version of the *araM* gene using the EcoRV and BmgBI	
pSP001	Codon-optimized GGGPS and DGGGPS genes from *M. maripaludis* cloned into pRSF-duet vector using the primers 11, 12, 39 and 40	Jain *et al*.(4)
pAC027	Codon optimized *carS* gene from *A. fulgidus* cloned into pSP001 vector using primers 583 and 584	This study
pAC029	*pssA* gene from *B. subtilis* cloned into the pAC027 vector using the vector using primers 585 and 586	This study
pSJ103	Codon optimized GGGPS and DGGGPS genes from *M. maripaludis* cloned into pRSF-duet vector using the primers 11 and 12	Jain *et al*.(4)
pME001	*fadD* gene from *E. coli* MG1655 cloned into pRSF-Duet-1 vector using the primers PrME001 and PrME002	This study
pME002	*plsB* gene from *E. coli* MG1655 cloned into pet28b vector using the primers PrME003 and PrME004	This study

Table S5. Oligonucleotide primers used in the present study.

Primers name	Primer sequence 5' ⟶ 3'	Restriction enzyme site
BG3606	GGCATGCCATGCAAACGGAACACG	SphI
BG3599	GCTGCAGTTATTTAAGCTGGGTAAATGCA	PstI
BG3600	GCTGCAGAGGAGATATACATATGGCAACCACTCATTTGG	PstI
BG3601	GTCTAGATCATTTTGTTGCCTTAATGAGTAGCG	XbaI
BG3602	GTCTAGAGGAGATATACTGATGAGTTTTGATATTGCCAAATAC	XbaI
BG3603	GCGGTACCTTATGCCAGCCAGGCC	KpnI
BG4429	GACGCGTACGGTGTCTTTTTTACCTGTTTGACC	BsiWI
BG4430	GACGCTTAAGCTACCTCTGGTGAAGGAGTTGG	AflII
BG4885	CTGACCCTGCCAGGCTACCGCCTGTTAGCGTAAACCACCACATAACTATGGCTACCTCTGGT GAAGGAGTTG	
BG4886	CTGTAATTCTTCAATTTGTTGTGTACGGGTTTTCATGTGCAGATGCTCGGCAGTGAGCGCAA CGCAATT	
BG3899	GAACGAATTCAGCCCGAATGACGGTCTGC	EcoRI
BG3900	GAATCTTAAGGCGCGACCAGTTCCTGAG	AflII
BG3901	GCTGAGATCTGATGAAACCAACTACGGTAATTGG	BglII
BG3902	CTTACTCGAGAAAGACATGGCGCTAGAG	XhoI
70	GCGCCATATGAATCGTATCGCAGCTGAC	NdeI
71	GCGCCTCGAGTTAGTGATGATGGTGGTGATGTTCATATAGACCATGGTTGATCAGCG	XhoI
11	GCGCGAATTCATGCATCACCACCACC	EcoRI
12	GCGCAAGCTTTCATTTTTTGGACAGC	HindIII
39	TCTTTACCTCTCTTATACTTAACTAATATACTAAGATGGG	blunt
40	CATATGGGCAGCCATCACCATCATCACCACAGCG	blunt
583	CGCGCCATGGATGCTGGATCTGATTCTGAAAACCATTTG	NcoI
584	CGCGGGATCCTTAGTGATGGTGATGGTGGTGATG	BamHI
585	GCGCGCGGCCGCATGAATTACATCCCCTGTATG	NotI
586	CGCGCTTAAGTTAGTGATGGTGATGGTGGTG	AflII
PrME001	TACTAGGAATTCATGAAGAAGGTTTGGCTTAACCG	EcoRI
PrME002	AGTCATGCGGCCGCTCAGTGGTGGTGGTGGTGGTGGGCTTTATTGTCCACTTTGC	NotI
PrME003	CATCCTGCCCATGGCCGGCTGGCCACGAATTTACTAC	NcoI
PrME004	CTTCATGATTCTCGAGCCCTTCGCCCTGCGTCGCAC	XhoI

4. Supplementary References

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