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Novel olfactory ligands via terpene synthases

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Supporting Information

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1. General Information

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. All were of analytical quality or better and used as received unless otherwise stated.

^{1}H, ^{31}P and ^{13}C NMR-spectra were measured on a Bruker Avance III 600 NMR spectrometer, a Bruker Avance 500 NMR spectrometer or a Bruker Avance DPX400 NMR spectrometer and are reported as chemical shifts in parts per million downfield from tetramethylsilane, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (to the nearest 0.5 Hz) and assignment, respectively. Assignments are made to the limitations of COSY, DEPT 90/135, gradient HSQC and gradient HMBC spectra. $CDCl_3$ was filtered through basic alumina prior to use in NMR spectroscopy. EI^+ mass spectra were measured on a Micromass LCT premiere XE mass spectrometer. GCMS was performed on a Hewlett Packard 6890 GC fitted with a J&W Scientific DB-5MS column (30 m x 0.25 mm internal diameter) and a Micromass GCT Premiere detecting in the range *m/z* 50-800 in EI^+ mode. scanning once a second with a scan time of 0.9 s. Injections were performed in split mode (split ratio 5:1) at 50 °C. Chromatograms were begun with an oven temperature of 50 °C (unless otherwise stated) rising at 4 °C min^{-1} for 25 min (up to 150 °C) and then at 20 °C min^{-1} for 5 min (250 °C final temperature).

2. Protein preparation and purification

Recombinant germacrene D synthase and mutants were overproduced in *E. coli* (DE3)Star as C-terminal His-tagged fusion proteins and purified by Ni^{2+}-affinity chromatography as previously described.[1]

Site directed mutagenesis of recombinant GDS

The Quickchange site-directed mutagenesis kit (Stratagene) was used to introduce the desired mutations according to the manufacturer's instructions. The primers used for mutagenesis were as follows:

5' CTGGTAGAGCTGTACTTTGCGGTACTGGGCGTTTATTTC 3' and

5' GAAATAAACGCCCAGTACCGCAAAGTACAGCTCTACCAG 3' for W275A

5' CTGGTAGAGCTGTACTTTCTGGTACTGGGCGTTTATTTC 3' and

5' GAAATAAACGCCCAGTACCAGAAAGTACAGCTCTACCAG 3' for W275L

5' GGTAGAGCTGTACTTTTTCGTACTGGGCGTTTATTTC 3' and

5' GAAATAAACGCCCAGTACGAAAAAGTACAGCTCTACC 3' for W275F

5' CGTGATCGACATGCTGGCGAAGAATGACGACAACC 3' and

5' GGTTGTCGTCATTCTTCGCCAGCATGTCGATCACG 3' for Y524A

5' GCGTGATCGACATGCTGCTGAAGAATGACGACAACC 3' and

5' GGTTGTCGTCATTCTTCAGCAGCATGTCGATCACGC 3' for Y524L

5' GTGATCGACATGCTGTTCAAGAATGACGACAAC 3' and

5' GTTGTCGTCATTCTTGAACAGCATGTCGATCAC 3' for Y524F

5' GAATCTGACGGGTGGCAGCAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTGCTGCCACCCGTCAGATTC 3' for Y406S

GAATCTGACGGGTGGCGGCAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTGCCGCCACCCGTCAGATTC 3' for Y406G

5' GAATCTGACGGGTGGCGCGAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTCGCGCCACCCGTCAGATTC 3' for Y406A

5' GAATCTGACGGGTGGCGTGAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTCACGCCACCCGTCAGATTC 3'for Y406V

5' GAATCTGACGGGTGGCATTAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTAATGCCACCCGTCAGATTC 3' for Y406I

5' GAATCTGACGGGTGGCCTGAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTCAGGCCACCCGTCAGATTC 3' for Y406L

5' GAATCTGACGGGTGGCTTTAAAATGCTGACGACG 3' and

5' CGTCGTCAGCATTTTAAAGCCACCCGTCAGATTC 3' for Y406F

5' CTGACGGGTGGCTGGAAAATGCTGACGAC 3' and

5' GTCGTCAGCATTTTCCAGCCACCCGTCAG 3' for Y406W

Plasmids were purified from overnight cultures (10 mL LB medium containing ampicillin 50 μmol/mL) using the QIAGEN miniprep kit as described by the manufacturer. Mutations were

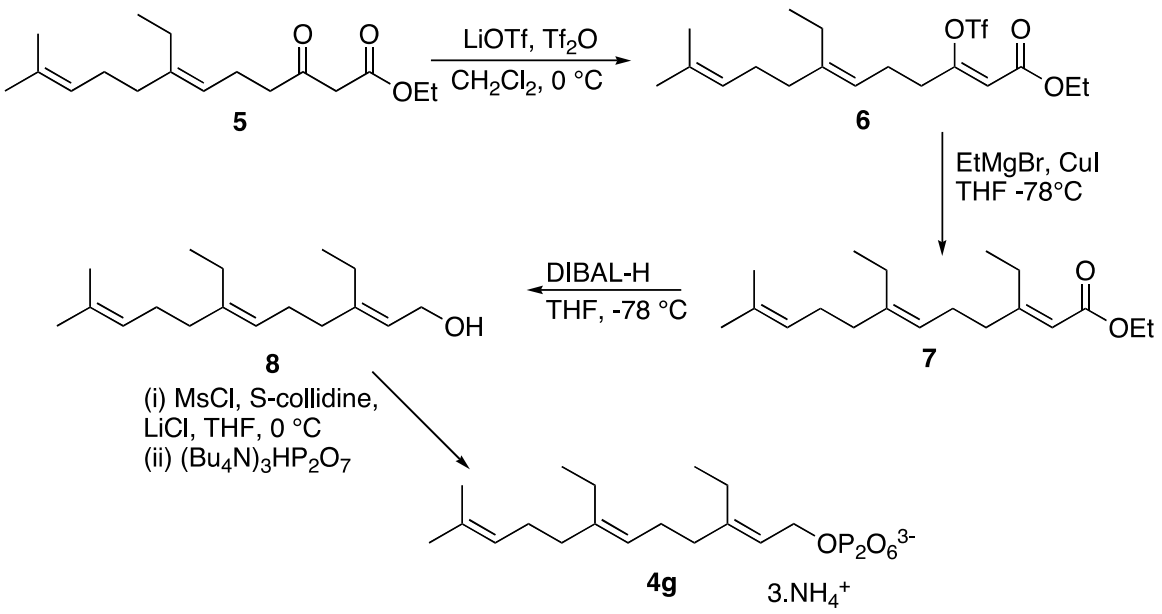
confirmed by DNA sequence analysis using the internal Walesbiogrid facilities (School of Bioscience, Cardiff University, UK).

3. Preparation of FDP analogues

Farnesyl diphosphate,[2] 12-methylfarnesyl diphosphate,[3] 14-methylfarnesyl diphosphate,[1] 14-fluorofarnesyl diphosphate,[4] 15-fluorofarnesyl diphosphate,[5] 15-methylfarnesyl diphosphate[1] and 6-fluorofarnesyl diphosphate[4] were prepared accordin to literature methods.

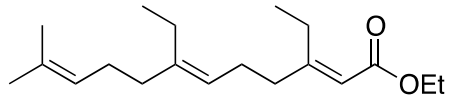
14,15-Dimethylfarnesyl diphoshate (4g)

The title compound was prepared from β-ketoester **5** according to reaction Scheme S1.[3]



Scheme S1

(2*E*,6*E*) Ethyl 3,7-diethyl-11-methyldodeca-2,6,10-trienoate (7)

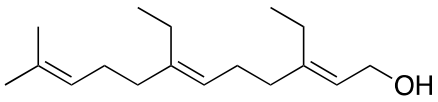


To a stirred solution of **5**[3] (0.35 g, 1.50 mmol) and lithium trifluoromethanesulfonate (0.78 g, 5.0 mmol) in dry CH_2Cl_2 (38 mL) under argon at 0 °C was added triethylamine (0.7 mL, 5.0 mmol) followed by trifluoromethanesulfonic anhydride (0.32 mL, 1.90 mmol). The mixture was stirred at 0 °C for 2 h before quenching with the addition of saturated NH_4Cl solution (20 mL). This mixture was diluted with CH_2Cl_2 (20 mL) and the separated aqueous phase was extracted with CH_2Cl_2 (2 x 10 mL). The pooled organic extracts were washed with water (30 mL) and brine (30 mL) before drying ($MgSO_4$), filtration and concentration under reduced pressure. This gave the enol triflate **6** as dark oil that was used directly without further purification (0.58 g, 83%).

To a stirred suspension of CuI (0.95 g, 5.00 mmol) in THF (12 mL) at 0 °C was added drop-wise, ethylmagnesium bromide (3.0 M solution in diethyl ether, 3.33 mL, 10.0 mmol). The solution was stirred for 30 minutes, whereupon an opaque black colour formed. The stirred reaction mixture was then cooled to -78 °C and a solution of **6** (0.58 g, 1.25 mmol) in anhydrous THF (4 mL) was added via a needle and the reaction was stirred at this temperature for 2.5 h before quenching by addition of saturated aqueous NH_4Cl solution (20 mL). Resulting emulsions were dissolved by addition of concentrated aqueous NH_4OH solution and stirring overnight. The separated aqueous layer was extracted with ethyl acetate (3 x 10 mL) and the combined organic extracts were washed with water (2 x 30 mL) and brine (30 mL) before drying ($MgSO_4$), filtration and concentration under reduced pressure. The residual oil was purified by flash chromatography on silica gel (19:1 hexane;ethyl acetate). The title compound was isolated as colourless oil (185 mg, 52%).

HRMS (*m/z* ES^+): calcd. for $C_{19}H_{32}O_2$ 292.2402; found 292.2407; δ_H (400 MHz, $CDCl_3$) 0.95 (3 H, t, *J* = 7.5 Hz, C=CHCH$_2$C*H*$_3$), 1.07 (3 H, t, *J* = 7.5 C=CHCH$_2$C*H*$_3$), 1.27 (3 H, t, *J* = 7.0 Hz, OCH$_2$C*H*$_3$), 1.59 (3 H, s, C*H*$_3$C=CH), 1.67 (3 H, s, C*H*$_3$C=CH), 1.98-2.05 and 2.03-2.04 (12 H, m, 2 x CH$_2$CH$_2$ and 2 x CH=CC*H*$_2$CH$_3$), 4.25 (2 H, q, *J* = 7.0 Hz, OC*H*$_2$CH$_3$), 5.01-5.20 (2 H, m, 2 x C=C*H*), 5.72 (1 H , s, C=C*H*CO$_2$Et); δ_C (62.5 MHz, $CDCl_3$) 12.97, 13.18, 14.29, 17.68 and 23.15 (*C*H$_3$), 25.34, 25.67, 25.78, 26.84, 36.43, 38,27 and 59.13 (CH$_2$), 114.79, 122.51 and 124.31 (3 x C=*C*H), 131.33 141.91 and 165.53 (3 x *C*=CH), 166.45 (C=O).

(2*E*,6*E*) 3,7-Diethyl-11-methyldodeca-2,6,10-trienol (8)

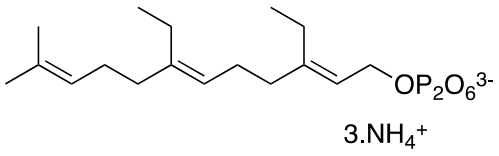


To a stirred suspension of **7** (0.18 mg, 0.60 mmol) in toluene (3.1 mL) at -78 °C was added DIBAL-H (1.5 M in toluene, 1.30 mL, 1.80 mmol), the solution was stirred at this temperature for 2 h. The reaction was quenched by addition of 2 M HCl (10 mL), diluted with CH_2Cl_2 (10 mL) and stirred for 1 h at room temperature. The aqueous layer was extracted with CH_2Cl_2 (2 x 10 mL) and the pooled organic layers were washed with aqueous saturated $NaHCO_3$ solution (3 x 10 mL), brine (2 x 15 mL), dried over $MgSO_4$ and then concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (eluting with 3:1 hexane-ethyl acetate) to give the title compound as a colourless oil (0.16 mg, 73% yield).

