Supporting Information for:

Probing the Mechanism of 1,4-Conjugate Elimination Reactions Catalyzed by Terpene Synthases

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1. Materials. Recombinant (*E*)-β-farnesene synthase (EBFS) from *Mentha* x *piperita* was produced as previously described for aristolochene synthase (PR-AS)[1] using competent cells of *E. coli* XL1 Blue and BL21-CodonPlus-RP. The QIAprep spin Miniprep was used for plasmid purification. Protein expression was induced with isopropyl-thio-D-galactopyranoside (IPTG). The pre-packed DEAE Sepharose Fast Flow anion exchange resin was used for protein purification. A prestained protein size marker (14.4-116) kDa was used to identify proteins by 10% SDS-gel. The Amicon-YM30 membranes were used for protein concentration. [1-^{3}H]FDP (20 Ci/mmol) was purchased from American Radiolabeled Chemicals. Unlabelled FDP was synthesized following Poulter's two steps (chlorination/diphosphorylation) protocol[2] with modifications[3] from commercially available all *trans*-farnesol. Commercial [1-^{3}H]FDP was diluted by adding cold FPP to give a final specific activity of 30 mCi/mmol. 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.

2. General Methods. Protein concentrations were determined by the Bradford method[4] using commercial reagents and commercial bovine serum albumin as the calibration standard. 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. The program uses an injection port 100 °C; split ratio 5:1; initial temperature 50 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. ^{1}H, ^{13}C, ^{31}P and ^{19}F NMR spectra were recorded on 500, 400 or 300 NMR spectrometers as specified and are presented in parts per million (δ scale) downfield from tetramethylsilane (TMS), 85% H_3PO_4 or $CFCl_3$. The following solvents and reference values in ppm were used in obtaining the NMR data: $CDCl_3$ (^{1}H, 7.26, ^{13}C 77.0), D_2O (^{1}H, 4.79). The purity of purified compounds was judged to be > 95% by TLC and/or GC analyses and NMR spectra 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. Flash

chromatography was performed according to the method of Still.[1] Reverse phase HPLC was performed on a system comprising of a Dionex P680 pump and a Dionex UVD170U detector unit.

3. Synthetic Procedures. (2*Z*, 6*E*)-FDP (**cis-3**) and (2*Z*, 6*E*)-2F-FDP (**2F-3**) were synthesized from the corresponding primary allylic alcohol as previously described.[6,7] 3CH_2F-FDP (**3CH_2F-3**) and 3CF_3-FDP (**3CF_3-3**) was prepared exactly as described by Dolence and Poulter.[8] The final diphosphorylation reactions were carried out according to the method developed by Poulter and coworkers.[9] (3*RS*)-*trans*-nerolidyl diphosphate (NDP)[10] was prepared from commertial (±)-*trans*-nerolidol following the Cramer-Danilov protocol[11] as described by Karp et at[12] with modifications.[13] The resulting silica gel purified $(Bu)_4N^+$ form of NDP was converted to the NH_4^+ salt by ion change chromatography (Dowex 50W-X8). (2*Z*, 6*E*)-2F-[1-^{3}H]Farnesyl diphosphate (activity 1.41 mCi/μmol) was synthesized following standardprocedures[10,14] via (2*Z*, 6*E*)-2F-[1-^{3}H]farnesol, prepared from 2F-farnesal and sodium borotritide (5 mCi, activity 15Ci/mmol) similarly as described for 2F-[1-^{3}H]geraniol,[15] and [1-^{3}H]-*trans*-*trans*-farnesol.[10,14] The diphosphorylation reaction of (2*Z*, 6*E*)-2F-[1-^{3}H]farnesol was carried out as described by Poulter.[10] (3*RS*)-(1*Z*)-*trans*-[1-^{3}H]Nerolidyl diphosphate[12] (activity 0.76 mCi/mmol) was prepared from (1*Z*)-*trans*-[1-^{3}H]nerolidol following the Cramer-Danilov protocol[11] as described by Karp *et at*[12] with modifications.[13] (1*Z*)-*trans*-[1-^{3}H]Nerolidol was synthesized essentially as described by Cane[16] via the γ-*cis*-vinylic metallation procedure first described by Julia[17], using ^3H_2O (activity 100 mCi/mL).[18]

An authentic sample of (*E*)-β-farnesene (**6**) was prepared from geranyl bromide according to Poppe *et al*[19] with modifications.[20] In addition, an authentic sample of (*E*)-β-, (*Z*)- α- and (*E*)- α-farnesenes was generated as a mixture exactly as recently described.[21] Commercial farnesol, nerolidol and (-)-α-bisabolol were also used as authentic standards in GC-MS measurements.

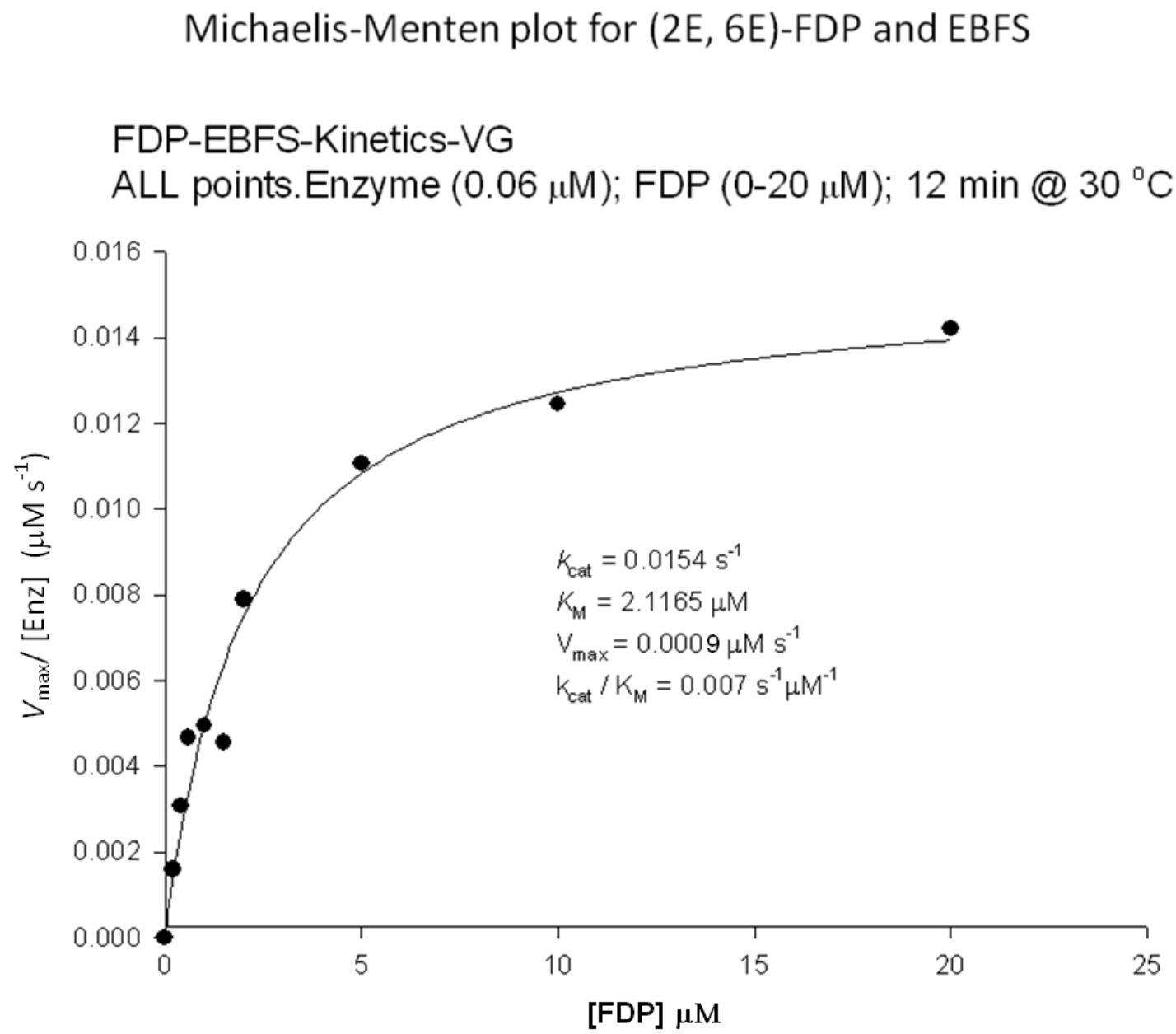
An authentic sample of 2F-farnesenes (3 isomers including (*E*)-β-2F-farnesene) and β-2F-bisabolenes (5 isomers) was generated by solvolysis [2,4,6-collidine in acetone/H_2O (7:3), room temperature, overnight] from 2F-farnesyl methanesulfonate.[22] An authentic sample of (*E*)-β-2F-farnesene and β-2F-bisabolene as a 1:7 mixture respectively was obtained by preparative TLC $AgNO_3$-silica purification of the original hydrocarbon mixture.

4. Expression and purification of recombinant (*E*)-β-farnesene synthase (EBFS) from *Mentha x piperita*. Individual *E. coli*. BL21-CodonPlus-RP colonies harboring cDNA plasmids for wild-type EBFS were grown overnight in 100 mL of LB medium containing 0.1 mg/mL ampicillin. The resulting culture was used to inoculate more LB medium (6 x 500 mL, 0.1 mg/mL ampicillin) and the cells were grown at 37 °C to an OD_{600} of 0.6. After cooling to 16 °C, protein expression was induced with 0.5 mM IPTG and left with gentle shaking (150 rpm) overnight.

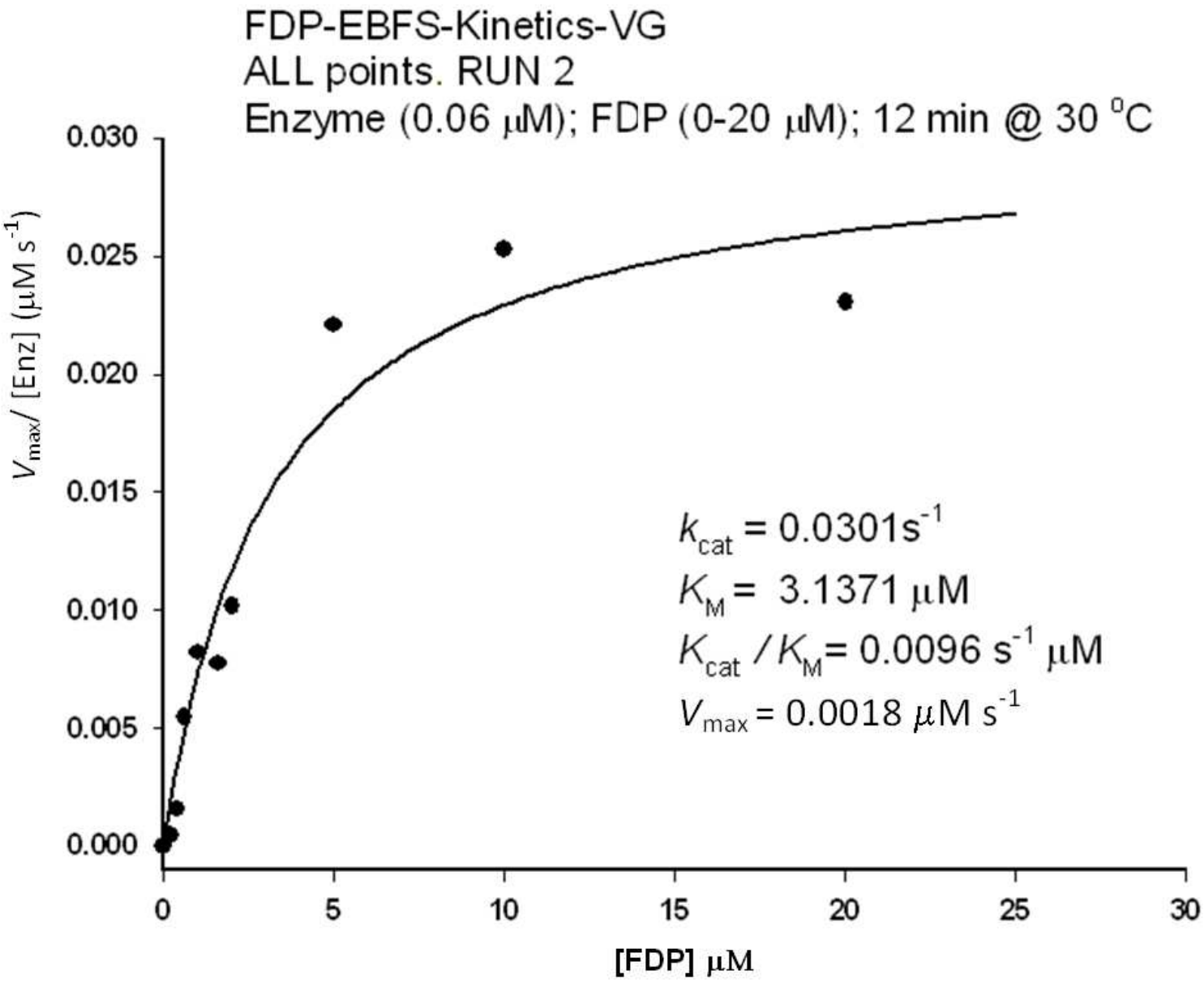
Cells were harvested by centrifugation (4500 rpm, 15 min at 4 °C). The cells were suspended in 40 mL of cell lysis buffer (6 mM EDTA, 5 mM β-mercaptoethanol, 20 mM MOPS pH 7.2) and disrupted by sonication (2-3 min Amp 40%, pulse 5 s on followed by 10 s pulse off) at 0 °C. The resulting suspension was centrifuged (15000 rpm, 4 °C, 20 min) and the supernatant discarded. Protein was then extracted from the inclusion bodies[23] and purified by FPLC (Fast Protein Liquid Chromatography) using DEAE Fast Flow. Fractions containing the terpene synthase (purity < 95% pure as judged by SDS-PAGE) were combined, dialyzed against dialysis buffer (20 mM MOPS, 5 mM, β-mercaptoethanol, pH 7.2) and concentrated to a final volume of 6 mL using an ultracell YM30 filter. According to the Bradford protein assay[4] the yield was 5 mg protein/L of LB media. The protein was aliquoted and stored at -20 °C.

