Supporting Information for:

Mechanism of germacradien-4-ol synthase controlled water capture

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1. Materials. A prestained protein size marker (6.5-175) kDa was used to identify proteins by 12% SDS-gel. The Amicon-YM30 membranes were used for protein concentration. [1-^{3}H]-FDP (20 Ci/mmol) was purchased from American Radiolabeled Chemicals. Commercial [1-^{3}H]-FDP was diluted by adding cold FDP to give a final specific activity of 24000 dpm/μM. For synthetic procedures, all chemicals and solvents were obtained from commercial vendors and used without further purification unless otherwise noted. Anhydrous tetrahydrofuran (THF), diethyl ether, toluene and acetonitrile were obtained from a MBraun SPS800 solvent purification system. Dichloromethane, and triethylamine were distilled from calcium hydride and KOH under nitrogen respectively. EtOH was distilled from calcium oxide**.**

^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 500 NMR spectrometer or a Bruker Fourier300 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 (to the nearest 0.5 Hz) and assignment, respectively. ^{1}H, ^{13}C and ^{31}P NMR spectra were measured on a Bruker Avance 500 NMR spectrometer and are reported as chemical shift downfield from tetramethylsilane (^{1}H and ^{13}C) or 85% H_3PO_4 (^{31}P), coupling constant where appropriate and assignment. Assignments are made to the limitations of COSY, DEPT 90/135, gradient HSQC and gradient HMBC spectra. ^{31}P NMR spectra were recorded on a Jeol Eclipse +300 NMR spectrometer or a Bruker Avance 500 NMR spectrometer.

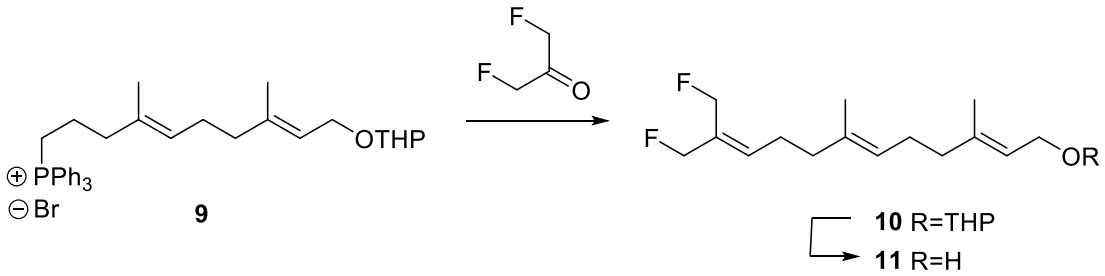
2. General Methods. GC-MS analysis of incubation products was performed on a Hewlett Packard 6890 GC apparatus fitted with a J&W scientific DB-5MS column (30 m x 0.25 mm internal diameter) and a Micromass GCT Premiere detecting in the range *m/z* 50-800 in the EI^{+} mode with scanning once a second with a scan time of 0.9 s. Method 1: The program uses an injection port temperature of 100 °C; split ratio 5:1; initial temperature 50 °C hold 1 min, ramp of 4 °C/min to 150 °C hold 15 min, ramp of 20 °C/min to 250 °C hold 3 min. High-resolution ES^{-} mass spectra were measured on a Micromass LCT premiere XE spectrometer fitted with a Waters 1525 Micro binary HPLC pump. The purity of purified compounds was judged to be > 95% by TLC and/or GC analyses and NMR spectroscopic analysis. High-resolution ES^{-} mass spectra were measured on a Micromass LCT premiere XE spectrometer fitted with a Waters 1525 Micro binary HPLC pump.

Thin layer chromatography was performed on pre-coated aluminium plates of silica G/UV_{254}. TLC visualizations were performed with 4.2% ammonium molybdate and 0.2% ceric sulfate in 5 % H_2SO_4, or 0.1 % berberine hydrochloride in EtOH or UV light. Reverse phase HPLC was performed on a system comprising of a Dionex P680 pump and a Dionex UVD170U detector unit.

3. Synthetic Procedures. (*2E, 6E*)-farnesyl diphosphate (**1**) was synthesized from commercial (*2E, 6E*)-farnesol using the method described by Poulter.[1,2] (*RS*)-trans-nerolidyl diphosphate (NDP, **6**) was

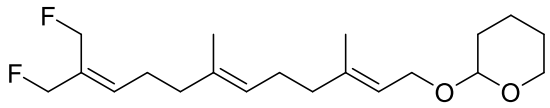
prepared from commercial (±)-trans-nerolidol following the Cramer-Danilov protocol[3-5] as described by Karp *et al.*[6] with modifications.[7] The resulting silica gel purified $(Bu)_4N^+$ form of NDP was converted to the NH_4^+ salt by ion exchange chromatography (Dowex 50W-X8). (2*Z*, 6*E*)-2F-Farnesyl diphosphate (**1b**) was synthesized as previously described.[8] 15F_3-Farnesyl diphosphate (**1c**) was synthesized as previously reported.[9] (3*RS*)-(1*Z*)-trans-[1-^{3}H]-Nerolidyl diphosphate[10] (activity 0.76 mCi/mmol) was prepared from (1*Z*)-trans-[1-^{3}H]-nerolidol following the Cramer-Danilov protocol[3-5] as described by Karp *et al.*[6] with modifications.[7] (1*Z*)-trans-[1-^{3}H]-Nerolidol was synthesized essentially as described by Cane[10] via the γ-cis-vinylic metallation procedure first described by Julia,[11] using ^3H_2O (activity 100 mCi/mL). (*2E, 6E*)-[*12,13*-F_2] farnesyl diphosphate (**1d**) was synthesized from (*2E, 6E*)-[*12,13*-F_2]-farnesol (difluorofarnesol) using the method described by Poulter.[2,3] [1,1-$^2H_2$]farnesol and (1*R*)-[1-^{2}H]farnesol were prepared following the procedures reported by Cane[12] and phosphorylated using the method described by Poulter.[1,2] to give **1e** and **1f** respectively.

Difluorofarnesol was synthesized by Wittig reaction between difluoroacetone and the triphenylphosphonium bromide (**9**), which was prepared as previously reported,[13] Scheme S1.



Scheme S1. Synthesis of difluorofarnesol **11**

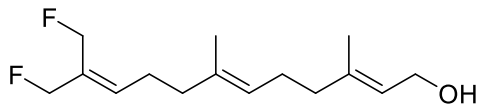
2-(((2E,6E)-12-Fluoro-11-(fluoromethyl)-3,7-dimethyldodeca-2,6,10-trien-1-yl)oxy)tetrahydro-2H-pyran 8



A stirred solution of **9** (140 mg, 0.23 mmol) in anhydrous THF (20 mL) was cooled to -78 °C and n-BuLi (100 μL 2.5 M, 0.25 mmol) added, developing a deep yellow colour as the ylide was formed. Difluoroacetone (23 mg, 19 μL, 0.25 mmol) was then added dropwise and the reaction was stirred at -78 °C for 2 h before being allowed to warm to -20 °C and quenched with water and Et_2O (10 mL, 1:1). The aqueous layer was separated and further washed with Et_2O (3 x 5 mL) and the combined ethereal extracts were washed with brine (20 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by column chromatography on silica (20% EtOAc in Hexane, R_f 0.5) yielded the title compound as a colourless oil (38 mg, 47%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.89 – 5.79 (m, 1 H), 5.40 – 5.31 (m, 1 H), 5.16 – 5.09 (m, 1 H), 4.99 (d, 2 H, *J* 47.6), 4.87 (d, 2 H, *J* 47.6), 4.62 (t, 1 H, *J* 3.5), 4.13 (ddd, 2 H, *J* 68.0, 11.9, 7.2), 3.95 – 3.46 (m, 2 H), 2.42 – 1.96 (m, 6 H), 1.96 – 1.46 (m, 6 H), 1.69 (s, 3 H), 1.60 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 140.19, 133.89, 125.23, 120.80, 98.00, 84.91 (d, *J* 168.0),

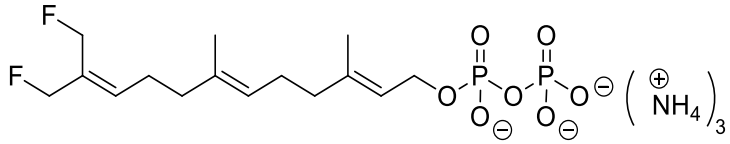
77.63 (d, *J* 156.8), 63.80, 62.46, 39.62, 30.84, 26.34, 25.62, 19.77, 16.56, 16.07; ^{19}F NMR (283 MHz, $CDCl_3$) δ -211.84 (t, *J* 47.6), -216.85 (t, *J* 47.6); HRMS (ES^+, $[M + Na]^+$) found 365.2257, $C_{20}H_{32}O_2F_2Na$ requires 365.2268.