HRMS (*m/z* APCI [M+H^+-H_2O]) calcd. for $C_{17}H_{29}$ 233.2269; found 233.2275,; δ_H (400 MHz, $CDCl_3$) 0.94-1.01 (6 H, m, 2 x CH$_2$C*H*$_3$), 1.60 (3 H, s, CH=CC*H*$_3$), 1.68 (3 H, s, CH=CC*H*$_3$)

1.97-2.13 (12 H, m, 6 x CH_2), 4.16 (2 H, d, *J* = 7.0 Hz, CH_2O), 5.09 (2 H, m, 2 x C=CH), 5.38 (1 H, t, *J* = 7.0 Hz, C=C*H*CH_2O). δ_C (62.5 MHz, $CDCl_3$) 13.20, 13.65, 17.68 and 23.21 (CH_3), 23.54, 25.65, 26.22, 26.96, 36.51 and 36.75 (CH_2), 59.09 (CH_2OH), 122.89, 123.43 and 124.46 (C=*C*H), 131.26, 141.25 and 145.69 (*C*=CH).

Trisammonium (2*E*,6*E*)-3,7-diethyl-11-methyldodeca-2,6,10-trienyl diphosphate (4g)



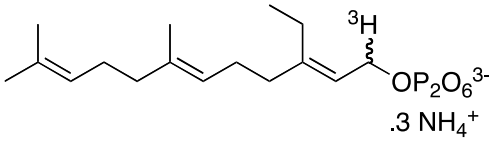
A stirred suspension of LiCl (0.27 g, 6.4 mmol) in anhydrous DMF (5.3 mL) was cooled to 0 °C (ice bath) and then S-collidine (0.3 mL, 2.4 mmol) and methanesulfonyl chloride (50 µL, 0.64 mmol) were added. The solution was stirred for 15 min during which time a white cloudy precipitate formed. Alcohol **8** (100 mg, 0.40 mmol) was added drop-wise as a solution in anhydrous DMF (1 mL) and the reaction was stirred to 0 °C for 3 h. The mixture was diluted with cold pentane (4 mL) then poured onto ice (25 g) and the resulting aqueous layer was extracted with pentane (3 x 10 mL). The pooled organic layers were washed with saturated $CuSO_4$ solution (3 x 10 mL), saturated $NaHSO_4$ solution (2 x 10 mL) and brine (2 x 10 mL) before drying ($MgSO_4$) and filtration. The solution was concentrated under reduced pressure and the resulting crude allylic chloride was used directly without further purification.

To a solution of the crude allylic chloride in anhydrous CH_3CN (1 mL) was added tris-(tetrabutylammonium) hydrogendiphosphate (0.7g, 1.8 mmol) and the mixture was stirred at room temperature for 15 h. The solvent was removed under reduced pressure and the residue

was dissolved in ion-exchange buffer (25 mM NH_4HCO_3 containing 2% *i*-PrOH, 1 mL). This solution was slowly passed through a column containing 30 equiv. of DOWEX 50W-X8 (100-200 mesh) cation exchange resin (NH_4^+ form) that had been pre-equilibrated with two column volumes of ion-exchange buffer. The column was eluted with two column volumes of ion-exchange buffer at a flow rate of one column volume per 15 min. Once ion exchange was complete, fractions containing product (as judged by TLC in 6:3:1 *i*-PrOH:c.NH_3:H_2O, staining with Hanessian's stain) was lyophilized to dryness. The white solid was triturated with MeOH (3 x 10 mL) and the organic extracts were concentrated to dryness affording a yellow solid that was cleaned with Et_2O (3 x 3 mL) to give the title compound as a white solid (64mg, 36%). The residue from the trituration was further purified by reverse-phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient to 100% B over 5 min.; solvent A: 25 mM NH_4HCO_3 in water, solvent B: CH_3CN, flow rate 5.0 mL/min, detecting at 220 nm, retention time 39.3 min).[6] Once purification was complete the solution was again lyophilized to dryness giving a further batch of the title compound as a fluffy white solid (34 mg, 18.9% yield).

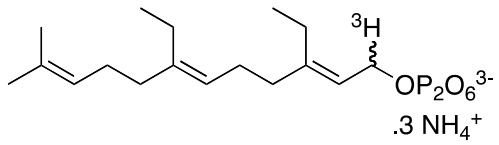
HRMS (*m/z* ES^-) calcd. for $C_{17}H_{31}O_7P_2$ 409.2545; found 409.2539; δ_H (400 MHz, D_2O) 0.8-0.91 (6 H, m, 2 x CH_2CH_3), 1.50 (3 H, s, C=CCH_3), 1.56 (3H, s, C=CCH_3) 1.92-2.03 (12 H, m, 6 x CH_2), 4.37 (2H, m, CH_2O), 5.07 (2 H, t, J = 6.0 Hz, 2 x C=C*H*), 5.32 (1H, t, , J = 6.5 Hz, C=C*H*CH_2O); δ_P (202.5 MHz, ^2H_2O) -10.41 (d, J_{PP} = 22.5 Hz), -8.30 (d, J_{PP} = 22.5 Hz).

Trisammonium [1-^{3}H]-(2*E*,6*E*)-3-ethyl-7,11-dimethyldodeca-2,6,10-trienyl diphosphate (4f)



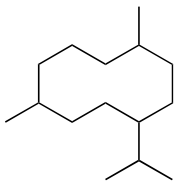
To a stirred solution of (2*E*,6*E*)-3-ethyl-7,11-dimethyldodeca-2,6,10-trienol[1] (40 mg, 0.17 mmol) in hexane (10 mL) was added activated MnO_2 and the suspension was stirred for 1 hr. The mixture was directly applied to a column of flash silica, eluting with 4:1 hexane-ethyl acetate. Fractions containing aldehyde were pooled and concentrated under reduced pressure to give 31 mg (78%) of the desired compound. This was directly dissolved in MeOH (0.5 mL) containing NaB^3H_4 (25 mCi, 15 Ci/mmol). The resulting solution was stirred under N_2 at 0 °C for 4 h then $NaBH_4$ (5 mg, 0.14 mmol) was added followed by stirring at 0 °C for a further 2 h. Brine (1 mL) was added and the mixture was extracted with pentane (3 x 1 mL). The pooled organic layers were filtered through a short pad of anhydrous $MgSO_4$ to yield 15-methyl farnesol (31 mg, 1.35 mCi). The resulting tritiated alcohol was transformed into the trisammonium diphosphate as described for **4g**, yielding the title compound as a white solid (specific activity 3.92 mCi/mmol). Analytical data was identical to the non-labelled compound.[1]

Trisammonium [1-^{3}H]-(2*E*,6*E*)-3,7-diethyl-11-methyldodeca-2,6,10-trienyl diphosphate (4g)



The title compound was prepared in an identical procedure to that described above using alcohol **8** (40 mg, 0.17 mmol). Tritiated **4g** was isolated with a specific activity of 8.47 mCi/mmol. Analytical data were identical to the unlabelled material **4g**.

Germacrane mixture (3)

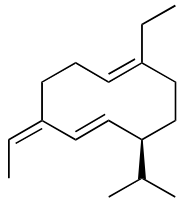


The pooled organic extracts from three preparative scale incubations of FDP with GDS[1] were concentrated to dryness by removal of the solvent under a gentle stream of nitrogen. The residue was dissolved in ethanol (2 mL) and PtO_2 (5-10% on C, 0.5 mg) was added. This mixture was stirred under an atmosphere of hydrogen gas for 16 h then the mixture was filtered through a small silica column to remove the catalyst. GC/MS analysis showed the product to be a mixture of all possible diastereoisomers of the germacrane skeleton with a trace impurity of cadinane, 0.7 mg. HRMS (*m/z*, EI^+) calcd. for $C_{15}H_{28}$ 208.2191; found 208.2185; *m/z* (EI^+) 208.2 (M^+, 2%), 167.2 (50), 151.2 (9), 138.1 (3), 125.1 (21), 111.1 (88), 91.1 (100), 83.1 (63), 69.1 (80), 55.1 (63).

4. Enzyme Incubations

Incubations of FDP analogues with GDS-His_6 and Y406F GDS-His_6 were optimised to generate maximum conversions as previously described.[1,3]

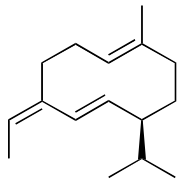
(*S*)-14,15-Dimethylgermacrene D (1g)



For production of (*S*)-14,15-dimethylgermacrene D 14,15-dimethyl-FDP (**4g**) (19 mg, 0.40 mM final concentration) and Y406F-GDS-His_6 (12 μM final concentration) were mixed in incubation buffer (20 mM Tris, 5 mM βME, and 10 mM $MgCl_2$, pH 7.5, 10% glycerol for GDS, 200 mL final volume) overlaid with pentane (10 mL). The mixture was gently agitated for 5 days at room temperature and then the separated aqueous layer was thoroughly extracted with further portions of pentane until no product could be detected by GCMS. The pooled pentane extracts were concentrated to dryness and the residue was purified by preparative thin layer chromatography on silica gel impregnated with 1% $AgNO_3$, eluting with 5% acetone in pentane. The title compound was isolated as a colourless oil (14 mg, 73%). HRMS (*m/z*, EI^+) calcd. for $C_{17}H_{28}$ 232.2191; found 232.2191; δ_H (600 MHz, $CDCl_3$) 0.79 (3 H, d, *J* = 7.0 Hz, $(CH_3)_2CH$), 0.85 (3 H, d, *J* = 7.0 Hz, $(CH_3)_2CH$), 0.86 – 0.90 (2 H, m, $(CH_3)_2CHCHCH_2$), 0.94 (3 H, t, *J* = 7.5 Hz, CH_3CH_2), 1.37-1.42 (1 H, m, $(CH_3)_2CH$), 1.68 (3 H, d, *J* = 6.0 Hz, CH_3CH=C), 1.70-1.75 (2 H, m, CH_2CEt), 1.85-1.90 (1 H, m, 1 x CH_2CH=CEt), 1.97-2.02 (1 H, m, 1 x CH_2CH=CEt), 2.02-2.09 (1 H, m, $(CH_3)_2CHCH$), 2.14-2.20 (1 H, m, 1 x CH_3CH=CCH_2), 2.25-2.32 (2 H, m, CH_3CH_2), 2.36-2.43 (1 H, m, 1 x CH_2C=CEt), 2.44-2.52 (1 H, m, 1 x CH_3CH=CCH_2), 5.03 (1 H, dd, *J* = 6 and 11 Hz, CH_3CH_2C=CH), 5.08 (1 H, dd, *J* = 10 and 16 Hz, CH_3CH=CCH=CH), 5.38 (1 H, q, *J* = 6.0

Hz, CH_3CH=C), 5.72 (1 H, d, *J* = 6.0 Hz, CH_3CH=CC*H*=CH) ; δ_C (150 MHz, $CDCl_3$) 12.66 (CH_3CH_2), 13.18 (CH_3CH=C), 14.13 (1 x $(CH_3)_2CH$), 19.28 (1 x $(CH_3)_2CH$) 20.75 ($(CH_3)_2CHCHCH_2$) 21.30 (CH_3CH_2), 22.70 (CH_2CEt), 27.09 (CH_2CH=CEt), 32.83 ($(CH_3)_2CH$), 36.96 (CH_3CH=CCH_2), 52.57 ($(CH_3)_2CHCH$), 120.0 (CH_3CH=C), 130.1 (*C*H=CEt), 133.6 (CH=*C*HCHCH$(CH_3)_2$), 136.1 (*C*H=CHCHCH$(CH_3)_2$), 139.4 (CH=*C*Et), 140.2 (CH_3CH=*C*) ; *m/z* (EI^+), 232.2 (22%, M^+), 203.2 (8, $[M\text{-}Et]^+$), 189.2 (100, $[M\text{-}(CH_3)_2CH]^+$), 175.2 (2), 161.1 (11), 147.1 (30), 133.1 (31), 119.1 (33), 105.1 (25), 91.1 (29), 79.1 (18), 67.1 (7), 55.1 (4).

