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Supporting Information

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High-Throughput Screening for Terpene-Synthase-Cyclization Activity and Directed Evolution of a Terpene Synthase**

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Supporting Information

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Instrumentation

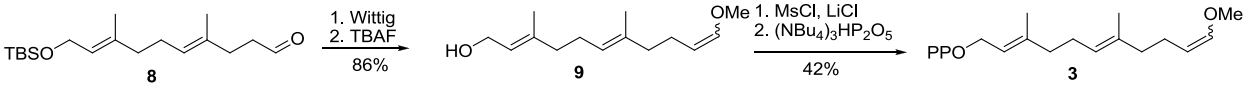
NMR measurements were performed on Varian instruments at 400 or 500 MHz for proton spectra, 161 MHz for phosphorous NMR, and 100 or 125 MHz for carbon spectra. Chemical shifts are given in ppm values and are not solvent referenced unless otherwise noted. Gas chromatography (GC) was performed on a Shimadzu GC-17A instrument equipped with an FID detector. The columns used were an HP-5 column (Agilent, 30 m x 0.32 mm x 0.25 μm film) and a CyclosilB column (Agilent, 30 m x 0.32 mm x 0.25 μm film, with a 10 m x 0.32 mm inert glass SGE guard column). Absorbance data from 96-well microtitre plates was collected on a Tecan infinite M200. Proteins were purified on an ÄKTAxpress with 5 ml HisTrap columns (GE Healthcare Life Sciences).

General Procedures

Reactions performed under Ar were done using flame dried glassware and standard syringe and septa techniques unless otherwise noted. Removal of solvent *in vacuo* refers to use of a Büchi rotary evaporator. Reagents were purchased from VWR, Fisher, or Aldrich and generally used without purification. TLC was performed with aluminum-backed silica gel 60 F_{254} analytical plates from EMD. Visualization of compound spots was done with a short wave UV lamp followed by staining with anisaldehyde. Flash chromatography was performed on Geduran silica gel 60 (40-63 μm) from EMD. Alcohol oxidase (AOX) was obtained from MP Biomedicals. Farnesyl diphosphate (FPP) was synthesized from farnesol according to the method of Poulter.[1] Primers were purchased from Integrated DNA Technologies (IDT). *E. cloni* Express electrocompetent *E. coli* BL21 (DE3) was purchased from Lucigen. Alkaline phosphatase, Taq polymerase, T4 ligase, and restriction enzymes DpnI, XhoI, and NdeI were purchased from New England Biolabs (NEB). BcBOT2 and SSCG_02150 in pET28a(+) were gifts from David E. Cane at Brown University. TB refers to terrific broth from RPI, and LB refers to Luria broth from the same vendor.

Synthesis of substrate 3

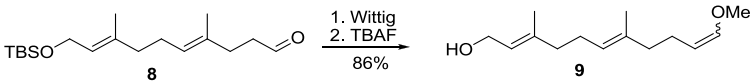
Overview of the synthetic scheme:



8 **9** **3**

Aldehyde **8** is a known compound, and was made from commercial farnesol acetate according to published procedures.[2]

(2*E*,6*E*)-11-methoxy-3,7-dimethylundeca-2,6,10-trien-1-ol (9)

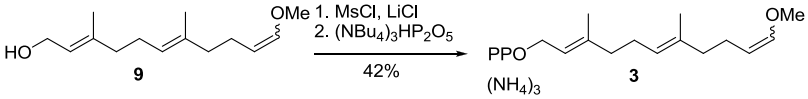


8 **9**

The Wittig reagent (methoxymethyl)triphenylphosphonium chloride (9.9 g, 28.9 mmol) was suspended in 90 mL of dry THF in a 250 mL flame-dried round bottom flask under Ar. Potassium *t*-butoxide (3.23 g, 28.8 mmol) was added in one portion, and the reaction was magnetically stirred at room temp for 15 min. The reaction mixture (deep red at this point) was cooled with an ice bath. A solution of aldehyde **8** (4.5 g, 14.5 mmol) in 45 mL of dry THF was added dropwise as the ice bath cooling and magnetic stirring were continued. The cooling bath was then removed, and the reaction was stirred at room temp for 45 min. The reaction was quenched with aqueous NH_4Cl, and the product was extracted into hexanes with ca. 5% EtOAc. The organic layer was washed with water and brine, dried over sodium sulfate, and concentrated *in vacuo*. Ph_3PO was removed by trituration in pentane, and the filtered pentane solution was concentrated *in vacuo* to give a nearly colorless liquid. This liquid was dissolved in 55 mL of dry THF under Ar in a septum-sealed 250 mL round bottom flask. The solution was magnetically stirred at room temp as 24 mL of 1 M TBAF in THF was added via syringe. The reaction was stirred for 3 h at room temp. The reaction mixture was then partitioned between Et_2O and water, and the organic phase was washed with brine and dried over sodium sulfate. The organic phase was concentrated *in vacuo*, and the residue was purified by silica gel chromatography using EtOAc in hexanes (1:3) with 1% triethyl amine. The product was collected as a viscous colorless liquid. Yield: 2.79 g (86%).

^{1}H NMR (400 MHz, C_6D_6) δ 6.29 (d, *J* = 12.6 Hz, 0.78 H, major isomer), 5.71 (dt, *J* = 6.2, 1.5 Hz, 0.49 H, minor isomer), 5.41-5.37 (m, 1 H), 5.24-5.17 (m, 1 H), 4.73-4.61 (m, 0.66 H, major isomer), 4.43-4.38 (td, *J* = 7.2, 6.3 Hz, 0.40 H, minor isomer), 4.02-3.94 (m, 2 H), 3.18 (s, 1.76 H), 3.12 (s, 1.08 H), 2.46-2.38 (m, 1 H), 2.17-2.06 (3 H), 2.05-1.93 (m, 5 H), 1.57 (s, 1.03 H), 1.53 (s, 1.68 H), 1.47-1.43 (2 overlapping s, 3 H). ^{13}C NMR (101 MHz, C_6D_6) δ 147.26, 146.18, 137.72, 137.51, 134.88, 134.47, 124.70, 124.56, 124.37, 124.06, 106.05, 102.09, 58.98, 58.60, 55.08, 40.99, 39.77, 39.47, 39.45, 26.38, 26.28, 26.22, 22.72, 15.74, 15.66, 15.56. HRMS (EI+) m/z: (M+) found 224.1777, calc for $C_{14}H_{24}O_2$ 224.1776.

((2*E*,6*E*)-11-methoxy-3,7-dimethylundeca-2,6,10-trienyloxy)diphosphate (3)[1]



Step 1: A 250 mL flame dried round bottom flask was charged with the alcohol **9** (1.0 g, 4.5 mmol) and dry DMF (50 mL). LiCl (1.89 g, 45 mmol) was added, and the mixture was magnetically stirred at room temp under Ar as *sym*-collidine (5.0 mL, 38 mmol) was added. Stirring was continued until a solution formed. The reaction was then cooled with an ice bath until it became cloudy. Mesyl chloride (1.05 mL, 13.6 mmol) was then added dropwise, and the reaction was stirred for 90 min in the ice bath. The reaction mixture was partitioned between pentane and water. The pentane layer was washed four times with aqueous copper (II) sulfate, once with brine, and once with saturated aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate and concentrated *in vacuo* to give a colorless liquid.

