Supporting information

Structural basis of catalysis in the bacterial monoterpene synthases linalool synthase and 1,8-cineole synthase

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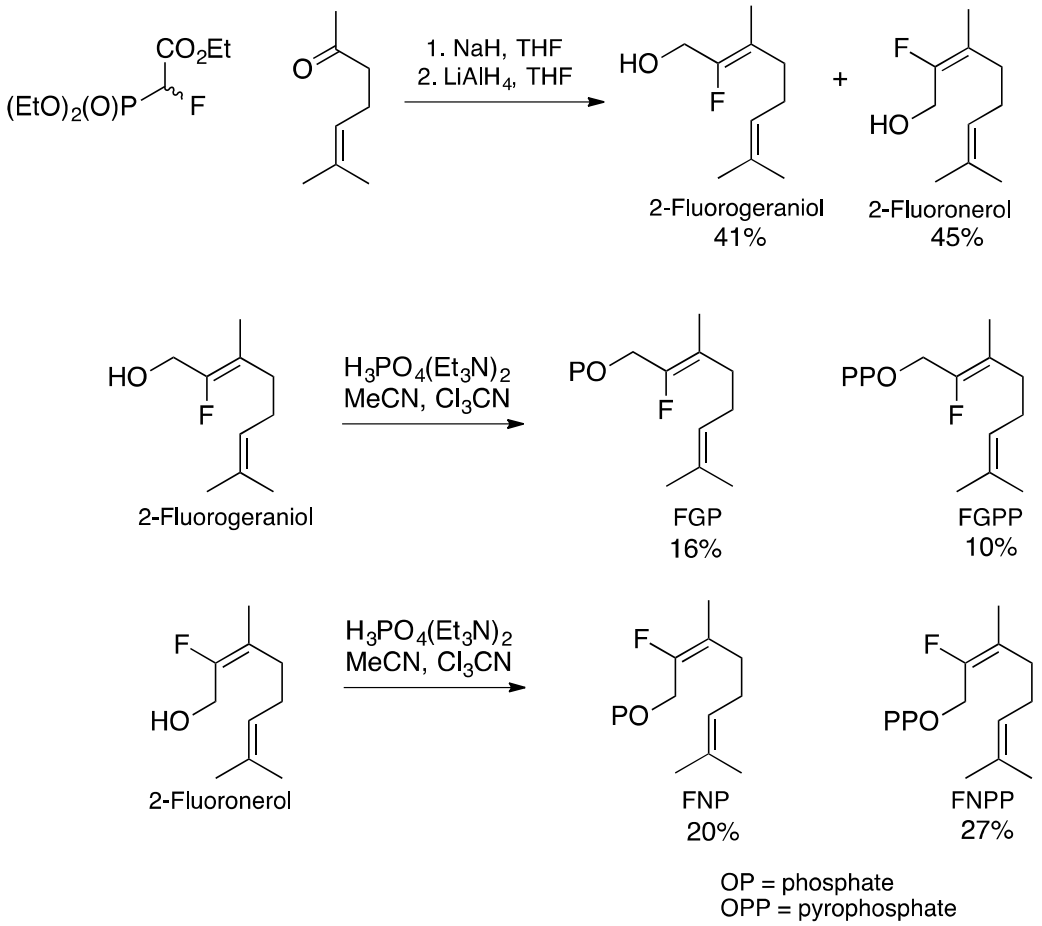
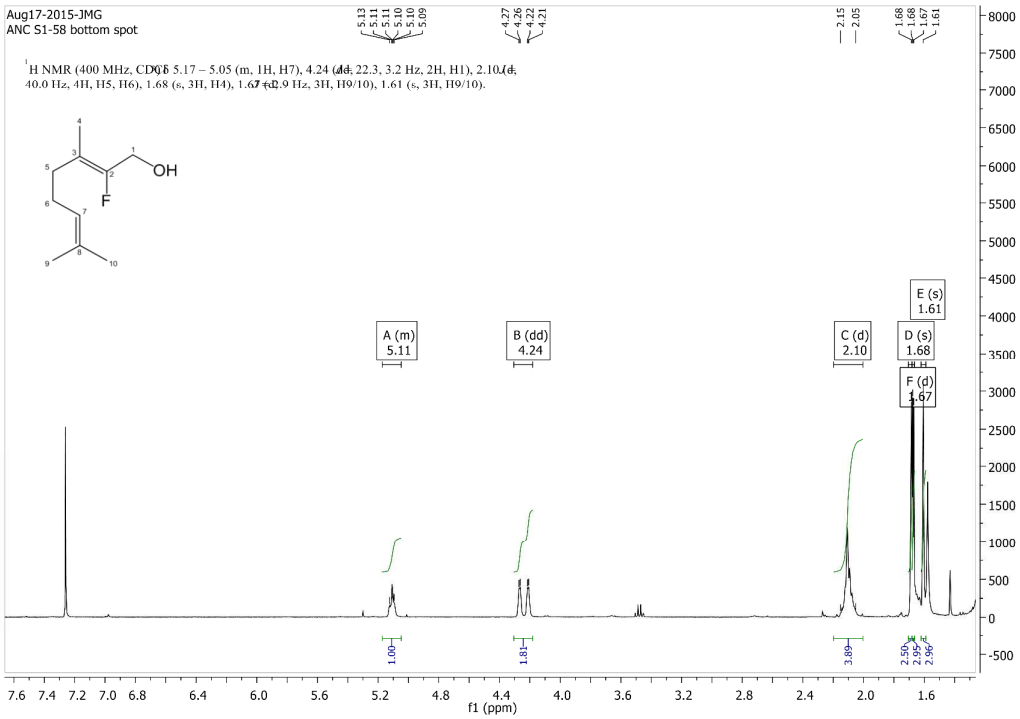
Extended EPR results and discussion

Comparisons of EPR spectra of Mn^{2+} ion substituted bCinS/bLinS with and without FGPP along with standard $MnCl_2$ samples show a clearly resolved multiplet structure for 1:1 bCinS/bLinS: $MnCl_2$ in the presence of FGPP. The observation of a multiplet structure indicates that degeneracy of the six-line EPR spectrum, often associated with free/unbound Mn^{2+} ion, was either lifted by the strong zero-field splitting of the Mn^{2+} ion or the '*g*-anisotropy' or combination of both (**Figure 3**; black asterisks). The intense EPR transitions centered around g ~ 2.0 (**Figure 3**; black/blue asterisks) were mainly due to the transitions from $|m_s, -½\rangle \rightarrow |m_s, +½\rangle$ manifold. The weak hyperfine structures that appear above and below the $g \sim 2.0$ region (**Figure 3**; blue arrows) are due to the transitions between the higher spin manifolds, $|m_s, -5/2\rangle \rightarrow |m_s, -3/2\rangle$ and $|m_s, -3/2\rangle \rightarrow |m_s, -½\rangle$ respectively. The multiplet structure observed for bCinS and bLinS directly probes the local/first coordination sphere of the Mn^{2+} ion, which was clearly different from the reference samples $[Mn(H_2O)_6]^{2+}$ (black dotted spectrum) and bCinS/bLinS without FGPP (black dashed spectrum). The aforementioned two samples predominantly showed a six-line EPR pattern with half-field transitions in the range of 80-150 mT, whereas for 1:1 bCinS/bLinS-FGPP: $MnCl_2$, it occurred between 120-180 mT and trailed towards high magnetic field (**Figures S6 and S7**). The intensity of the multiplet structure and the half-field transitions between 120-180 mT (**Figures S6 and S7**; red downward arrows) remained as a plateau for the samples when the concentration of the Mn^{2+} ion relative to bCinS-FGPP/bLinS-FGPP was more than 3. The presence of additional equivalents of Mn^{2+} ion was observed as free/unbound Mn^{2+} ion, which predominantly contributes to the six-line EPR pattern around the $g = 2$ region (**Figure 3**; black asterisks). In addition, new half-field transitions were observed at low magnetic fields between 80-150 mT (**Figures S6 and S7**; blue arrows) and the intensity increased linearly. The observed half-field transitions were consistent with the free/unbound Mn^{2+} ion EPR signal. All these observations are consistent with bCinS and bLinS having 3 metal binding sites.

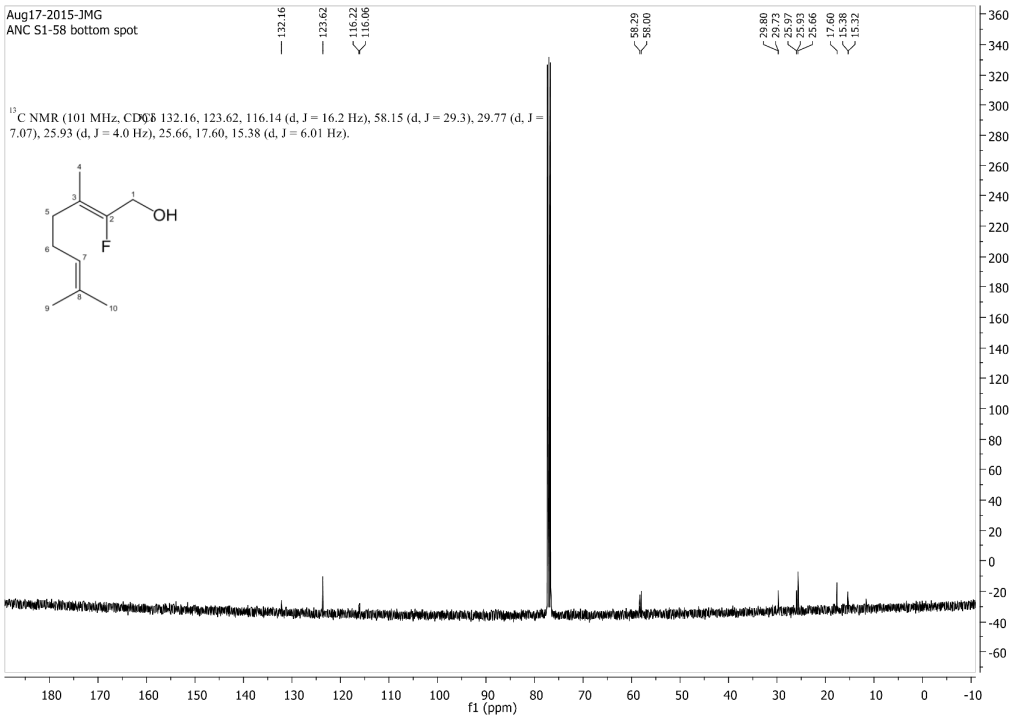


Scheme S1: Synthesis of FGP, FGPP, FNP and FNPP.