(*S*)-15-Dimethyl germacrene D (1f)



The title compound was produced in similar fashion to **1g**. 15-methyl-FDP (**4f**) (19 mg, 0.40 mM final concentration) and Y406F-GDS-His_6 (12 μM final concentration) were mixed in incubation buffer (20 mM Tris, 5 mM βME, and 10 mM $MgCl_2$, pH 7.5, 10% glycerol for GDS, 200 mL final volume) overlaid with pentane (10 mL). The mixture was gently agitated for 5 days at room temperature and then the separated aqueous layer was thoroughly extracted with further portions of pentane until no product could be detected by GCMS. The pooled pentane extracts were concentrated to dryness and the residue was purified by preparative thin layer chromatography on silica gel impregnated with 1% $AgNO_3$, eluting with 5% acetone in pentane. The title compound was isolated as a colourless oil (8 mg, 45%). HRMS (*m/z*, EI^+) calcd. for $C_{17}H_{28}$ 232.2191; found 232.2191; δ_H (600 MHz, $CDCl_3$) 0.72 – 0.80 (3 H, m, $(CH_3)_2CHCHCH_2$), 0.83 (3 H, d, *J* = 7.0 Hz, $(CH_3)_2CH$), 0.90 (3 H, d, *J* = 7.0

Hz, (CH_3)$_2$CH), 1.56 (3 H, s, CH_3C=CH), 1.71 (3 H, d, J = 7.0 Hz, CH_3CH=C), 1.95-2.05, 2.16-2.18, 2.21-2.25, 2.30-2.37 and 2.52-2.54 (7 H, m, allylic CHs), 5.14-5.18 (2 H, m, CH_3CH=CCH=C*H* and CH_3C=C*H*), 5.42 (1 H, q, J = 7.0 Hz, CH_3C*H*=C), 5.76 (1 H, d, J = 6.0 Hz, CH_3CH=CC*H*=CH) ; *m/z* (EI$^+$), 218.2 (28%, M$^+$), 203.2 (10, [M-CH_3]$^+$), 189.2 (9), 175.1 (100), 143.1 (18), 133.1 (20), 119.1 (22), 105.1 (25), 91.1 (20), 79.1 (10), 67.1 (4), 55.1 (3).

5. Enzyme Kinetics

Kinetic assays were carried out as for the wild-type enzyme previously described (with the concentration of GDS-His$_6$ or mutants at 40 - 80 nM).[1]

Steady-state parameters (K_M and k_{cat}) were obtained by direct fitting of the data to the equation v = k_{cat}[E] / (K_M + S) with the Systat SigmaPlot package (Systat Sigmaplot 10.0, 2007. Sigmaplot for Windows Version 10.0, Build 10.0.0.54, 2006, Systat Software Inc. 1735, Technology Drive, Ste 430, San Jose, CA 95110, USA. Molecular Operating Environment (MOE 2004.03) Chemical Computing Group, Inc., 1255 University St. Suite 1600, Montreal, Quebec, Canada. H3B)

GDS	K_M (µM)		k_{cat} (s^{-1})		k_{cat}/K_M 10^3 ($M^{-1}.s^{-1}$)	
	value	error	value	error	value	error
WT	3.60	± 0.26	0.0094	± 0.0002	2.612	± 0.191
Y524F	5.34	± 0.43	0.0143	± 0.0004	2.669	± 0.227
W275F	2.06	± 0.21	0.0082	± 0.0002	3.971	± 0.405

Table S1. Kinetics parameters of Y524F and W275F mutants

GDS	K_M (µM)		k_{cat} (s^{-1})		k_{cat}/K_M 10^3 ($M^{-1}.s^{-1}$)	
	value	error	value	error	value	error
WT	3.60	± 0.26	0.0094	± 0.0002	2.612	± 0.191
Y406W	3.14	± 0.26	0.0005	± 0.00001	0.139	± 0.012
Y406F	12.75	± 0.81	0.0853	± 0.0003	6.692	± 0.426
Y406L	8.13	± 0.53	0.0543	± 0.0012	6.679	± 0.459
Y406I	4.37	± 0.44	0.0319	± 0.0010	7.299	± 0.782
Y406V	4.17	± 0.57	0.0131	± 0.0003	4.363	± 0.394

Y406A	1.46	± 0.09	0.0132	± 0.0002	9.043	± 0.562
Y406S	--	--	--	--	--	--
Y406G	--	--	--	--	--	--

Table S2. Kinetics parameters of GDS-Y406 variants

	GDS-His$_6$						Y406F-GDS-His$_6$					
	K_M μM		k_{cat} s^{-1}		k_{cat}/K_M M^{-1} s^{-1}		K_M μM		k_{cat} s^{-1}		k_{cat}/K_M M^{-1}.s^{-1}	
	value	error	value	error	value	error	value	error	value	error	value	error
4a	3.60	± 0.26	0.0094	± 0.0002	2600	± 200	12.75	± 0.81	0.0853	± 0.0003	6700	± 400
4g	2.63	± 0.39	0.0043	± 0.0004	1600	± 300	12.39	± 3.85	0.0228	± 0.0028	1800	± 600
4f	5.02	± 1.62	0.0068	± 0.0008	1400	± 500	5.82	± 1.20	0.0222	± 0.0011	3800	± 800

Table S3. Turnover kinetics of **4a**, **4g**, and **4f** with GDS-His$_6$ and GDS- Y406F.

6. Electrophysiology

Electroantennogram (EAG) recordings were made using Ag-AgCl glass electrodes filled with saline solution [composition as in Maddrell[7] but without glucose]. The head of an alate virginoparous grain aphid, *Sitobion avenae*, was excised and placed within the indifferent electrode and the tips of both antennae were removed before they were inserted into the recording electrode. The signals were passed through a high impedance amplifier (UN-06, Syntech, Hilversum, The Netherlands) and analysed using a customized software package (Syntech).

The coupled gas chromatography-electrophysiology system (GC-EAG), in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously.[10] Separation of the required germacrene D analogue and any contaminants present in the sample was achieved on an Agilent 6890 GC equipped with a cool on-column inlet and an FID, using an HP-1 (50 m x 0.32 mm, O.D. x 0.52 μm,

phase thickness) column with helium as carrier gas (flow rate of 2.5 ml/min). The oven temperature was maintained at 30 °C for 2 minutes and then ramped at 15 °/minute to 250 °C.

The outputs from the EAG amplifier and the FID were monitored simultaneously and analysed using the Syntech software package. Peaks eluting from the GC column were judged to be active if they elicited EAG activity in three or more coupled runs.

GC-EAG

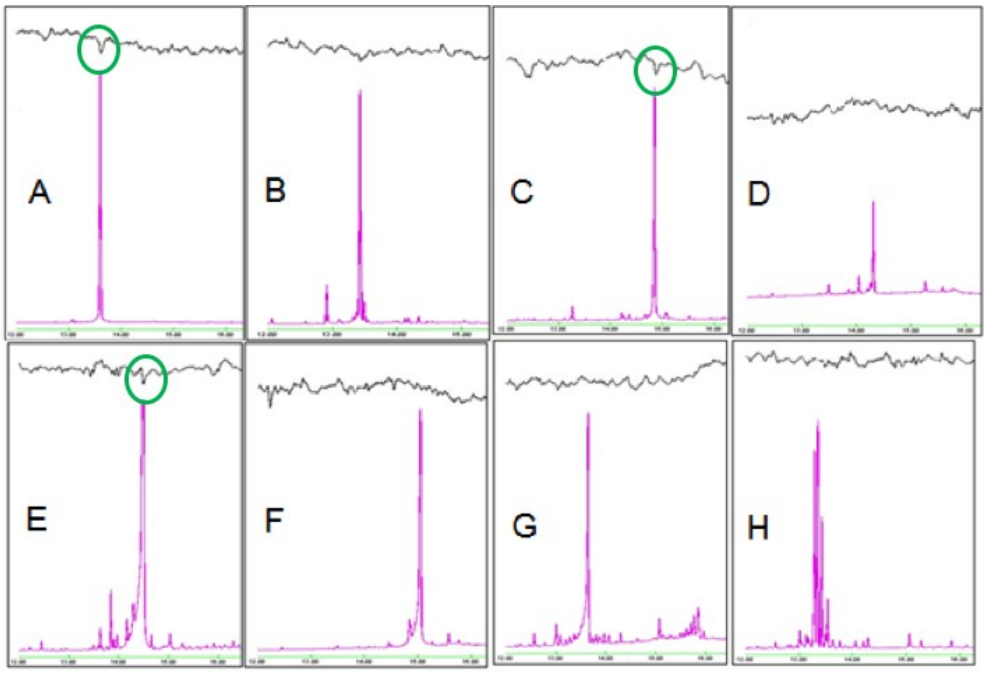
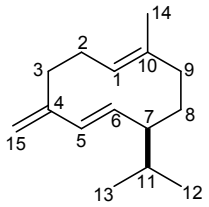


Figure S1. Coupled gas chromatography-electroantennography (GC-EAG) with the grain aphid, *Sitobion avenae*. Upper traces = EAG response, lower traces = GC response. A – (*S*)-germacrene D (**1**); B – (*R*)-germacrene D (**2**); C – 14-methylgermacrene D (**1c**); D – 12-methylgermacrene D (**1b**); E – 15-methylgermacrene D (**1f**); F – 14,15-dimethylgermacrene D; G – 1-fluorogermacrene D (**1h**); H – germacrane (**3**).

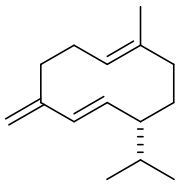
7. Behavioural Assay

The responses of individual grain aphids, *Sitobion avenae*, to test compounds were observed using a Perspex four-arm olfactometer[8,9] (Figure S2), which was maintained at 23 °C and lit from above. A filter paper disc was laid in the bottom section of the olfactometer to provide traction for the aphid and the middle and top sections were fitted into place very tightly to give a good seal. The four arms, consisting of the barrels of disposable 10 mL syringes (Plastipak), were fitted tightly into the holes of the middle section, and filtered air was drawn through them and into the body of the olfactometer through a tube inserted into a hole in the centre of the top section and attached to a pump. The measured total flow rate was 200 mL/min and it was assumed that the flow rate through each arm was 50 mL/min. The three control arms each contained a filter paper strip to which had been applied 10μL hexane which had been allowed to evaporate for 30s. The treatment arm contained a filter paper strip to which the test compound in 10μL of hexane (20 – 200 ng μL^{-1}) had been applied and left for 30 s for the hexane to evaporate. A single aphid was introduced through the central hole and the suction tube quickly reinserted. The time spent in each arm and in the central zone were recorded, using specialist software (OLFA, Udine, Italy), for the next 16 minutes. The olfactometer was rotated through 90° every 2 min to eliminate any directional bias. Each assay comprised 10 replicates and the mean time spent in treated and control arms were compared using a paired *t*-test (Genstat).