Step 2: In a separate 250 mL round bottom flask under Ar was combined flame dried crushed 3 Å molecular sieves (8 g), tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (6.3 g) and 40 mL of dry acetonitrile. The combination of the components was exothermic, and an ice bath was used for several minutes to keep the reaction from overheating. The residue from step 1 was dissolved in 10 mL of dry acetontrile, and was added via syringe to the reaction. The reaction was stirred for 90 min at room temp. The reaction mixture was then filtered through a coarse sintered glass filter, followed by a second filtration through a medium frit sintered glass filter. Slight cloudiness remained in the filtrate. The filtrates were washed with pentanes, and then were concentrated *in vacuo*. The remaining thick caramel-colored residue was dissolved in about 10 mL of anion exchange buffer (2.0 g NH_4HCO_3 in water with 2% isopropanol), and was passed through a 4 x 15 cm Dowex 50W-X8 (100-200 mesh, ammonium form) column using more anion exchange buffer. The anion exchange buffer was passed through the column slowly with gravity, and the first 100 mL of eluent was collected and lyophilized overnight. The resulting solid was triturated with methanol (5 x 10 mL) to extract the product. The methanol extract was concentrated *in vacuo*, and the off-white solid product was subjected to azeotropic removal of methanol. The solid was treated with dichloromethane, followed by removal of solvents *in vacuo*. It was important to mix the solid and dichloromethane well with a spatula until a gel-like consistency was obtained. This azeotrope was conducted 3-4 times, followed by removal of remaining solvent under high vacuum (*ca*. 1 mmHg) for 3 h. The remaining solid was stored at -20 °C until use. Yield: 0.82 g (42%).

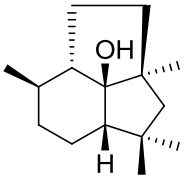
^{1}H NMR (400 MHz, CD_3OD) δ 6.03 (d, *J* = 12.4 Hz, 0.57 H, major isomer), 5.63 (app. d, *J* = 6.4 Hz, 0.24 H, minor isomer), 5.18 (t, *J* = 6.4 Hz, 1 H), 4.90-4.85 (m, 1 H), 4.48-4.42 (m, 1 H), 4.26 (t, *J* = 6.6 Hz, 2 H), 3.29 (s, 0.70 H, minor isomer), 3.20 (s, 1.9 H, major isomer), 1.90-1.84 (m, 3 H), 1.80-1.70 (m, 5 H), 1.44 (s, 3 H), 1.35 (s, 3 H). ^{13}C NMR (101 MHz, CD_3OD) δ 148.3, 140.8, 135.9, 125.4, 122.3, 107.0, 103.5, 63.8, 56.1, 42.2, 40.7, 27.50, 27.39, 23.4, 16.5, 16.1. ^{31}P NMR (161 MHz, CD_3OD)δ -9.39 (d, J = 19 Hz), -10.0 (d, J = 18 Hz). HRMS (ES-) m/z: (the alkyl diphosphate anion of **3** was observed as the doubly protonated form with a single negative charge) found 383.1025, calc for $C_{14}H_{25}O_8P_2$ 383.1007.

Overexpression and purification of BcBOT2 and SSCG_02150

The proteins were overexpressed in BL21(DE3). An overnight culture (10 mL) at 37 °C in TB containing the corresponding antibiotic was used to inoculate 1L of TB with the antibiotic. After 4 hours of growth at 37 °C and 250 rpm, the temperature was dropped to 25 °C and protein production was induced with addition of IPTG up to an end concentration of 100 μM. After 16 hours of incubation the cells were harvested by centrifugation at 5000 x g and 4 °C for 20 minutes. The cell pellets were resuspended in three volumes per cell pellet mass of 20 mM Tris pH 7.6, 300 mM NaCl, 5 mM DTT, 20 mM imidazole and frozen at -20°C overnight. Subsequent to thawing, the cells were lysed by sonication. The lysate was then cleared by centrifugation at 25000 x g for 30 minutes and filtering through a 0.2 μm filter. Since the enzymes were hexahistidine-tagged, the soluble fraction of the cell lysate was passed through a Ni-NTA column and washed with 20 mM Tris pH 7.6, 300 mM NaCl, 5 mM DTT, 20 mM

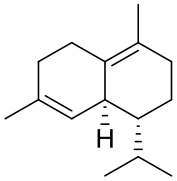
imidazole. The proteins were eluted from the column using a gradient running up to 20 mM Tris pH 7.6, 300 mM NaCl, 5 mM DTT, 500 mM imidazole. For BcBOT2, the fractions containing protein were pooled and the buffer was exchanged to 50mM PIPES, 10mM $MgCl_2$, 100mM NaCl, 2 mM DTT, pH 7.6. After addition of 25% glycerol (end concentration), the SSCG_02150 was aliquoted, frozen on dry ice and stored at -80 °C until further use.
The proteins were tested for production of their natural compounds in GC-scale reactions (see below) with FPP as the substrate. The identity of the product of the BcBOT2 reaction was confirmed by scaling up the reaction, isolating the product, and confirming its identity by NMR. The GC-scale reaction of SSCG_02150 with FPP in the presence of 10% betaine gave a single product as expected, and the product was not further characterized. The products are shown below.

BcBOT2



presilphiperfolan-8β-ol

SSCG_02150



(-)-δ-cadinene

Bioreactions for GC analysis

A 2 mL GC vial was placed on ice. Standard buffer was added first, followed by purified enzyme, and then by a 10 mM solution of either surrogate substrate **3** or FPP. The total volume of these three components totaled 500 μL, and the volumes changed depending on the experiment. A typical experiment to test the fidelity of the product mixture of the enzymes with FPP includes 10 μL of enzyme (100 μM stock solution, for a final concentration of 2 μM), 15 μL of FPP (10 mM stock solution in 25 mM NH_4HCO_3), and 475 μL standard buffer (or alternative buffer if noted). The reaction is overlayed with 500 μL of pentante, hexanes, or EtOAc containing 200 μM dodecane as an internal standard. The reaction is sealed with a septum cap, and incubated at the desired temp and time. The reactions are worked up by vortexing, separating the layers (by centrifugation when required), and filtering the organic layer through a plug of sodium sulfate. The filtrate is placed in a GC-vial containing a low-volume insert, and is then analyzed by GC.

Preparatory scale reaction of synthetic substrate 3

In a 250 mL glass bottle, 100 mL of standard buffer (50 mM PIPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, pH 7.5) was combined with 200 μL of purified BcBOT2 (100 μM stock solution, 0.2 μM final concentration) and 4 mL of substrate **3** (10 mM stock solution in 25 mM NH_4HCO_3, 0.4 mM final concentration, 0.04 mmol). The reaction mixture was overlayed with 100 mL of pentane, and the reaction was sealed and left at room temp without stirring. After 24 h, another portion of 200 μL of BcBOT2 was added, followed by another 100 μL portion the following day (both 100 μM stock solution). After a total of 72 h, the reaction was worked up by saturating the aqueous layer with NaCl, shaking vigorously, and separating the layers. The organic layer was dried over Na_2SO_4, filtered, and concentrated to a few mL *in vacuo* with the rotary evaporator bath filled with ice water. Final removal of the solvent was done under a stream of Ar in a 20 mL vial on ice to give the product as a colorless semi-solid. Yield: about 6 mg (~75% yield). Germacrenes and similar compounds are a mixture of several conformations in solution, making NMR spectra of these compounds very complex and rarely amenable to deconvolution.[3] The NMR spectra of **7** is similar to that of germacrenes in reference 4, and is shown below. Additionally, there is the expected peak in the aldehyde range and no remaining methoxy peaks. Furthermore, the identity of **7** is supported by its thermal rearrangement behavior below. HRMS (EI+) *m/z*: (M+) found 192.1564, calc for $C_{13}H_{20}O$ 192.1514.