5. Steady-State kinetic parameters of EBFS. Kinetics assays were carried out according to the standard, linear range, micro-assay procedure developed for limonene and bornyl diphosphate synthases with modifications.[12] This protocol involved the incubation of varying amounts of [1-^{3}H]FDP (specific activity 30 mCi/mmol), (2*Z*, 6*E*)-2F-[1-^{3}H]farnesyl diphosphate (activity 1.41 mCi/μmol) or (3*RS*)-(1*Z*)-*trans*-[1-^{3}H]nerolidyl diphosphate (activity 0.76 mCi/mmol) at pH 7.5 with fixed concentrations of purified EBFS (60-300 nM) in 20 mM MOPS buffer containing 1 mM DTT and 5 mM $MgCl_2$ at pH 7.2. The reaction mixtures containing buffer, FDP and protein were prepared on ice in a total volume of 250 μL and were overlaid with ca 0.85 mL of HPLC-grade hexane prior to incubation. The assay mixtures were incubated at 30 °C for 12 min. The reactions were immediately ice-cooled and quenched by addition of 200 μL of 100 mM EDTA (pH 8.5) and brief vortexing. The hexane overlay and two additional 1 mL hexane extracts were passed through a short pipette column containing silica gel. The column was washed with additional hexane (1 mL) and the combined filtrates were analyzed by liquid scintillation counting using 15 mL of scintillation cocktail. The observation of non-enzymatic tritiated products arising from incubations of (3RS)-(1Z)-trans-[1-^{3}H]nerolidyl diphosphate in the absence of enzyme requires a mandatory background substation for accuracy of the measurement. Steady-State kinetic parameters for wild-type EBFS were obtained by direct fitting of the data to the Michaelis-Menten equation by nonlinear least squares regression in conjunction with the graphical procedures developed by Lineweaver-Burk[24] using the commercial SigmaPlot package (Systat Software). The calculated values for K_M, k_{cat} and k_{cat}/K_M of 1.79 ± 0.16 μM, 0.028 ± 0.002 s^{-1} and (1.56 ± 0. 17)x10^4 $M^{-1}s^{-1}$ when using [1-^{3}H]FDP are in good agreement with those parameters obtained earlier for EBFS.[25,26] For (2*Z*, 6*E*)-2F-[1-^{3}H]farnesyl diphosphate the following kinetic parameters were obtained K_M, k_{cat} and k_{cat}/K_M of 1.63 ± 0.18 μM, 0.0002 ± 0.00005 s^{-1} and (0.012 ± 0.003)x10^4 $M^{-1}s^{-1}$. For (3*RS*)-(1*Z*)-*trans*-[1-^{3}H]nerolidyl diphosphate the following kinetic parameters were obtained K_M, k_{cat} and k_{cat}/K_M of 25.01 ± 4.2 μM, 0.023 ± 0.001 s^{-1} and (0.092 ± 0.011)x10^4 $M^{-1}s^{-1}$.