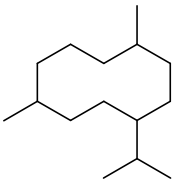
Olfactometry



1



2



3

Compound	Dose (µg)	Time (min.) spent in:- Control arms (mean of 3)	Treatment arm	Significance (ρ)
(*S*)-(-)-germacrene D (**1**)*	0.2	2.31 (0.26)	1.14 (0.25)	0.005
	1.0	2.67 (0.190	1.63 (0.35)	0.007
	2.0	2.29 (0.41)	0.50 (0.14)	0.012
1-fluorogermacrene D (**1h**)*	1.0	2.54 (0.27)	2.62 (0.50)	0.447
	2.0	2.60 (0.26)	2.46 (0.39)	0.402
12-methylgermacrene D (**1b**)*	0.9	2.21 (0.42)	1.20 (0.24)	0.039
	1.2	2.66 (0.17)	2.13 (0.25)	0.075
14-methylgermacrene D (**1c**)*	0.2	2.21 (0.27)	2.41 (0.73)	0.409
	1.0	2.61 (0.28)	3.27 (0.68)	0.225
	2.0	2.01 (0.23)	1.65 (0.39)	0.209
15-methylgermacrene D (**1f**)*	0.8	2.61 (0.26)	1.38 (0.38)	0.008
	1.0	2.52 (0.23)	1.92 (0.31)	0.094
14,15-dimethylgermacrene D (**1g**)*	0.8	2.60 (0.22)	3.38 (0.38)	0.069
	1.0	2.27 (0.11)	2.77 (0.31)	0.052
	1.0	2.39 (0.20)	2.97 (0.40)	0.032
	1.2	1.96 (0.21)	2.92 (0.23)	0.001
(*R*)-(+)-germacrene D (**2**)	1.0	2.57 (0.19)	3.15 (0.53)	0.447
	2.0	1.86 (0.29)	2.65 (0.52)	0.108
germacrane (**3**)	1.0	2.45 (0.30)	2.04 (0.58)	0.265
	2.0	2.39 (0.24)	2.38 (0.50)	0.485

* Analogue prepared from FDP or analogue using GDS.

Table S4. Behavioural response of cereal aphids, *Sitobion avenae*, to germacrene D analogues using 4-way olfactometer (Figure S2). Data were recorded as mean (±SE) time spent in control (solvent only) or treatment arms and were analysed using Students T-test.

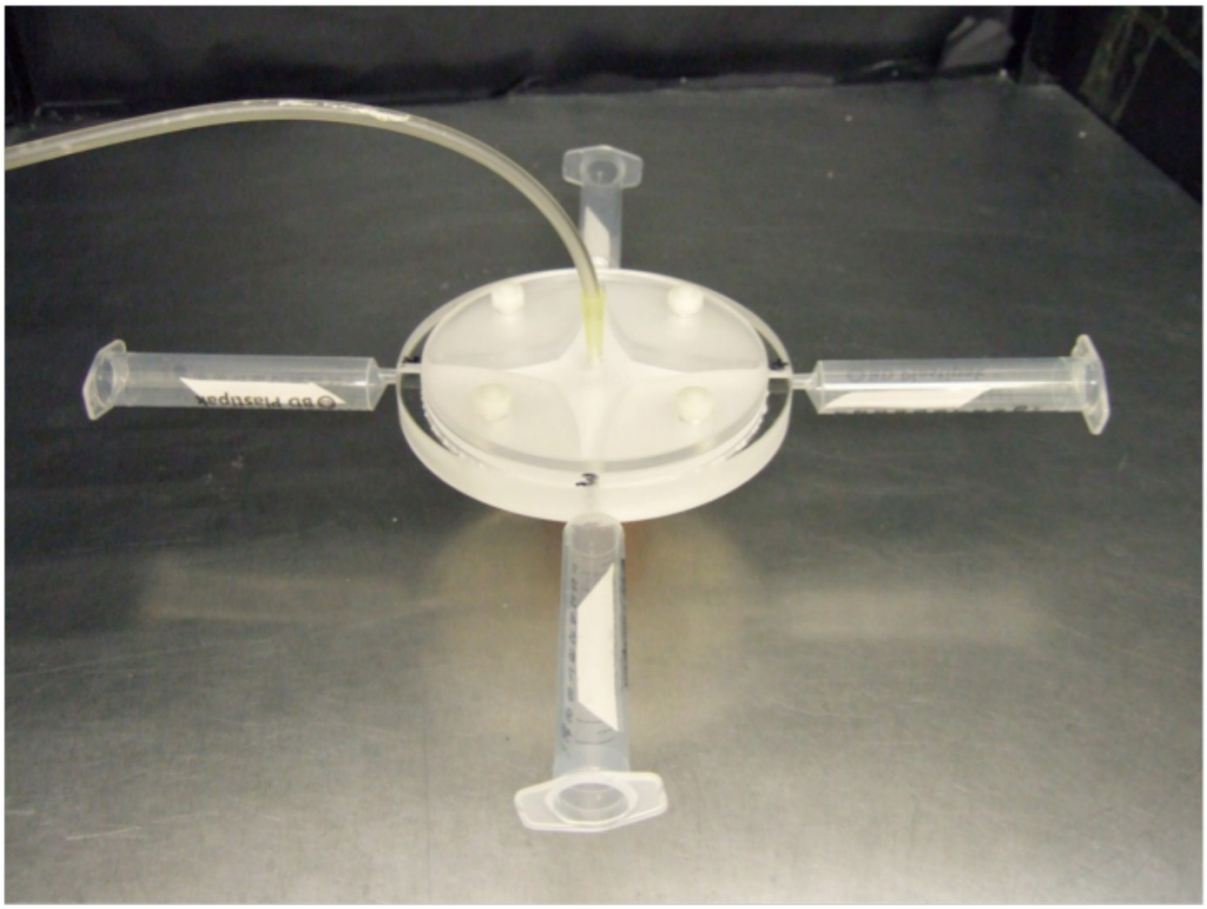
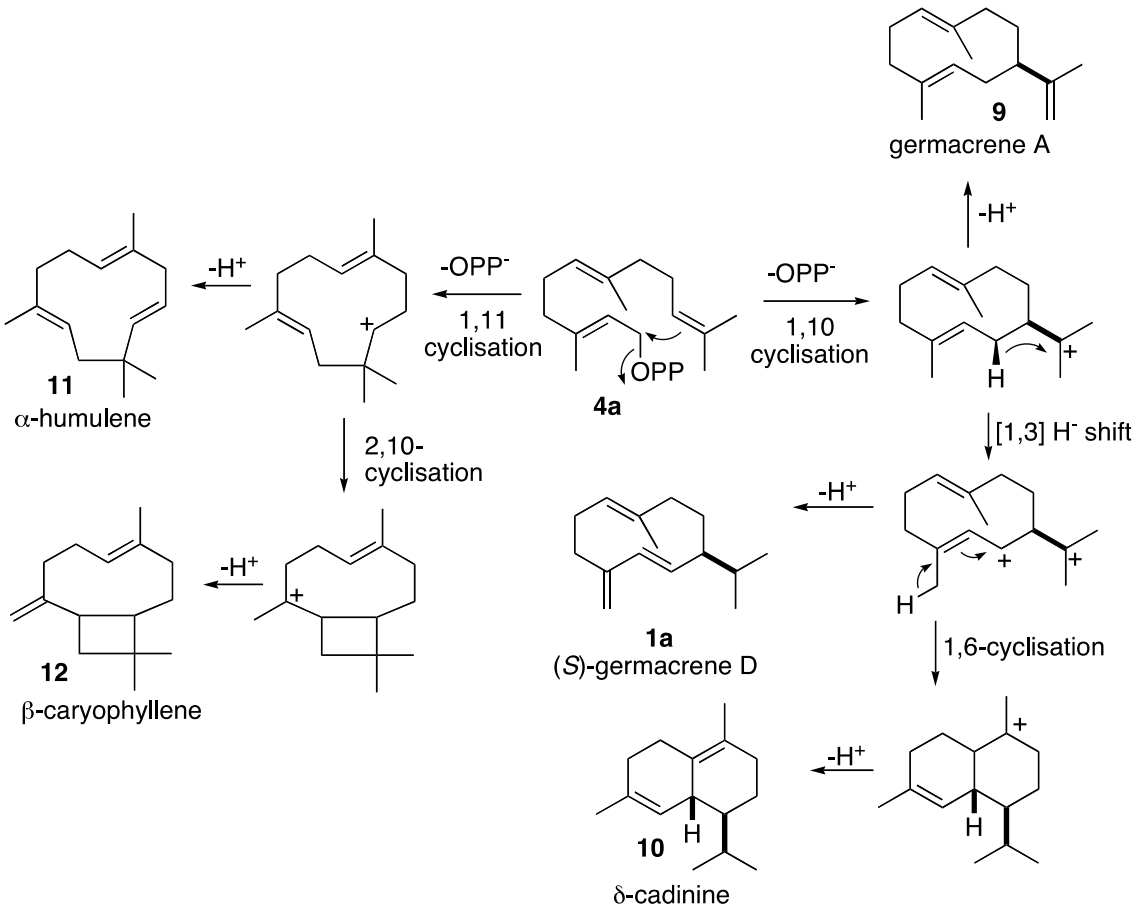


Figure S2. Pettersson 4-way olfactometer.

8. GC-MS Analysis



Scheme S2. Alternative GDS-catalysed cyclisation pathways for GDS mutants.

GDS	1a	12	11	9	10	unknown
WT	100	--	--	--	--	--
Y406W	90	--	10	--	--	--
Y406F	100	--	--	--	--	--
Y406L	100	--	--	--	--	--
Y406I	79	3	--	--	4	12
Y406V	70	4	2.5	--	7	16.5
Y406A	46	6	3	3	10	32

Table S5. Product distribution in pentane extracts from incubation of FDP with Y406 mutants.

Gas chromatograms for the products isolated from turnover of 14-methylfarnesyl diphosphate, 14-fluorofarnesyl diphosphate, 15-fluorofarnesyl diphosphate and 6-fluorofarnesyl diphosphate were as previously published. [1]

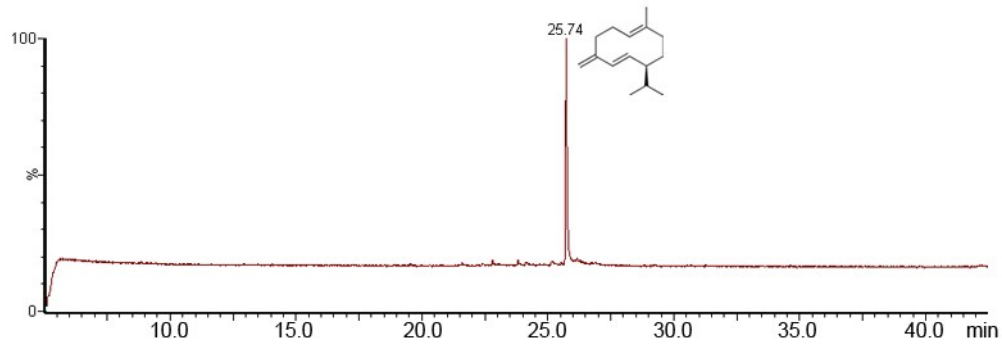


Figure S3. Gas chromatogram of the pentane extractable products from incubation of farnesyl diphosphate (**4a**) with Y406F-GDS-$His_{6.}$ **1a** eluted at 25.74 min.