Cope rearrangement of 7

The terpene germacrene A and similar structures are known to undergo Cope rearrangements upon exposure to heat.[3] These Cope rearrangements occur in the injection ports of GC instruments when these ports are heated to near or at 250 °C. By analogy, product **7** was expected to rearrange to a diasteromeric mixture of aldehydes in a hot injector port.

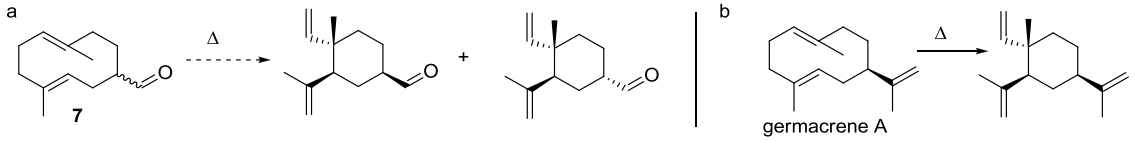


Figure S1. a) Proposed Cope rearrangement of **7**. b) Analogous known Cope rearrangement of germacrene A into elemene.

The ability of **7** to undergo a Cope rearrangement was explored to support the identity of the product. The injector temperature of the GC was set at 195, 210, and 250 °C for three successive injections of **7**. The GC runs were done on an HP-5 column with the initial temp held at 60 °C for 4 min, and then a temp ramp of 15 °C per min to 280 °C followed by a 3 min hold at this temp. With the injector at 195 °C, GC analysis of **7** showed predominantly one product with an elution time of about 11.2 min, with a minor amount of two products eluting at around 10 min (Figure S2). Upon raising the temperature to 210 °C, the initially predominant product increasingly rearranged to the two formerly minor peaks. Further heating to 250 °C resulted in almost complete rearrangement of the peak at 11.2 min to the two peaks at about 10 min. These data support the identity of the peak at 11.2 min as product **7**, which is then transformed by high temperatures into the two smaller peaks at around 10 min (corresponding to the two Cope product diastereomers indicated in Figure S1 a).

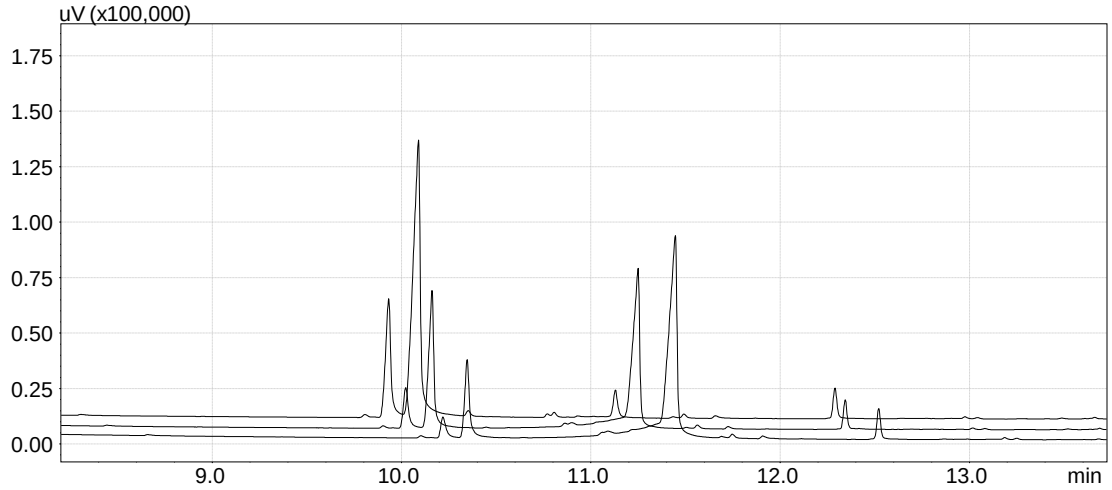


Figure S2. Thermal rearrangement of **7** in the GC injector port. Injector temp: 250 °C in back, 210 °C in the middle, 195 °C in front.

Assay validation with methanol standards

A dilution series was made by dissolving known concentrations of methanol (from 16 to 500 μM) in buffer (50 mM PIPES, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.5). The dilution series was put into a 96-well microtiter plate with three repeats per concentration, and 200 μL of total volume per well. Then 10 μL per well of a solution of dilute alcohol oxidase (AOX, 50 μL of AOX stock solution dissolved in 950 μL of the same buffer from above) was added. The microtiter plates were shaken at room temp for 5 min at 600 rpm on a table top shaker, and then left to react undisturbed for 5 min more. Purpald® solution (Aldrich, 351 mg dissolved in 15 mL of 2 N NaOH, 50 μL per well) was added and shaking was resumed for about 5 min, and then the plates were left undisturbed for 30 min.

The plates were briefly centrifuged at 5000 rpm to remove bubbles, and the absorbances of the wells were read with a plate reader at 550 nm.

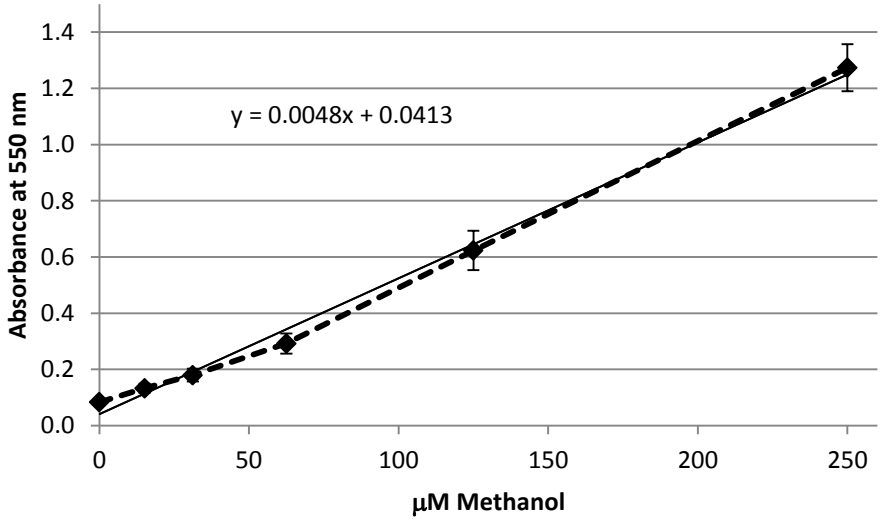


Figure S3. Screen response for methanol standards

Materials

Standard Buffer: 50 mM PIPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, pH 7.6

Lysis Buffer: Standard buffer, 0 .5 mg/mL Lysozyme, 0.02 mg/mL DNase I

Dilute AOX: AOX Stock solution from frozen stock (MP Biomedicals) dissolved in 950 μL of 100 mM KPi, pH 8.0.

Purpald Solution: 351mg solid Purpald® (Aldrich) in 15 ml 2N NaOH

EDTA: 0.5 M pH 8.0 aqueous (NaOH to adjust pH)

Surrogate 3 stock solution: 10 mM Surrogate **3** in 25 mM NH_4HCO_3. This stock solution is stable for at least several months if frozen at -20 °C, or at least several weeks if stored at 4 °C.