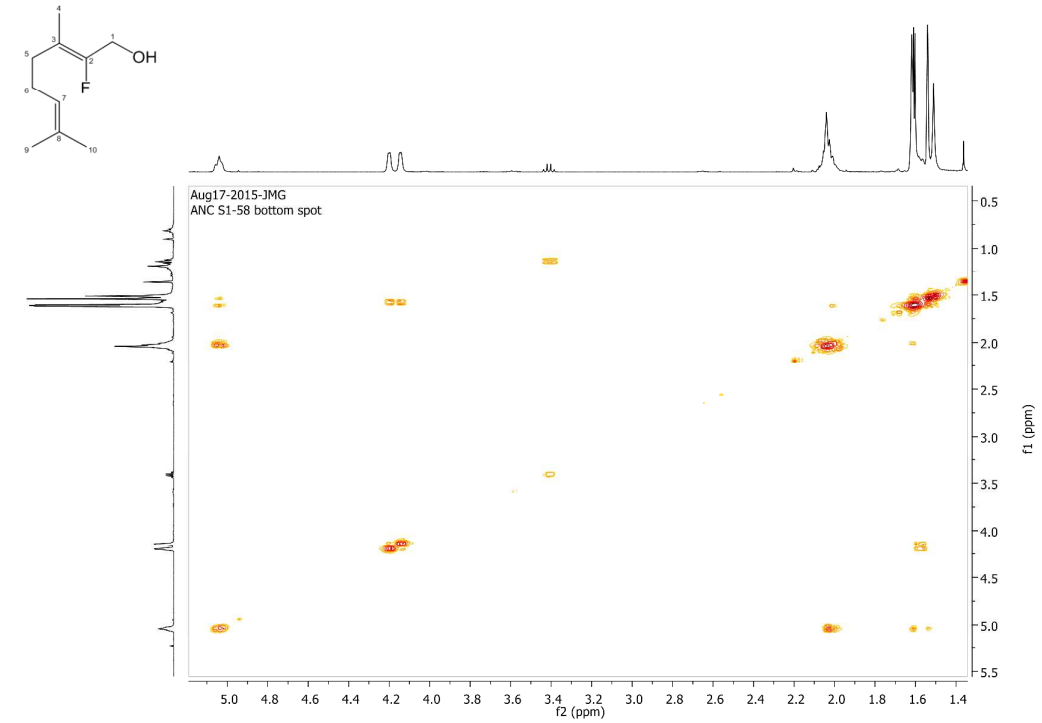
A


Aug17-2015-JMG
ANC S1-58 bottom spot
A (m) 5.11
B (dd) 4.24
C (d) 2.10
D (s) 1.68
E (s) 1.61
F (d) 1.67
f1 (ppm)

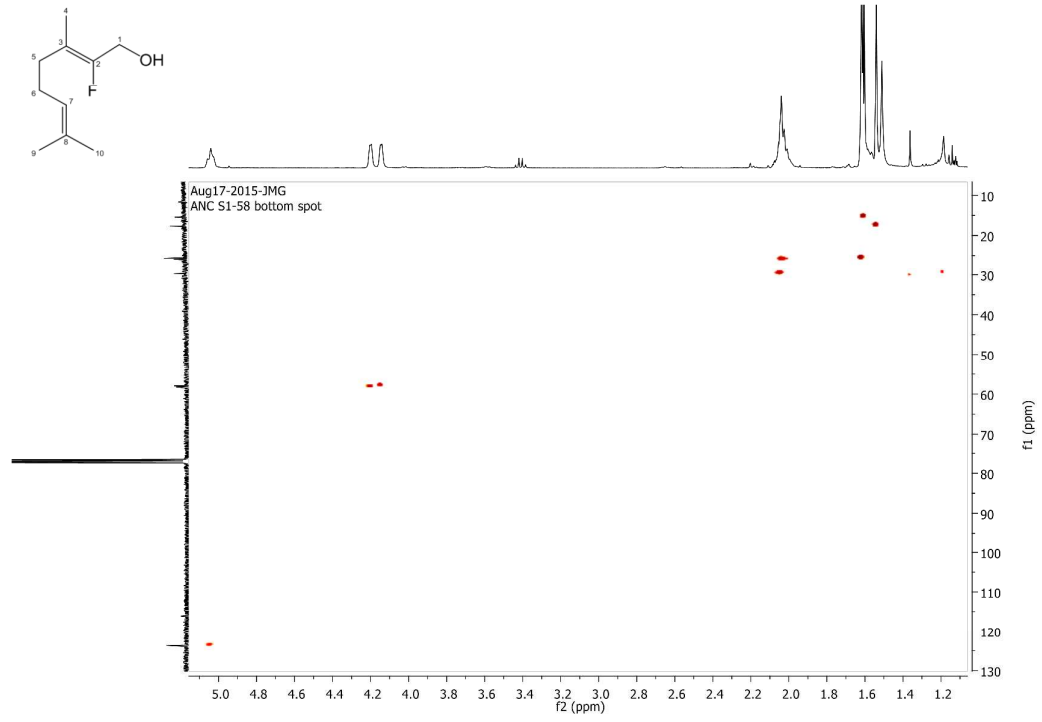
B


Aug17-2015-JMG
ANC S1-58 bottom spot
f1 (ppm)

C


OH
F
Aug17-2015-JMG
ANC S1-58 bottom spot
f2 (ppm)
f1 (ppm)

D


OH
F
Aug17-2015-JMG
ANC S1-58 bottom spot
f2 (ppm)
f1 (ppm)

E

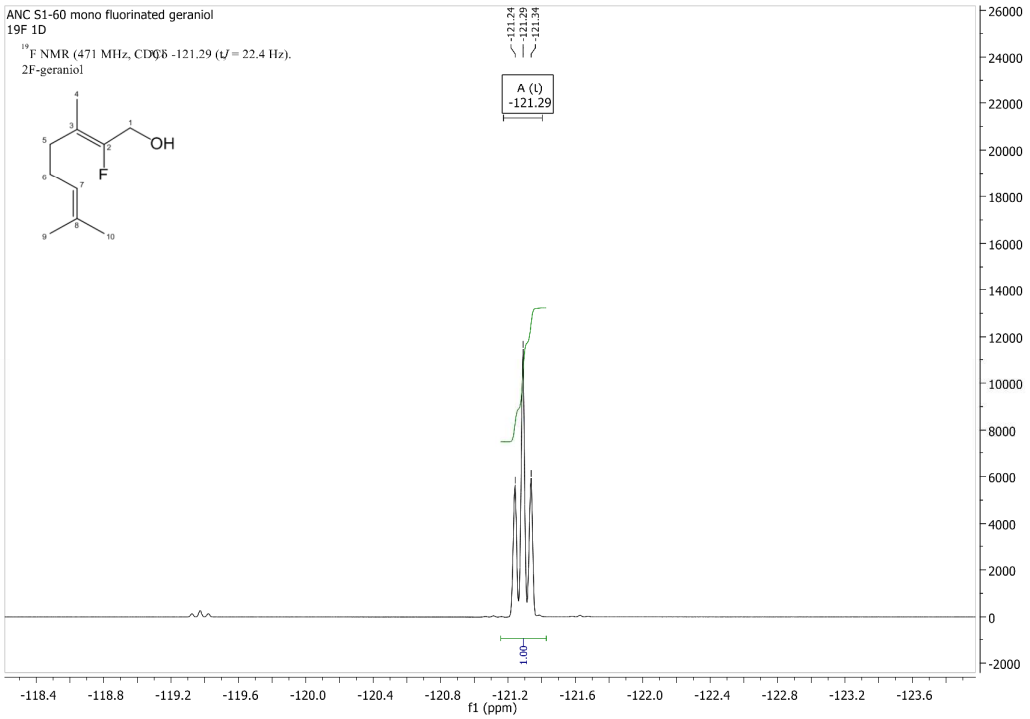
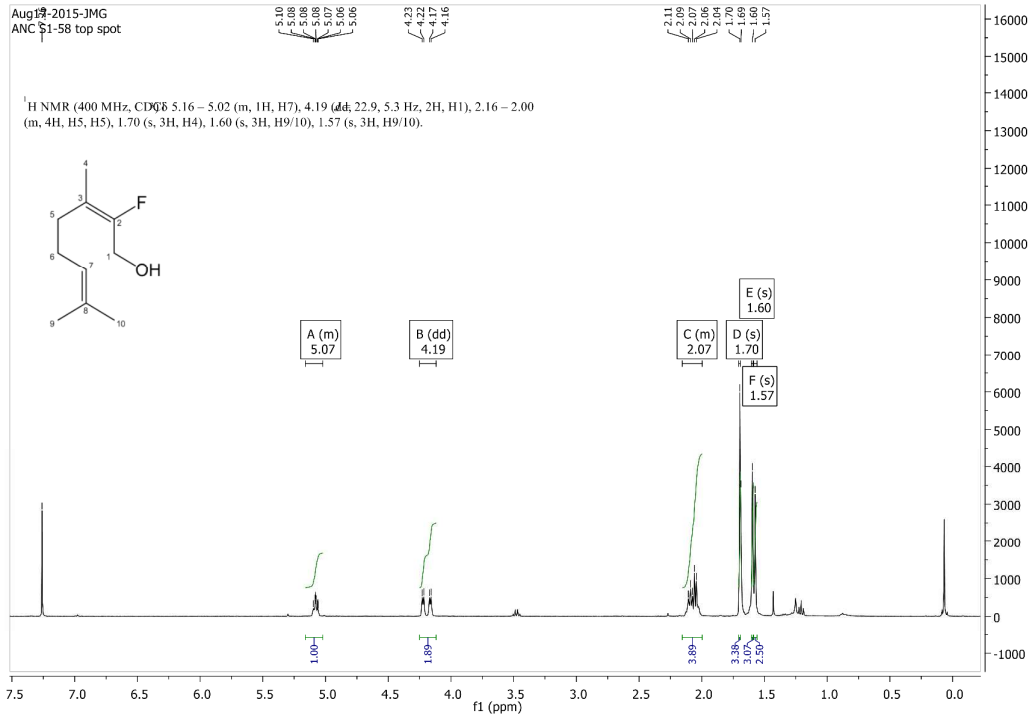
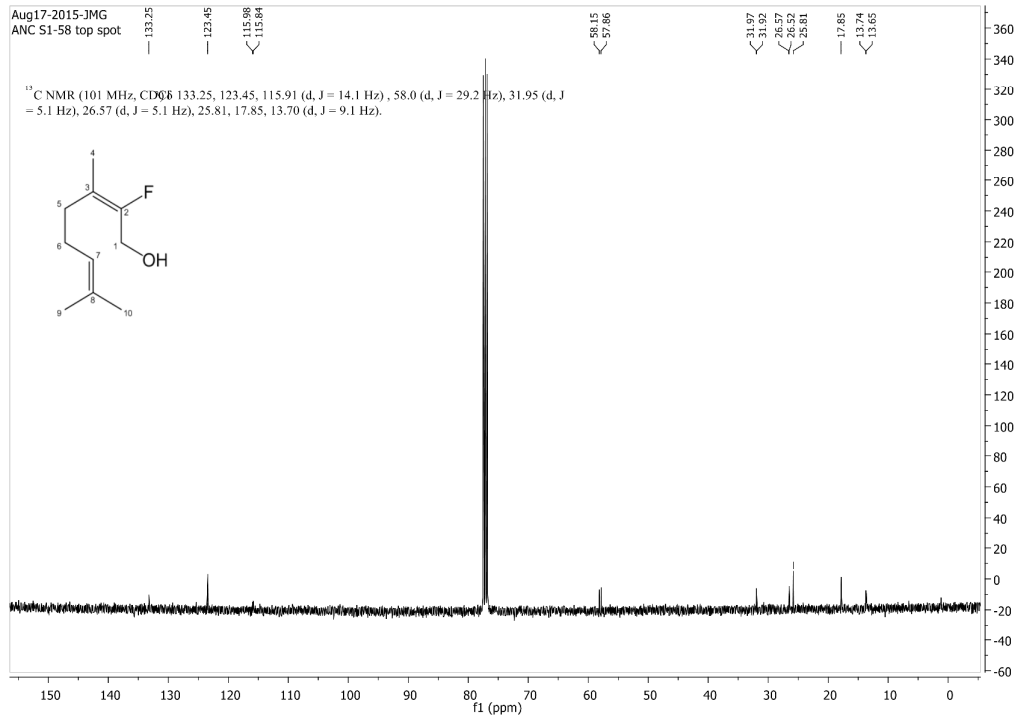


Figure S1. NMR spectra of 2-fluorogeraniol. A) ^{1}H NMR. B) ^{13}C NMR. C) ^{1}H - ^{1}H COSY. D) ^{1}H -^{13}C HSQC. E) ^{19}F NMR.