6. Michaelis-Menten Plots

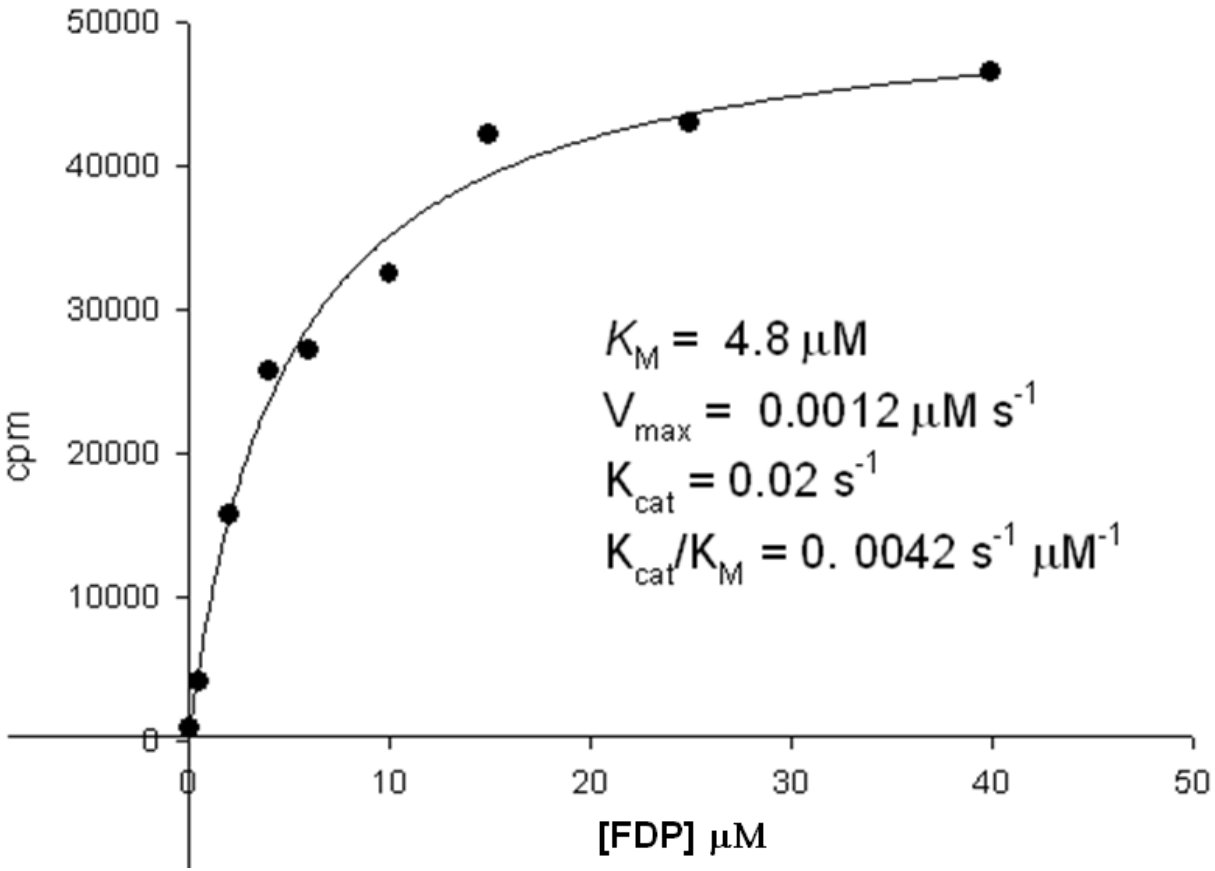


Michaelis-Menten plot for (2E, 6E)-FDP and EBFS

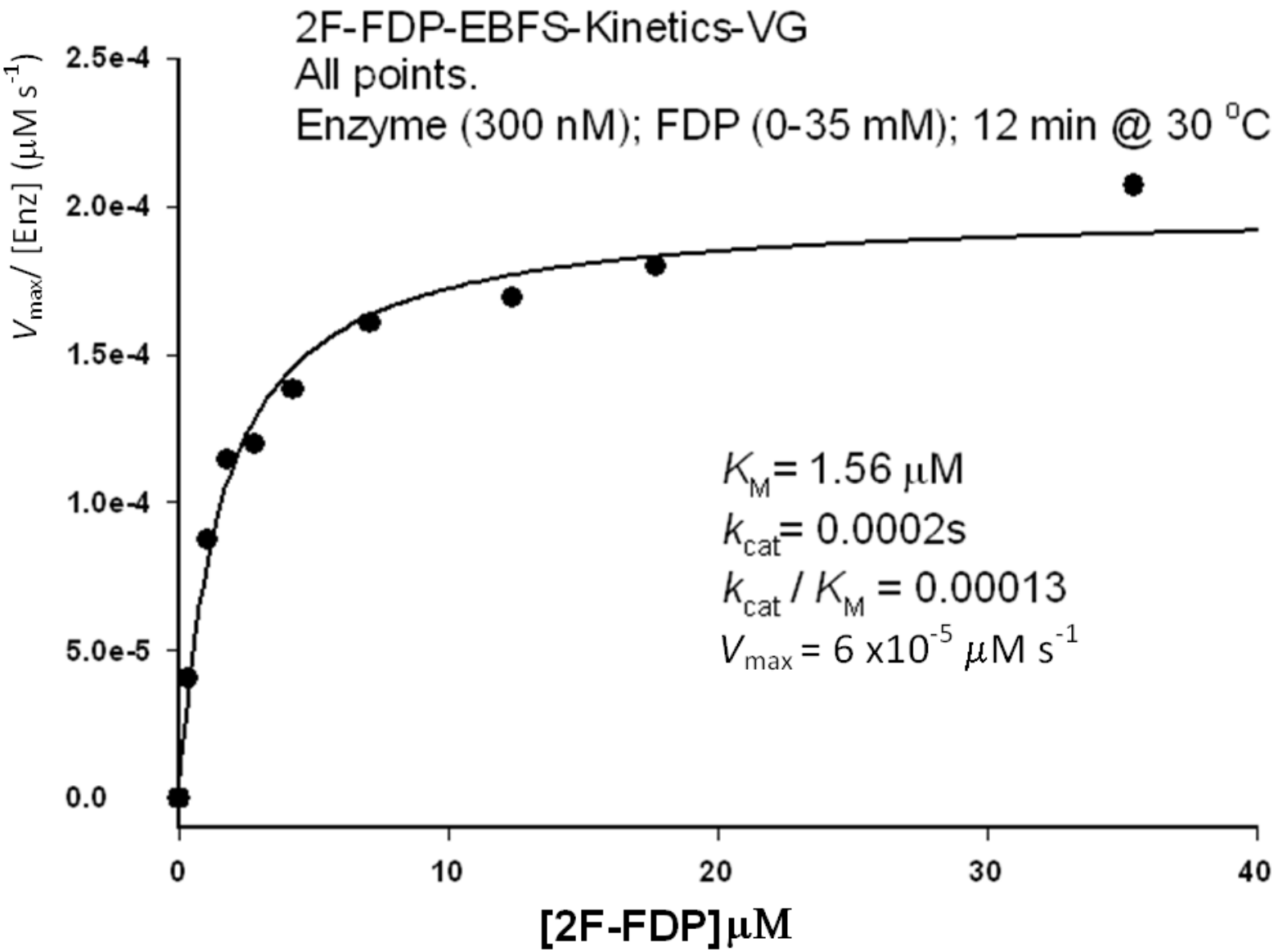


Michaelis-Menten plot for (2E, 6E)-FDP and EBFS

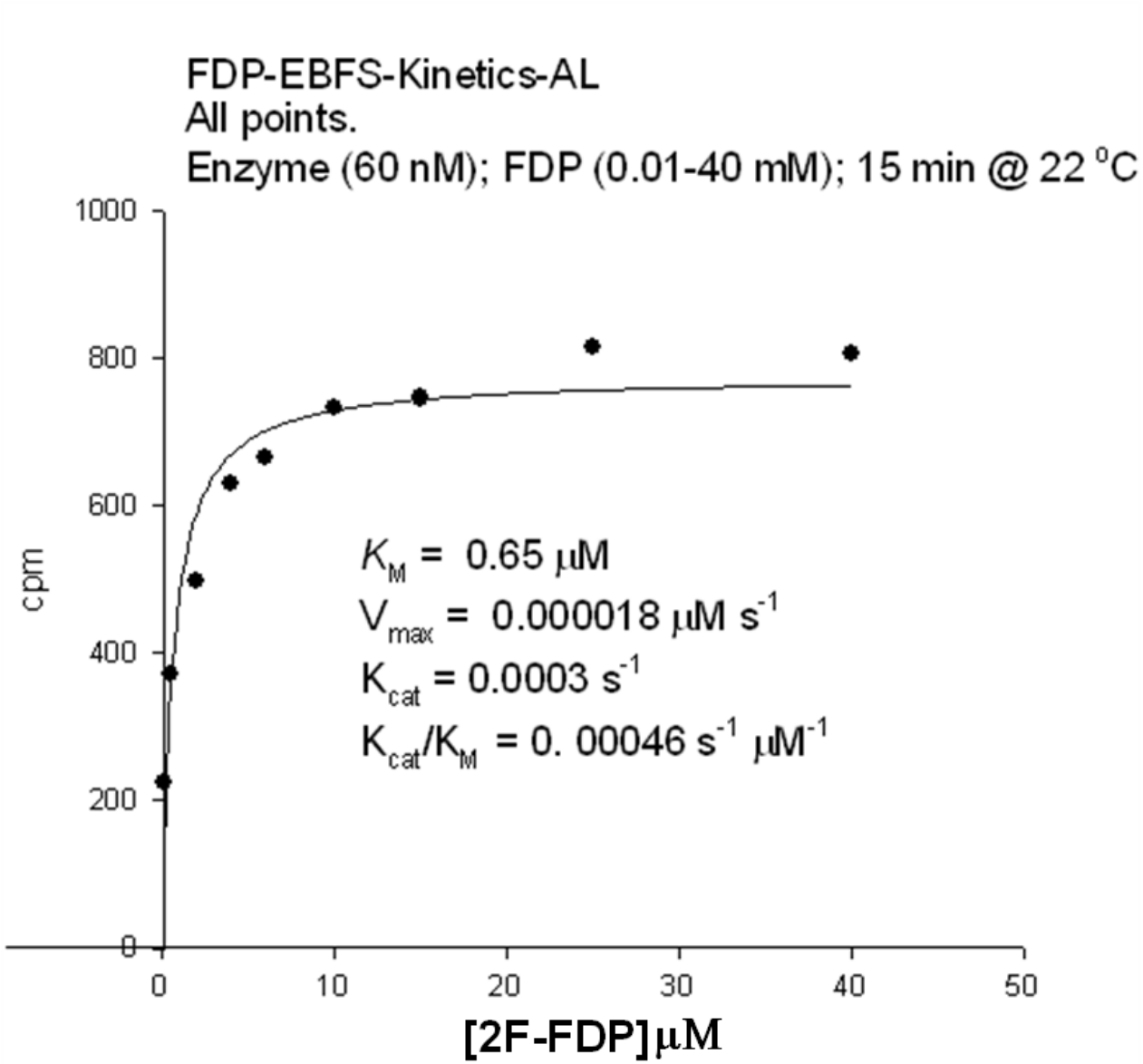
FDP-EBFS-Kinetics-AL
All points.
18.05.2012. Enzyme (60 nM); FDP (0.01-40 mM); 15 min @ .22 ^{o} C



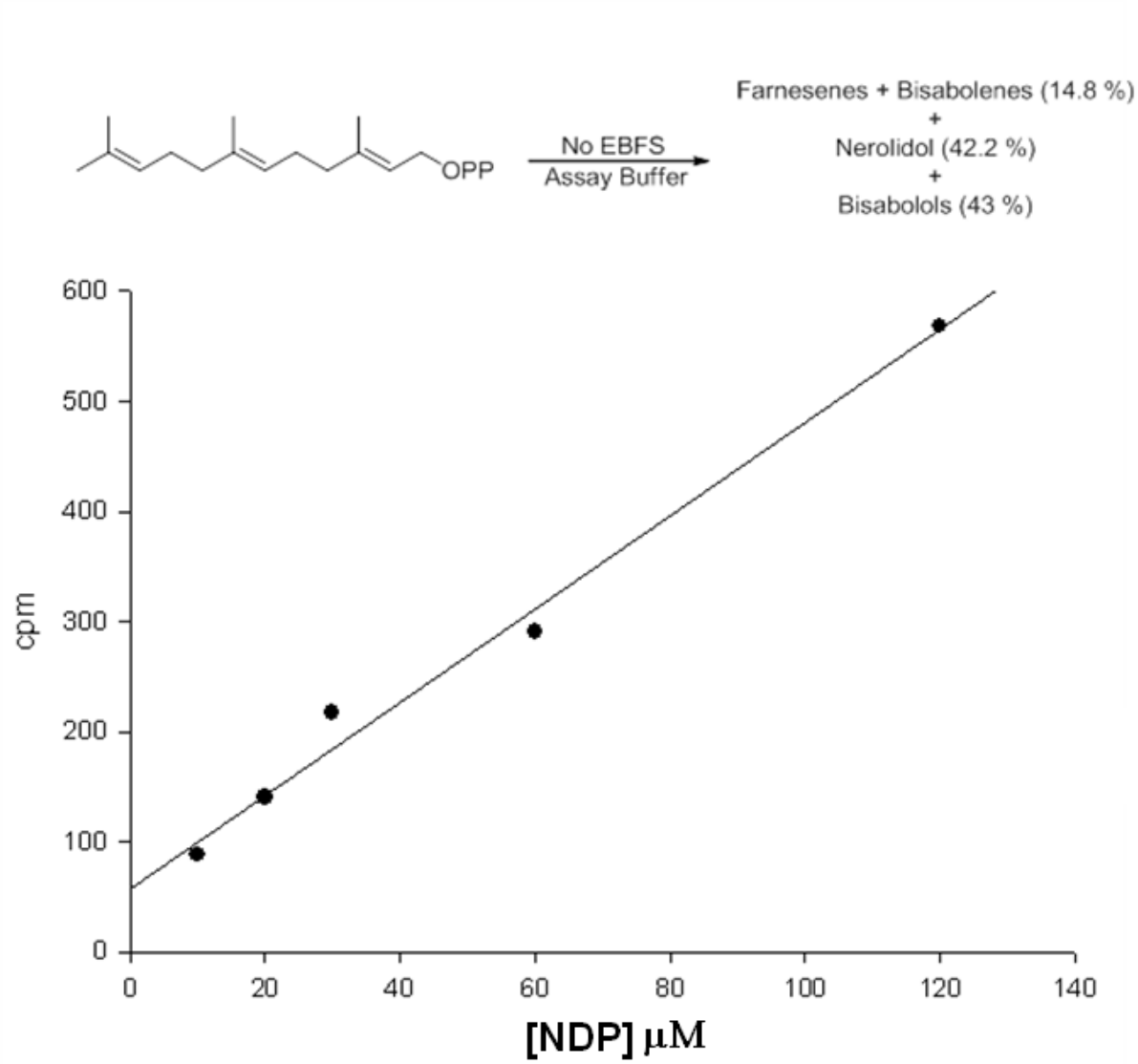
Michaelis-Menten plot for (2Z, 6E)-2F-FDP and EBFS



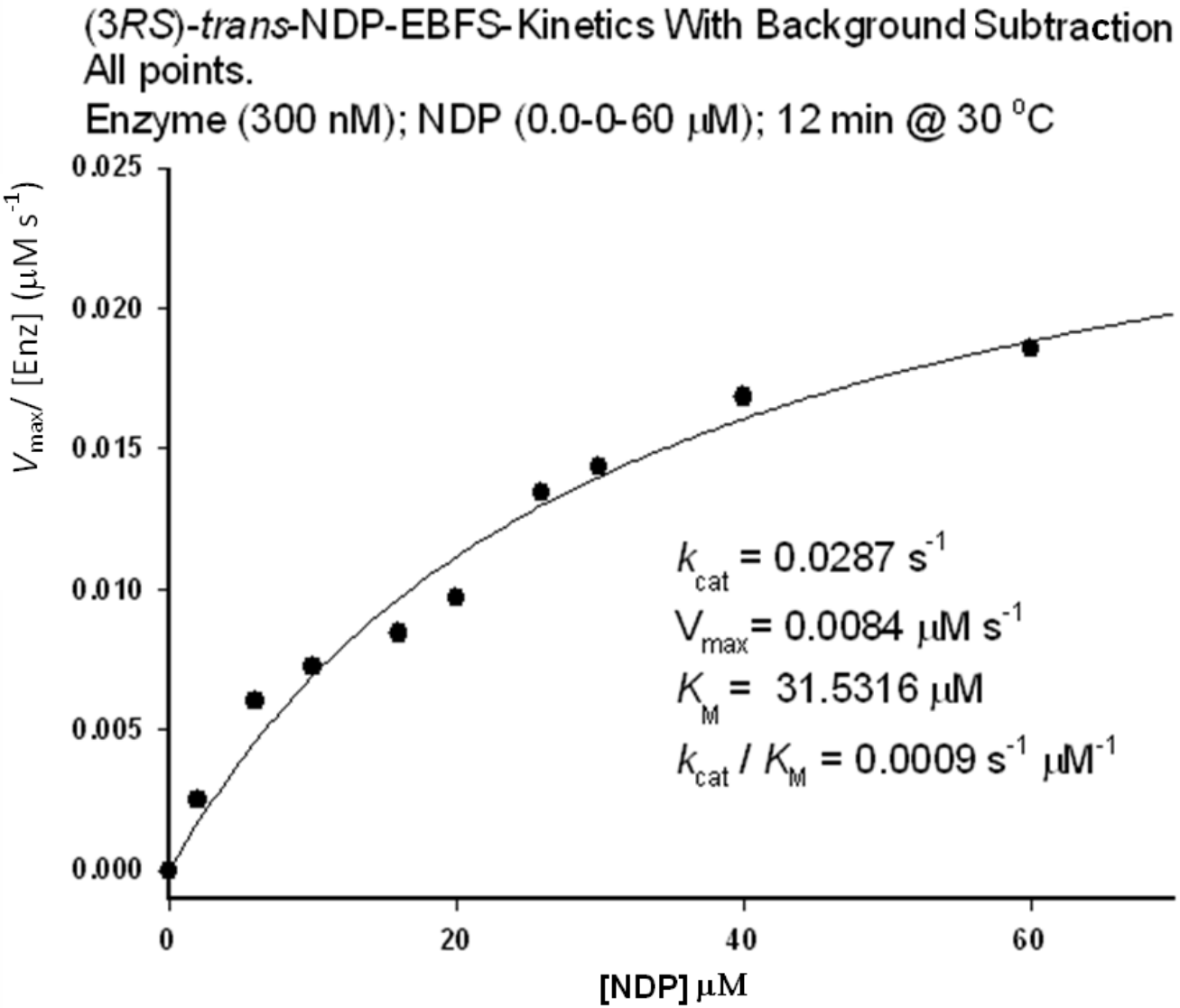
Michaelis-Menten plot for (2Z, 6E)-2F-FDP and EBFS



NDP-EBFS Calibration Plot accounting for the Non-Enzymatic (Hydrolysis) Products observed under Incubation conditions with no enzyme.BACKGROUND