Screening for thermostability

Libraries of 96-well deep well plates stored at – 20 °C containing cell pellets were thawed for 20 – 30 min and then treated with 300 μL per well of lysis buffer (50 mM PIPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, pH 7.6, 0.5 mg/mL lysozyme, 0.02 mg/mL DNase I). The cell pellets were resuspended by vortexing, and the plates were incubated at 37 °C for 60 min. The cell debris was separated by centrifugation at 5000 x g at 4 °C for 15 min, and 100 μL from each well was transferred to 96-well PCR plates. The PCR plates were sealed with Microseal B film from Bio-rad and then placed in a thermocycler or water bath for 10 min at a temperature which was optimized to almost complete inactivation of BcBOT2. After this heat treatment, the PCR plates were immediately placed on ice. The seals were removed, and each well was treated with 100 μL of 0.5 mM surrogate substrate **3** in standard buffer (50 mM PIPES, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, pH 7.6). This solution was made immediately prior to use. The reactions were aged at room temp for 1-2 h, and then the contents of the PCR plates were transferred to 96-well microtiter plates. Then 10 μL per well of a solution of dilute alcohol oxidase (AOX, 50 μL of AOX stock solution from MP Biomedicals dissolved in cold 950 μL of 0.1 M potassium phosphate buffer pH 8.0) was added. The microtiter plates were shaken at room temp for 10 min at 600 rpm on a table top shaker. Purpald® solution (Aldrich, 351 mg dissolved in 15 mL of 2 N NaOH, 50 μL per well) was added and shaking was resumed for 30 min. The plates were briefly centrifuged at 3-5000 rpm to remove bubbles, and the absorbances of the wells were

read with a plate reader at 550 nm. Background absorbance was typically 0.2 or below, with active enzymes sometimes giving absorbances exceeding 1.

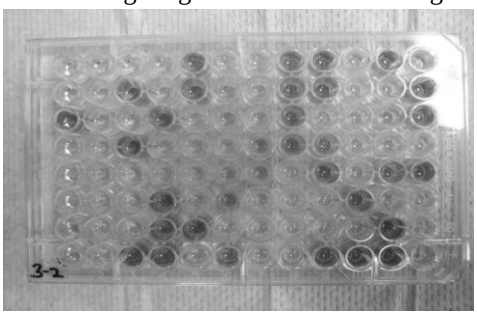


Figure S4. An example of a 96-well microtiter plate with wells containing active terpene synthases indicated by the blue-purple color of the product.

Growth of libraries

A library of BcBOT2 mutants was made by error-prone PCR using primers BcBOT2Fwd and BcBOT2RevV2. The template was the BcBOT2 gene in pET28a(+) vector as supplied by David E. Cane, and Taq (Roche) was used in the supplied buffer for reactions along with 200 μM $MnCl_2$ and 0.4 mM of each dNTP (dNTP mixture from Roche). These conditions gave a mutation rate of 3 base pair mutations per gene. The insert obtained from this epPCR was digested with DnpI (NEB) to remove remaining template, and the insert was double digested with XhoI and NdeI (both NEB). The vector pET28a(+) was double digested with XhoI and NdeI, and was dephosphorylated with calf intestinal phosphatase (NEB). Gel purification was used after each digestion, and a gel extraction kit (Promega) was used to isolate the DNA. Ligation of the insert and backbone was carried out with T4 ligase (NEB) at 16 °C overnight, and the DNA was isolated with a clean and concentrate kit (Zymo Research). Electro-competent BL21 (DE3) cells were transformed with 1 μL of the cleaned ligation mixture, and individual colonies were picked by hand using toothpicks into 96-well shallow well plates (1 mL volume) containing 300 μL per well of LB media containing 0.05 mg/mL of kanamycin. The colonies were grown overnight at 37 °C with shaking at 225 rpm and 80% rh. Then, 50 μL aliquots of culture were transferred to wells in 96-well deep well plates containing 800 mL per well of media (LB for the intitial library, TB for subsequent libraries) containing 0.05 mg/mL of kanamycin. At this time, cryostocks of the libraries were made in 96-well microtitre plates in 20% glycerol. In the deep well expression plates, the cultures were grown at 37 °C for 4 h, and then the temperature was reduced (to 18 °C for the initial library, 25 °C for subsequent libraries). At the same time the protein production was induced with 50 μL per well of IPTG in LB media for a final IPTG concentration of 0.5 mM. Shaking was continued for 24 h, and then the cells were pelleted by centrifugation at 3000 g for 10 min at 4 °C. The plates containing cell pellets were frozen at -20 °C overnight or longer until beginning the screening protocol.

T_{50} determinations

T_{50} data were obtained by filling a row in a 96-well PCR plate with 100 μL per well of enzyme at 4 μM in standard buffer. The PCR plate was sealed with Microseal B film, and a thermocycler was used to apply a temperature gradient for 10 min. The PCR plate was then immediately cooled on ice. Then, 100 μL of 0.5 mM surrogate substrate **3** in standard buffer was added, and the reactions were allowed to react at room temp or 30 °C for 1 h. The screening protocol using AOX and Purpald® was conducted as described. The T_{50} values were estimated as the temp at which the remaining active enzyme produced half the screen response, above background, as the maximum response of the non-denatured enzyme.

Product specificity analysis

The mutants were overexpressed and purified in the same manner as for BcBOT2, and the purified enzymes were used in bioreactions on a GC scale as described above. The product specificity of the mutants was compared to

BcBOT2 using FPP as substrate. The same product dominated, as seen in Figure S5, and only baseline side products were observed.

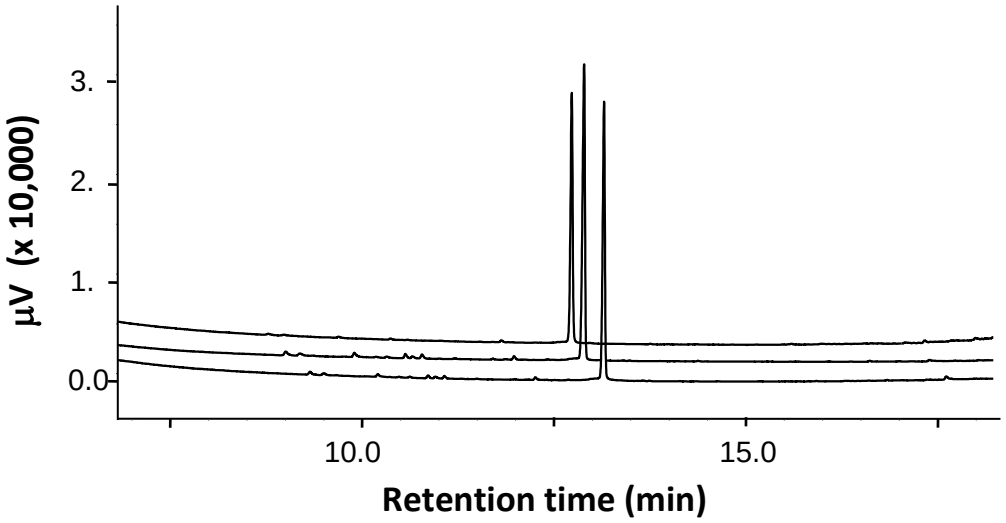


Figure S5. Gas chromatography traces of products from BcBOT2 and mutants. BcBOT2 in back, 19B7 middle, 9D6 in front.