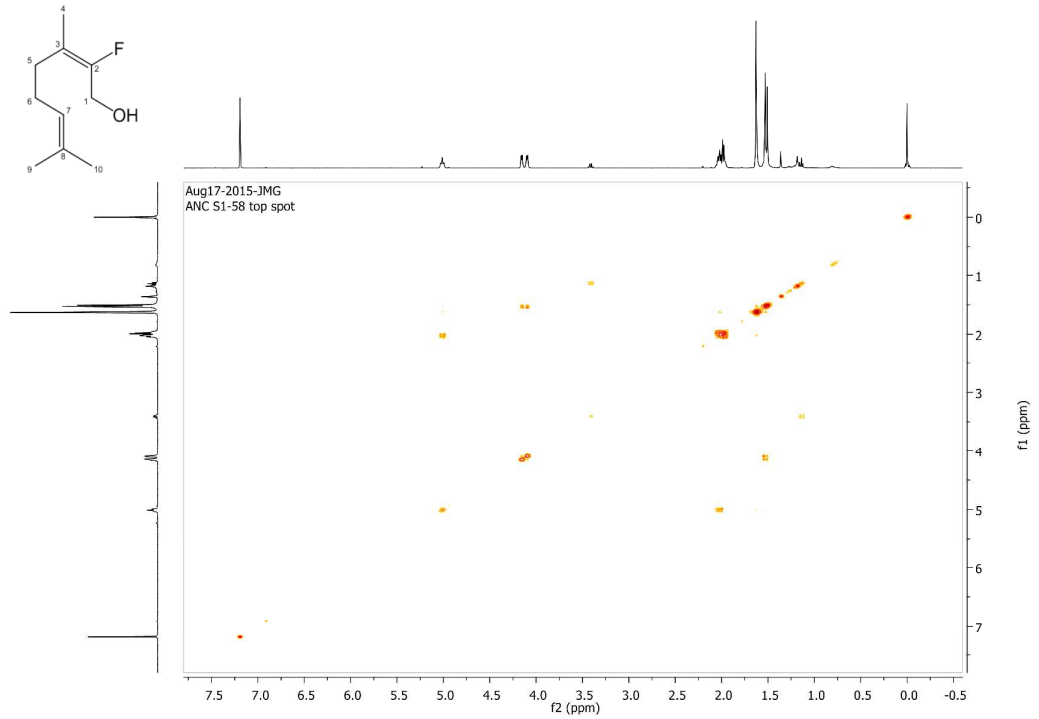
A


Aug17-2015-JMG
ANC S1-58 top spot
5.10
5.08
5.08
5.08
5.07
5.06
5.06
4.23
4.22
4.17
4.16
2.11
2.09
2.07
2.06
2.04
1.70
1.69
1.60
1.57
A (m) 5.07
B (dd) 4.19
C (m) 2.07
D (s) 1.70
E (s) 1.60
F (s) 1.57
1.00
1.89
3.89
3.38
3.07
2.50
f1 (ppm)

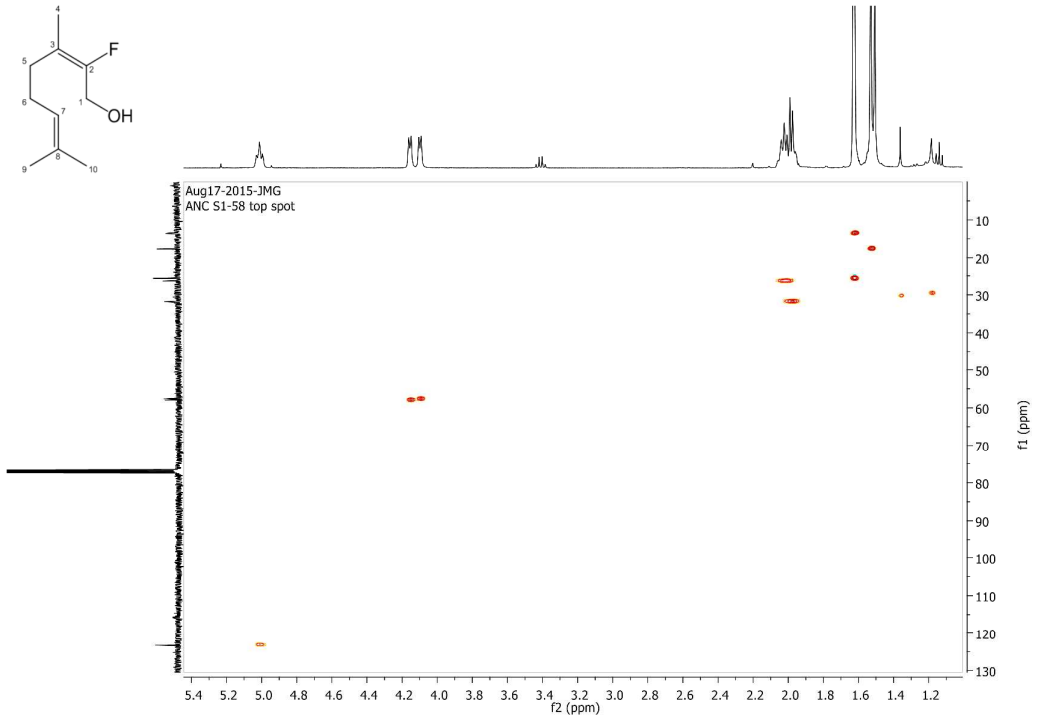
B


Aug17-2015-JMG
ANC S1-58 top spot
133.25
123.45
115.98
115.84
58.15
57.86
31.97
31.92
26.57
26.52
25.81
17.85
13.74
13.65
f1 (ppm)

C


F
OH
Aug17-2015-JMG
ANC S1-58 top spot
f2 (ppm)
f1 (ppm)

D


F
OH
Aug17-2015-JMG
ANC S1-58 top spot
f2 (ppm)
f1 (ppm)

E

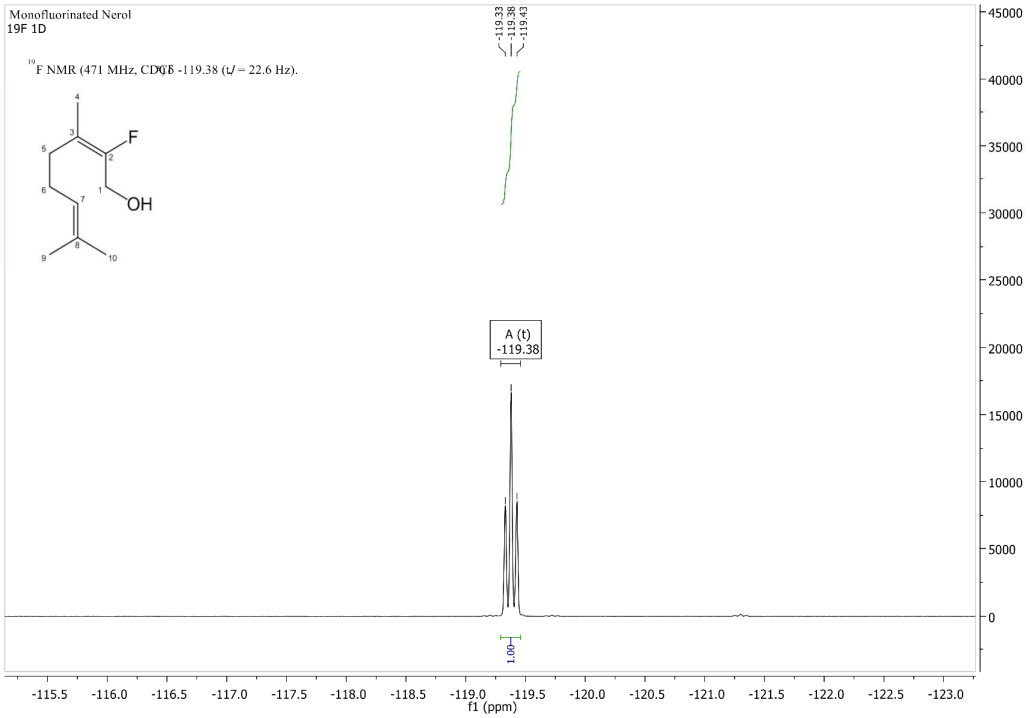
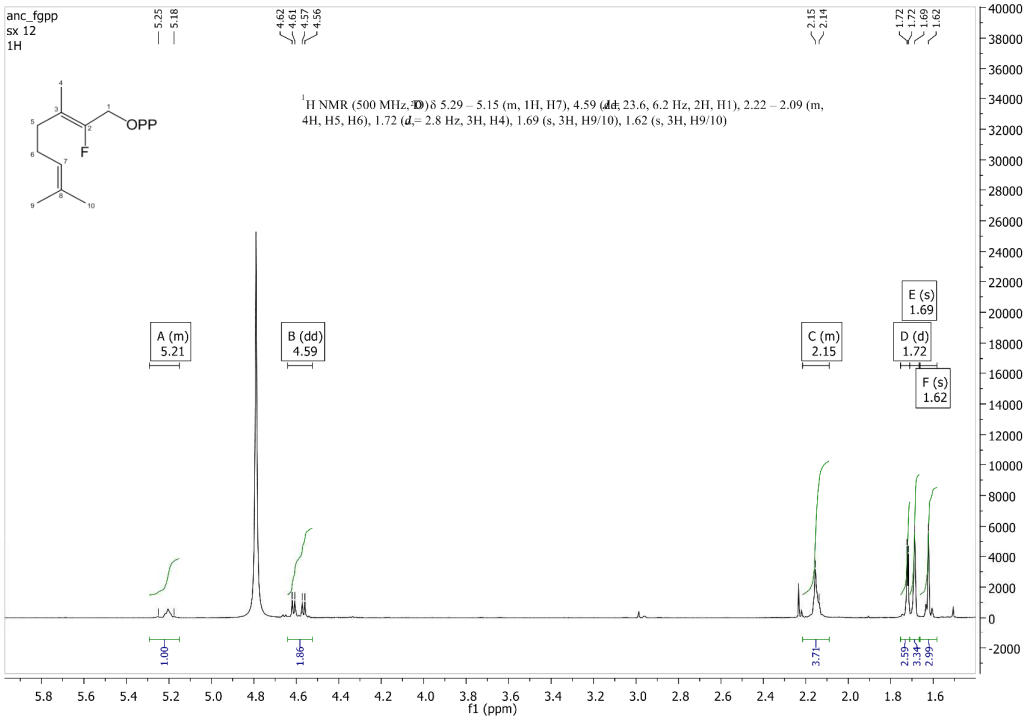


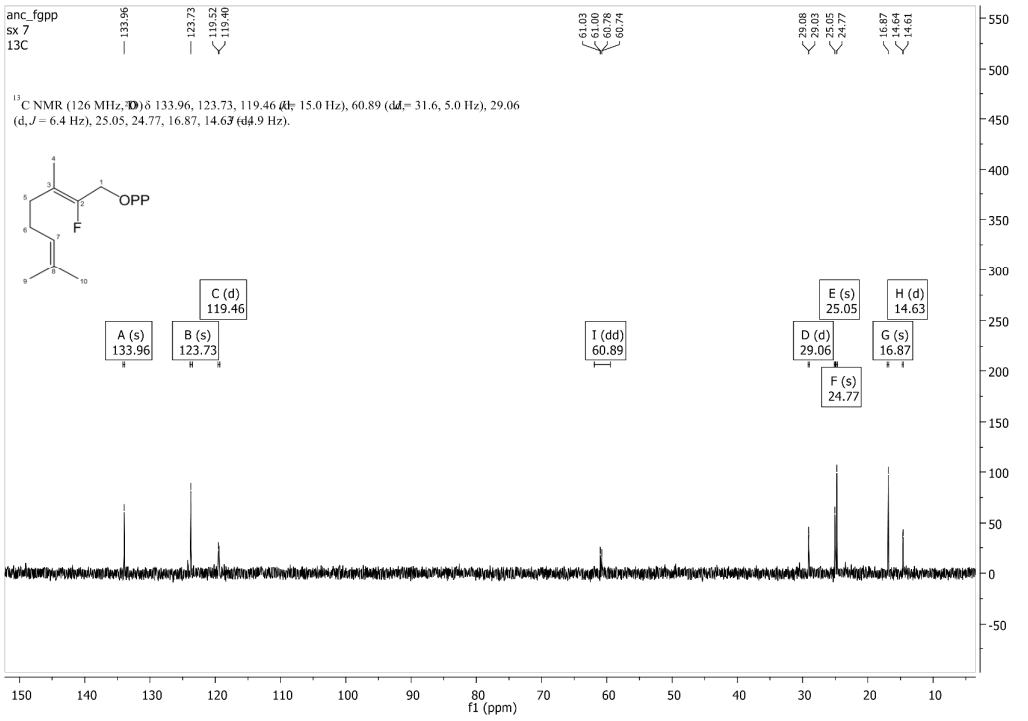
Figure S2. NMR spectra of 2-fluoronerol. A) ^{1}H NMR. B) ^{13}C NMR. C) ^{1}H -^{1}H COSY. D) ^{1}H -^{13}C HSQC. E) ^{19}F NMR.