(2E,6E)-12-Fluoro-11-(fluoromethyl)-3,7-dimethyldodeca-2,6,10-trien-1-ol 9



To a stirred solution of **10** (38 mg, 0.11 mmol) in methanol (10 mL) was added *p*-toluenesulfonic acid (1 mg, 0.0055 mmol) and the mixture was stirred at room temperature for 2 h. The volume of methanol was reduced by 80 % under reduced pressure, then saturated aqueous, $NaHCO_3$ (10 mL) and hexane (10 mL) were added. The separated aqueous layer was further washed with hexane (3 x 5 mL) and the combined organic fractions were washed with brine (10 mL), dried over anhydrous $MgSO_4$, then filtered and concentrated under reduced pressure. Purification by column chromatography on silica (20% EtOAc in Hexane, R_f 0.29) yielded the title compound in 91% yield (28 mg, 0.10 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.89 – 5.80 (m, 1 H), 5.45 – 5.37 (m, 1 H), 5.19 – 5.09 (m, 1 H), 4.99 (d, 2 H, *J* 47.6), 4.87 (d, 2 H, *J* 47.6), 4.16 (d, 2 H, *J* 6.9), 2.33 – 1.96 (m, 8 H), 1.68 (s, 3 H), 1.60 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.69, 133.99, 125.11, 123.55, 84.84 (d, *J* 164.3), 77.63 (d, *J* 168.0), 59.53, 39.51, 38.96, 26.31, 16.41, 16.06; ^{19}F NMR (283 MHz, $CDCl_3$) δ -214.24 (t, *J* 47.4), -219.22 (t, *J* 47.4); HRMS ($APCI^+$, $[M + Na]^+$) found 281.1683, $C_{15}H_{24}OF_2Na$ requires 281.1693.

(2E,6E)-12-Fluoro-11-(fluoromethyl)-3,7-dimethyldodeca-2,6,10-trien-1-yl tris-ammonium diphosphate 1d



The difluorofarnesyl diphosphate trisammonium salt was prepared from **11** using the method described by Poulter,[1,2] to give the title compound as a white solid in 59% yield (27.7 mg, 0.059 mmol). ^{1}H NMR (500 MHz, D_2O) δ 6.08 – 5.98 (m, 1 H), 5.46 (t, 1H, *J* = 7.3 Hz), 5.23 (t, 1 H, J = 6.9), 5.10 (d, 2 H, *J* 47.6), 4.95 (d, 2 H, *J* 47.6), 4.47 (t, 2 H, *J* 6.6), 2.40 – 2.03 (m, 8 H), 1.72 (s, 3 H), 1.62 (s, 3 H); ^{19}F NMR (283 MHz, D_2O) δ -207.11 (t, *J* 47.4), -212.67 (t, *J* 47.5); ^{31}P NMR (202 MHz, D2O) -6.17 (d, *J* 19.0), -10.18 (d, *J* 21). HRMS (ES^-, $[M - H]^-$) found 417.1037, $C_{15}H_{25}O_7F_2P_2$ requires 417.1044.

4. Site-directed mutagenesis. The Quickchange site-directed mutagenesis kit (Stratagene) was used to introduce the desired mutation according to the manufacturer instructions. Plasmids were purified from overnight LB/ampicillin cultures (5 mL) using the QIAGEN miniprep kit as described by the manufacturer. Mutations were confirmed by DNA sequence analysis using Eurofins MWG Operon's DNA sequencing service.

Table S1. Mutagenic primers, with the mutated codon underlined

Name	5′-Sequence-3′
GdolS-Y303F Fwd	CTGGCTCGCGGGTTTTCTCCACTGGGAGTC
GdolS-Y303F Rev	GACTCCCAGTGGAGAAAACCCGCGAGCCAG
GdolS-Y303I Fwd	CTCTGGCTCGCGGGTATTCTCCACTGGGAGTC
GdolS-Y303I Rev	GACTCCCAGTGGAGAATACCCGCGAGCCAGAG
GdolS-E307Q Fwd	GTTACCTCCACTGGCAGTCCCACACCCG
GdolS-E307Q Rev	CGGGTGTGGGACTGCCAGTGGAGGTAAC
GdolS-E307M Fwd	GTTACCTCCACTGGATGTCCCACACCCGC
GdolS-E307M Rev	GCGGGTGTGGGACATCCAGTGGAGGTAAC
GdolS-Y303T Fwd	CTCTGGCTCGCGGGTACCCTCCACTGGGAGTC
GdolS-Y303T Rev	GACTCCCAGTGGAGGGTACCCGCGAGCCAGAG
GdolS-D80E Fwd	CTACTTCCTCTTCGAAGACCAGTTCGACAG
GdolS-D80E Rev	CTGTCGAACTGGTCTTCGAAGAGGAAGTAG
GdolS-D81E Fwd	CCTCTTCGACGAACAGTTCGACAGCC
GdolS-D81E Rev	GGCTGTCGAACTGTTCGTCGAAGAGG
GdolS-D84E Fwd	CGACCAGTTCGAAAGCCCGCTCGGG
GdolS-D84E Rev	CCCGAGCGGGCTTTCGAACTGGTCG
GdolS-N218Q Fwd	CATCCCGTCGTTCACCCAGGACGTGCGCTCCTTC
GdolS-N218Q Rev	GAAGGAGCGCACGTCCTGGGTGAACGACGGGATG
GdolS-S222A Fwd	CCAATGACGTGCGCGCGTTCGCACAGGAGTC
GdolS-S222A Rev	GACTCCTGTGCGAACGCGCGCACGTCATTGG
GdolS-E226D Fwd	CTTCGCACAGGATTCCGAGCGCGGC
GdolS-E226D Rev	GCCGCGCTCGGAATCCTGTGCGAAG
GdolS-N218L Fwd	CATCCCGTCGTTCACCCTGGACGTGCGCTCCTTC
GdolS-N218L Rev	GAAGGAGCGCACGTCCAGGGTGAACGACGGGATG
GdolS-D80N Fwd	GGTTCTACTTCCTCTTCAATGACCAGTTCGACAGCC
GdolS-D80N Rev	GGCTGTCGAACTGGTCATTGAAGAGGAAGTAGAACC
GdolS-D81N Fwd	CTACTTCCTCTTCGACAATCAGTTCGACAGCCCGC
GdolS-D81N Rev	GCGGGCTGTCGAACTGATTGTCGAAGAGGAAGTAG
GdolS-D84N Fwd	GACGACCAGTTCAATAGCCCGCTCGG
GdolS-D84N Rev	CCGAGCGGGCTATTGAACTGGTCGTC
GdolS-N218E Fwd	CATCCCGTCGTTCACCGAAGACGTGCGCTCCTTC
GdolS-N218E Rev	GAAGGAGCGCACGTCTTCGGTGAACGACGGGATG
GdolS-N218T Fwd	CGTCGTTCACCACCGACGTGCGCTCC
GdolS-N218T Rev	GGAGCGCACGTCGGTGGTGAACGACG
GdolS-E248A Fwd	CTGCTCCACCGCAGAGGCCTG
GdolS-E248A Rev	CAGGCCTCTGCGGTGGAGCAG

5. Kinetic Plots.

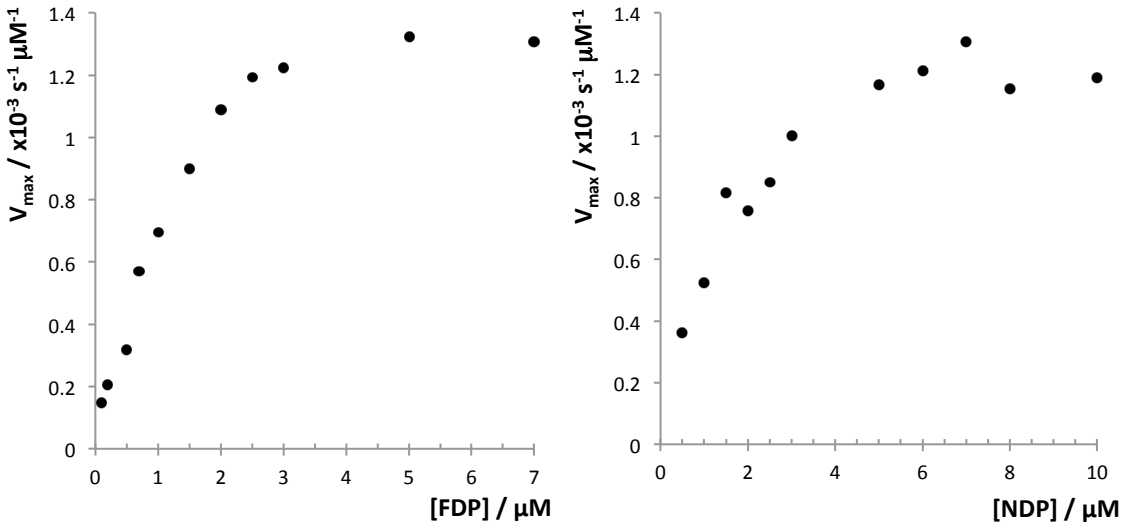


Figure S1. Representative Michaelis-Menten plot of GdolS with FDP (left) and NDP (right)