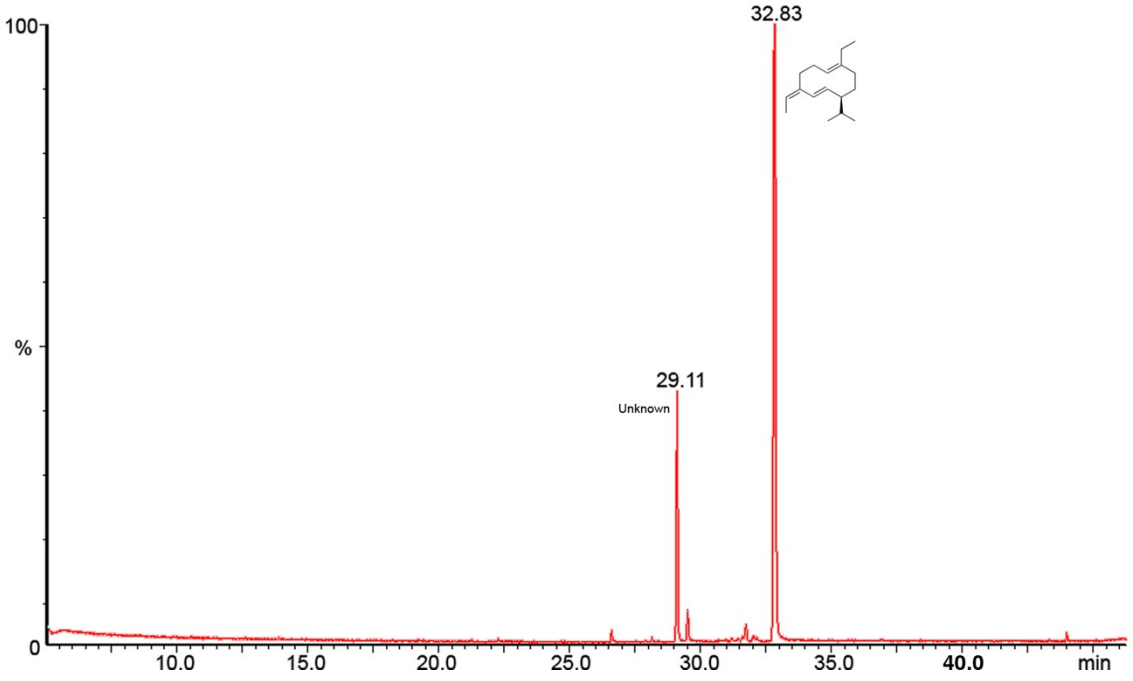


Figure S4. Gas chromatogram of the pentane extractable products from incubation of 14,15-dimethylfarnesyl diphosphate (**4g**) with Y406F-GDS-$His_{6.}$ **1g** eluted at 32.83 min.

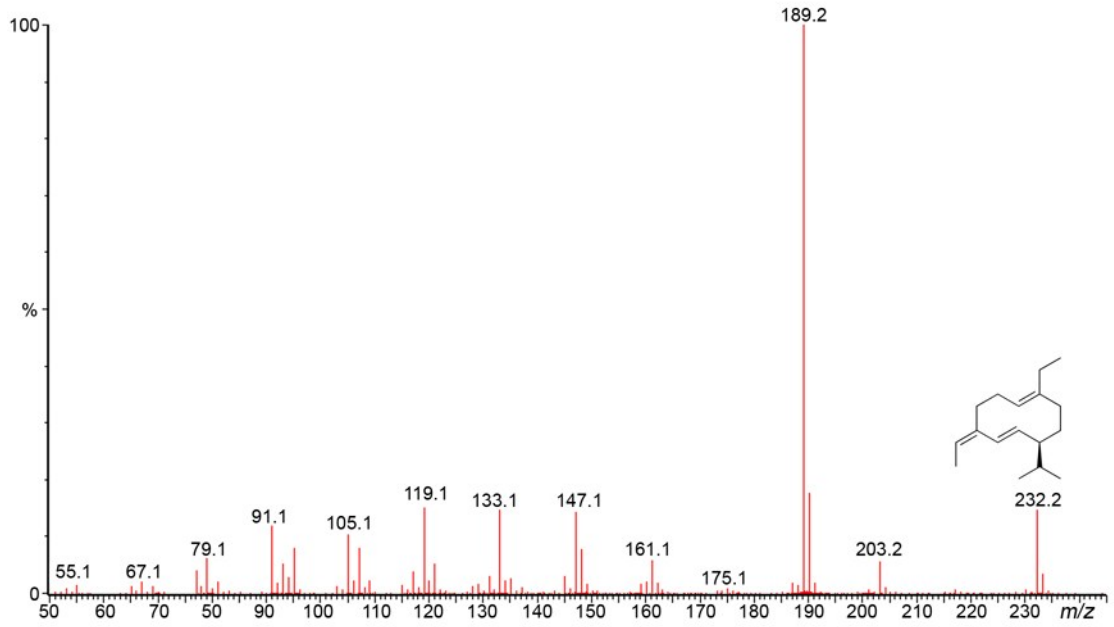


Figure S5. Mass spectrum of the product eluting at 32.83 min from incubation of 14,15-dimethylfarnesyl diphosphate with Y406F-GDS-$His_{6.}$

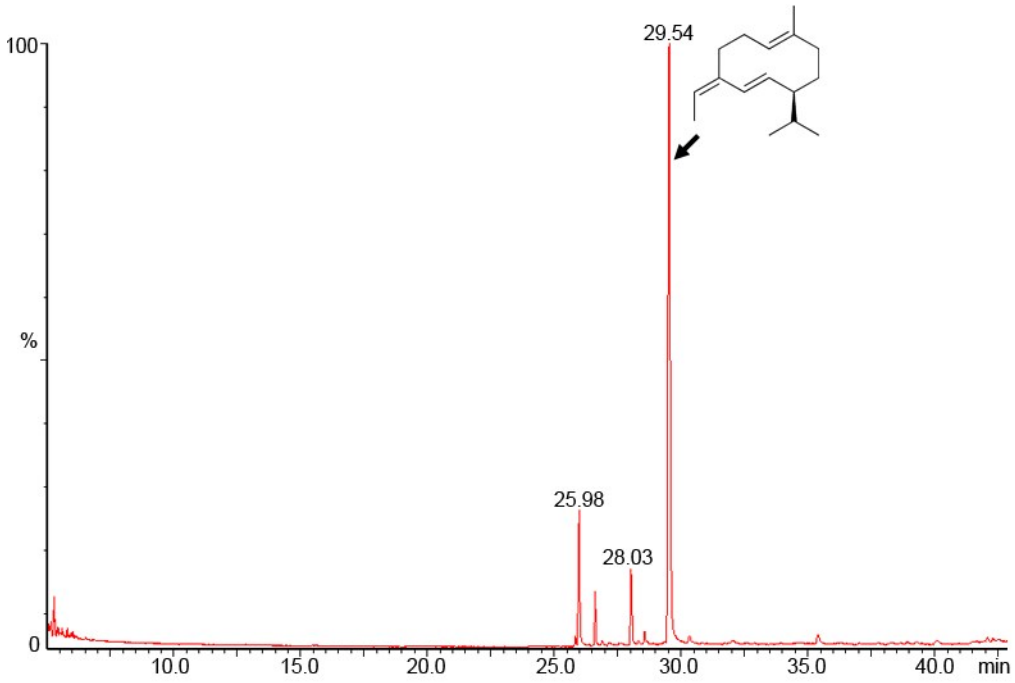


Figure S6. Gas chromatogram of the pentane extractable products from incubation of 15-dimethylfarnesyl diphosphate (**4f**) with Y406F-GDS-$His_{6.}$ **1f** eluted at 29.54 min.

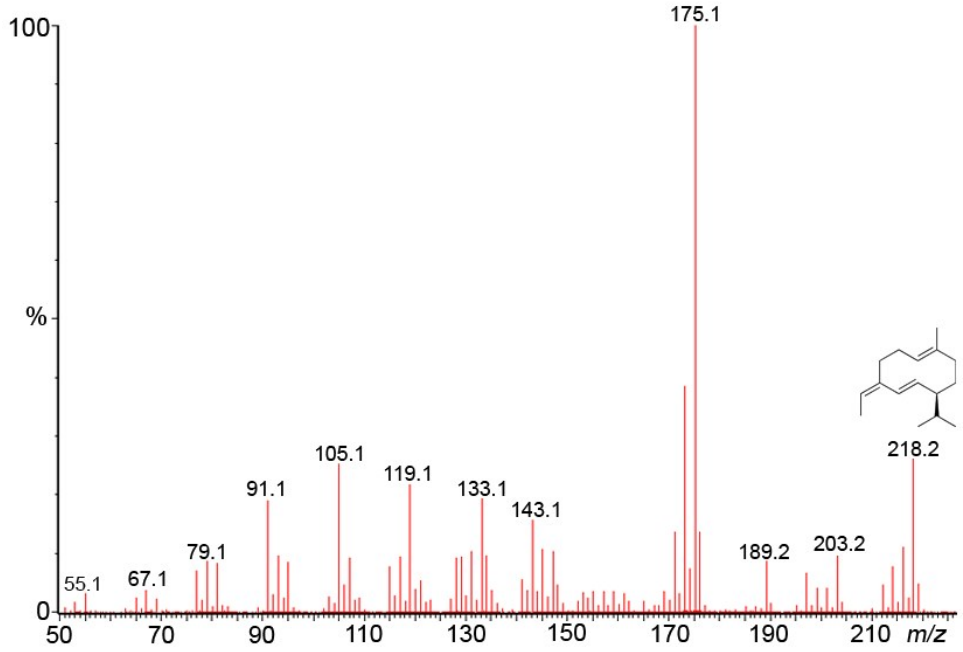


Figure S7. Mass spectrum of the product eluting at 29.54 min from incubation of 15-dimethylfarnesyl diphosphate with Y406F-GDS-His_6.

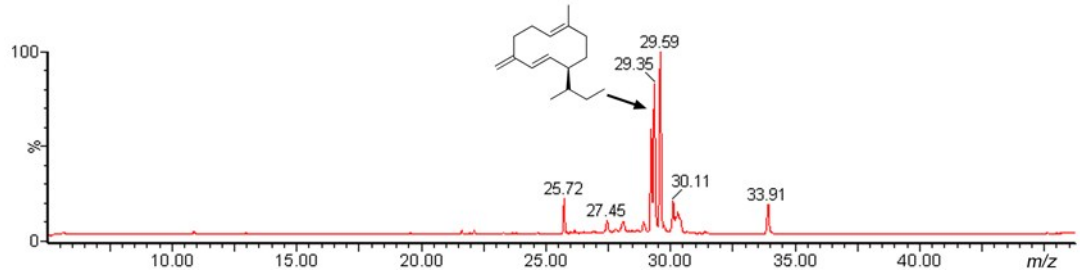


Figure S8. Gas chromatogram of the pentane extractable products from the incubation of 12-methylfarnesyl diphosphate. The germacrene D analogue **1b** eluted at 29.35 minutes.

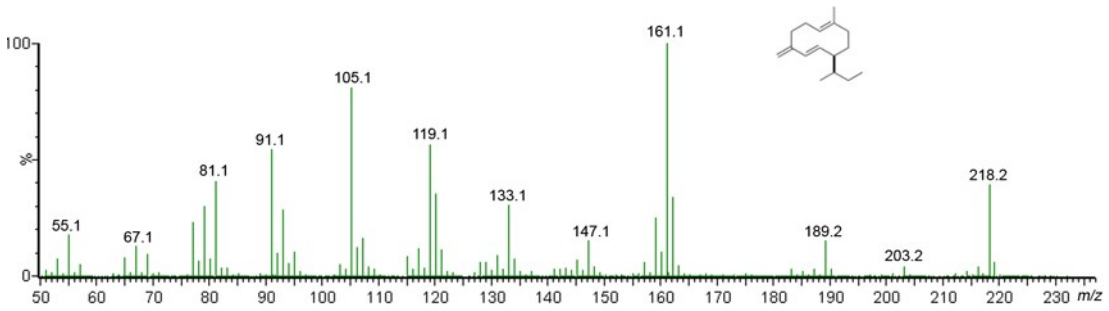


Figure S9. Mass spectrum (EI^+) of **1b**.