Comparing the relative levels of protein expression

Equal amounts (as measured by OD_{600}) of *E. coli* BL21(DE3) expressing the protein of interest were mixed in 1:1 ratio with SDS-PAGE sample buffer containing 500 mM TRIS pH 7.5, 600 mM glycine, 20% (v/v) glycerine, 4% (w/v) SDS and 50 µl/ml β-mercaptoethanol and incubated at 95°C for 10 minutes. After separation of the proteins on an Any kD™ Mini-PROTEAN® TGX™ Precast Gel (Bio-rad), the protein bands were stained with Coomassie Blue and the gel was digitalized. The corresponding picture was processed with ImageJ.[4] The lanes on the gel were converted into graphs, where the peak area corresponding to the protein of interest was integrated. The amount of protein loaded was normalized to a prominent band around 43kDa, most likely corresponding to *E. coli* EF-Tu.

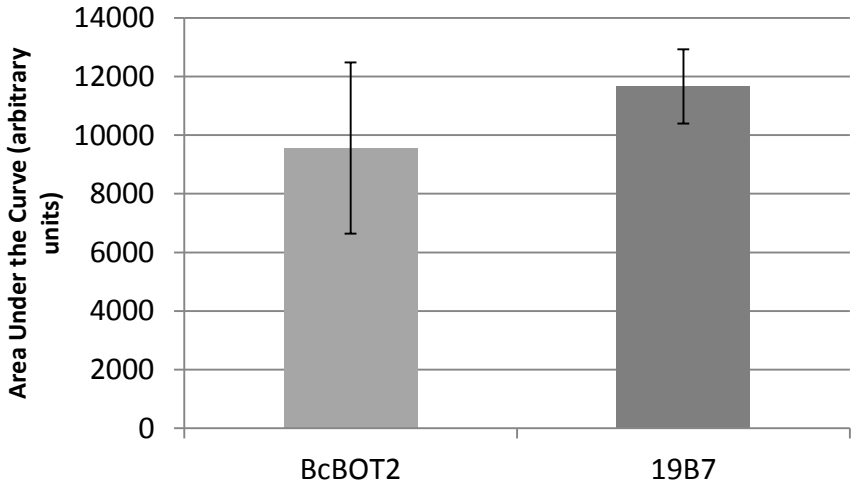


Figure S6. Expression of BcBOT2 and 19B7 as determined by imaging of an SDS-Page gel. The amounts of produced protein are statistically indistinguishable. This analysis was not carried out for 9D6, but the amounts obtained from 1 L expression cultures after purification were equal to or better than BcBOT2 on all of the several occassions when 9D6 was expressed.

Correlation of screen response with product formation for 19B7

In 2 mL GC vials on ice were combined standard buffer (489 μL), FPP (10 μL of a 10 mM stock solution for a final concentration of 200 μM), 19B7 (0.5 μL of a 100 μM stock solution in standard buffer, freshly thawed on ice, final concentration of 100 nM), and an overlayer of ethyl acetate (500 μL with 200 μM dodecane as an internal standard). The vials were then placed in a water bath at 29.5°C. At each time point, a vial was taken out and vortexed. The vial was placed on ice and 250 μL of the overlayer was filtered through a plug of sodium sulfate into a GC vial with an insert, and the sample was analysed by GC using a CyclosilB column. The temperature program began with a four minute hold at 75°C, followed by a ramp of 8.0°C per min to 250°C and a final hold at 250°C for 3 min. The product was quantified by corrected areas under the curve comparison to authentic product standards. A total of five analyses were done for each time point.

In a 20 mL scintillation vial in an ice bath was combined standard buffer (3.9 mL), 80 μL of **3** (10 mM of stock, for a final concentration of 200 μM), and 4 μL of 19B7 (100 μM stock solution in standard buffer, freshly thawed on ice, final concentration 100 nM). Immediately, the vial was placed in a water bath at 29.5°C. At each time point, 3 aliquots of 185 μL were removed and placed into individual wells of a microtiter plate and were mixed with 15 μL of 0.50 M EDTA (pH 8.0). After all of the time points were sampled, the AOX-Purpald® treatment was done as described for the assay. The responses were converted to methanol concentrations, after subtracting out background, by normalizing to the data in the section 'assay validation with methanol standards.'

Optimizing conditions for 02150 expression, lysis, and reactions

Single colonies of *E. coli* BL21 containing 02150 in pET-28a(+) were picked into a 96 well shallow well plate containing 300 μL of LB kan per well. The plate contained several blank wells. The plate was fitted with a plastic lid shaken overnight at 37 °C at 225 rpm and 80% rh. This plate was then used to inoculate 4 deep well 96-well plates containing 800 μL of LB kan in the top half of the plate and TB kan in the bottom. Innoculation was done with 40 μL per well of the starter culture. The plates were grown for 4 h at 37 °C at 225 rpm shaking and 80% rh. Then, two of the plates were cooled to 25 °C and the other two were cooled to 18 °C. All plates were induced with 50 μL of 9.0 mM IPTG in LB kan, and shaking was continued for 24 h at either 18 or 25 °C at 225 rpm without humidity control. The plates were then centrifuged at 3000 xg for 10 min at 4 °C, and the media was discarded. The plates containing the cell pellets were stored overnight or longer at – 20 °C.

The cell pellets in deep well 96-well plates obtained above were thawed at RT for about 20 min, and the cell pellets were then resuspended in 300 μL of lysis buffer containing either no additive, or 10% w/v of either sucrose or betaine. The suspensions were incubated for 1 h at 37 °C, and the cell debris was then pelleted at 5000 g for 15 min at 4 °C. From each well of the plates, 100 μL of lysate was transferred to the corresponding wells in microtiter plates. To these wells was added 100 μL of standard buffers with the same additives, if any, as in the lysis step. Additionally, these buffers contained 0.50 mM of **3**. The plates were sealed with Microseal B film and incubated for 37 °C for about 70 min. The screening protocol of AOX and Purpald® was carried out as described in the experimental section. Results are displayed in Table 1.

Confirmation of 02150 optimization by GC reactions

A 96-well plate of *E. coli* BL21 containing 02150 in pET-28a(+) was picked from individual colonies and expressed at 25 °C as in the preceding section. The frozen cell pellets were thawed, and were resuspended in 300 μL of lysis buffer containing either 10% w/v betaine or no betaine. The plate was incubated at 37 °C for 1 h, and the cell debris was then pelleted by centrifugation at 5000 xg at 4 °C for 15 min. Aliquots of 100 μL of lysate were transferred to wells in a 96-well microtitre plate. Then 100 μL per well of standard buffer containing 0.50 mmol of either FPP or **3** was added. The microtiter plate was sealed with Microseal B film and was incubated at 37 °C for 2 h. Then, the

contents of 3 wells per each condition were combined into a 1.7 mL centrifuge tube and extracted with 500 μL of hexanes. After vortexing briefly, the hexane layer separated and 250 μL of this layer was filtered through a plug of sodium sulfate into a GC vial containing a low-volume insert. GC analysis was done on the HP-5 column with the initial temp held at 75 °C for 6 min, then a temp ramp of 9.0 °C per min to 174 °C, then a second temp ramp of 35.0 °C per min to 280 °C followed by a 3 min hold at this temp. Each condition is represented by an average of three samples, and the relative product amount is shown in Figure S7 as uncorrected areas-under-the-curve.