A



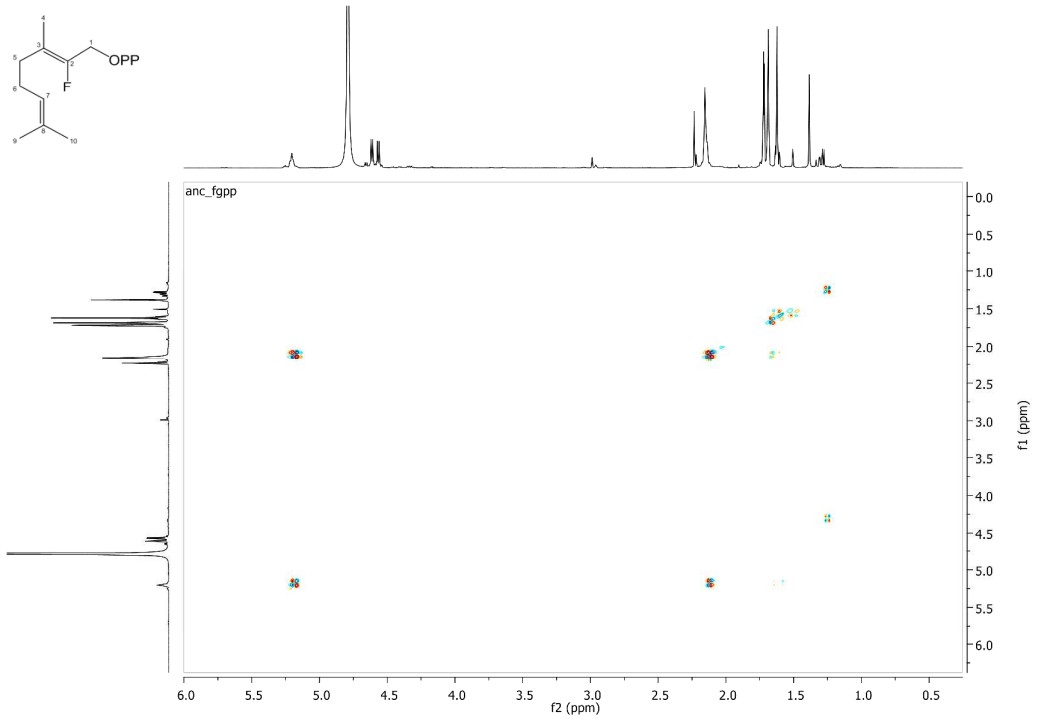
^{1}H NMR (500 MHz, D$_2$O) δ 5.29 – 5.15 (m, 1H, H7), 4.59 (dd, *J* = 23.6, 6.2 Hz, 2H, H1), 2.22 – 2.09 (m, 4H, H5, H6), 1.72 (d, *J* = 2.8 Hz, 3H, H4), 1.69 (s, 3H, H9/10), 1.62 (s, 3H, H9/10)

B

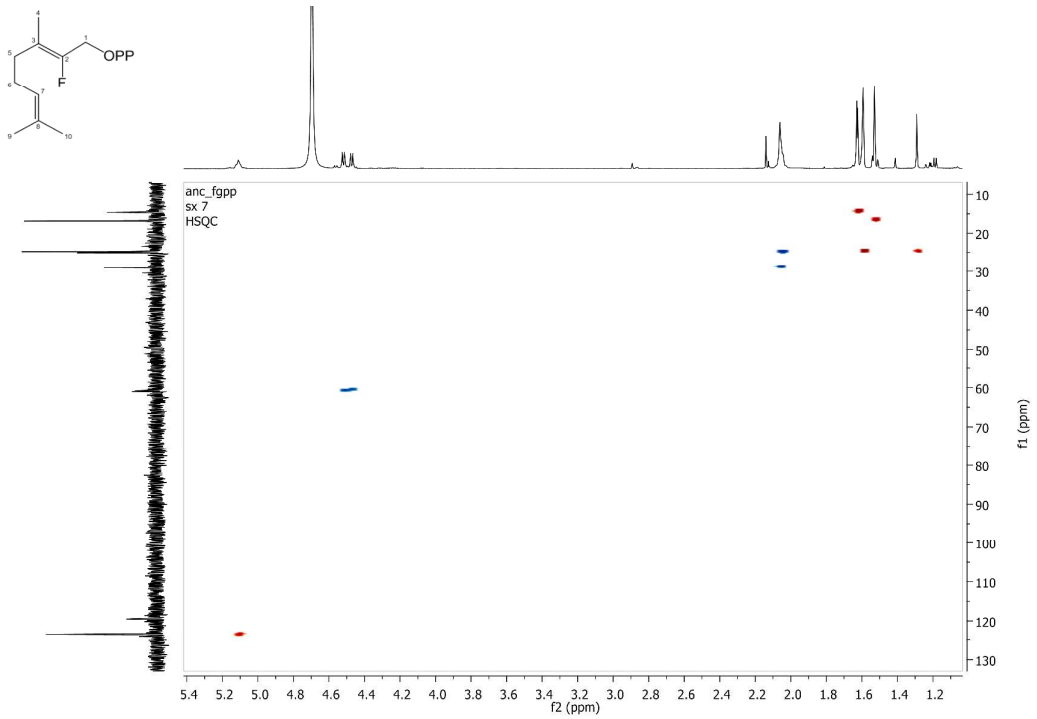


^{13}C NMR (126 MHz, D$_2$O) δ 133.96, 123.73, 119.46 (d, *J* = 15.0 Hz), 60.89 (dd, *J* = 31.6, 5.0 Hz), 29.06 (d, *J* = 6.4 Hz), 25.05, 24.77, 16.87, 14.63 (d, *J* = 4.9 Hz).

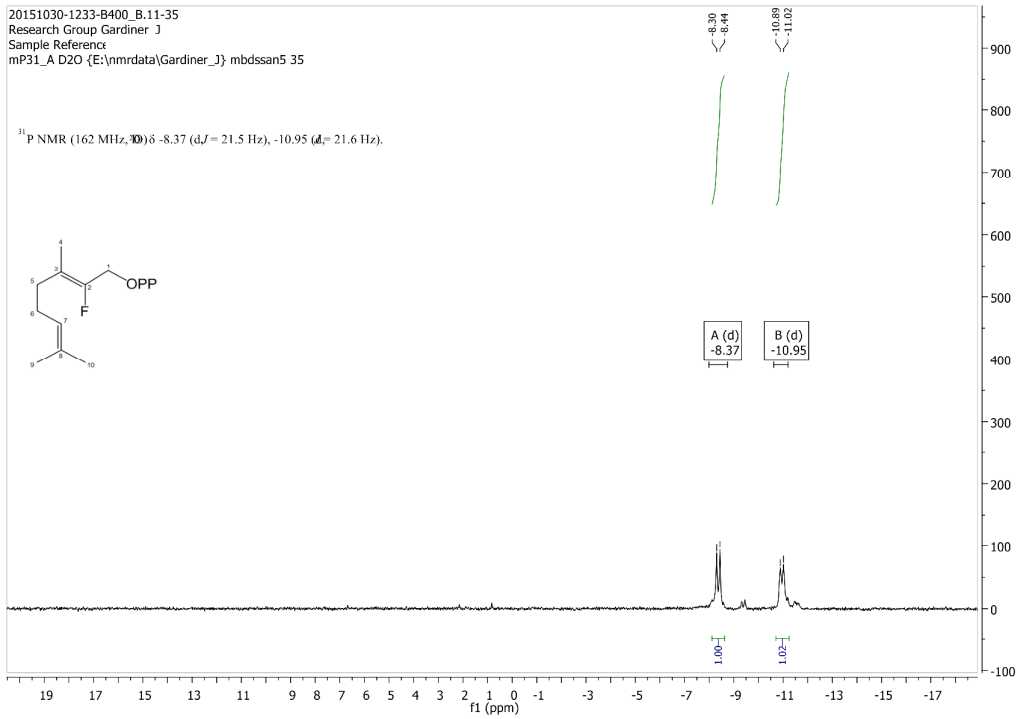
C



D



E


20151030-1233-B400_B.11-35
Research Group Gardiner J
Sample Reference
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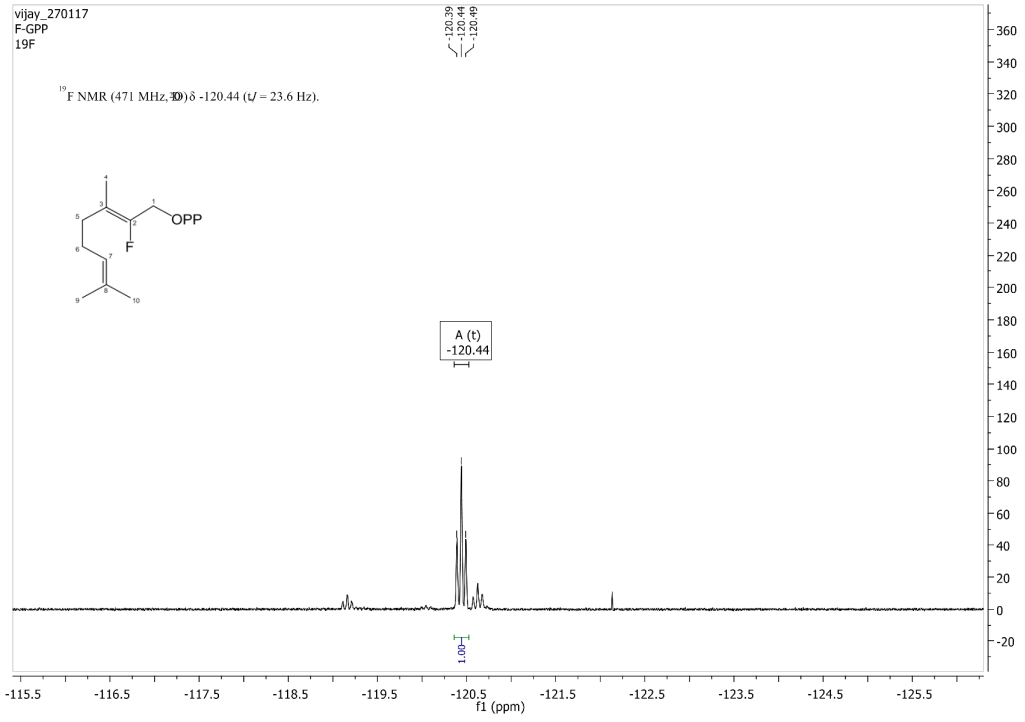
31P NMR (162 MHz, D2O) δ -8.37 (d, J = 21.5 Hz), -10.95 (d, J = 21.6 Hz).
-8.30
-8.44
-10.89
-11.02
OPP
F
A (d)
-8.37
B (d)
-10.95
1.00
1.02
f1 (ppm)