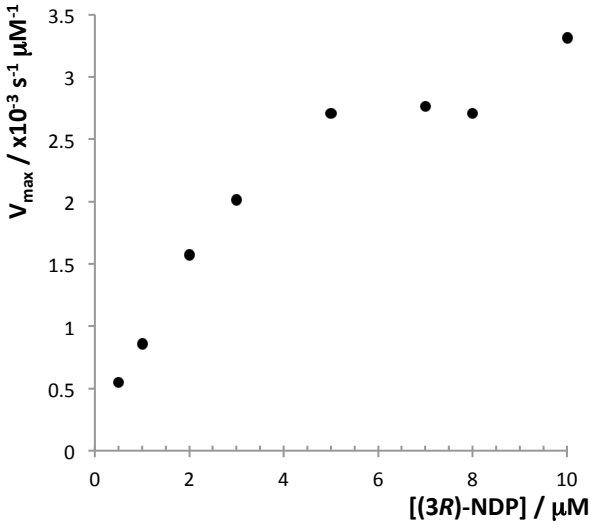


Figure S2. Representative Michaelis-Menten plot of GdolS with (3*R*)-NDP

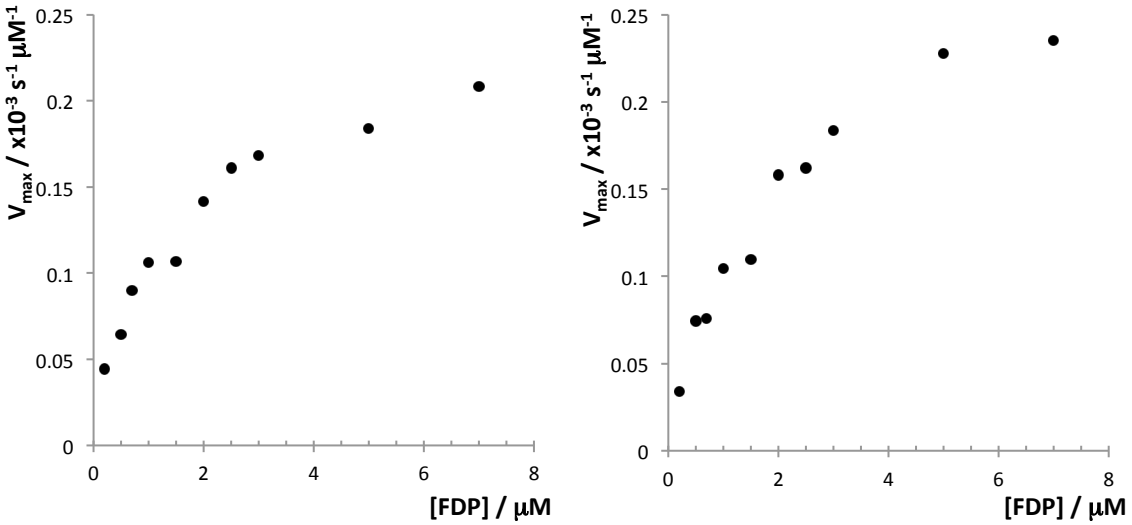


Figure S3. Representative Michaelis-Menten plot of GdolS-D81E and GdolS-D84E with FDP

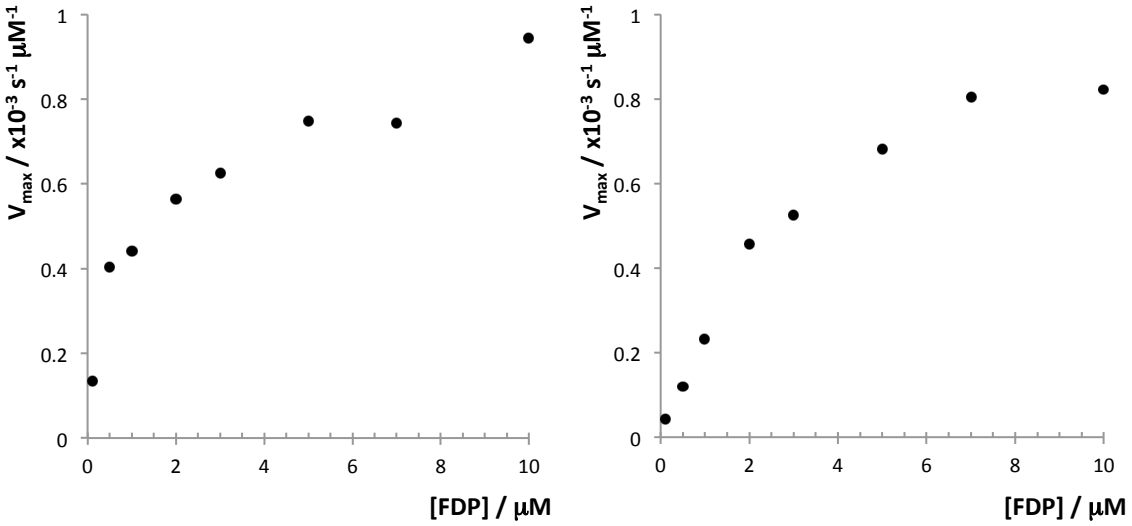


Figure S4. Representative Michaelis-Menten plot of GdolS-Y303F and GdolS-E307Q with FDP

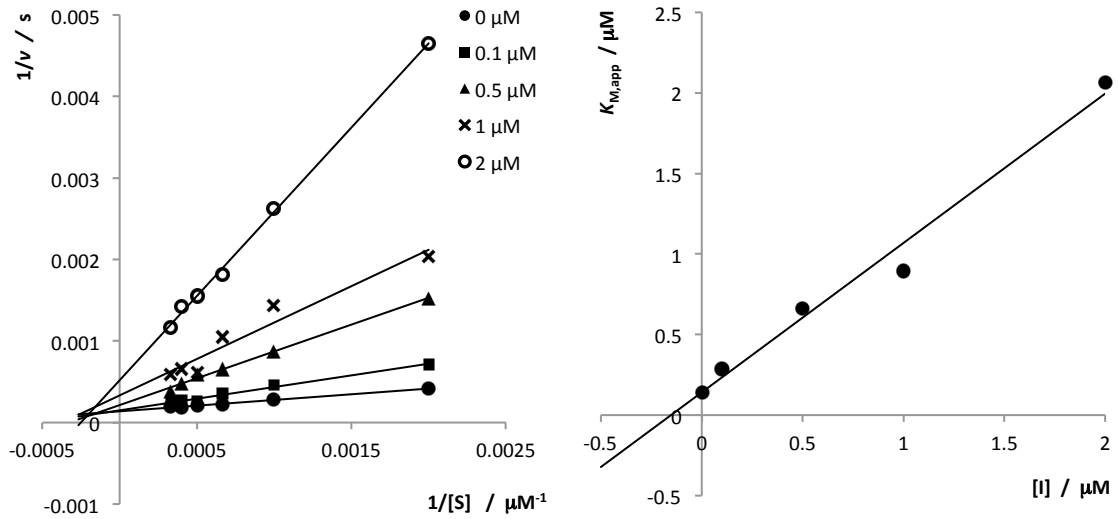


Figure S5. Double reciprocal plot for inhibition of GdolS catalysed turnover of **1a** at varying concentrations of **1b** (left) and plot of $K_{M,app}$ against concentration of inhibitor for calculation of K_i (right).