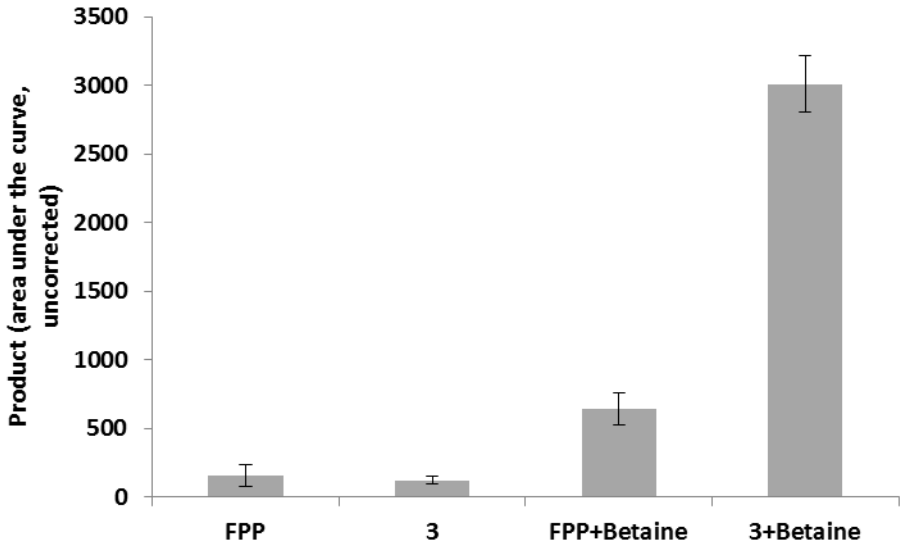


Figure S7. Relative product formation of 02150 with FPP and **3** in the presence and absence of 10% w/v betaine.

Primers

BcBOT2Fwd: GCAGCCATCATCATCATCATCACAGCAGCG

BcBOT2RevV2: GTGGTGGTGCTCGAGTTATGCAACTACAG

Nucleotide sequences

BcBOT2

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCAAT
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19B7
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Protein sequences
Mutated positions with respect to BcBOT2 are in underlined.

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VVA

19B7
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References

[1] V. J. Davisson, A. B. Woodside, T. R. Neal, K. E. Stremler, M. Muehlbacher, C. D. Poulter, *J. Org. Chem.* **2006**, *5*, 4768-4779.

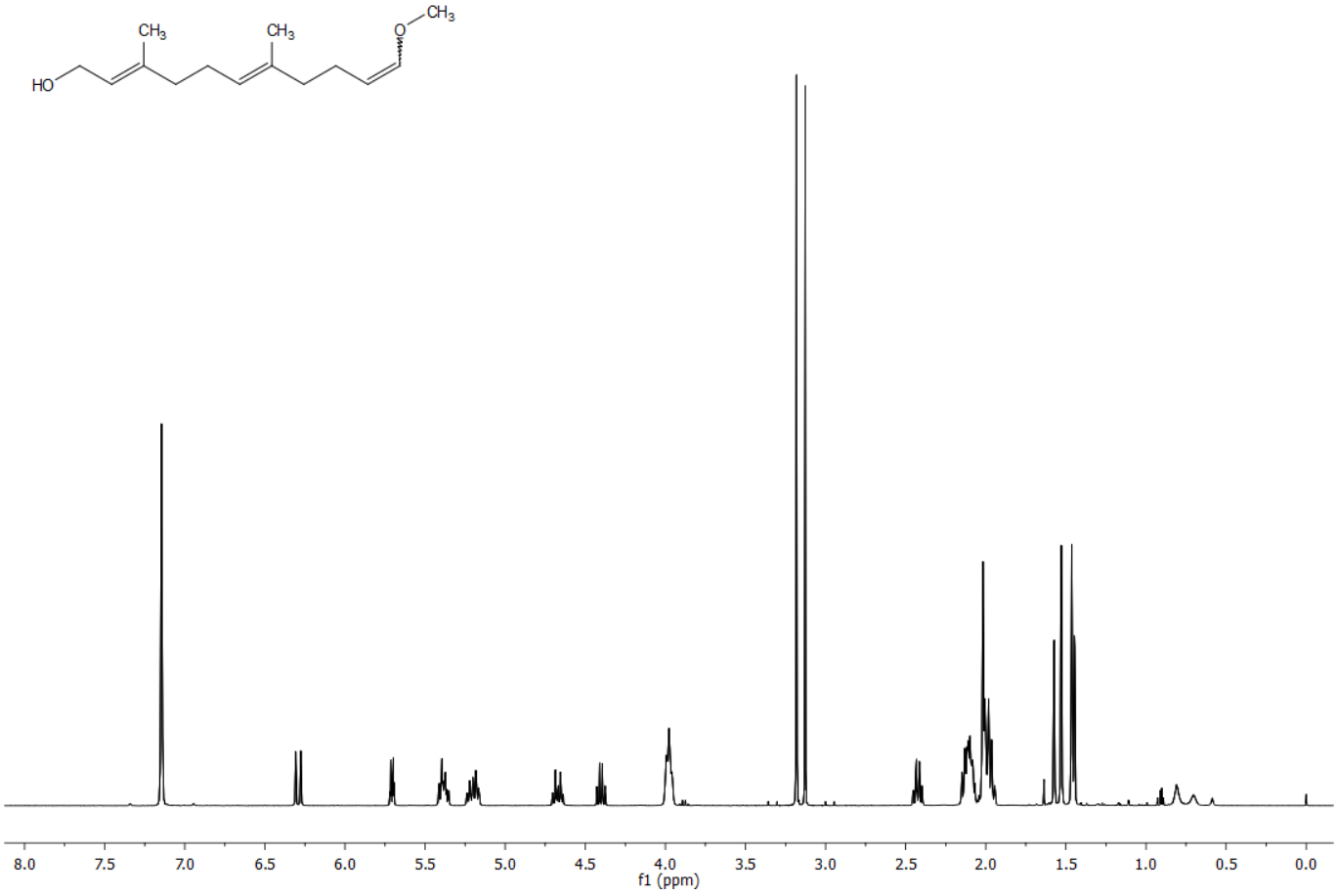
[2] a) R. P. Hanzlik, *Org. Syntheses*. **1988**, *Coll. Vol. 6*, p. 560; **1977**, *Vol. 56*, p. 112. b) Labadie, G. R.; Viswanathan, R.; Poulter, C. D. *J. Org. Chem*. **2007**, *72*, 9291-9297.

[3] a) J.-W. de Kraker, C. R. Franssen, A. de Groot, W. A. Konig, H. Bouwneester, *Plant Physiol.* **1998**, *117*, 1381-1392. b) J. A. Faraldos, S. Wu, J. Chappell, R. M. Coates, *Tetrahedron* **2007**, *63*, 7733-7742. c) O. Cascon, S. Touchet, D. J. Miller, V. Gonzalez, J. A. Faraldos, R. K. Allemann, *Chem.Commun*. **2012**, *48*, 9702-9704. d) J. A. Faraldos, Y. Zhao, P. E. O'Maille, J. P. Noel, R. M. Coates, *ChemBioChem* **2007**, *8*, 1826-1833. e) D. J. Miller, F. Yu, D. W. Knight, R. K. Allemann, *Org. Biomol. Chem*. **2009**, *7*, 962-975. f) D. J. Miller, F. Yu, R. K. Allemann, *ChemBioChem* **2007**, *8*, 1819-1825. g) W. N. Setzer, *J. Mol. Model*. **2008**, *14*, 335-342.