F


vijay_270117
F-GPP
19F
19F NMR (471 MHz, D2O) δ -120.44 (t, J = 23.6 Hz).
-120.39
-120.44
-120.49
OPP
F
A (t)
-120.44
1.00
f1 (ppm)

G

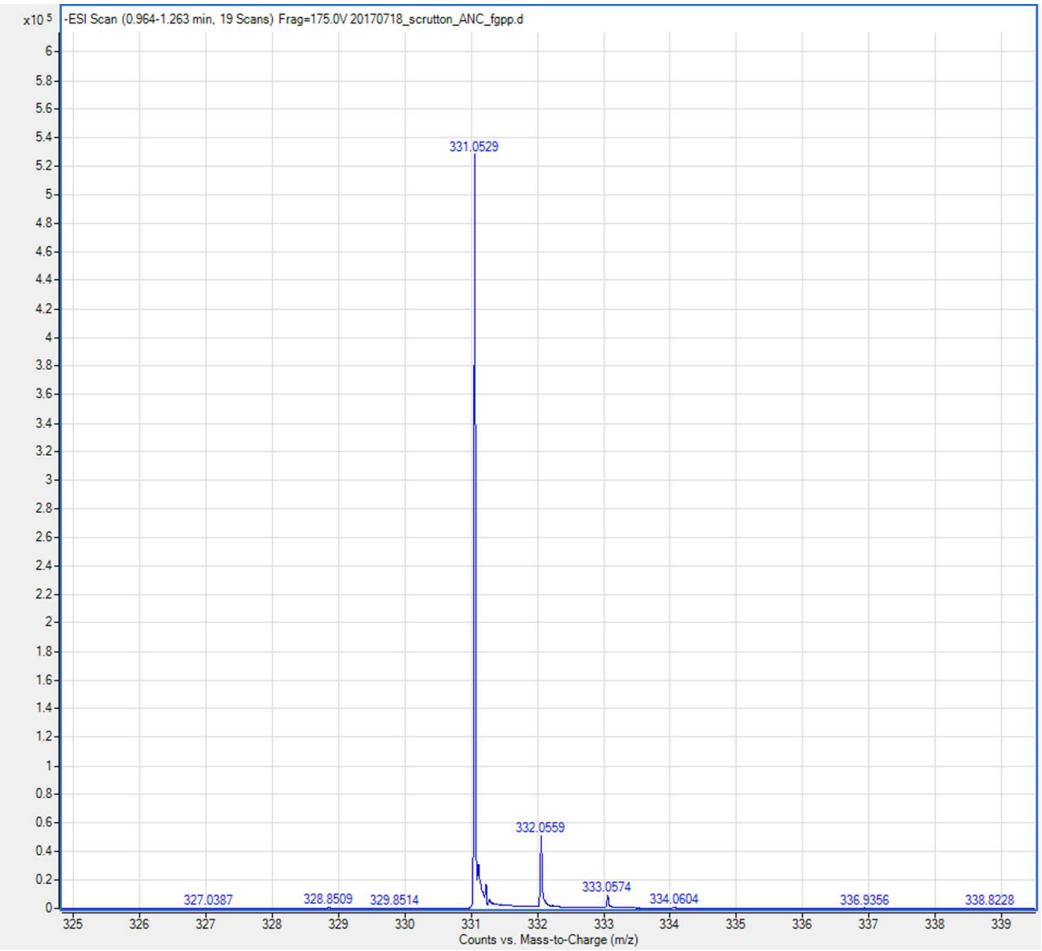
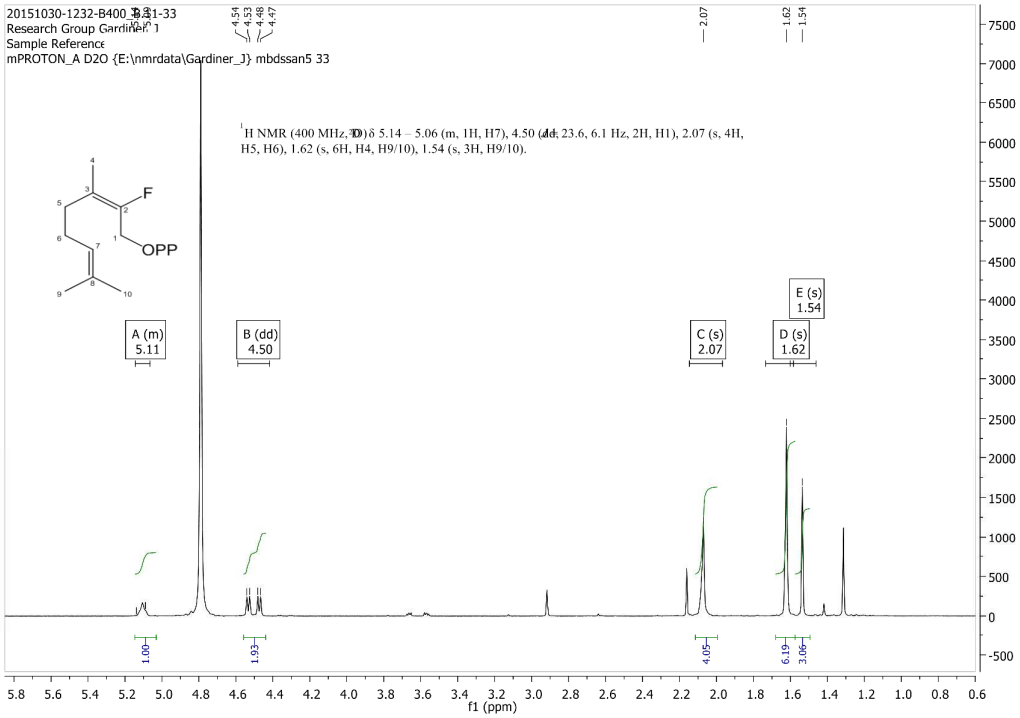


Figure S3. NMR and HRMS-ESI spectra of 2-fluorogeranyl pyrophosphate (FGPP). A) ^{1}H NMR. B) ^{13}C NMR. C) ^{1}H -^{1}H COSY. D) ^{1}H -^{13}C HSQC. E) ^{31}P NMR. F) ^{19}F NMR. G) HRMS-ESI.

A


20151030-1232-B400_3_51-33
Research Group Gardiner_1
Sample Reference
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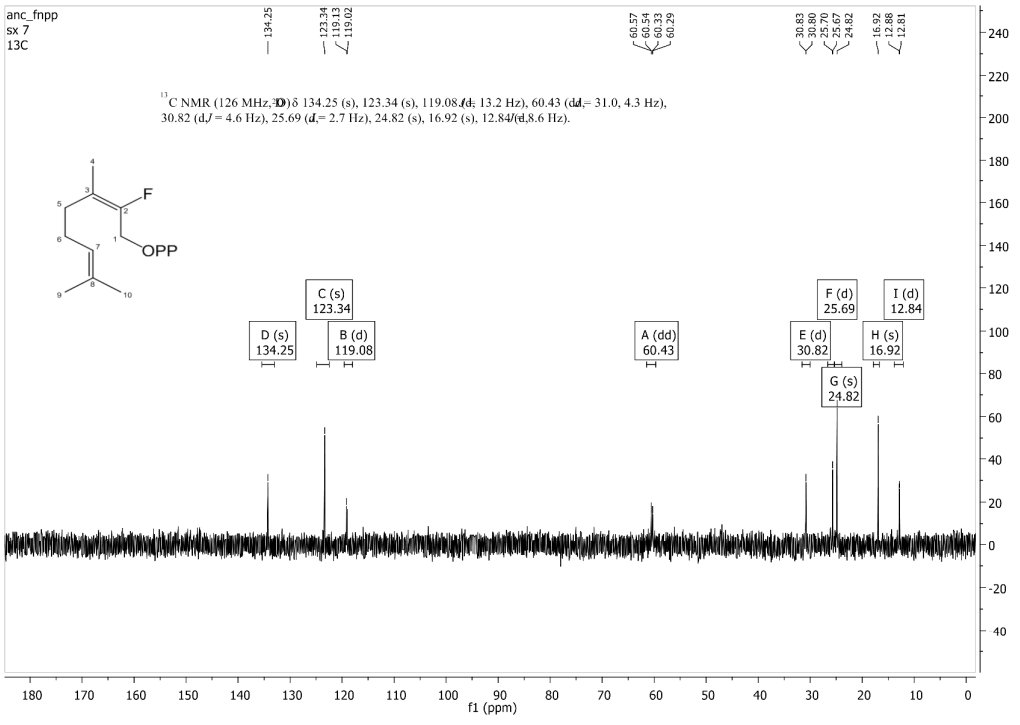
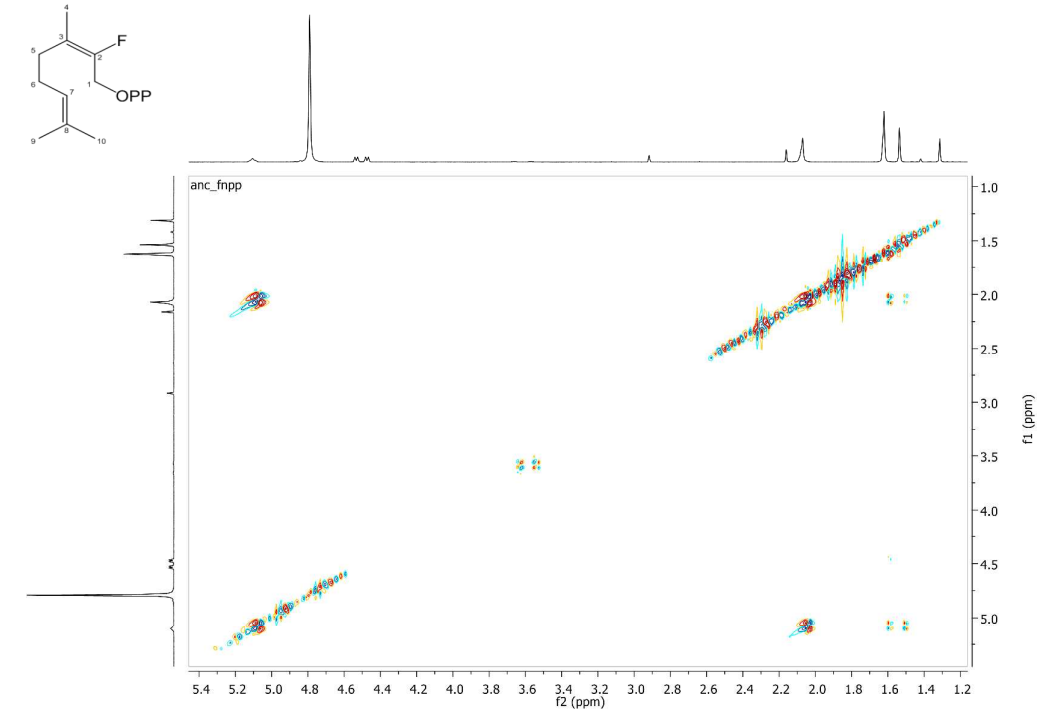
4.54
4.53
4.48
4.47
2.07
1.62
1.54
1H NMR (400 MHz, D2O) δ 5.14 – 5.06 (m, 1H, H7), 4.50 (dd, J = 23.6, 6.1 Hz, 2H, H1), 2.07 (s, 4H, H5, H6), 1.62 (s, 6H, H4, H9/10), 1.54 (s, 3H, H9/10).
F
OPP
A (m) 5.11
B (dd) 4.50
C (s) 2.07
D (s) 1.62
E (s) 1.54
1.00
1.93
4.05
6.19
3.06
f1 (ppm)