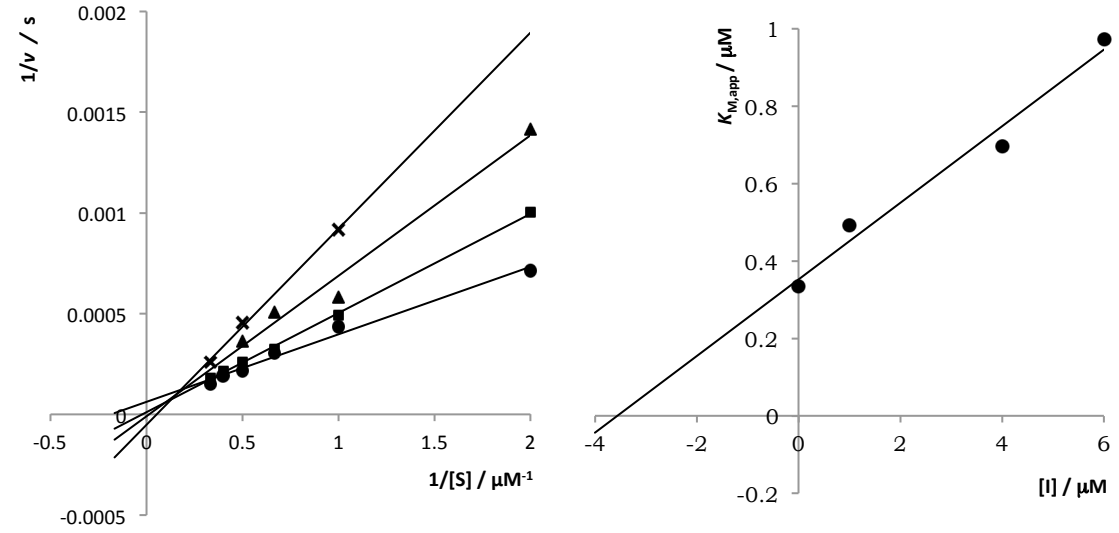


Figure S6. Double reciprocal plot for inhibition of GdolS catalysed turnover of **1a** at varying concentrations of **1c** (left) and plot of $K_{M,app}$ against concentration of inhibitor for calculation of K_i (right).

6. Analytical Incubation of GdolS with isoprenyl diphosphates. A solution of 1 μM GdolS and 200 μM isoprenyl diphosphate in incubation buffer (250 mL, 50 mM Tris, 5 mM βME, 5 mM $MgCl_2$, pH 8.0)was prepared. The aqueous layer was overlaid with HPLC grade pentane (0.5 mL) and the resulting mixture was gently agitated (6 - 18 h) at 25 °C. The incubations were repeated without enzyme as negative controls. The pentane extracts was then analyzed by gas chromatography-mass spectrometry (GC-MS) according to General Methods.

GdolS and FDP were incubated in buffer containing $H_2{}^{18}O$ to study incorporation only, not as kinetic experiments. For the incubation of FDP and GdolS in 50% $H_2{}^{18}O$ buffer (v/v), HEPES buffer (50 mM, pH 7.5, 125 μL) was diluted with $H_2{}^{18}O$ (125 μL). $MgCl_2$, GdolS and FDP in H_20 were added to final concentrations of 2.5 mM, 1 μM and 200 μM, respectively, resulting in a solution of 45 ± 10 mol%

$H_2{}^{18}O$. This resulted in approximately 65% incorporation of ^{18}O into germacradien-4-ol as judged by MS (Fig. S8).

Peaks labelled **δ** and **γ** are non-enzymatic resulting from rearrangement of **2** under mild acid.[14,15] δ-cadinene (**δ**) was identified by comparison with a genuine enzymatic sample;[16] γ-cadinene (γ) was putatively identified from the NIST mass spectra library.[17]

7. GC-MS Collection

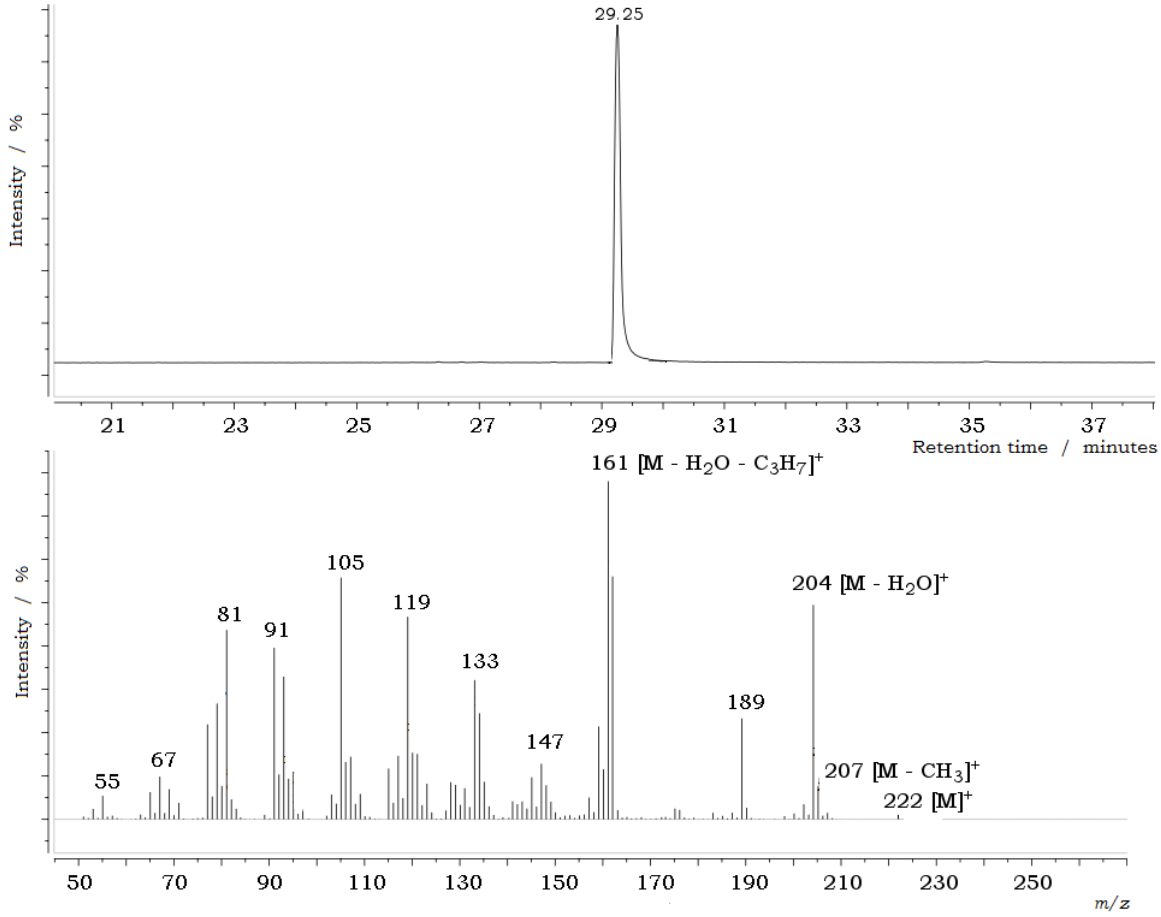


Figure S7. GC-MS (TOF-EI$^+$) analysis of the pentane extracted products of an overnight incubation of GdolS with **1a**. Top, gas chromatogram. Below, mass spectrum (EI$^+$) of the product at 29.25 minutes.

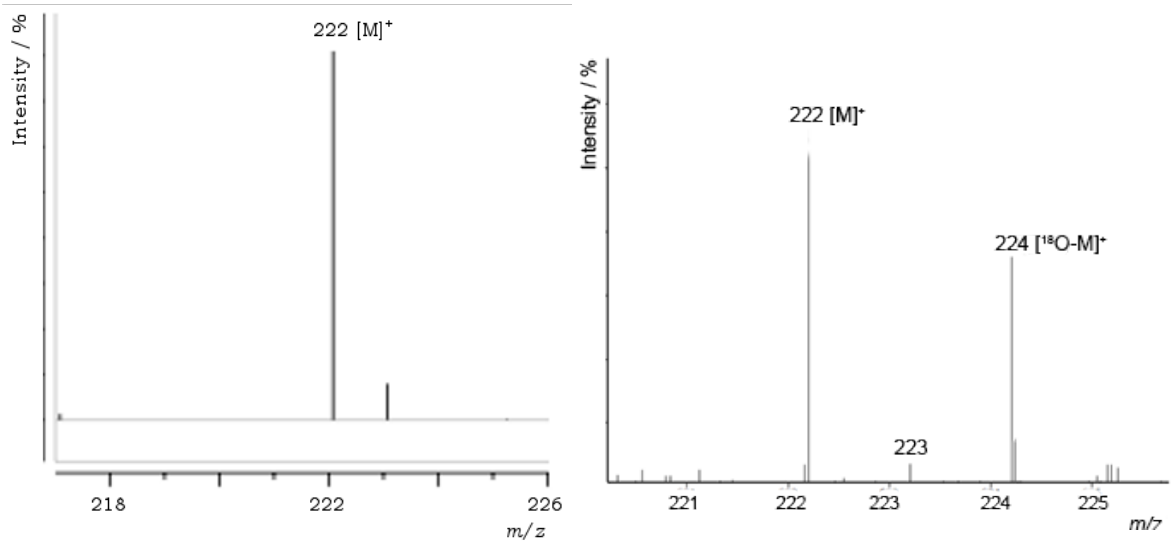


Figure S8. Mass spectra of germacradien-4-ol arising from incubation of GdolS and 1a in H_2O buffer (top left) and 50 % $H_2{}^{18}O$ buffer (top right) Showing expansion of the molecular ion.