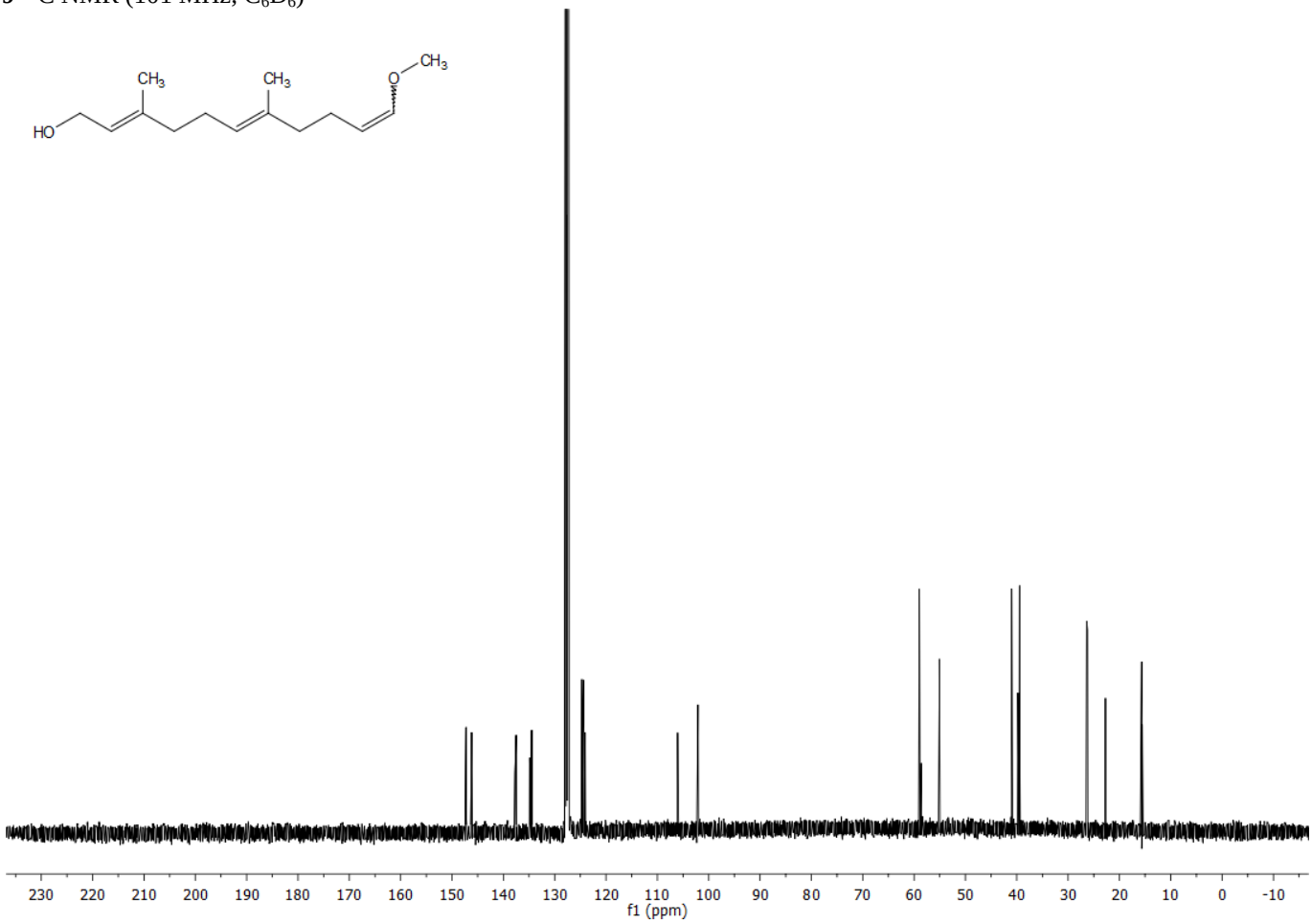
[4] Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, **1997-2008**.

NMR Spectra

9 ^{1}H NMR (400 MHz, C_6D_6)



9 ^{13}C NMR (101 MHz, C_6D_6)



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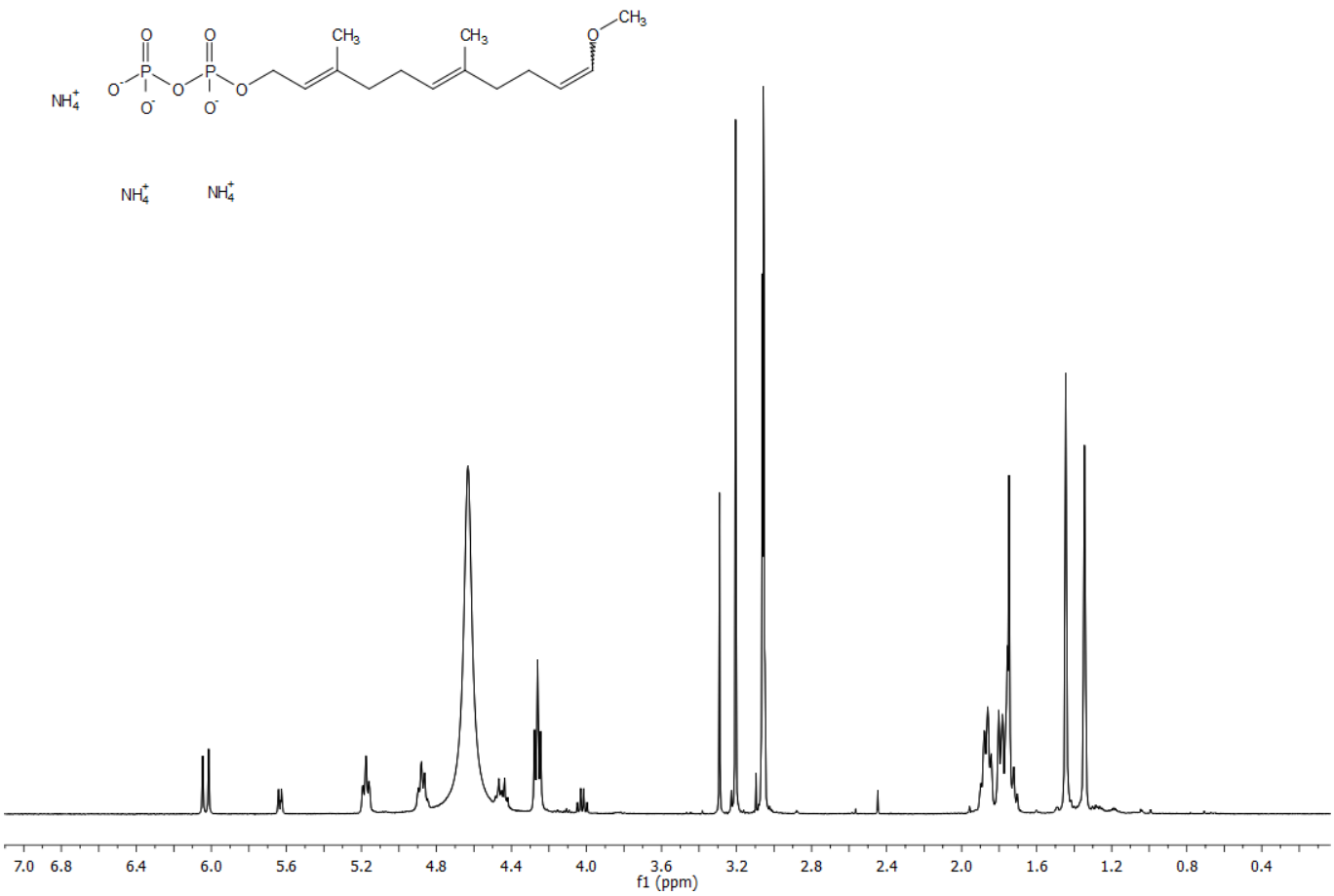