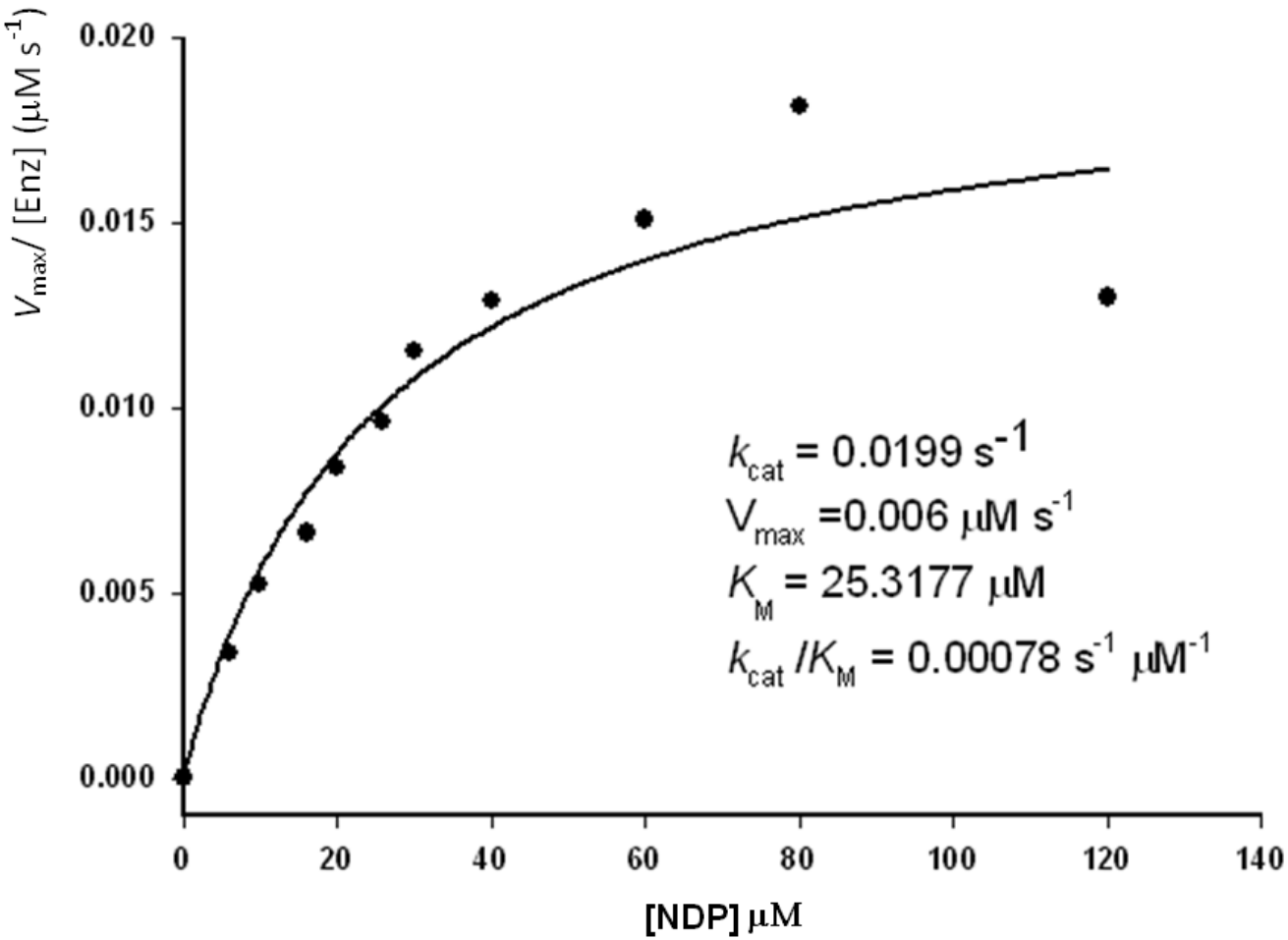


Michaelis-Menten plot for (3*RS*)-*trans*-NDP and EBFS



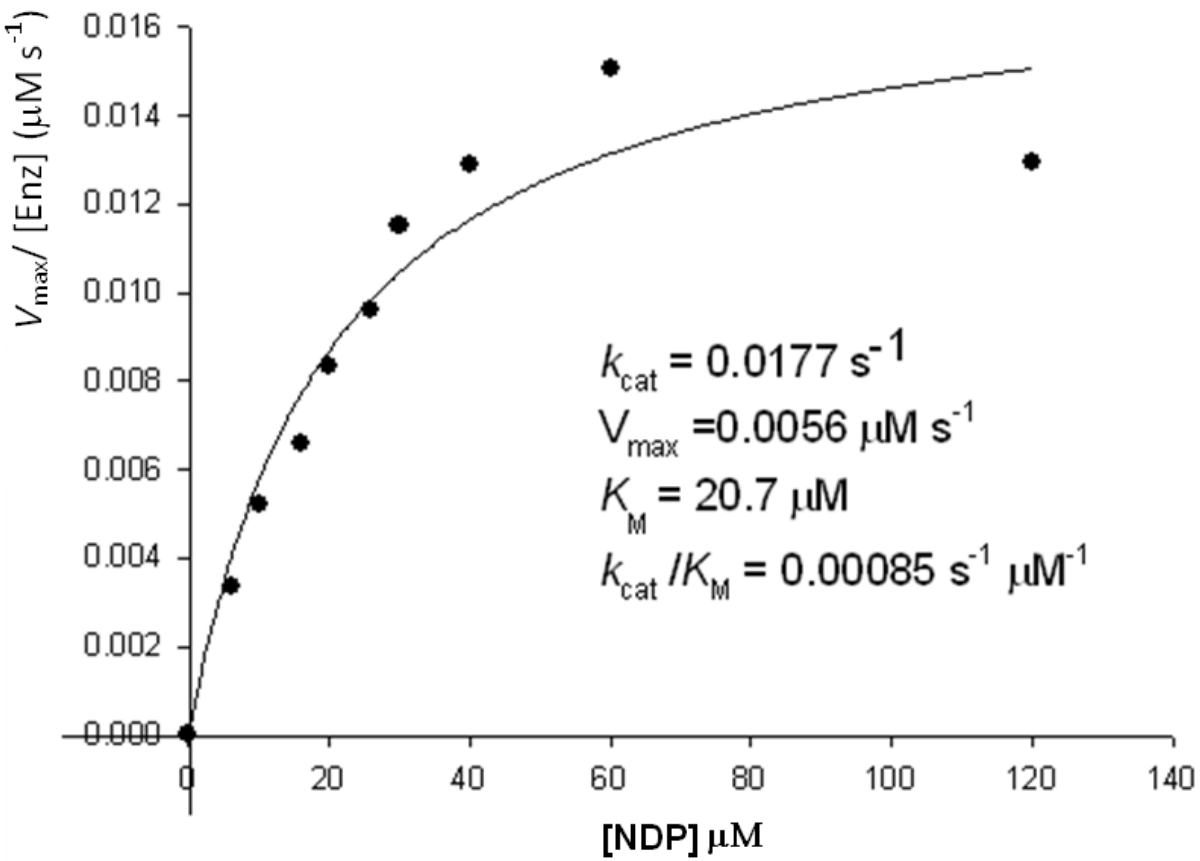
Michaelis-Menten plot for (3*RS*)-*trans*-NDP and EBFS

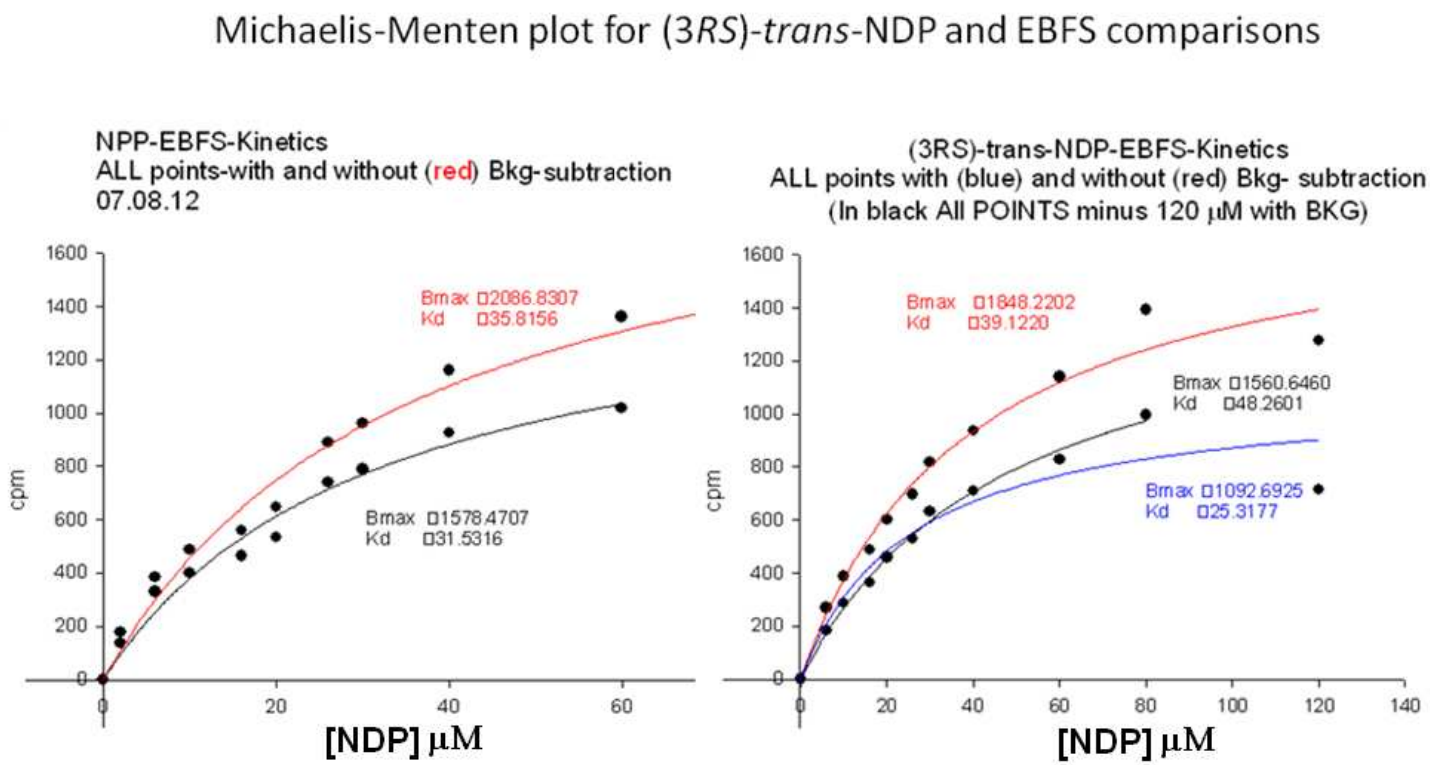
(3*RS*)-*trans*-NDP-EBFS-Kinetics With Background Subtraction
All points.
Enzyme (300 nM); NDP (0.0-0-120 μM); 20 min @ 30 °C



Michaelis-Menten plot for (3*RS*)-*trans*-NDP and EBFS

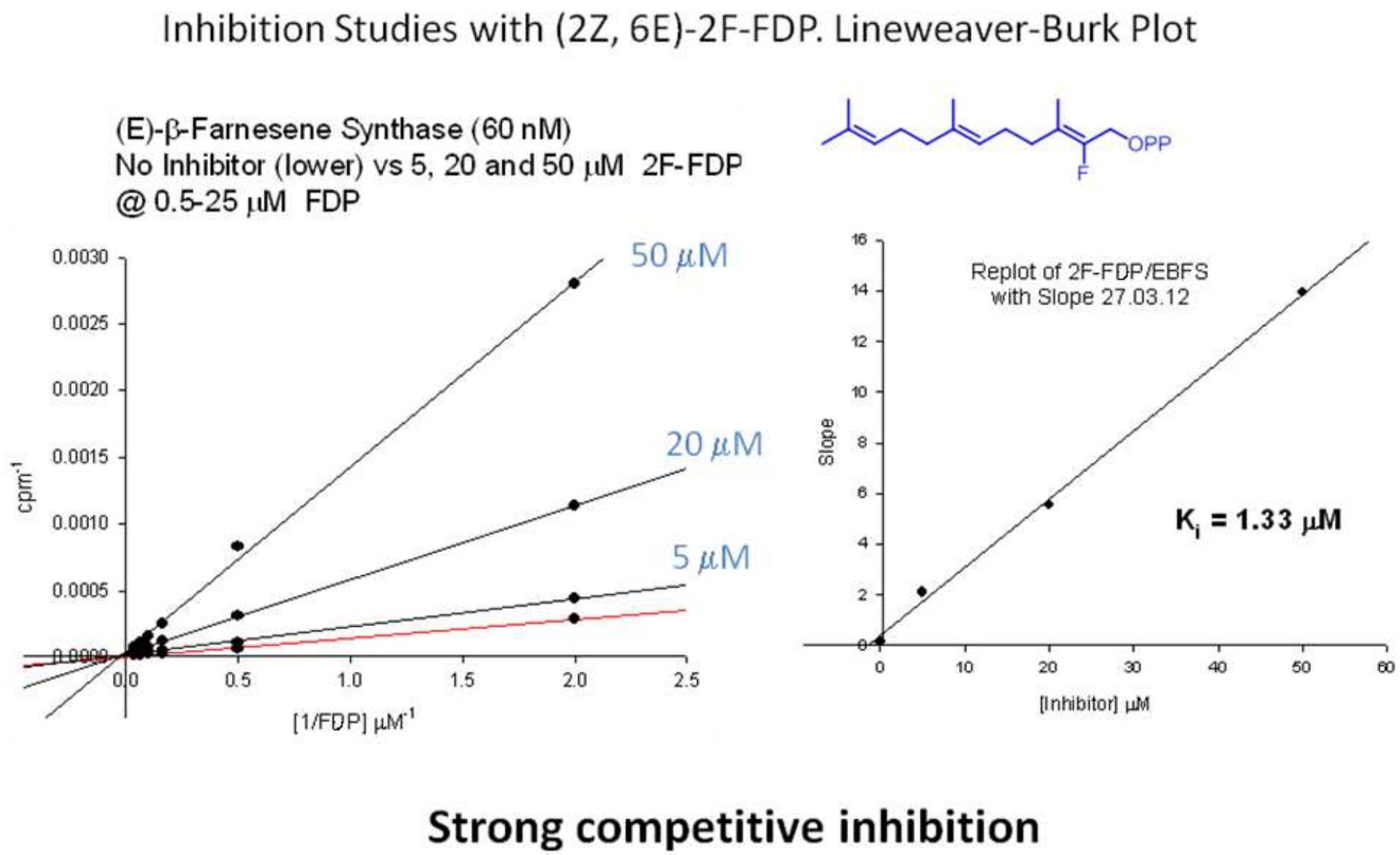
(3*RS*)-*trans*-NDP-EBFS-Kinetics.
One points less.
Enzyme (300 nM); NDP (0.0-0-120 μM); 20 min @ 30 °C