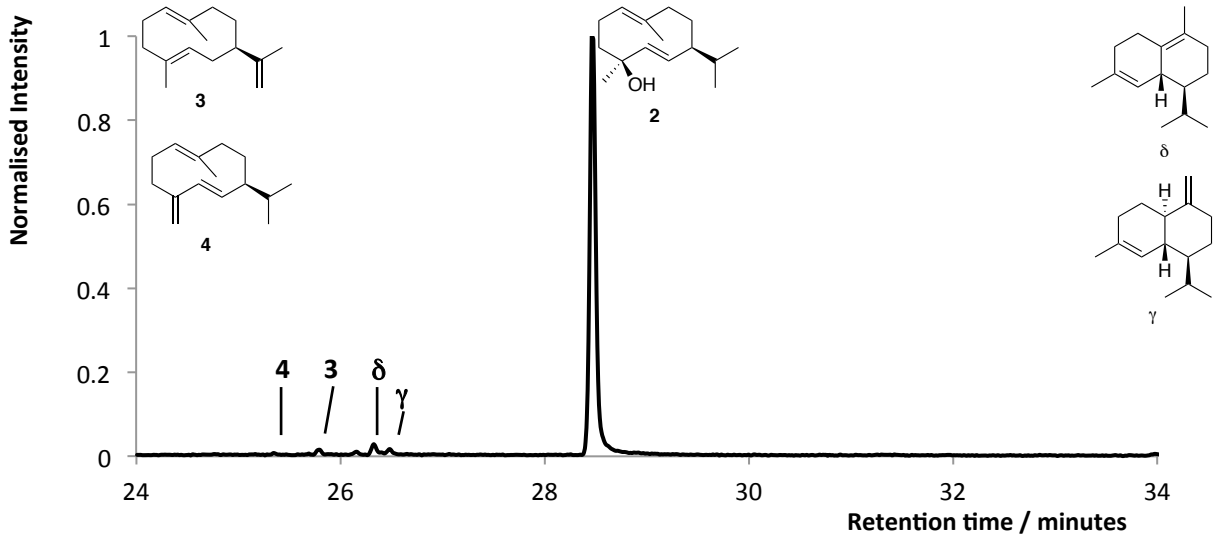


Figure S9. Total ion chromatogram (TOF-EI+) of the pentane extracted products of an overnight incubation of GdolS-D80E with **1a**.

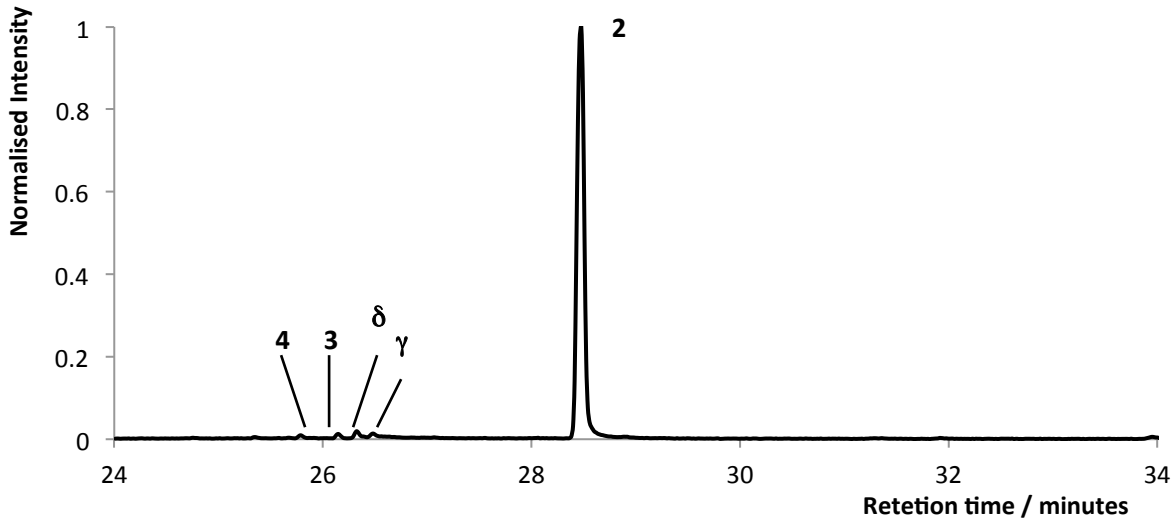


Figure S10. Total ion chromatogram (TOF-EI$^+$) of the pentane extractable products of an overnight incubation of GdolS-D81E with **1a**.

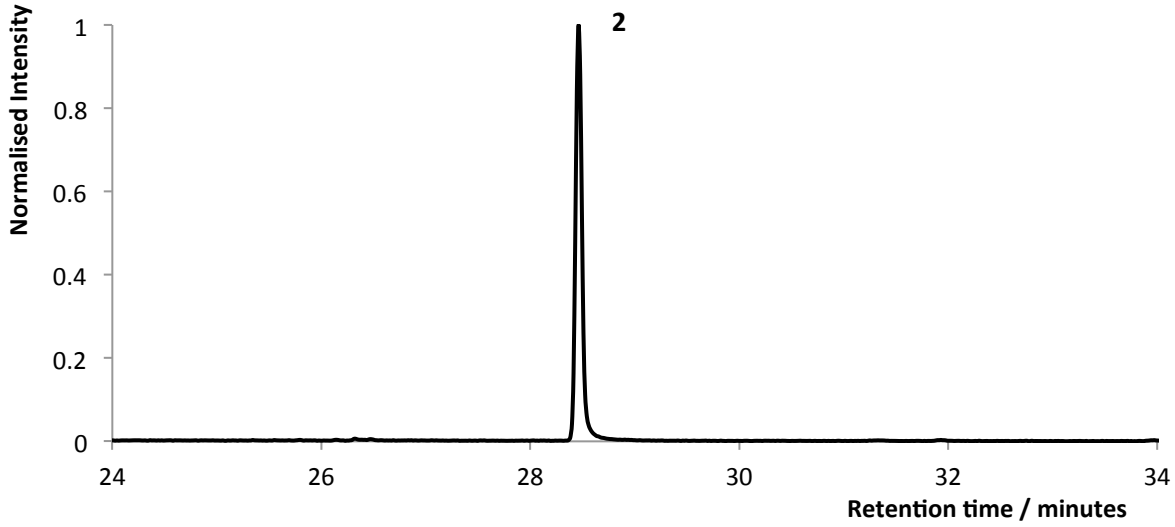


Figure S11. Total ion chromatogram (TOF-EI$^+$) of the pentane extractable products from an overnight incubation of GdolS-D81N with **1a**.

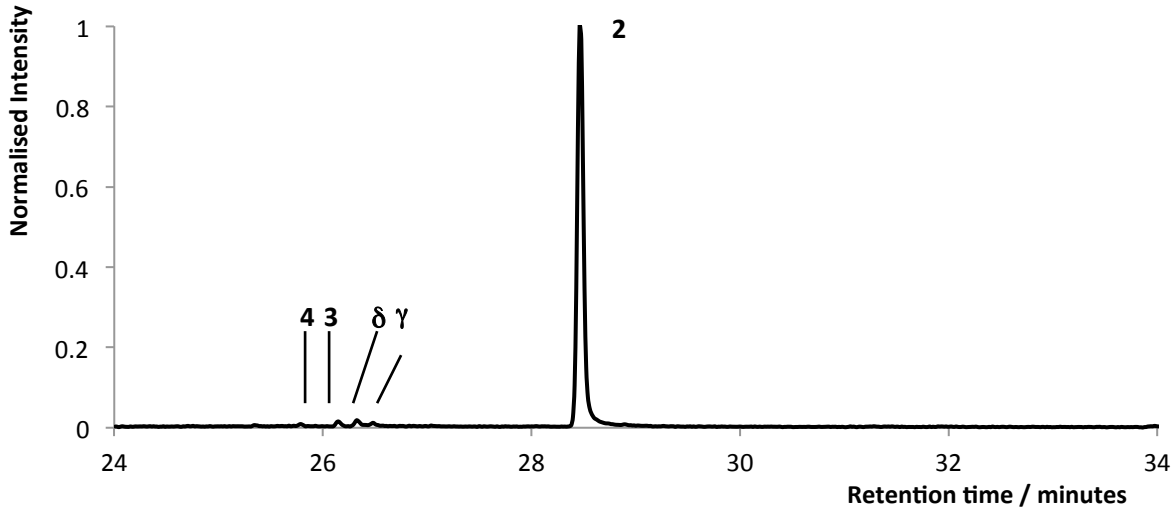


Figure S12. Total ion chromatogram (TOF-EI+) of the pentane extractable products from an overnight incubation of GdolS-D84E with **1a**.