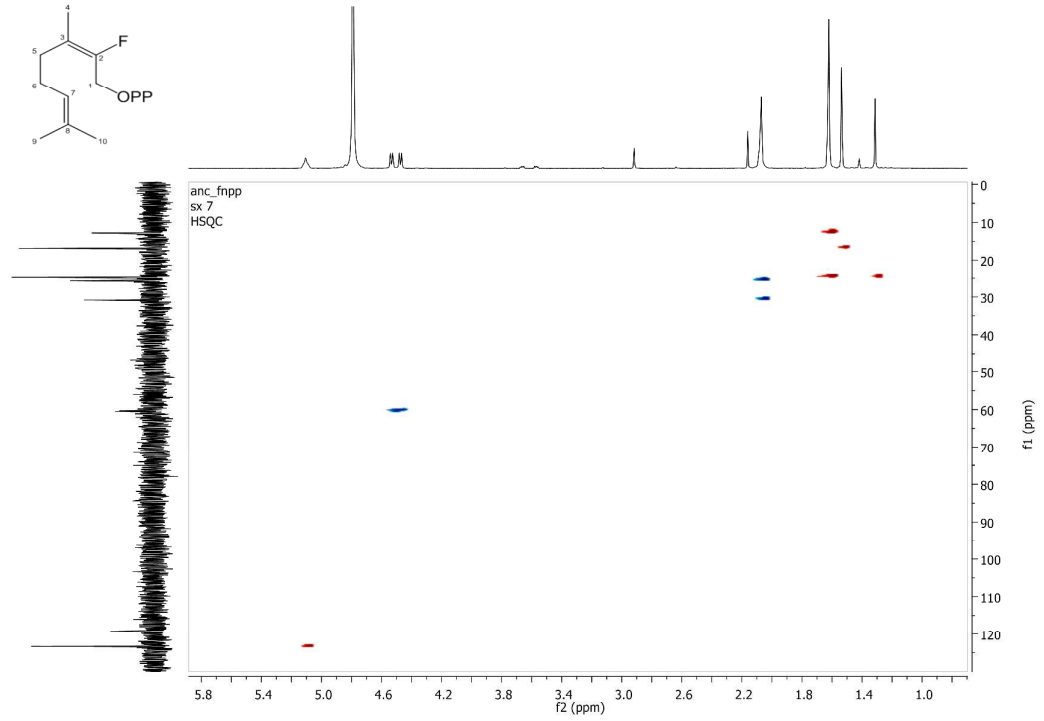
B


anc_fnpp
sx 7
13C
134.25
123.34
119.13
119.02
60.57
60.54
60.33
60.29
30.83
30.80
25.70
25.67
24.82
16.92
12.86
12.81
13C NMR (126 MHz, D2O) δ 134.25 (s), 123.34 (s), 119.08 (d, J = 13.2 Hz), 60.43 (dd, J = 31.0, 4.3 Hz), 30.82 (d, J = 4.6 Hz), 25.69 (d, J = 2.7 Hz), 24.82 (s), 16.92 (s), 12.84 (d, J = 8.6 Hz).
F
OPP
D (s) 134.25
C (s) 123.34
B (d) 119.08
A (dd) 60.43
E (d) 30.82
F (d) 25.69
G (s) 24.82
H (s) 16.92
I (d) 12.84
f1 (ppm)

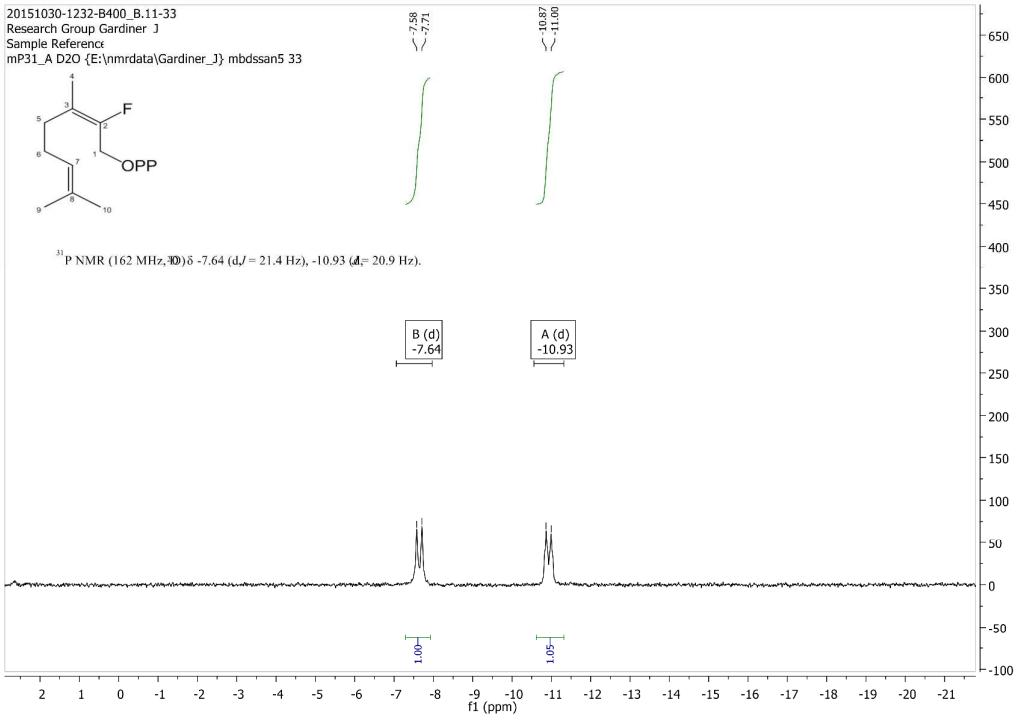
C



D



E


20151030-1232-B400_B.11-33
Research Group Gardiner J
Sample Reference
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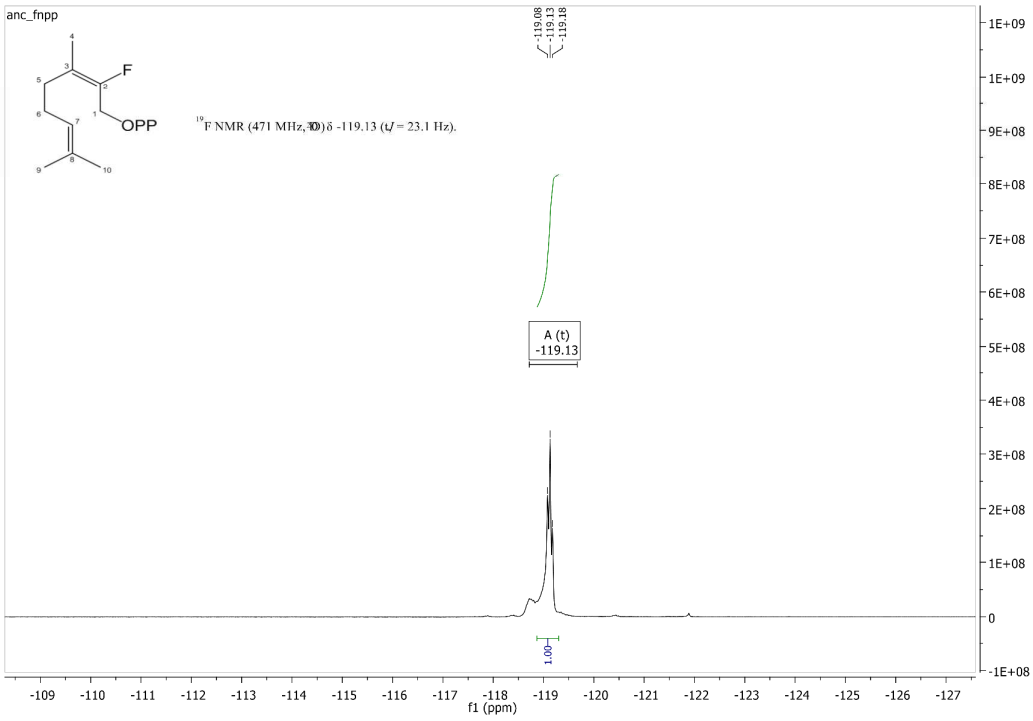
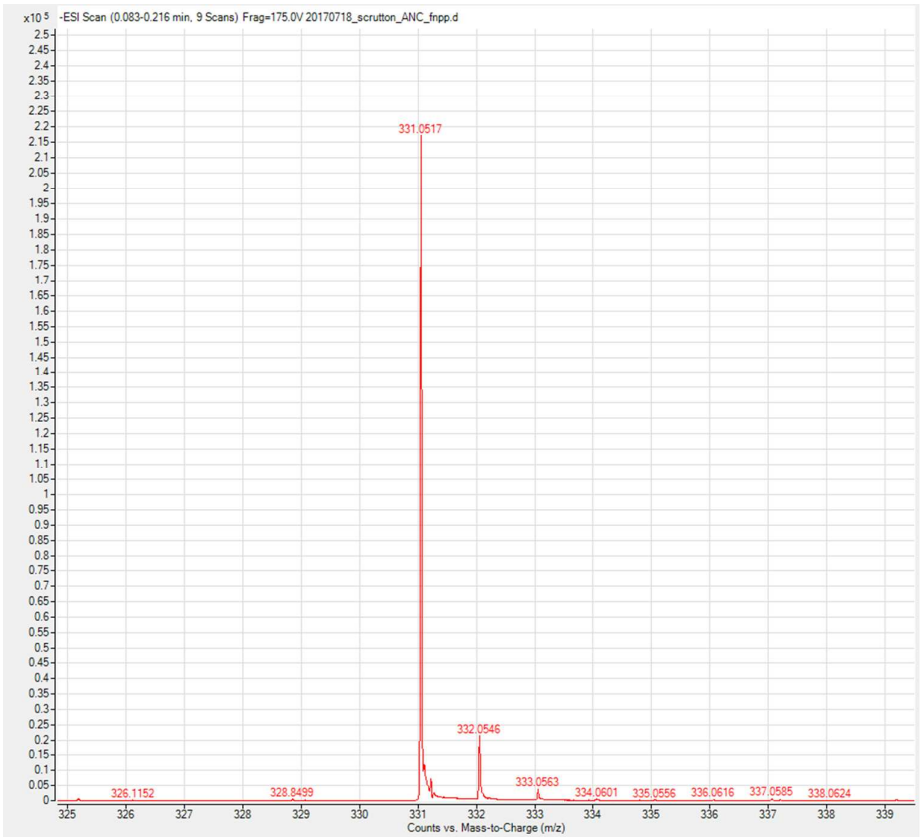
F
OPP
-7.58
-7.71
-10.87
-11.00
31P NMR (162 MHz, D2O) δ -7.64 (dJ = 21.4 Hz), -10.93 (dJ = 20.9 Hz).
B (d)
-7.64
A (d)
-10.93
1.00
1.05
f1 (ppm)

F


anc_fnpp
F
OPP
-119.08
-119.13
-119.18
19F NMR (471 MHz, D2O) δ -119.13 (dJ = 23.1 Hz).
A (t)
-119.13
1.00
f1 (ppm)

G


x10 5 -ESI Scan (0.083-0.216 min, 9 Scans) Frag=175.0V 20170718_scrutton_ANC_fnpp.d
331.0517
332.0546
333.0563
326.1152
328.8499
334.0601
335.0556
336.0616
337.0585
338.0624
Counts vs. Mass-to-Charge (m/z)