7. Inhibition studies using the fluorinated diphosphates. Kinetics assays were carried out according to the standard, linear range, micro-assay procedure developed for limonene and bornyl diphosphate synthases with modifications.[12] This protocol involved the incubation of varying amounts of [1-^{3}H]FDP (specific activity 30 mCi/mmol) with fixed concentration of purified EBFS (60 nM) in 20 mM MOPS buffer containing 10 mM $MgCl_2$, and 5 mM DTT at pH 7.2. The reactions mixtures containing buffer, inhibitor, FDP and protein were prepared on ice in a total volume of 250 μL, and initialized by the addition and overlaid with 0.8 mL of HPLC-grade hexane prior to incubation. The reactions were initiated by addition of the enzyme and the resulting mixtures were incubated at room temp (22 °C) for 15 min. The reactions were immediately ice-cooled and quenched by addition of 100 μL of EDTA (100 mM, pH 8.5) and brief vortexing. The hexane overlay and two additional 1 mL hexane extracts were passed through a short pipette column containing silica gel and $MgSO_4$. The column was washed with additional hexane (1 mL) and the combined filtrates were analyzed by liquid scintillation counting using 15 mL of Ecoscint O. Steady-State kinetic parameters were obtained according to the graphical procedure developed by Lineweaver-Burk.[24]

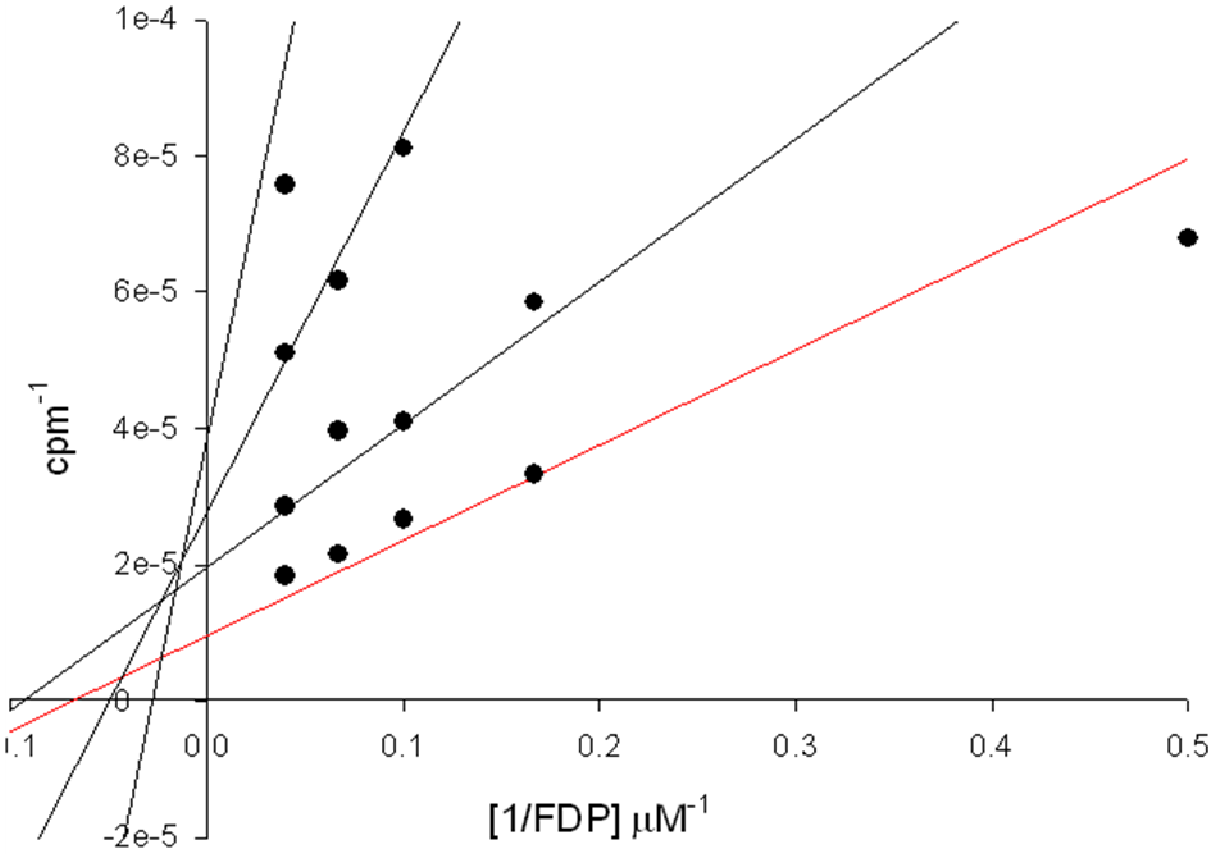
8. Lineweaver-Burk Plots.



Strong competitive inhibition

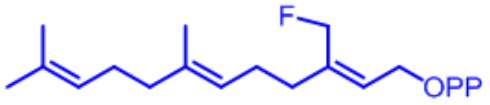
Inhibition Studies with (2Z, 6E)-2F-FDP.

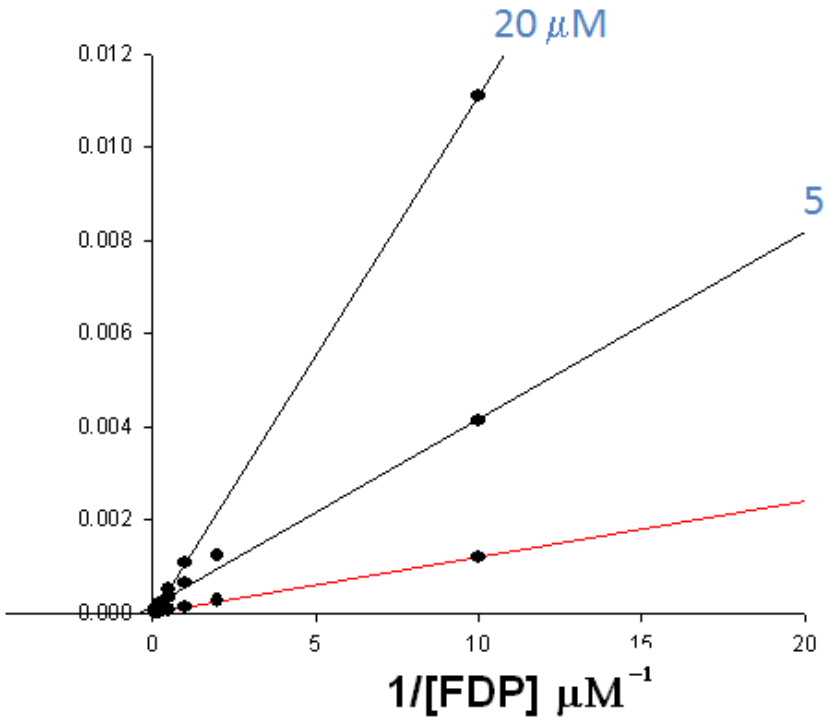
(E)-β-Farnesene Synthase (60 nM)
No Inhibitor (lower) vs 5, 20 and 50 μM 2F-FDP
@ 0.5-25 μM FDP. Interception Zoom In

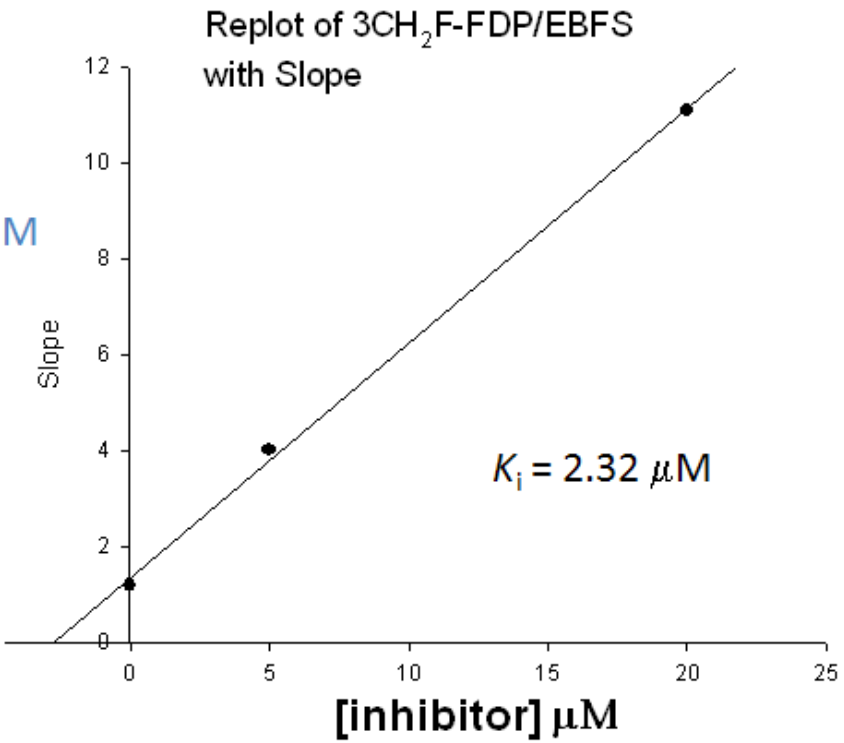


Inhibition Studies with $3CH_2F$-FDP. Lineweaver-Burk Plot

(E)-β-Farnesene Synthase (60 nM)
No Inhibitor (lower) vs 5, and 20 μM $3CH_2F$-FDP
@ 0.1-10 μM FDP