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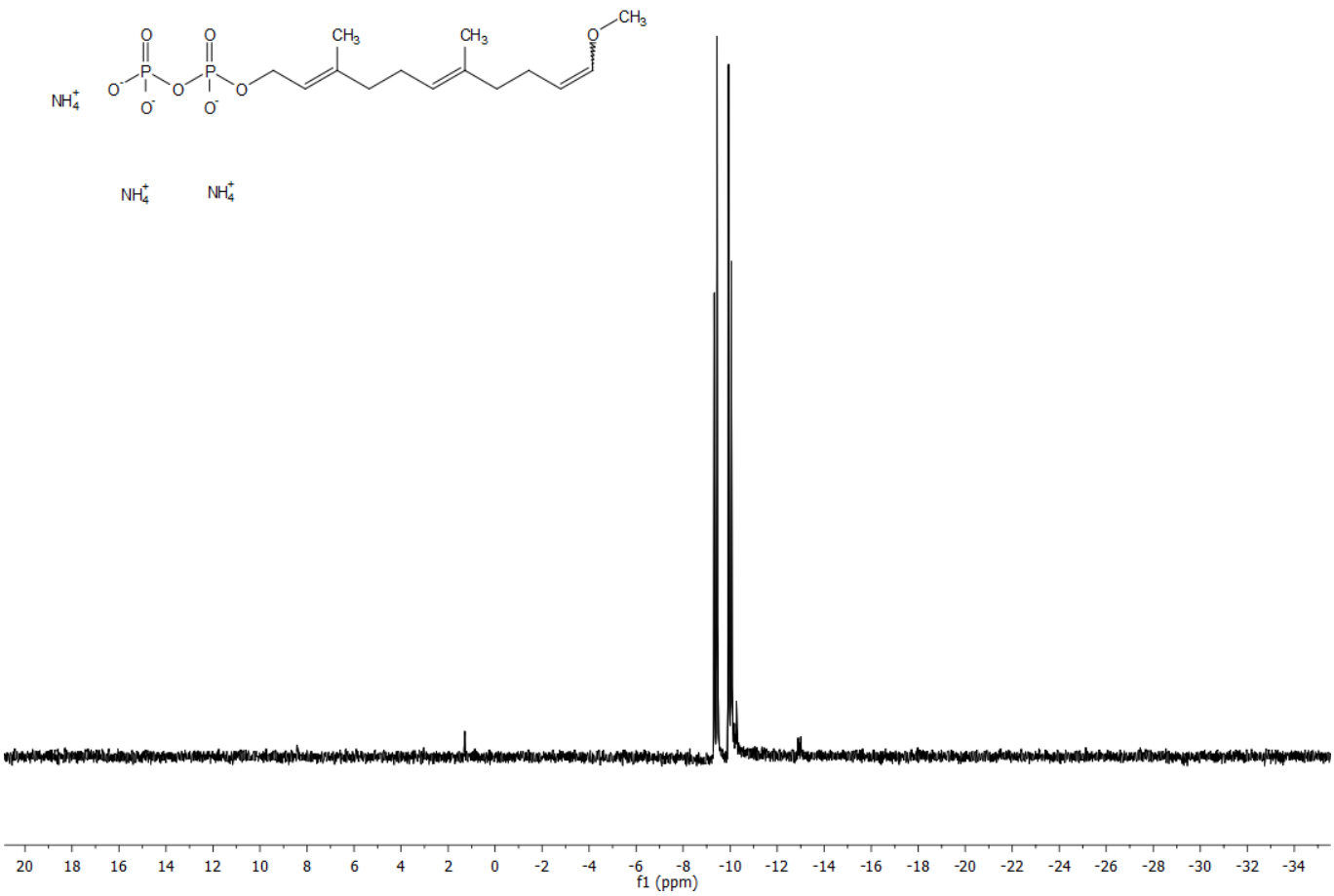

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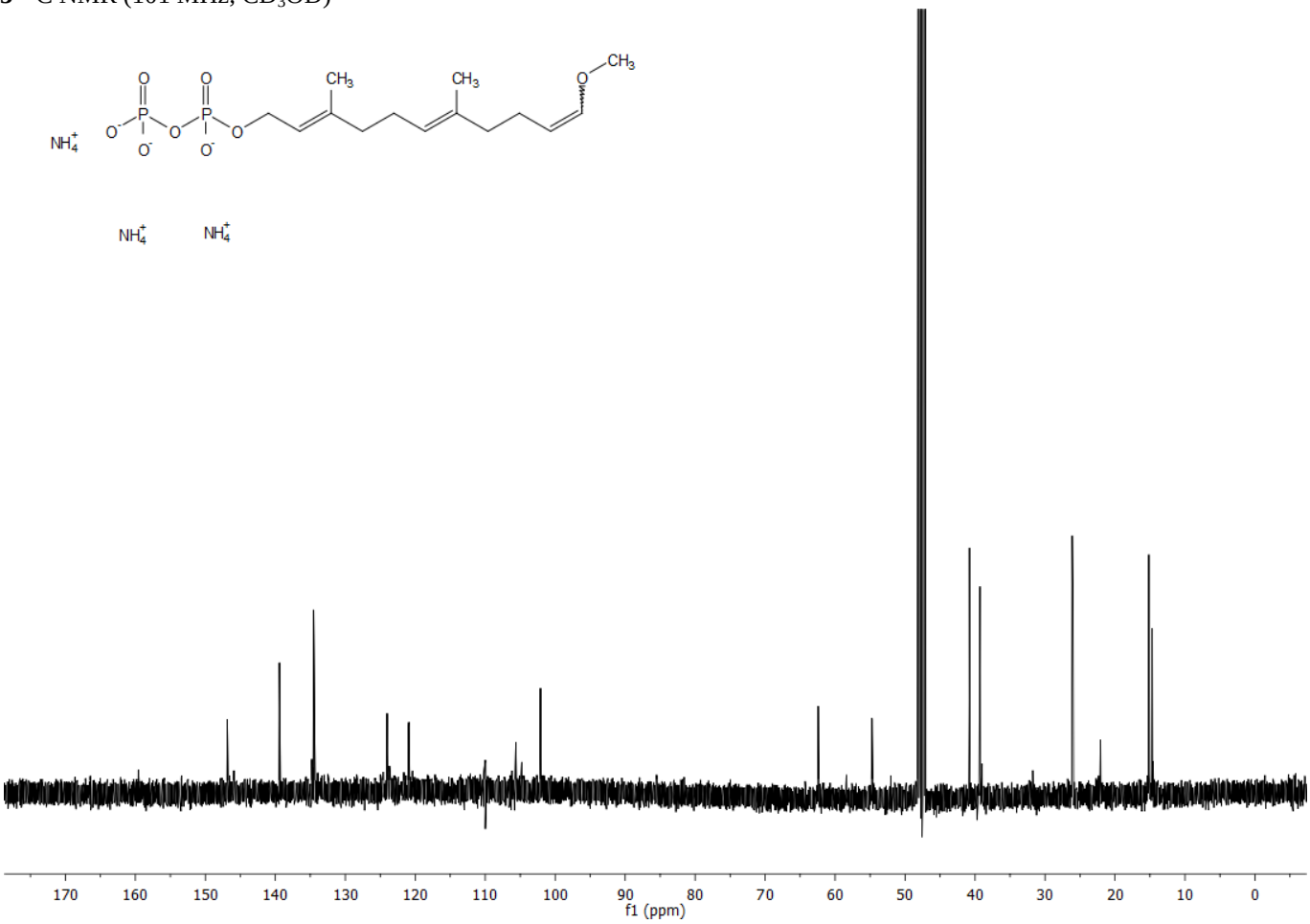

7 ^{1}H NMR (500 MHz, toluene-d_6)