H

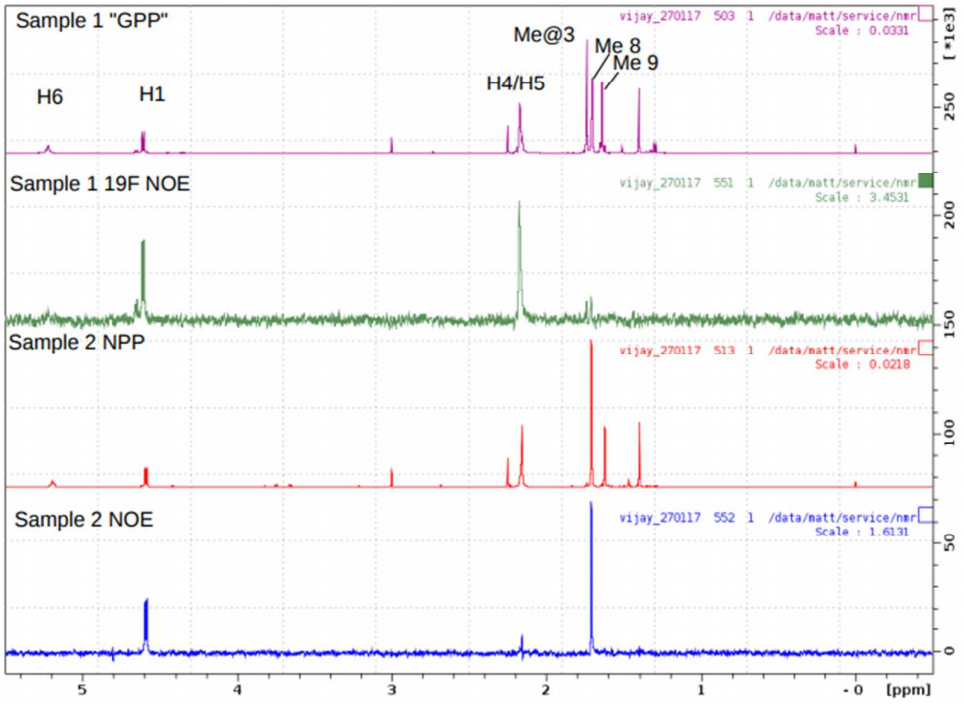


Figure S4. NMR and HRMS-ESI spectra of 2-fluoroneryl pyrophosphate (FNPP). A) ^{1}H NMR. B) ^{13}C NMR. C) ^{1}H -^{1}H COSY. D) ^{1}H -^{13}C HSQC. E) ^{31}P NMR. F) ^{19}F NMR. G) HRMS-ESI. H) Comparison of NOE data for FGPP and FNPP.

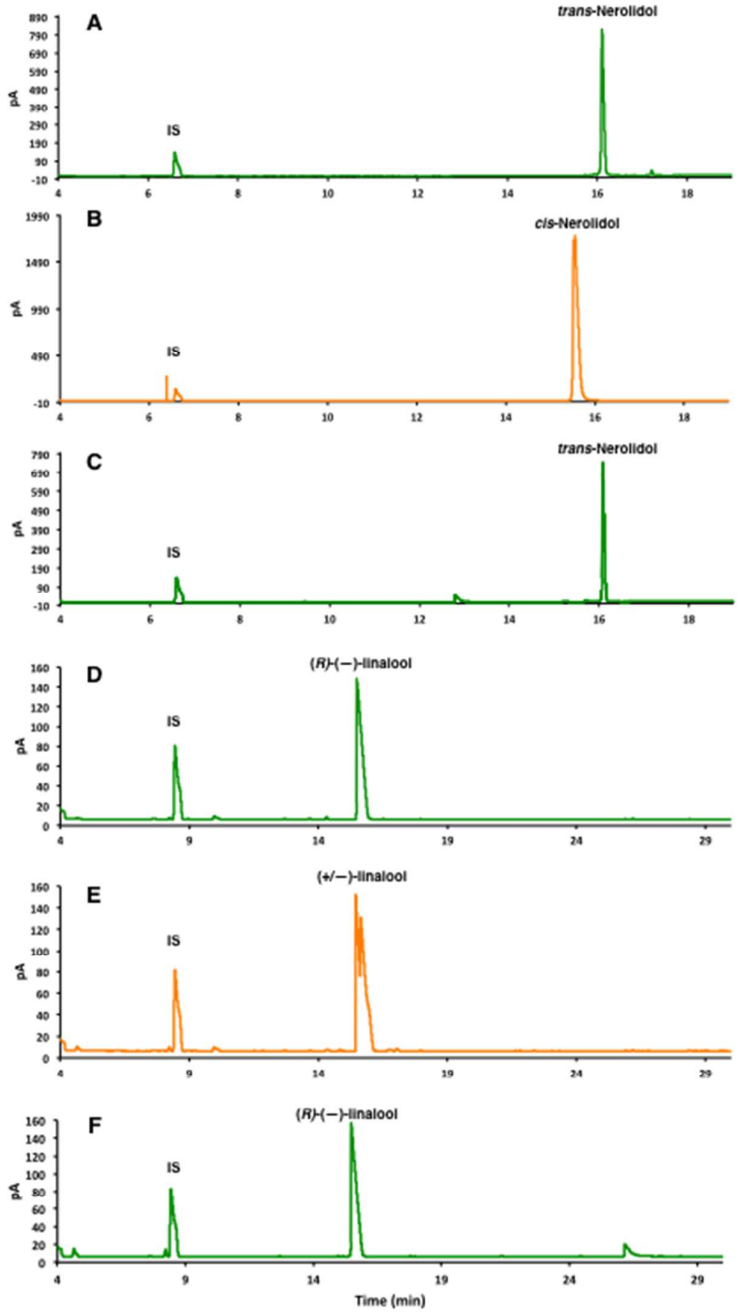


Figure S5. Gas chromatography analysis of bLinS with FPP and GPP. A) *trans*-Nerolidol standard. B) *cis*-Nerolidol standard. C) bLinS conversion of FPP (2 mM) *in vitro*. D) *R*-(–)-linalool standard. E) (+/–)-linalool standard. F) bLinS conversion of GPP (2 mM) *in vitro*. IS= internal standard (*sec*-butyl benezene)

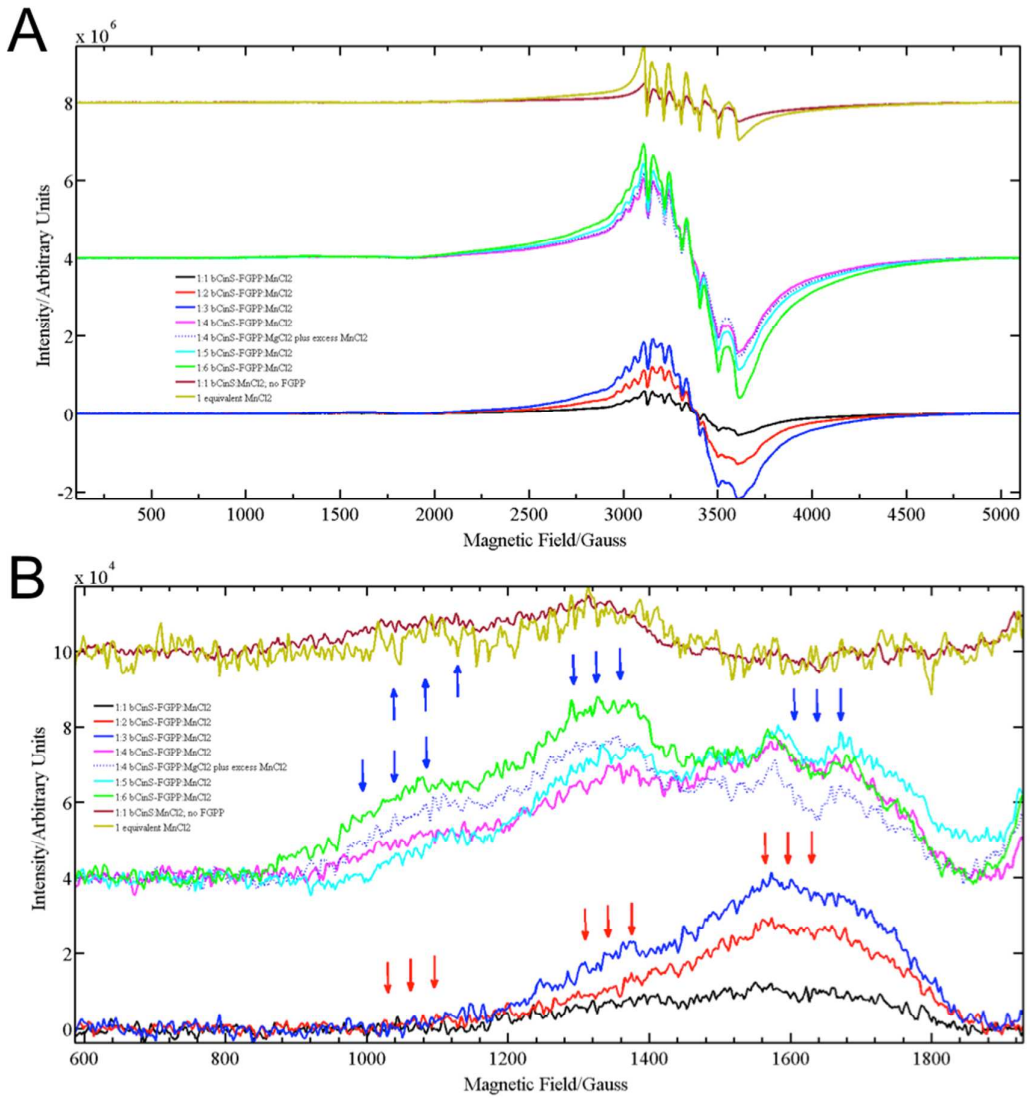


Figure S6. **cw-EPR spectra of bCinS.** Mn^{2+} substituted bCinS samples with varying equivalents of Mn^{2+} ion concentration with and without FGPP, measured as a frozen solution along with standard $MnCl_2$. A) Wide sweep from 10-510 mT. B) Expanded between 60-190 mT to show the half-field transitions of the Mn^{2+} ion under different coordination environments. The features that are highlighted with red and blue arrows are discussed in the text. Conditions: microwave power 36 dB; modulation amplitude 5 G; time constant 41 ms; conversion time 41 ms; sweep time 84 s; receiver gain 60 dB; average microwave frequency 9.384 GHz; temperature 20 K.