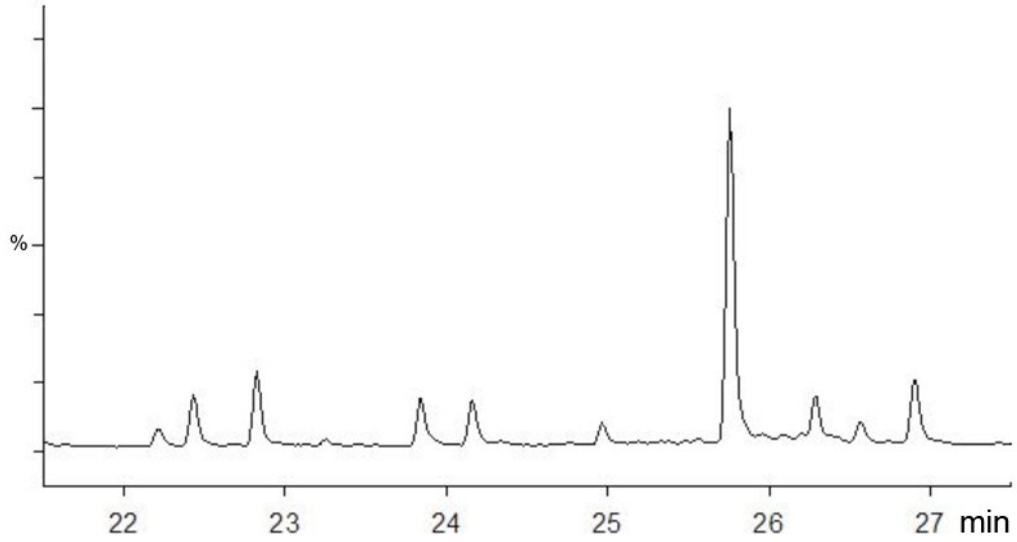


Figure S10. Representative total ion chromatogram of the pentane extractable products from incubation of FDP and GDS-Y406A : β-caryophyllene (retention time, 23.84 min), α-humulene (retention time, 24.96 min), germacrene D (retention time, 25.75 min), germacrene A (retention time, 26.56 min), δ-cadinene (retention time, 26.89 min) and unknown compounds (retention time, 22.22 min, 22.43 min, 22.82 min, 24.15 min and 29.69 min).

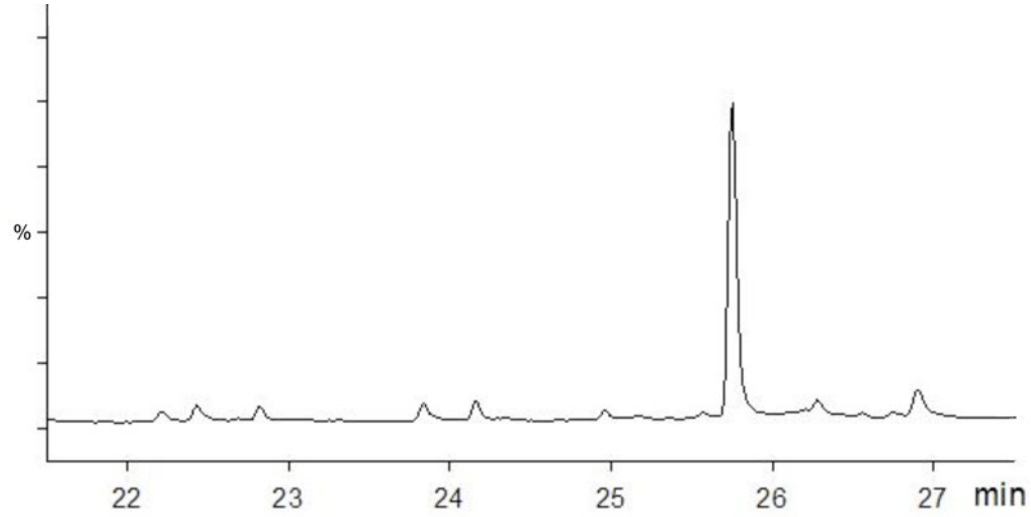


Figure S11. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and GDS-Y406V: (□□-caryophyllene (retention time, 23.84 min), α-humulene (retention time, 24.96 min), germacrene D (retention time, 25.75 min),δ-cadinene (retention time, 26.89 min) and unknown compounds (retention time, 22.22 min, 22.43 min, 22.82 min, 24.15 min and 29.69 min).

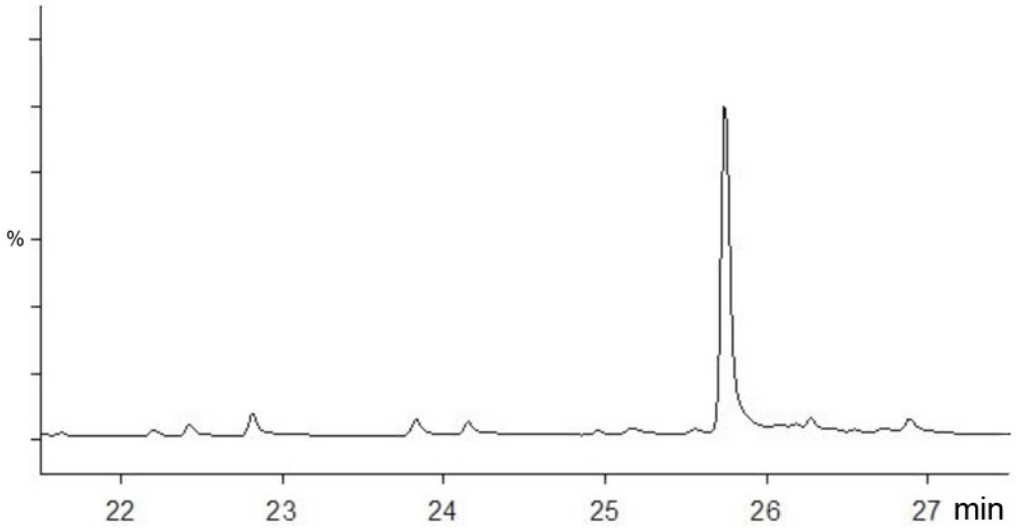


Figure S12. Representative total ion chromatogram of the pentane extractable products formed from incubation of FPP and GDS-Y406I: (*E*)-caryophyllene (retention time, 23.84 min), germacrene D (retention time, 25.75 min), δ-cadinene (retention time, 26.89 min) and unknown compounds (retention time, 22.22 min, 22.43 min, 22.82 min and 24.15 min).

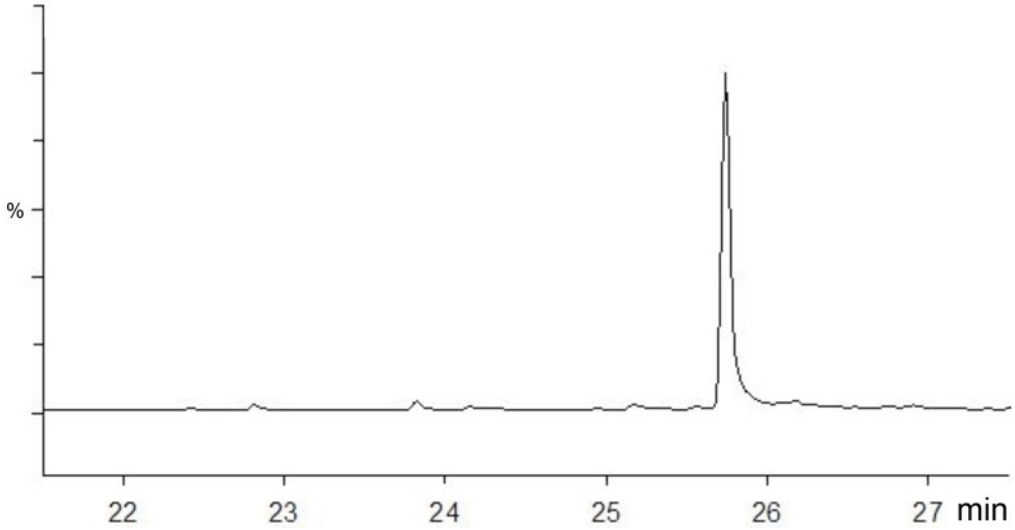


Figure S13. Representative total ion chromatogram of the pentane extractable products formed from incubation of FPP and GDS-Y406L: germacrene D (retention time, 25.75 min).

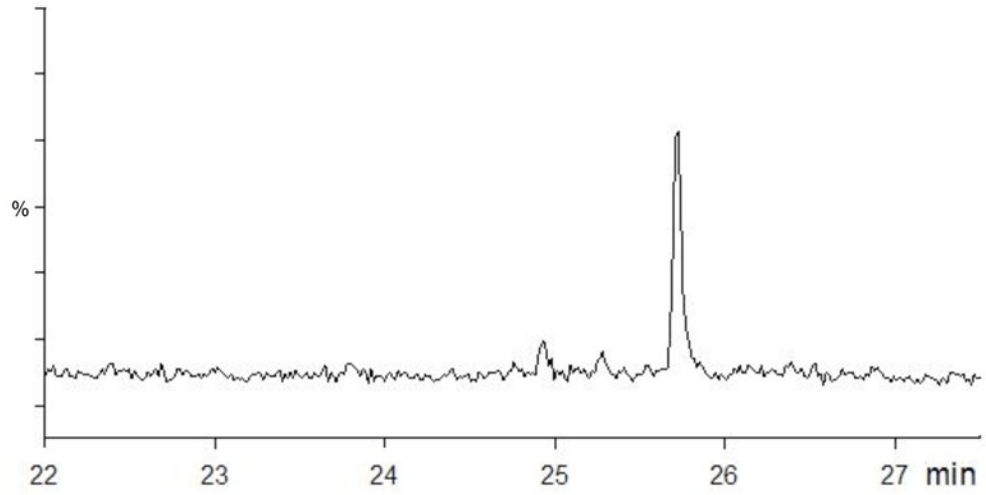


Figure S14. Representative total ion chromatogram of the pentane extractable products formed from incubation of FPP and GDS-Y406W: α-humulene (retention time, 24.96 min) and germacrene D (retention time, 25.75 min).