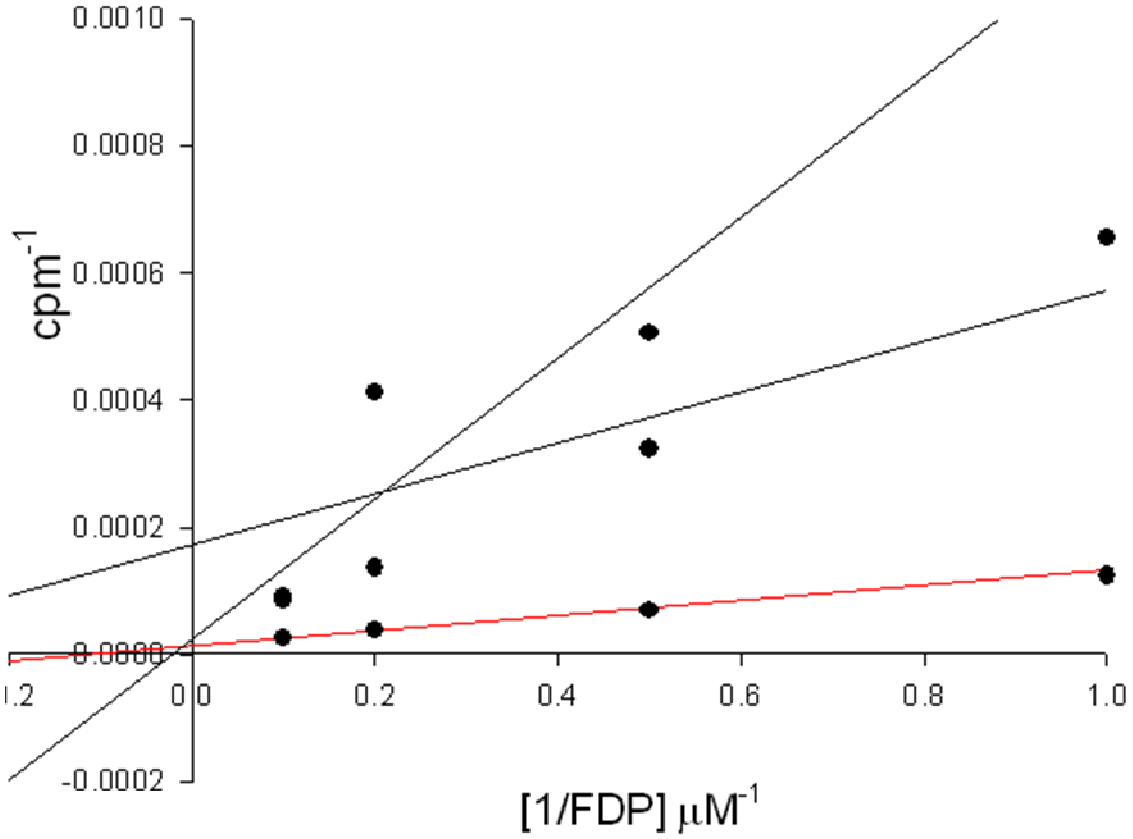




Strong competitive inhibition

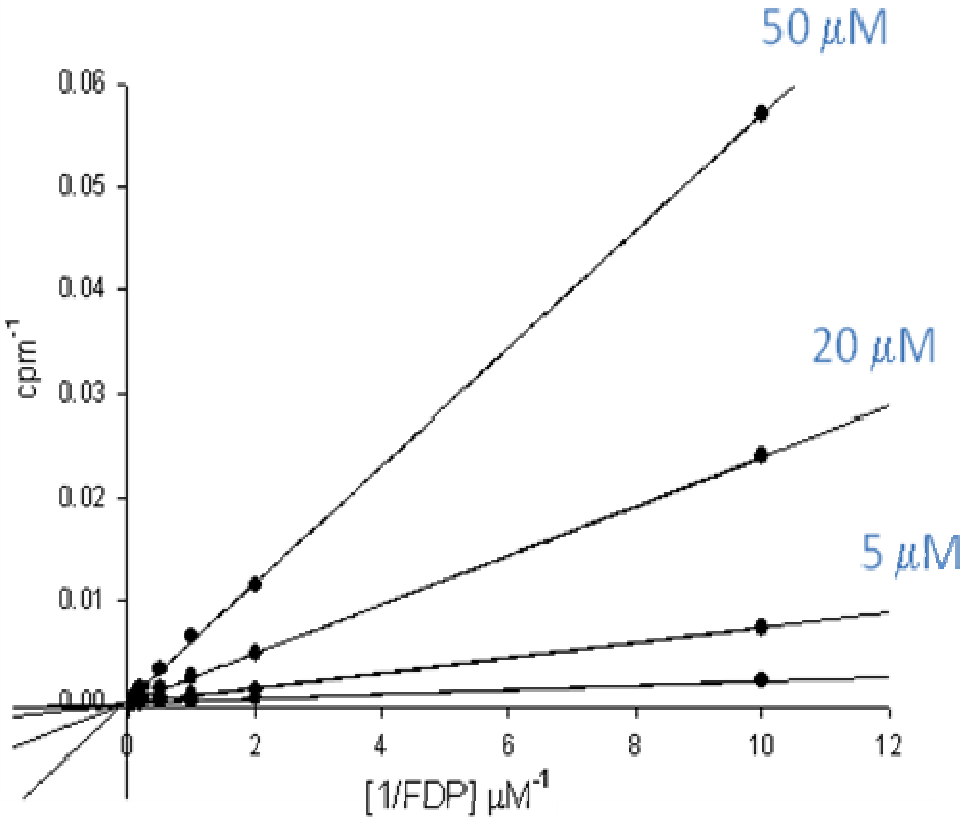
Inhibition Studies with $3CH_2F$-FDP

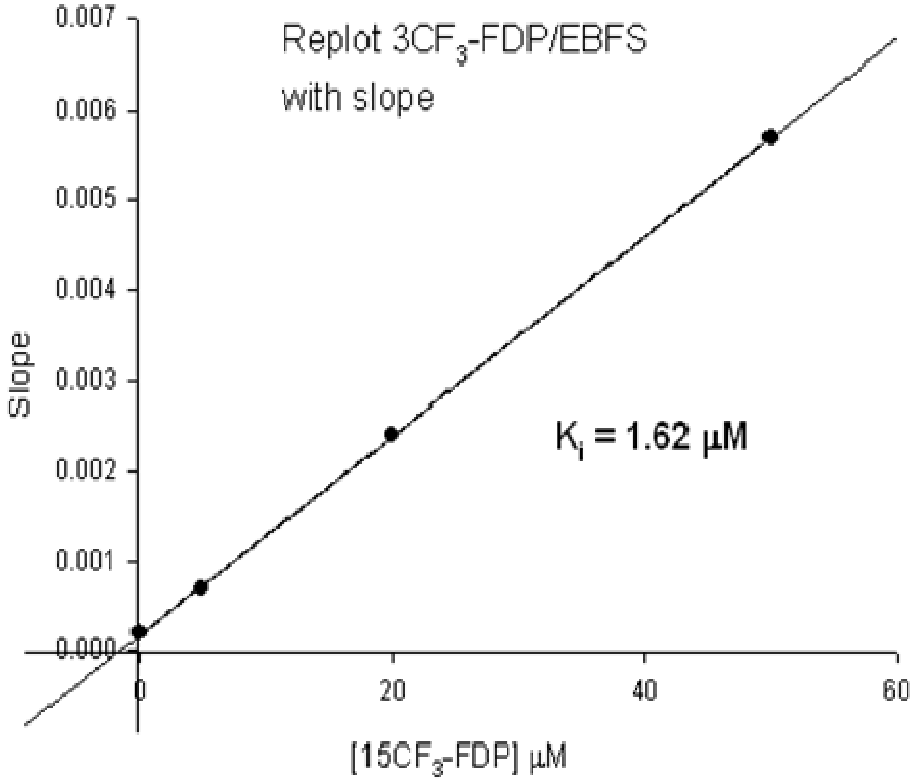
(E)-β-Farnesene Synthase (60 nM)
No Inhibitor (lower) vs 5, and 20 μM $3CH_2F$-FDP
@ 0.1-10 μM FDP.Interception Zoom In



Inhibition Studies with $3CF_3$-FDP. Lineweaver-Burk Plot

(E)-β-Farnesene Synthase (60 nM)
No Inhibitor (lower) vs 5, 20, 50 μM $3CF_3$-FDP
@ 0.5-10 μM FDP

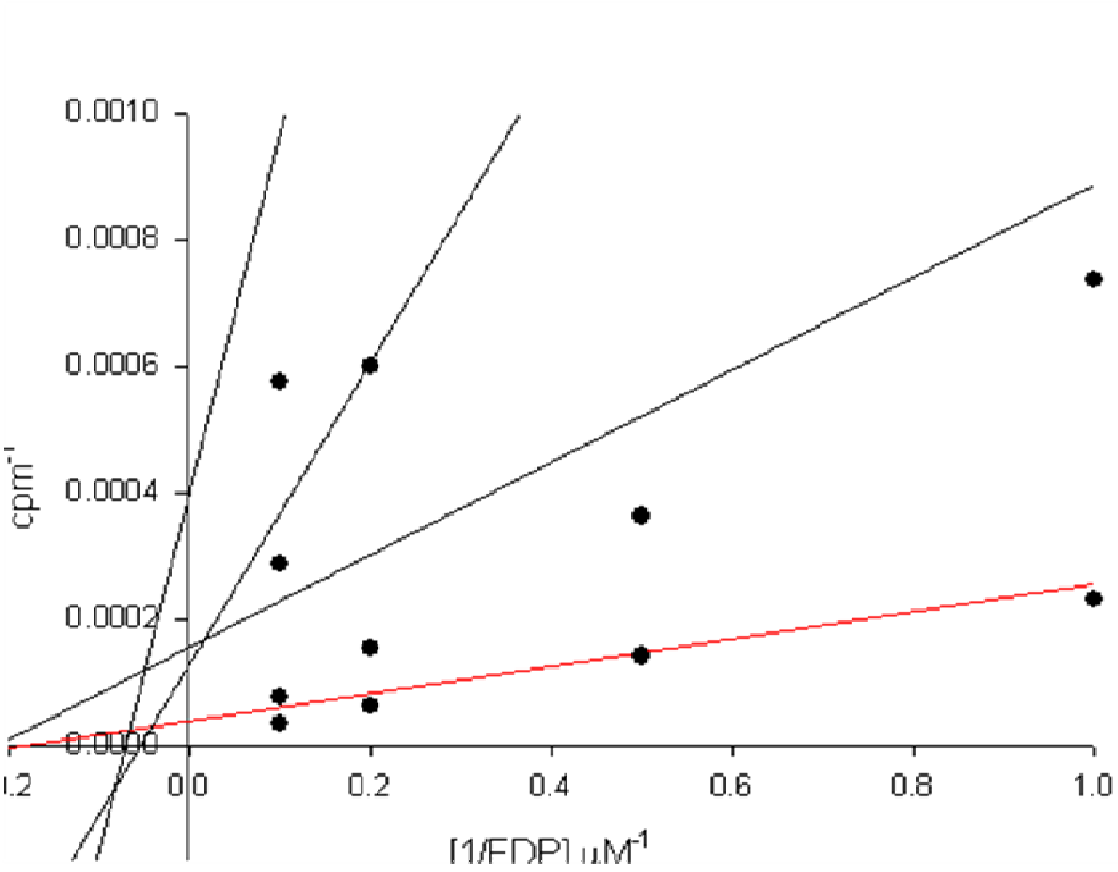




Strong competitive inhibition

Inhibition Studies with $3CF_3$-FDP

(E)-β-Farnesene Synthase (60 nM)
No Inhibitor (lower) vs 5, 20 and 50 μM $15CF_3$-FDP
@ 0.1-10 μM FDP. Interception Zoom In



9. Analytical Incubations of EBFS with FDP and FDP analogues. The prenyl diphosphate (25 μL, 10 mM) was added to assay buffer [350 μL, 20 mM MOPS, 5 mM DTT, and 5 mM $MgCl_2$ (pH 7.2)] follow by addition of EBFS (125 μL, 40 μM). The aqueous layer was overlaid with HPLC grade pentane (0.5 mL) and the resulting micture was incubated (18-24 h) at 25 °C. The enzymatic hydrocarbons were extracted with pentane (3 x 0.5 mL), and the resulting organic layer was through a short pad of silica gel (< 500 mg). The incubations were repeated without enzyme as negative controls. The filtered pentane extracts are then analyzed by gas chromatography-mass spectrometry (GC-MS) according to General Methods.

GC-MS profile of EBFS with (*E*, *E*)-farnesyl diphosphate (FDP). Incubation of purified EBFS with FDP in assay buffer gave (*E*)-β-farnesene (95%) as the dominant product, along with small amount of three other acyclic sesquiterpenes, (*Z*)-β-farnesene (1.5%), (3*Z,* 6*E*)-α-farnesene (1.3%) and (3*E,* 6*E*)-α-farnesene (0.2%) (Figure S8). The identity of the peaks for (*E*)-β-farnesene, (3*Z,*6 *E*)-α-farnesene and (3*E,* 6*E*)-α-farnesene was confirmed by comparing the GC retention time and MS spectrum of the enzymatic products with those obtained for authentic standards (Figure S9 and S10).[19,21] Enzymatic (*Z*)-β-farnesene was tentatively identified by comparing its mass spectrum with that provide in the NIST database. Another 5 sesquiterpene products (<2% in total) were also detected by GC-MS (Figure S8). The current product profile differs somewhat to that obtained previously from a partially purified preparation of recombinant EBFS and FDP, which was reported to yield (*E*)-β-farnesene (85%), (*Z*)-β-farnesene (8%), and δ-cadinene (5%) plus other minor sesquiterpene hydrocarbons.[25]

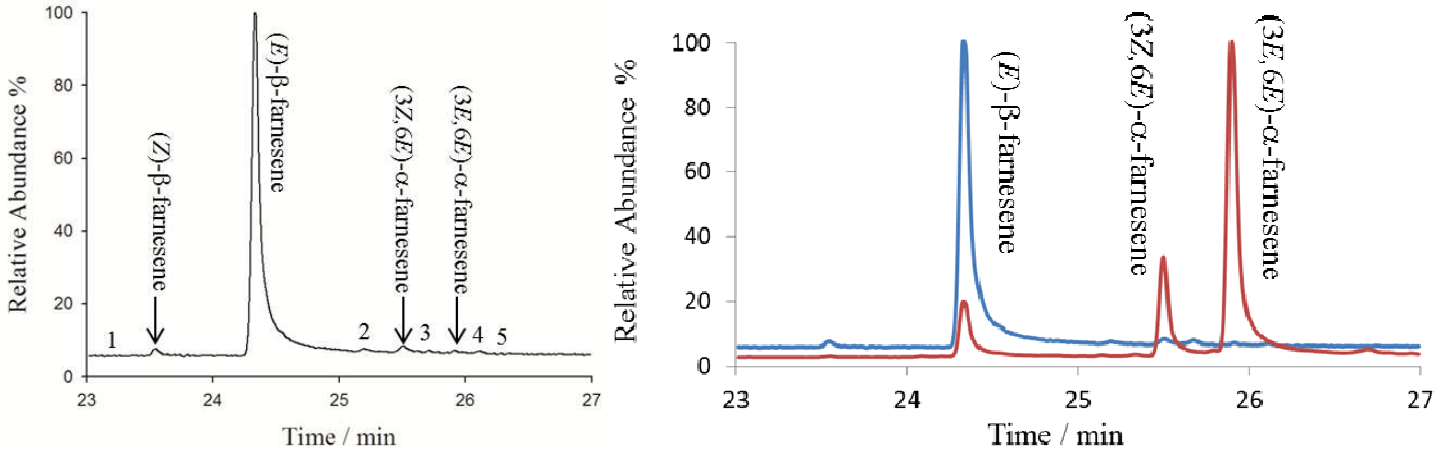


Figure S1. Left: GC trace for pentane-extractable products following incubation of 10 μM EBFS with 0.5 mM FDP in the presence of 5 mM Mg^{2+}. Peaks 1 to 5 are unknown sesquiterpene products. Right: Overlay of GC chromatograms for EBFS incubation products (blue) and synthetic (*E*)-β-farnesene, (*Z*)-α-farnesene and (*E*)-α-farnesene samples (red).