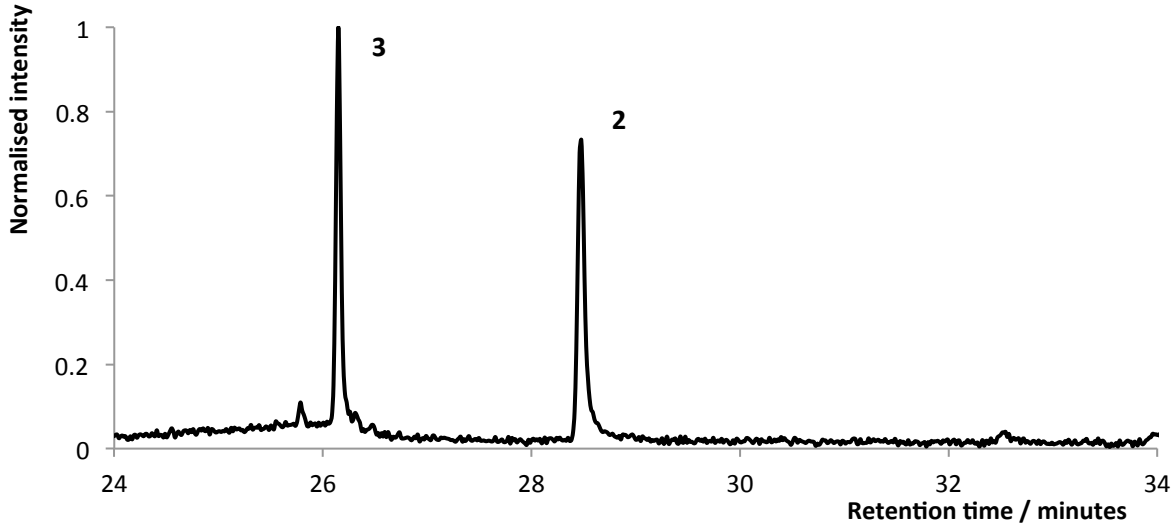


Figure S13. Total ion chromatogram (TOF-EI+) of the pentane extractable products from an overnight incubation of GdolS-N218Q with **1a**.

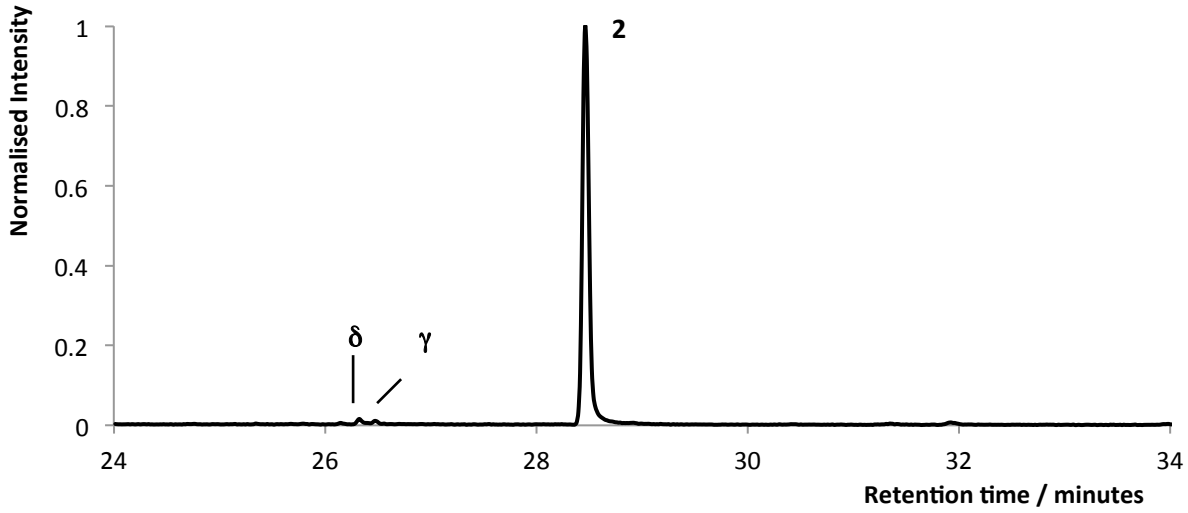


Figure S14. Total ion chromatogram (TOF-EI+) of the pentane extractable products from an overnight incubation of GdolS-N218L with **1a**.

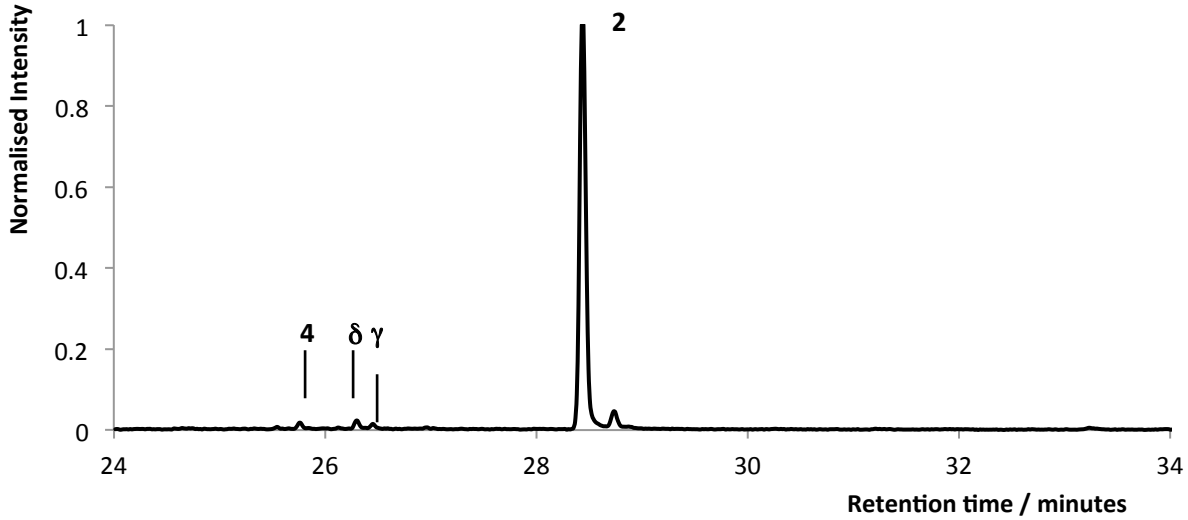


Figure S15. Total ion chromatogram (TOF-EI+) of the pentane extracted products of an overnight incubation of GdolS-S222A with **1a**.

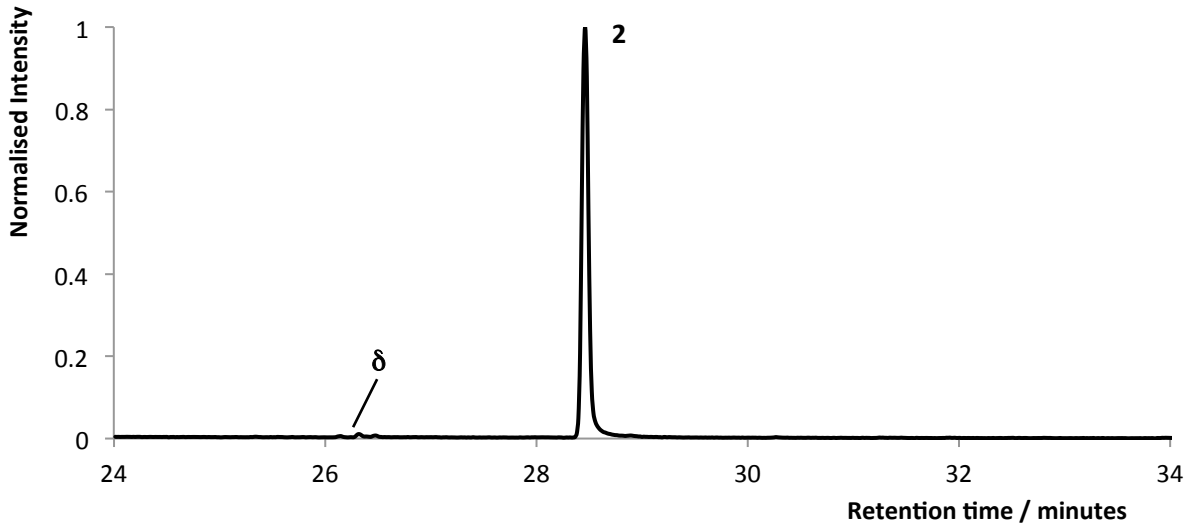


Figure S16. Total ion chromatogram (TOF-EI$^+$) of the pentane extracted products of an overnight incubation of GdolS-E226D with **1a**.