9. NMR Spectra

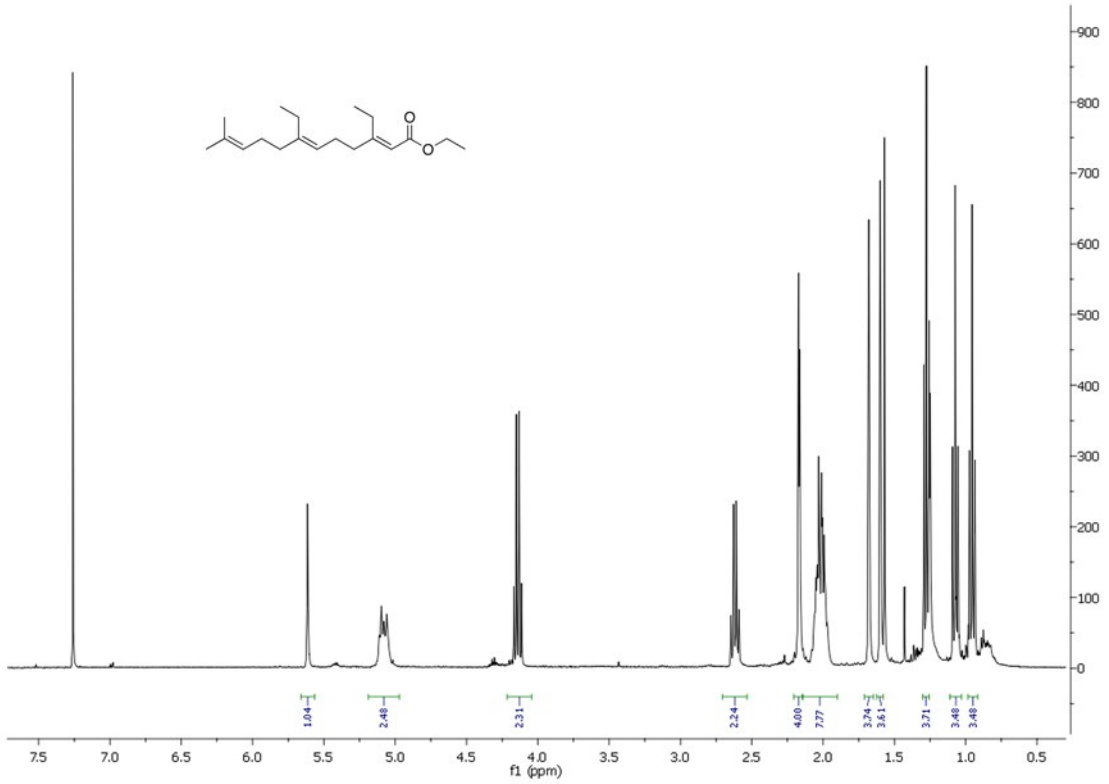


Figure S15. ^{1}H NMR-spectrum (400 MHz, $CDCl_3$) of **7**.

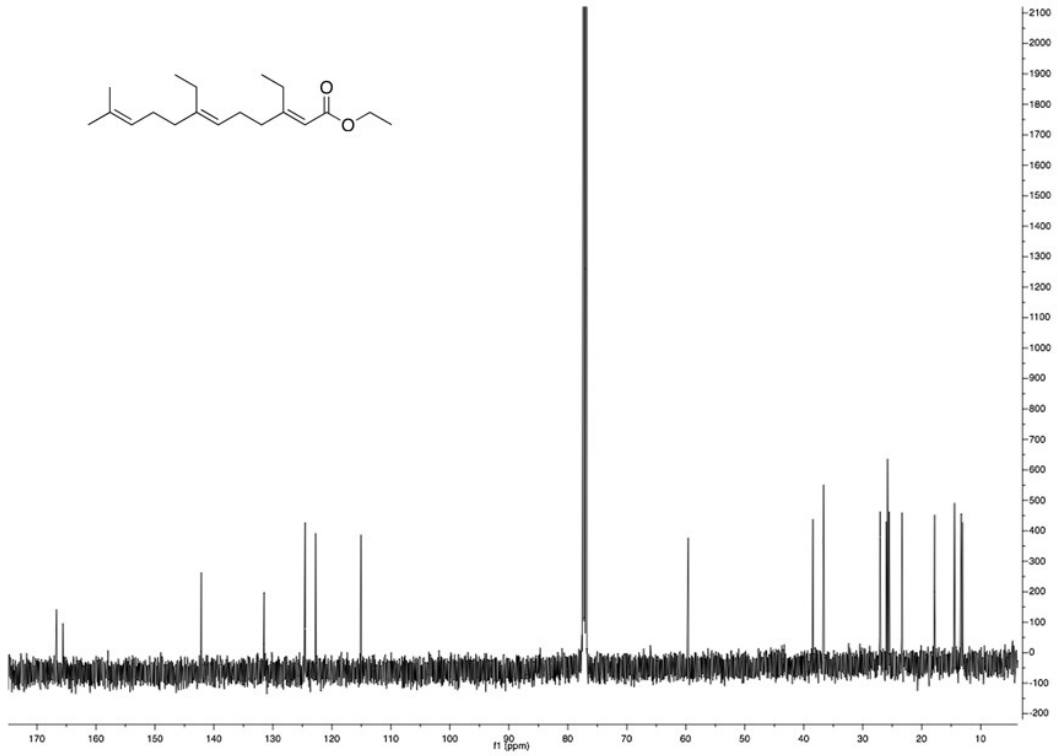


Figure S16. ^{13}C NMR-spectrum (100 MHz, $CDCl_3$) of **7**.

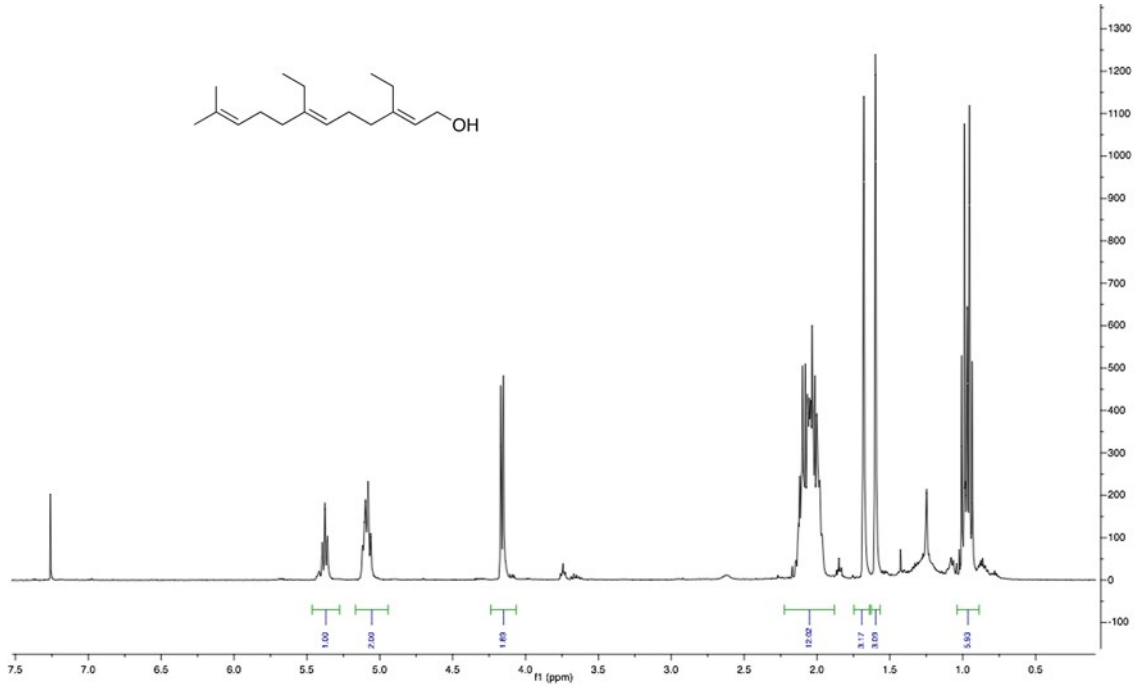


Figure S17. ^{1}H NMR-spectrum (400 MHz, $CDCl_3$) of **8**.

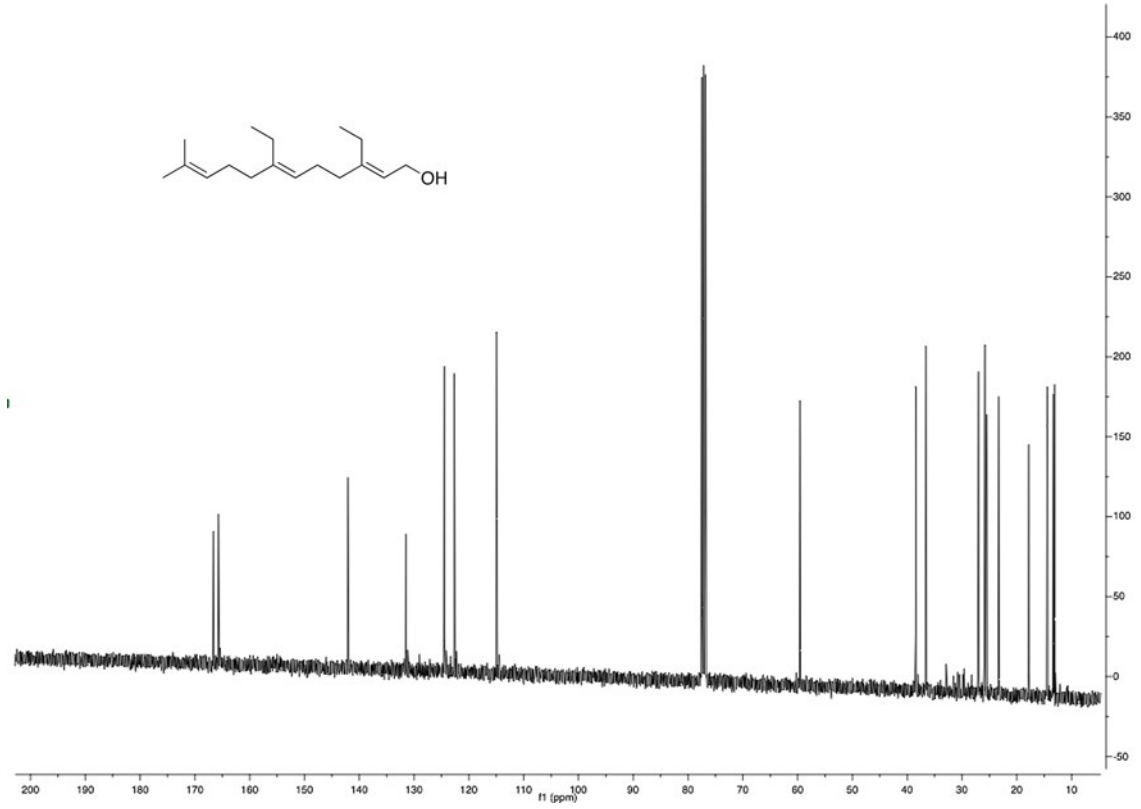


Figure S18. ^{13}C NMR-spectrum (100 MHz, $CDCl_3$) of **8**.

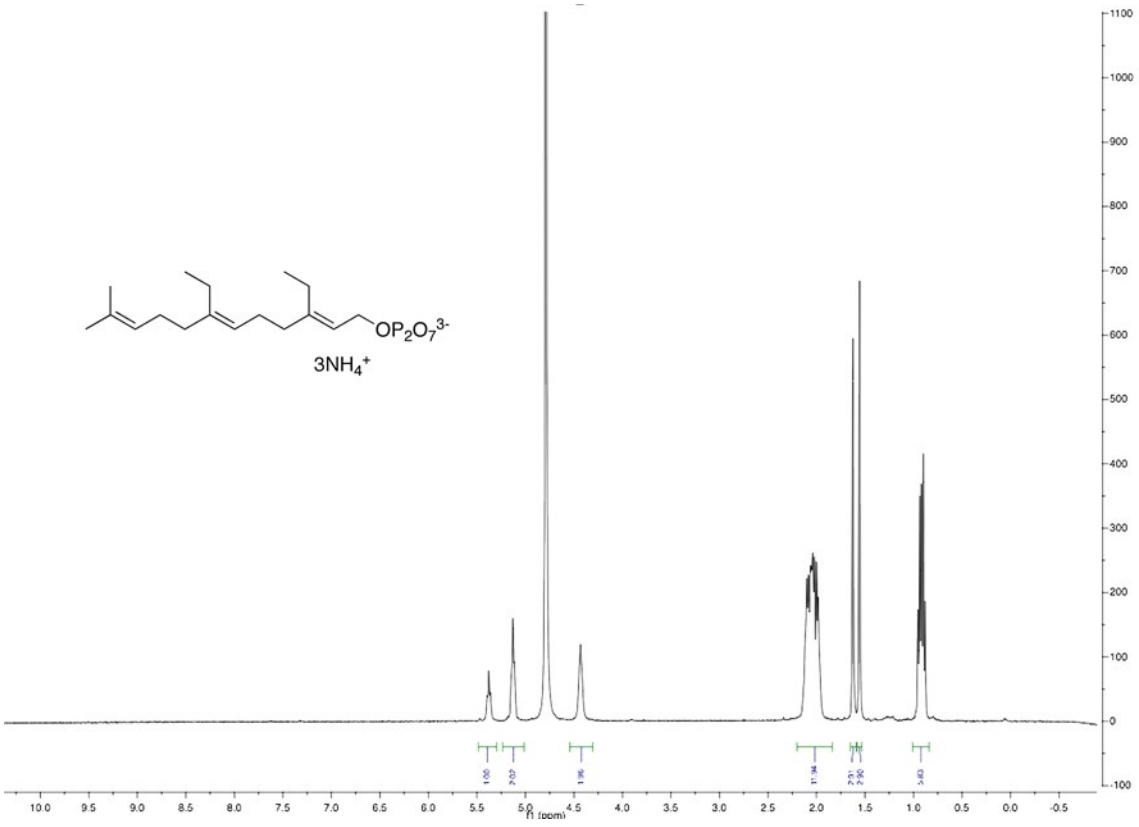


Figure S19. 400 MHz ^{1}H NMR-spectrum of **1g**.