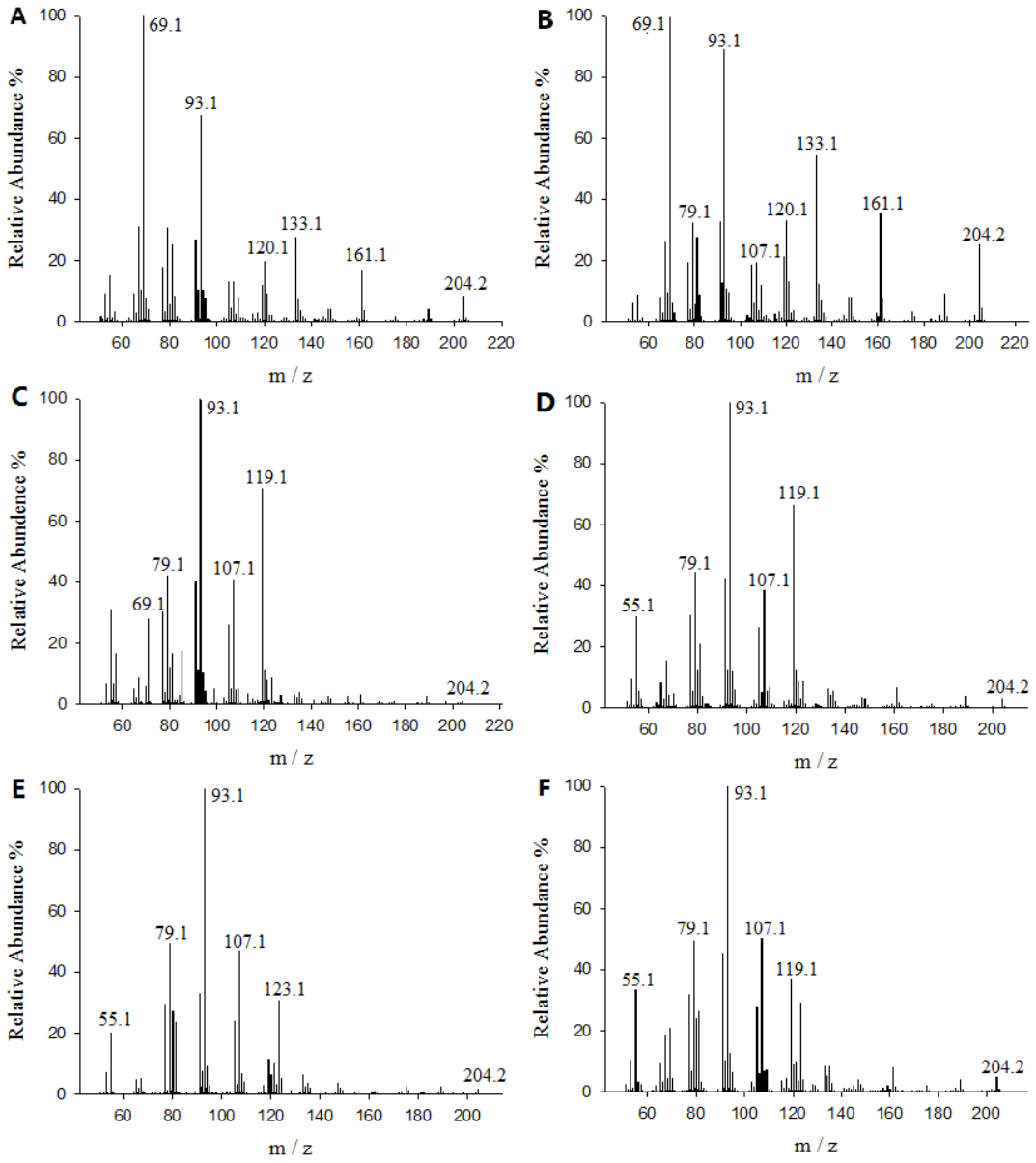


Figure S2. EI-mass spectrum of (*E*)-β-farnesene (enzymatic (A) and standard (B)), (Z)-α-farnesene (enzymatic (C) and standard (D)), and (E)-α-farnesene (enzymatic (E) and standard (F)).

11. Analytical Incubations of DMADP and isoprene synthase (IS). Enzymatic reactions were performed in 0.3 mL ReactiVials (Thermo Fisher, Waltham, MA) sealed with PTFE/silicone septa (SUPELCO, USA). Each reaction was initiated by adding 0.6 µmol unlabeled DMADP, (*Z*)-[4,4,4-$^{2}H_3$]DMADP, or (*E*)-[4,4,4-$^{2}H_3$]DMADP to a 30 µL reaction mixture containing 1 nmol ISPS in ISB buffer [50 mM Tris–HCl (pH 8.5), 20 mM $MgCl_2$, 5% glycerol, and 2 mM dithiothreitol]. After the reaction vial was incubated for 60 min at 35 °C, the reaction was stopped by removing the solution with a gas tight syringe (SGE Analytical Science, Australia) and washing the vial with 50 µL ISB buffer. A 10 µL sample of headspace gas was manually injected into a Thermo Scientific ISQ single quadrupole GC-MS system (Waltham, MA) equipped with a Stabilwax column (Restek, Bellefonte, PA) using helium as the carrier gas at a flow rate of 1.5 mL/min. The injection port was held at 230 °C with the following GC settings: 35 °C hold for 4 min, ramp to 230 °C at 50 °C/min. The MS parameters were as follows: ion source temperature = 200 °C using electron impact ionization (70 eV), scanning frequency = 4/s from m/z 45 to m/z 300. Isoprene is a very volatile organic compound (bp = 34 °C) and its elution time from the GC column is 1.7 - 3.0 min.

GC-MS profile of IS with (DMADP) and analogues

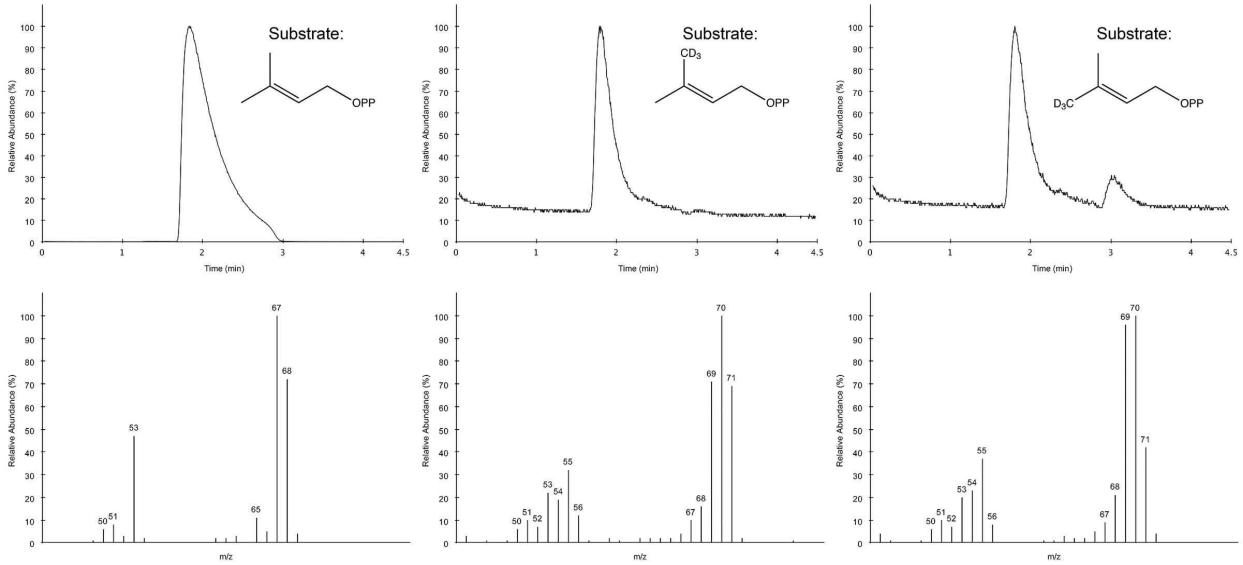
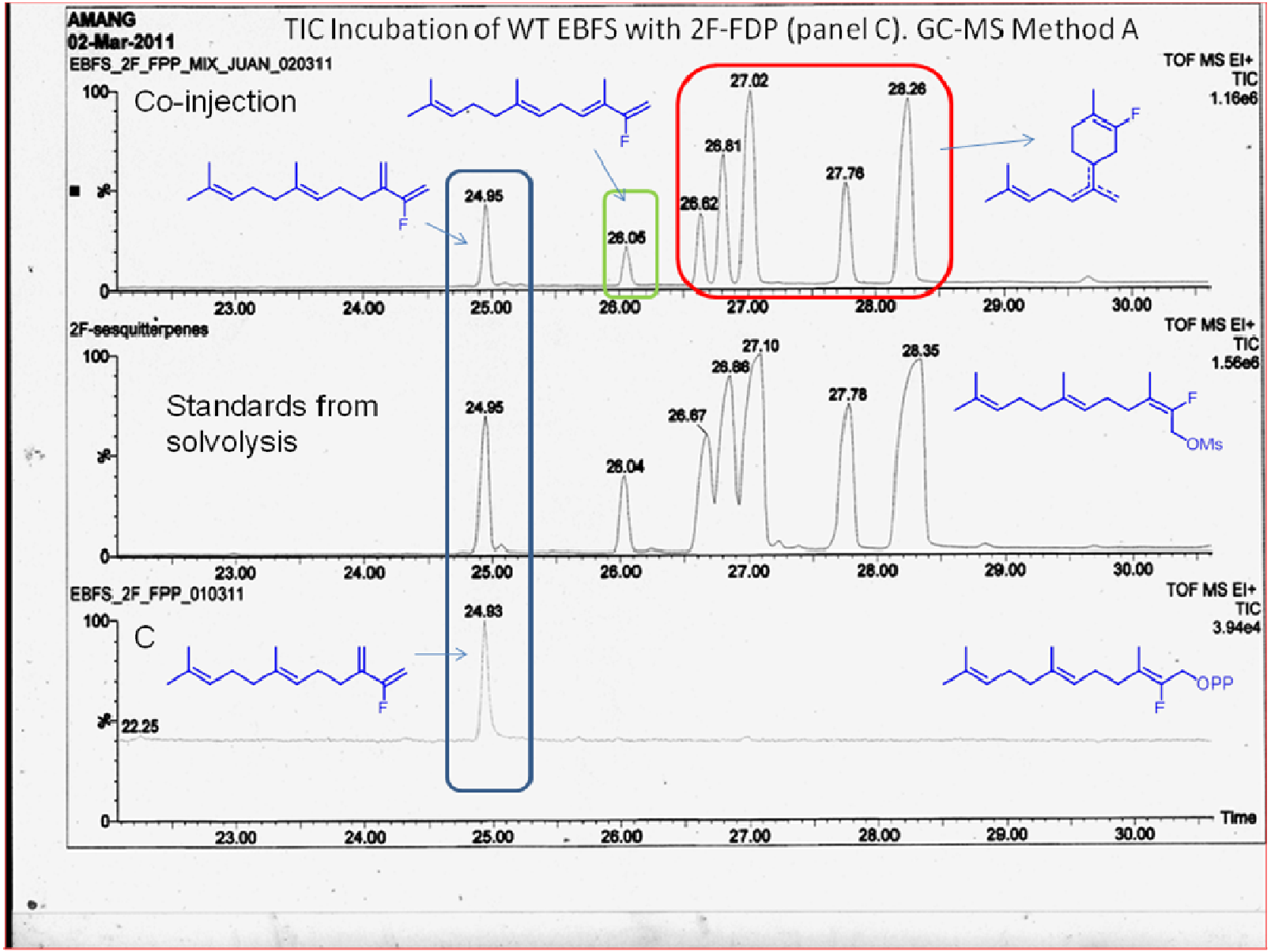