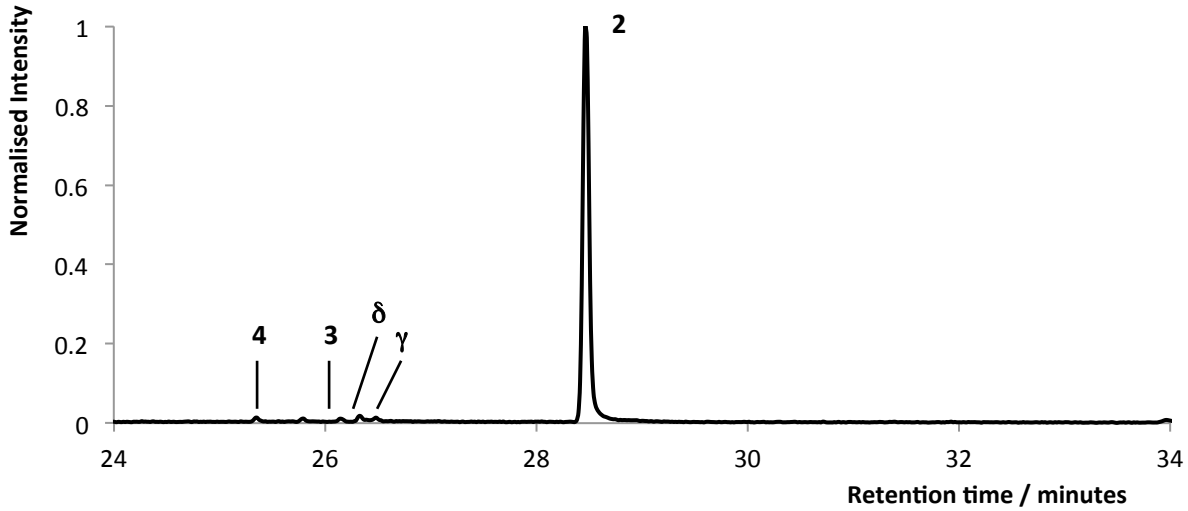


Figure S17. Total ion chromatogram (TOF-EI$^+$) of the pentane extracted products of an overnight incubation of GdolS-E307Q with **1a**.

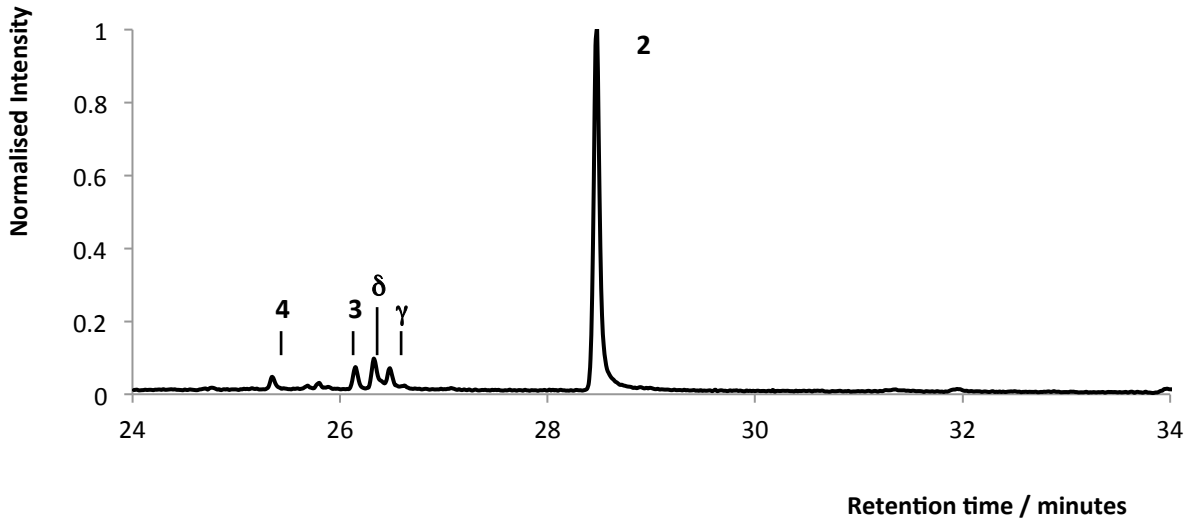


Figure S18. Total ion chromatogram (TOF-EI+) of the pentane extracted products of an overnight incubation of GdolS-E307M with **1a**.

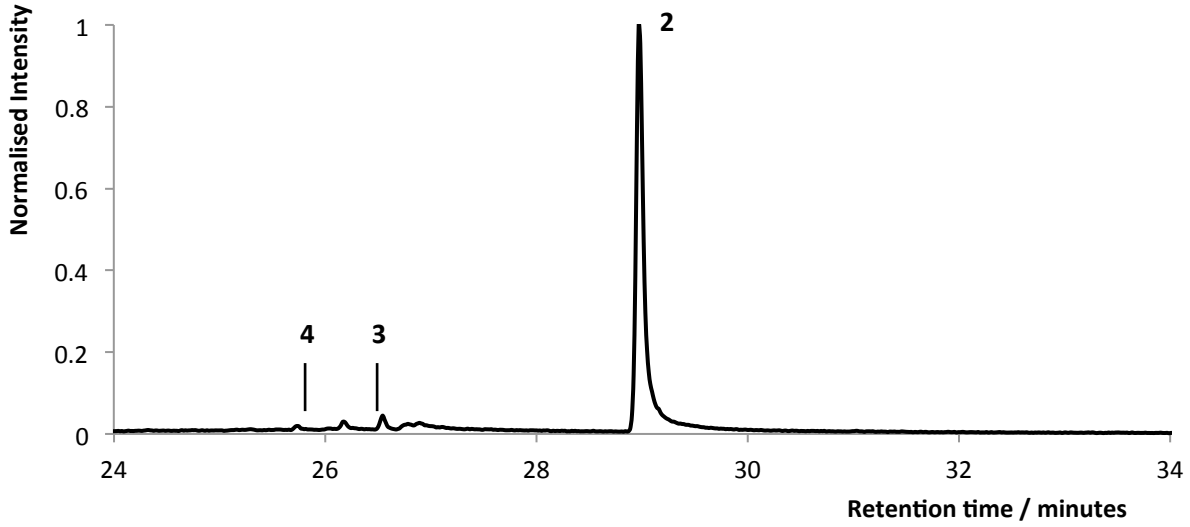


Figure S19. Total ion chromatogram (TOF-EI+) of the pentane extracted products of an overnight incubation of GdolS-Y303F with **1a**.

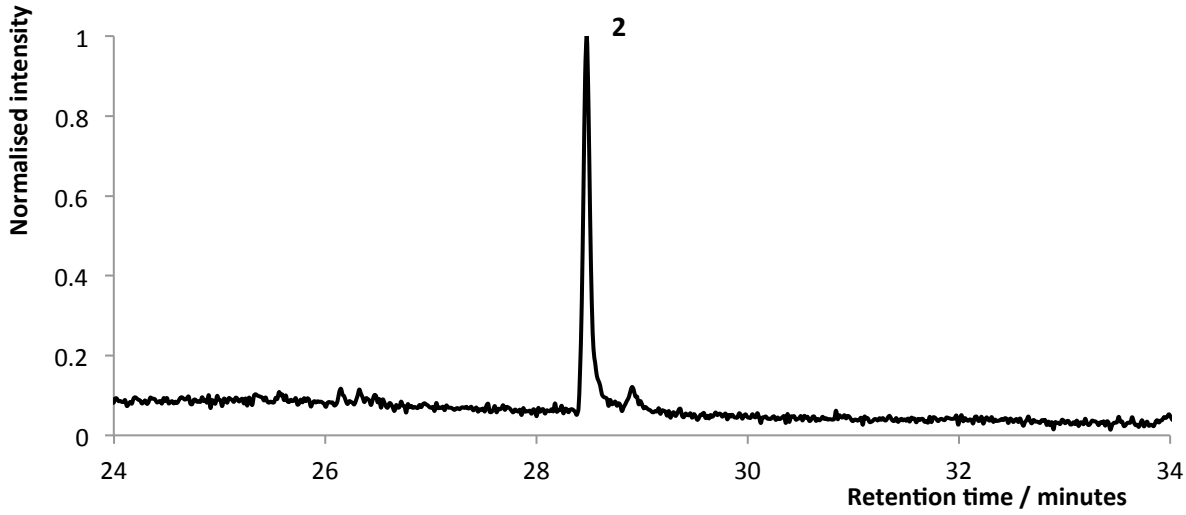


Figure S20. Total ion chromatogram (TOF-EI$^+$) of the pentane extracted products of an overnight incubation of GdolS-Y303I with **1a**.