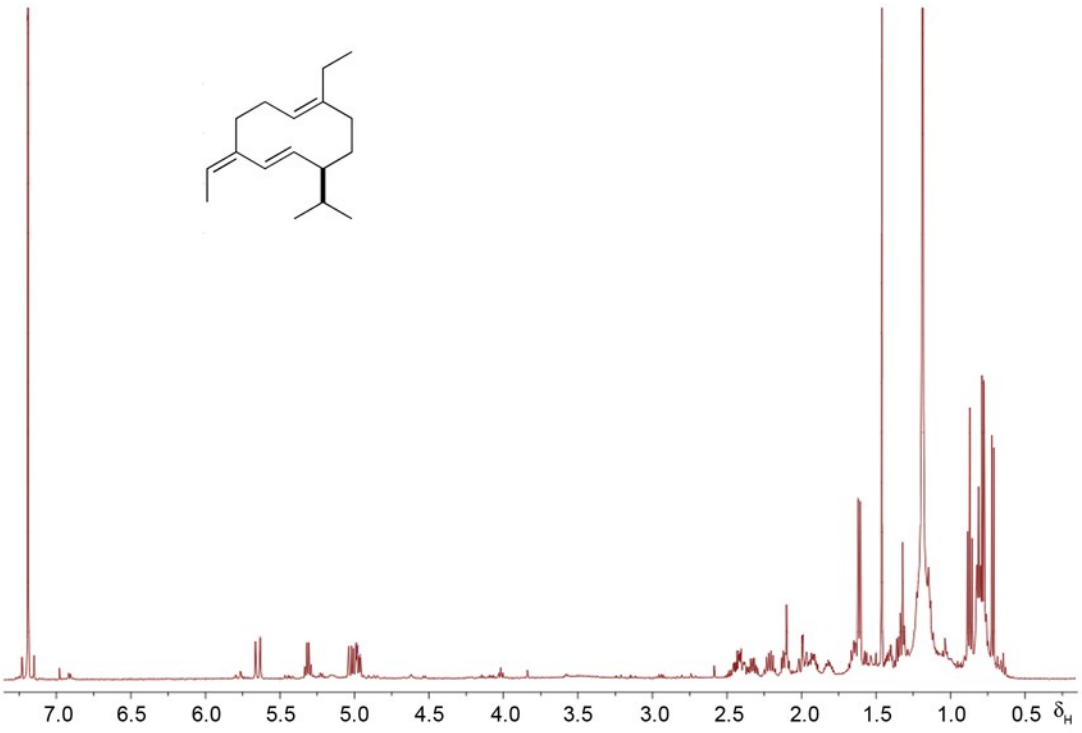


Figure S20. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of **1g**.

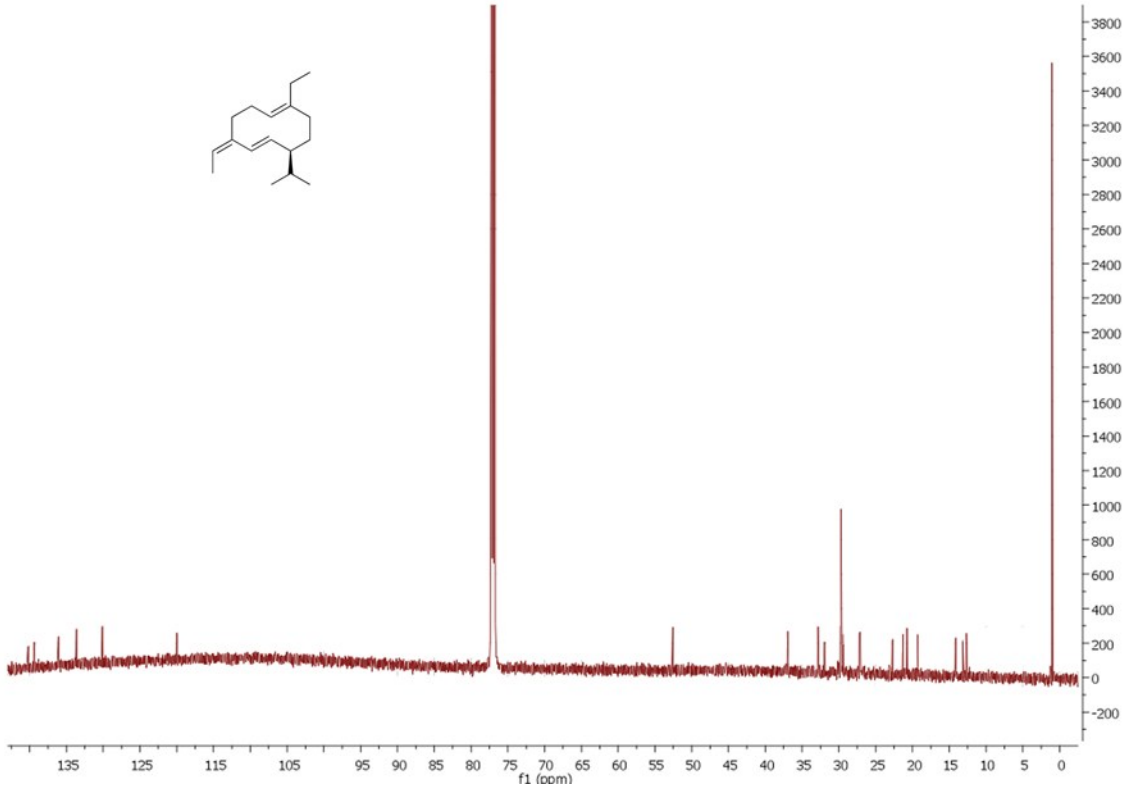


Figure S21. ^{13}C NMR-spectrum (150 MHz, $CDCl_3$) of **1g**.

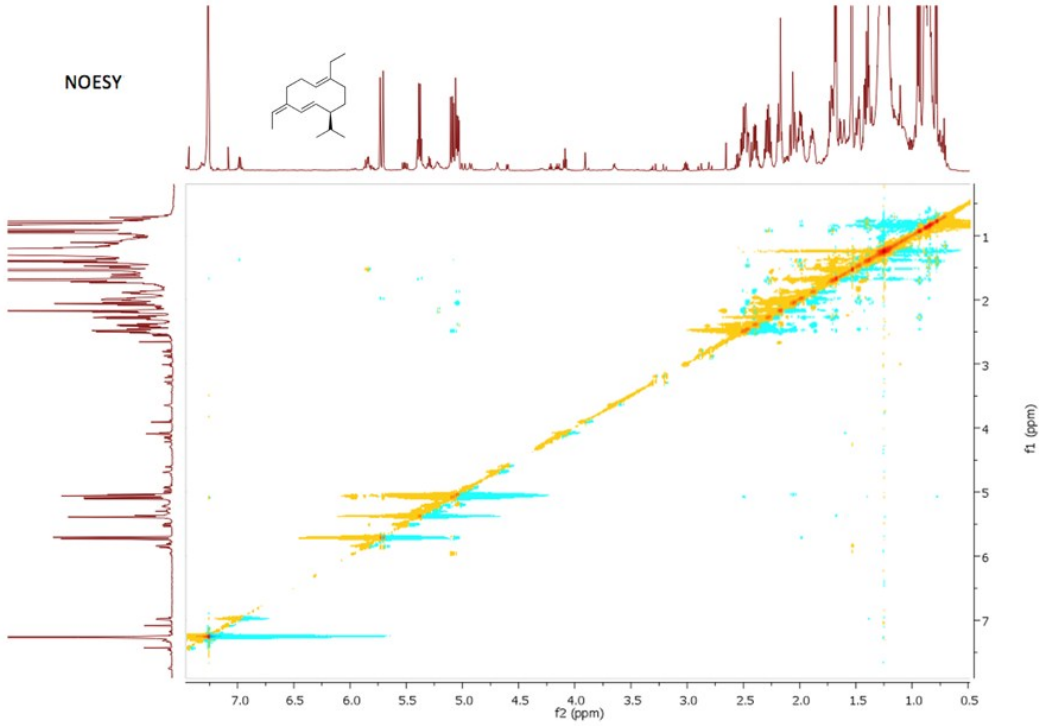


Figure S22. NOESY NMR-spectrum (600 MHz, $CDCl_3$) of **1g**.

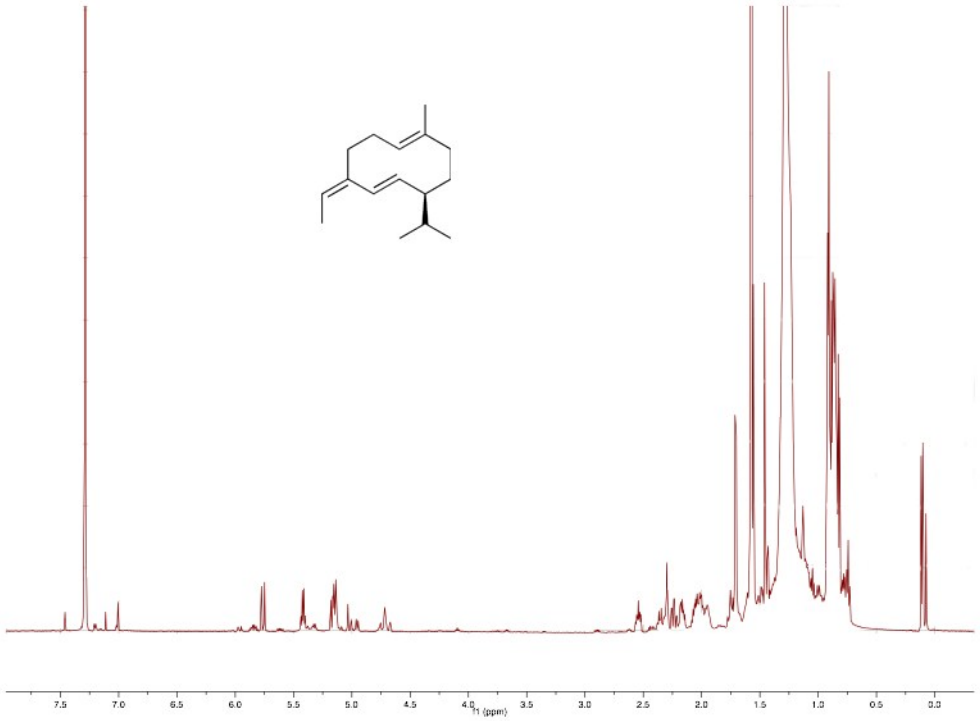


Figure S23. ^{1}H NMR-spectrum (600 MHz, $CDCl_3$) of **1f**

10. References.

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