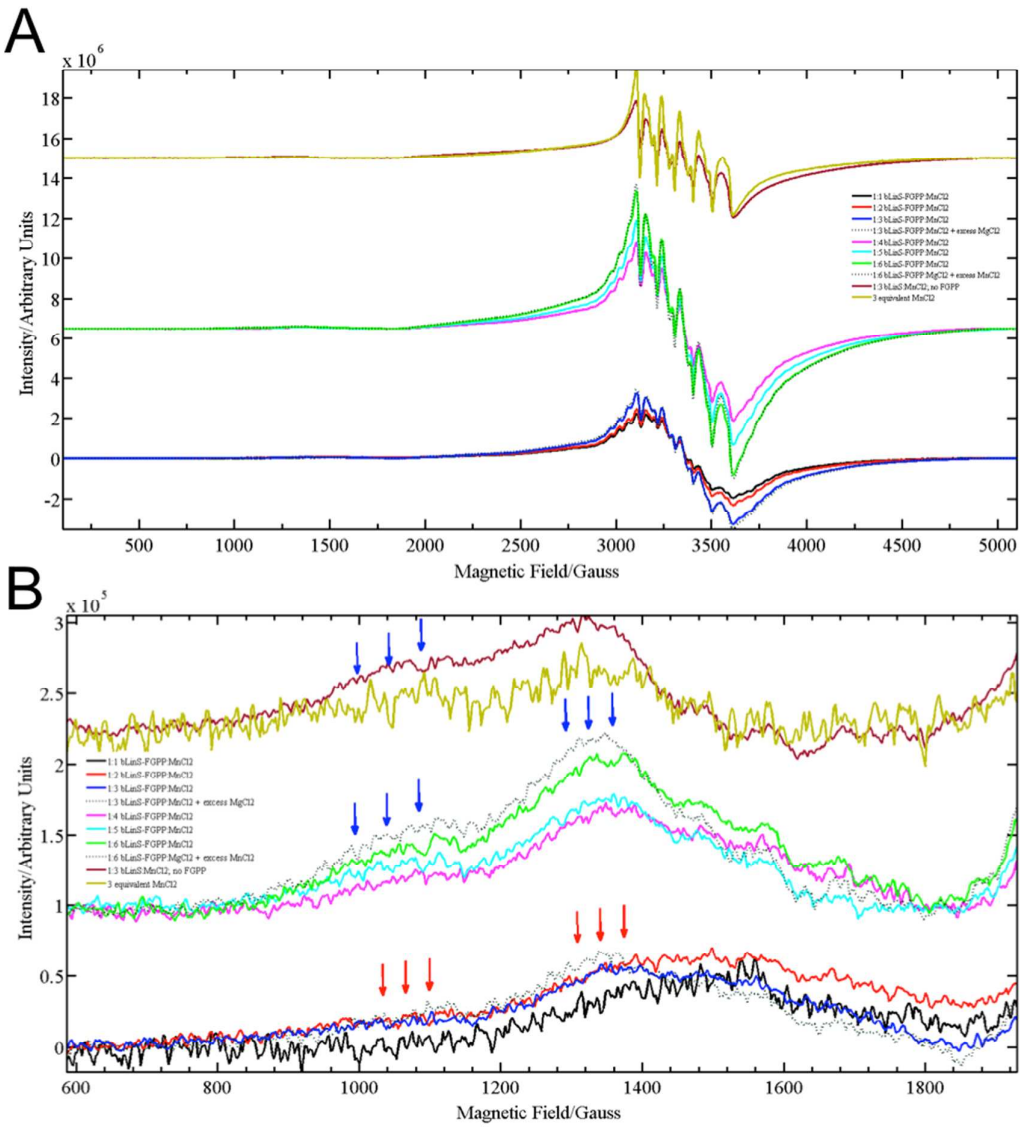


Figure S7. **cw-EPR spectra of bLinS.** Mn^{2+} substituted bLinS samples with varying equivalents of Mn^{2+} ion concentration with and without FGPP, measured as a frozen solution along with standard $MnCl_2$. A) Wide sweep from 10-510 mT. B) Expanded between 60-190 mT to show the half-field transitions of the Mn^{2+} ion under different coordination environments. The features that are highlighted with red and blue downward arrows are discussed in the text. Conditions: as in **Fig S2.**

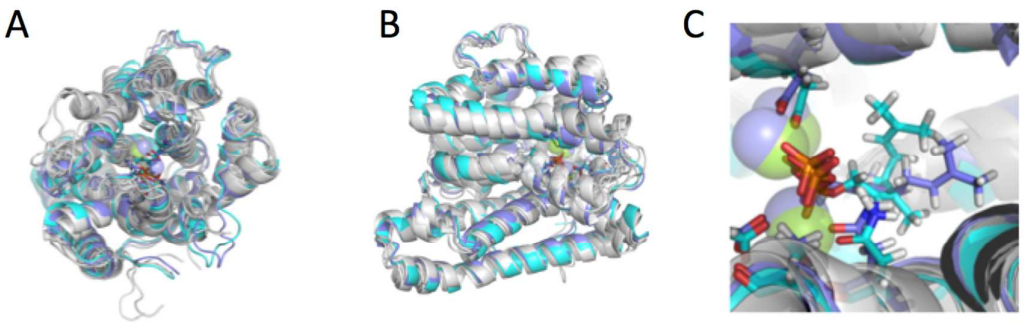


Figure S8. Comparison of structures from MD of *apo*-bCinS with MD of the ternary complex of bCinS with 3 Mg^{2+} ions and GPP or NPP. White: 4 clusters from 100 ns simulation of *apo*-bCinS, purple: the representative structure of the most populated cluster (after alignment on active site residues) from 100 ns simulations of bCinS with 3 Mg^{2+} ions and GPP, cyan the representative structure of the most populated cluster (after alignment on active site residues) from 100 ns simulations of bCinS with 3 Mg^{2+} ions and NPP. **A)** and **B)** show different views of the entire monomer, and **C)** shows the active site of bCinS. No significant structural changes are observed between the *apo* form and the ternary complex.

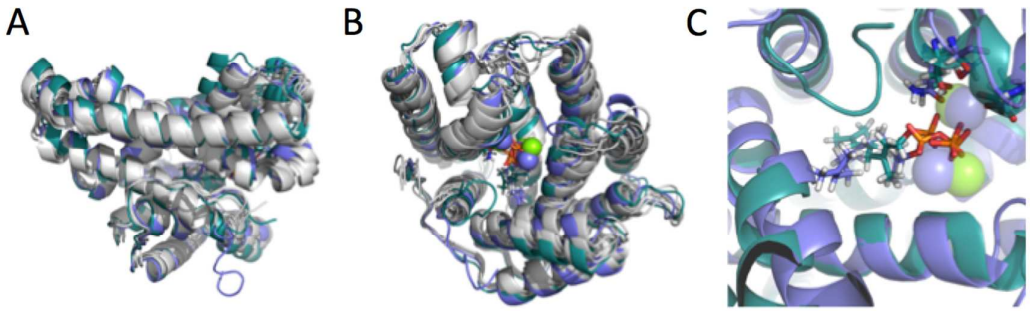


Figure S9. Comparison of structures from MD of *apo*-bLinS with MD of the ternary complex of bLinS with 3 Mg^{2+} ions and GPP or FPP. White: 4 clusters from 100 ns simulation of *apo*-LinS, teal: the representative structure of the most populated cluster (after alignment on active site residues) from 100 ns simulations of bLinS with 3 Mg^{2+} ions and GPP, purple: the representative structure of the most populated cluster (after alignment on active site residues) from 100 ns simulations of bLinS with 3 Mg^{2+} ions and FPP. **A)** and **B)** show different views of the entire monomer, and **C)** shows the active site of bLinS. No significant structural changes are observed between the *apo* form and the ternary complex. The larger FPP can easily be accommodated in the active site without any major structural rearrangements.

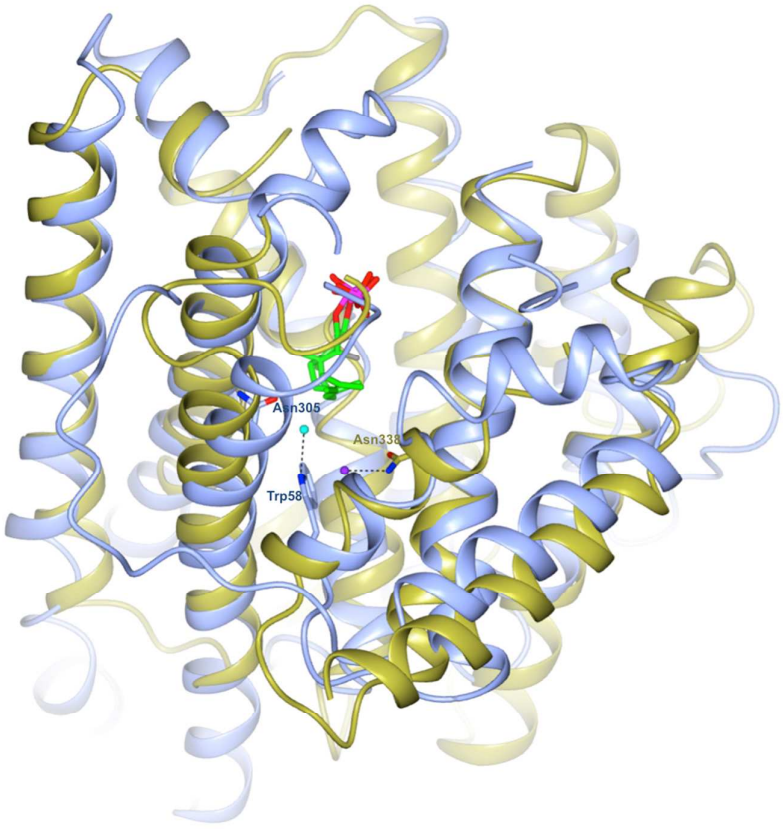


Figure S10: Structural overlay of bCinS and C-terminal domain of Sf-CinS1. In the bCinS-FNPP/FGPP structure (blue ribbon), residues Trp58 and Asn305 coordinate the water molecule (cyan sphere), which is proposed to be involved in water attack. In Sf-CinS1 (gold ribbon), Asn338 was identified to be crucial for 1,8-cineole synthesis and forms a hydrogen bond with a water molecule (purple sphere). Asn338 in Sf-CinS1 resides in a different helix and region of the active site compared to Asn305 in bCinS.

Table S1: Plasmids used in this study

Plasmid reference	Plasmid name	Description (Origin of replication, Antibiotic marker, Reference(s), Promoters and Operons)	Reference
pMVA	BbA5a-MTSAe-T1f-MBI(f)-T1002i	p15A, Kanr, PlacUV5, MTSA, T1, MBI-f, T1002	1
pGPPSmTC/S15	pBbB2a-trAgGPPS(co)-trSLimS_Ms	pBBR, Ampr, Ptet, trAgGPPS(co)- trSLimS_Ms	1
pGPPSmTC/S38	pBbB2a-trAgGPPS(co)-bLinS	pBBR, Ampr, Ptet, trAgGPPS(co)- bLinS	This study
pGPPSmTC/S39	pBbB2a-trAgGPPS(co)-bCinS	pBBR, Ampr, Ptet, trAgGPPS(co)- bCinS	This study

Table S2: bLinS and bCinS: homologous structures

Name	PDB	Organism	RMSD (Å) / Cα atoms
bLinS (chain A)			
Pentalenene synthase	1ps1	*Streptomyces exfoliatus*	1.52 / 283
Germacradien-4-ol synthase	5i1u	*Streptomyces citricolor*	1.59 / 276
Hedycaryol synthase	4mc3	*Actinomycete K. setae*	1.99 / 275
Geosmin synthase	5dz2	*Streptomyces coelicolor*	1.82 / 279
Epi-isozizaene synthase	4ltv	*Streptomyces coelicolor*	2.19 / 286
Selinadiene synthase	4okz	*Streptomyces pristinaespiralis*	1.83 / 265
2-Methylisoborneol synthase	4la6	*Streptomyces coelicolor*	2.09 / 268
bCinS			
Geosmin synthase	5dz2	*Streptomyces coelicolor*	1.54 / 296
Pentalenene synthase	1ps1	*Streptomyces exfoliatus*	1.80 / 292
Germacradien-4-ol synthase	5i1u	*Streptomyces citricolor*	1.80 / 283
Epi-isozizaene synthase	3kb9	*Streptomyces coelicolor*	1.94 / 304
Hedycaryol synthase	4mc3	*Actinomycete K. setae*	2.07 / 276
Aristolochene synthase	4kwd	*Aspergillus terreus*	2.28 / 280

REFERENCES

1. Leferink, N. G. H.; Jervis, A. J.; Zebec, Z.; Toogood, H. S.; Hay, S.; Takano, E.; Scrutton, N. S. *ChemistrySelect* **2016,** 1, 1893-1896.