8. 12,13-difluoro-(*E*)-β-farnesene

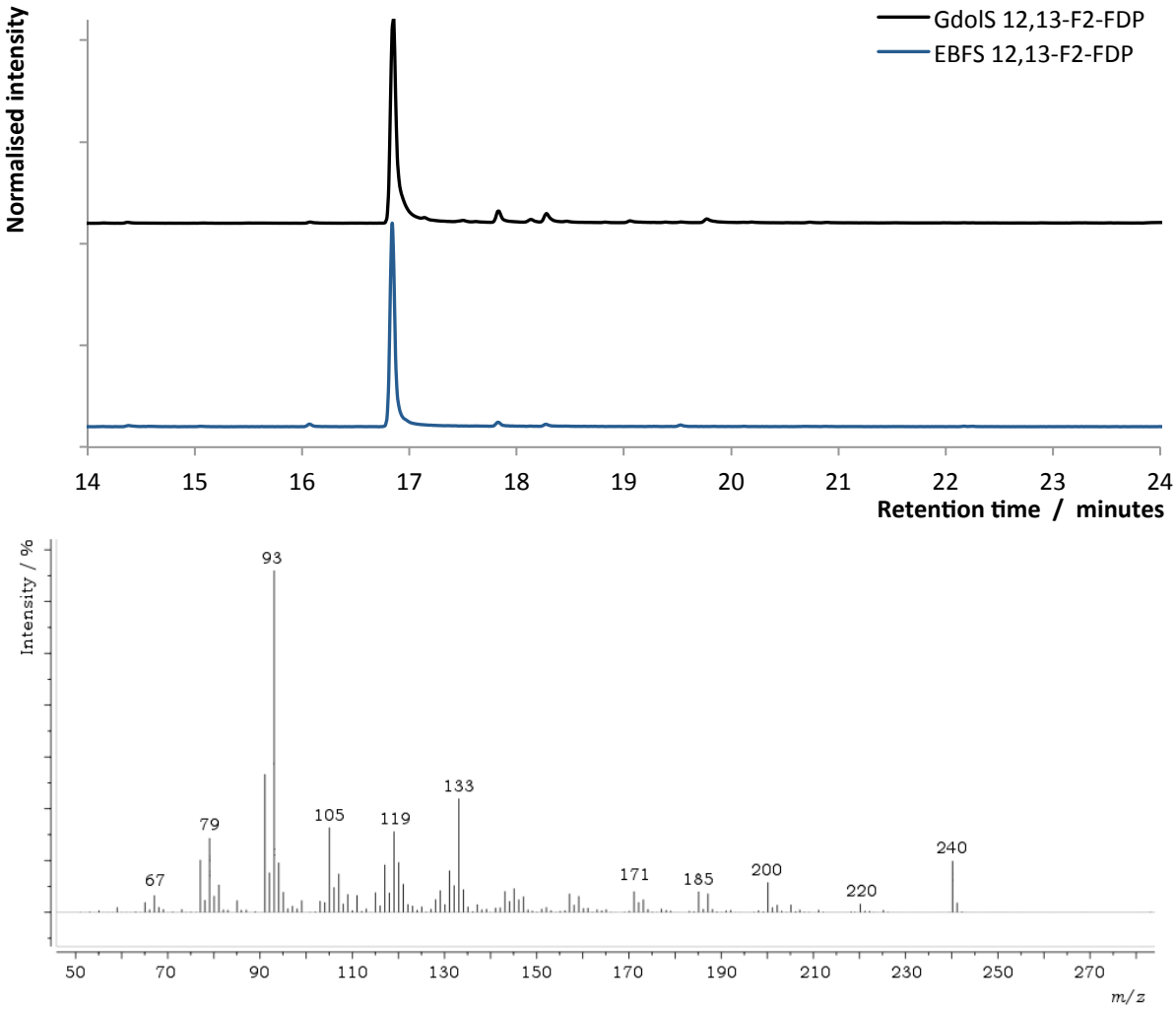


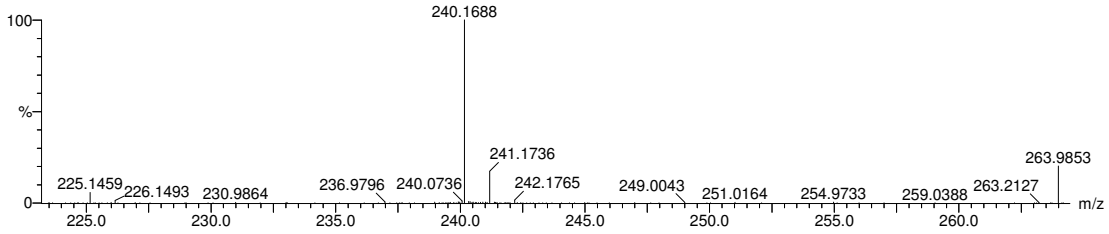
Figure S21. Total ion chromatogram (TOF-EI$^+$) of the pentane extracted products of overnight incubations of GdolS (Black) and (*E*)-β-farnesene synthase[18] (EBFS, blue) with **1d**. Top, Gas chromatogram. Below, mass spectrum of the 12,13-F_2-farnesene.

Elemental Composition Report **Page 1**

Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
4 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-15 H: 0-22 19F: 0-3

17-Jun-2015 DG School of Chemistry Cardiff University
DG_EBFS_12,13-F2_170615 743 (16.823) TOF MS EI+
6.31e+003



Minimum:				-1.5		
Maximum:		5.0	10.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
240.1688	240.1690	-0.2	-0.8	4.0	0.4	C15 H22 19F2

Figure S22. HR-MS (TOF-EI+) of 12,13-difluoro-(*E*)-β-farnesene.

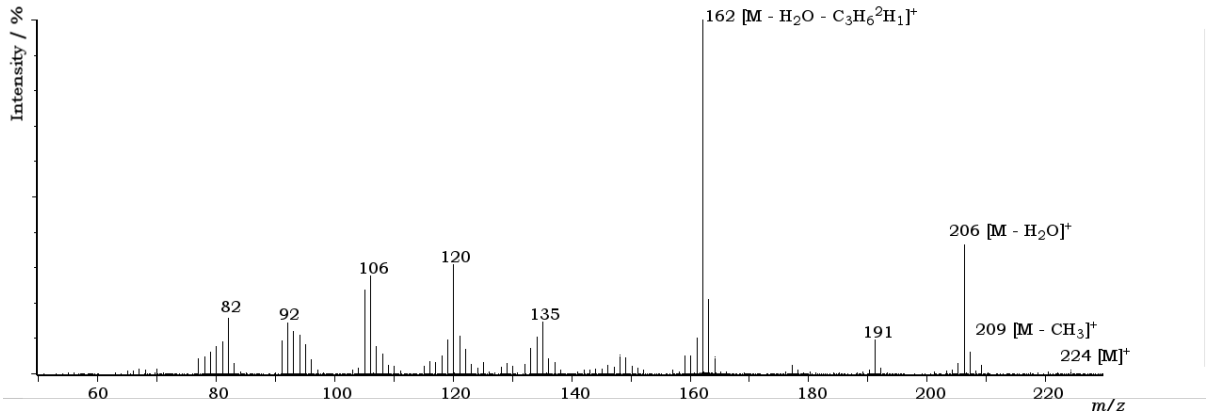


Figure S23. Mass spectrum (TOF-EI+) of **2** arising from incubation of GdolS and **1e**.

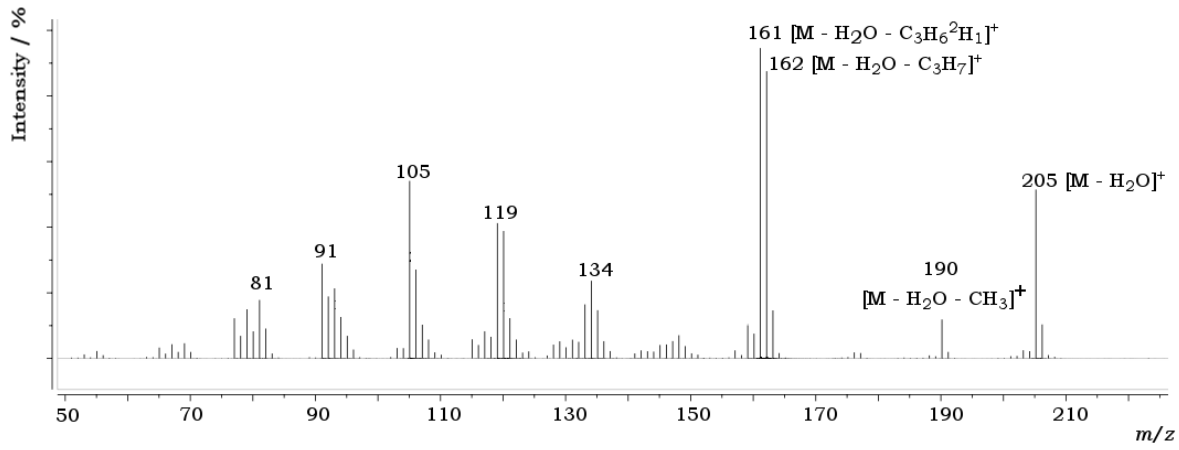


Figure S24. Mass spectrum (TOF-EI$^+$) of **2** arising from incubation of GdolS and **1f**.

9. References

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