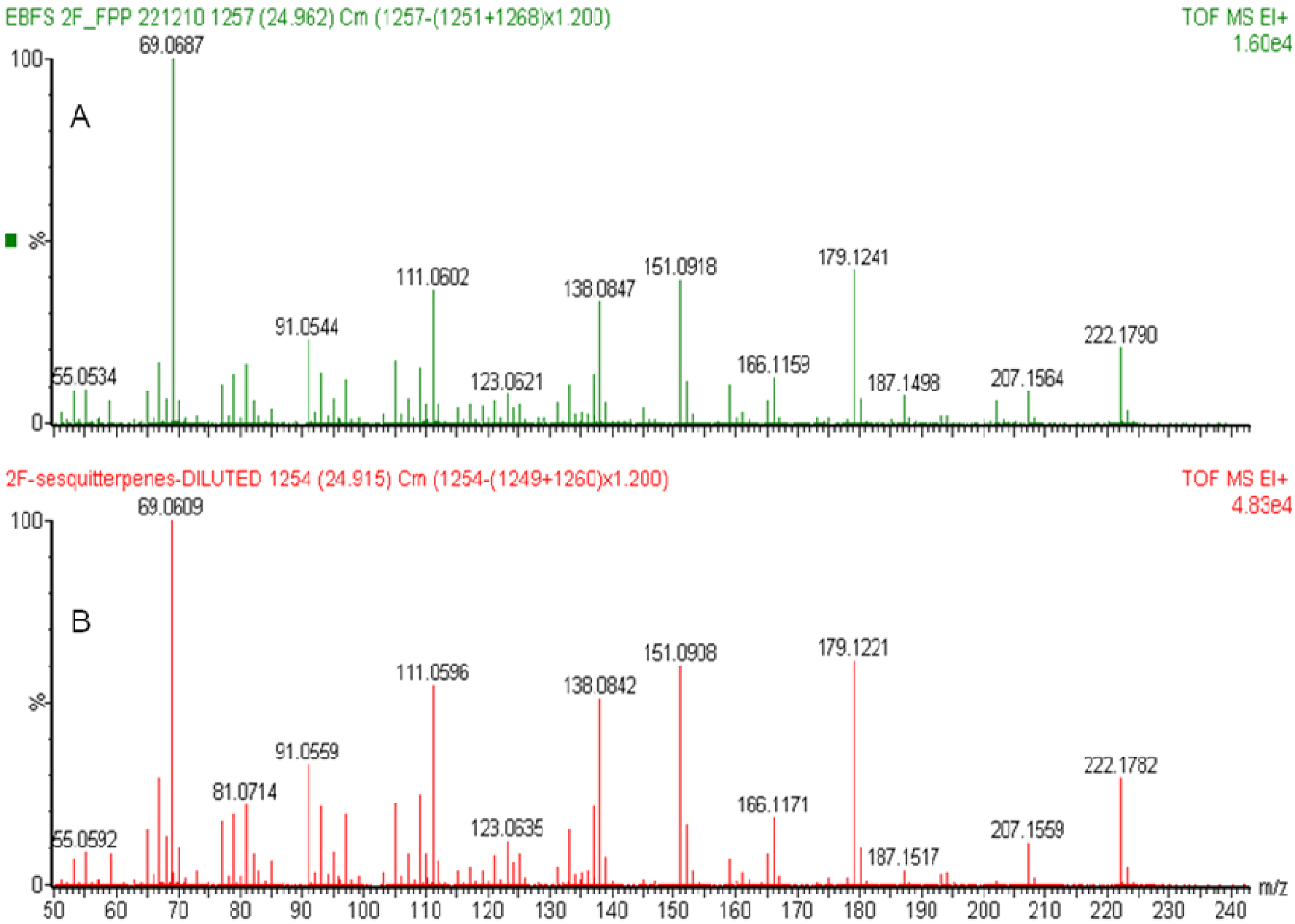
Figure S3: GC trace (upper panels) and the EI mass spectrum (lower panels) of isoprene produced by ISPS-catalyzed reactions using DMADP or its deuterated isomers; the small peak with retention time of ~3 min obtained from substrate (*E*)-[4,4,4-$^{2}H_3$]DMADP is believed to be an impurity. Regardless of whether (*Z*)-[4,4,4-$^{2}H_3$]DMADP or (*E*)-[4,4,4-$^{2}H_3$]DMADP is utilized as a substrate, both deuterated isoprene products with mass 70 and 71 are detected (along with the corresponding lower molecular weight fragments), which is consistent with the non-regiospecific elimination pathway

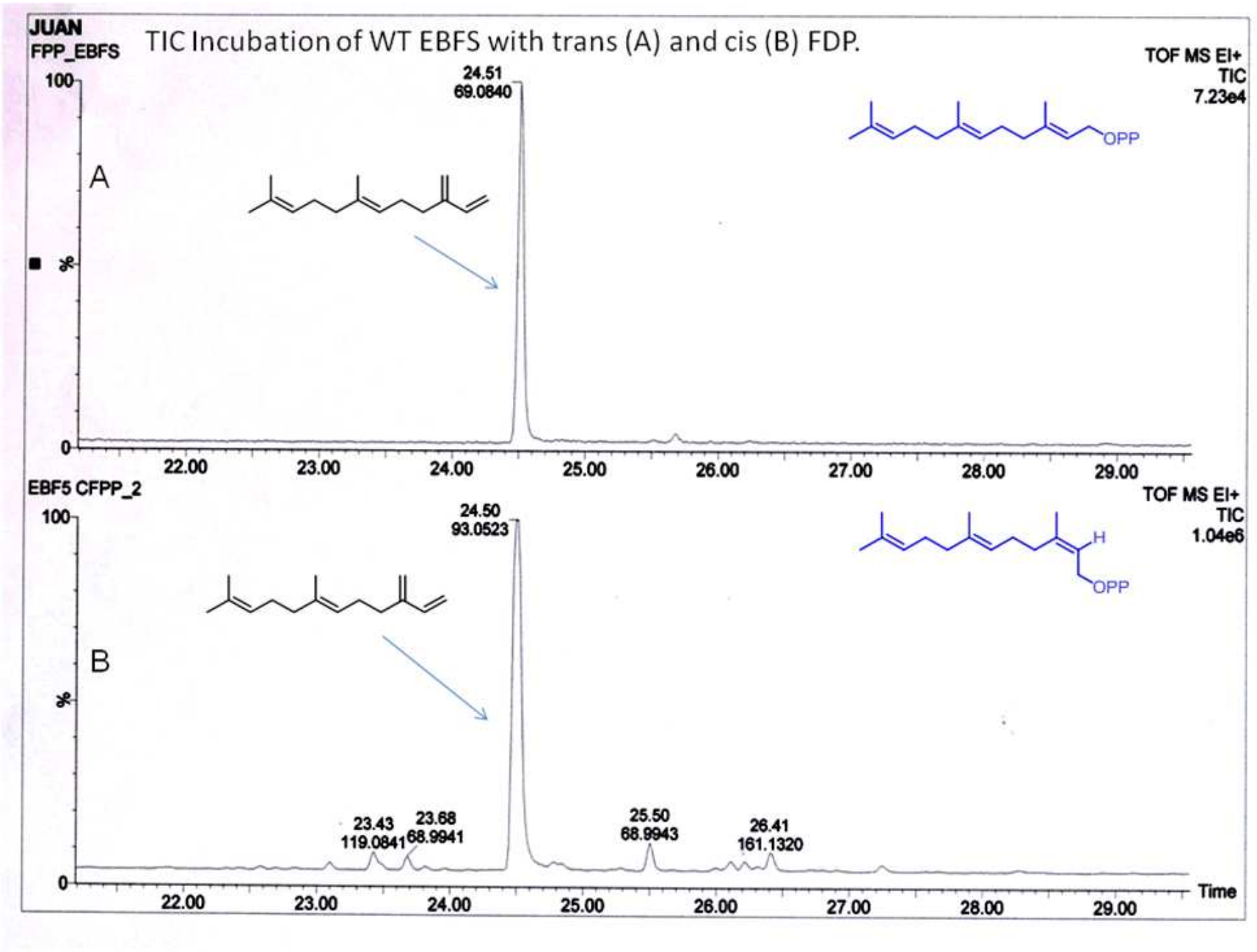
10. Collection of GC-MS

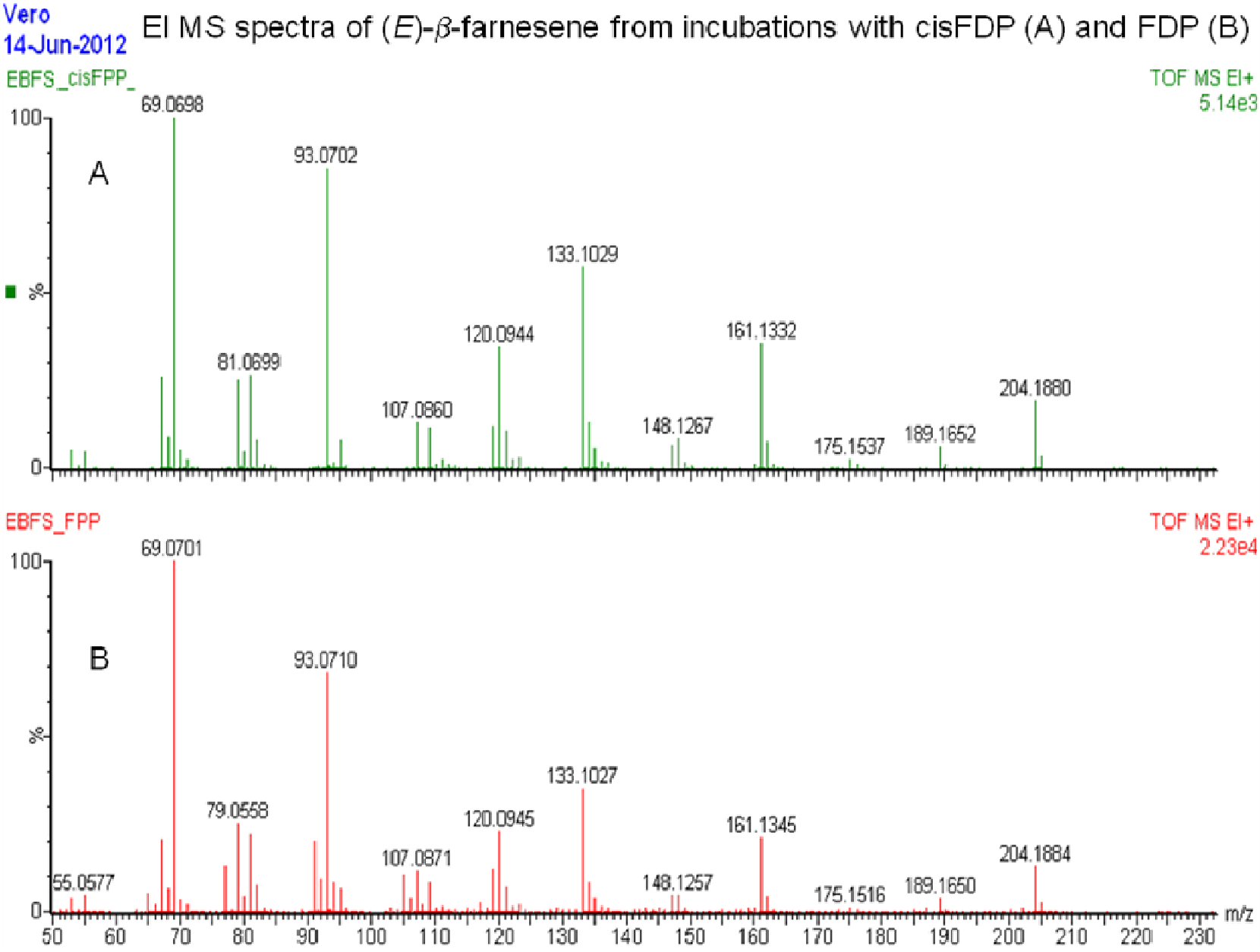


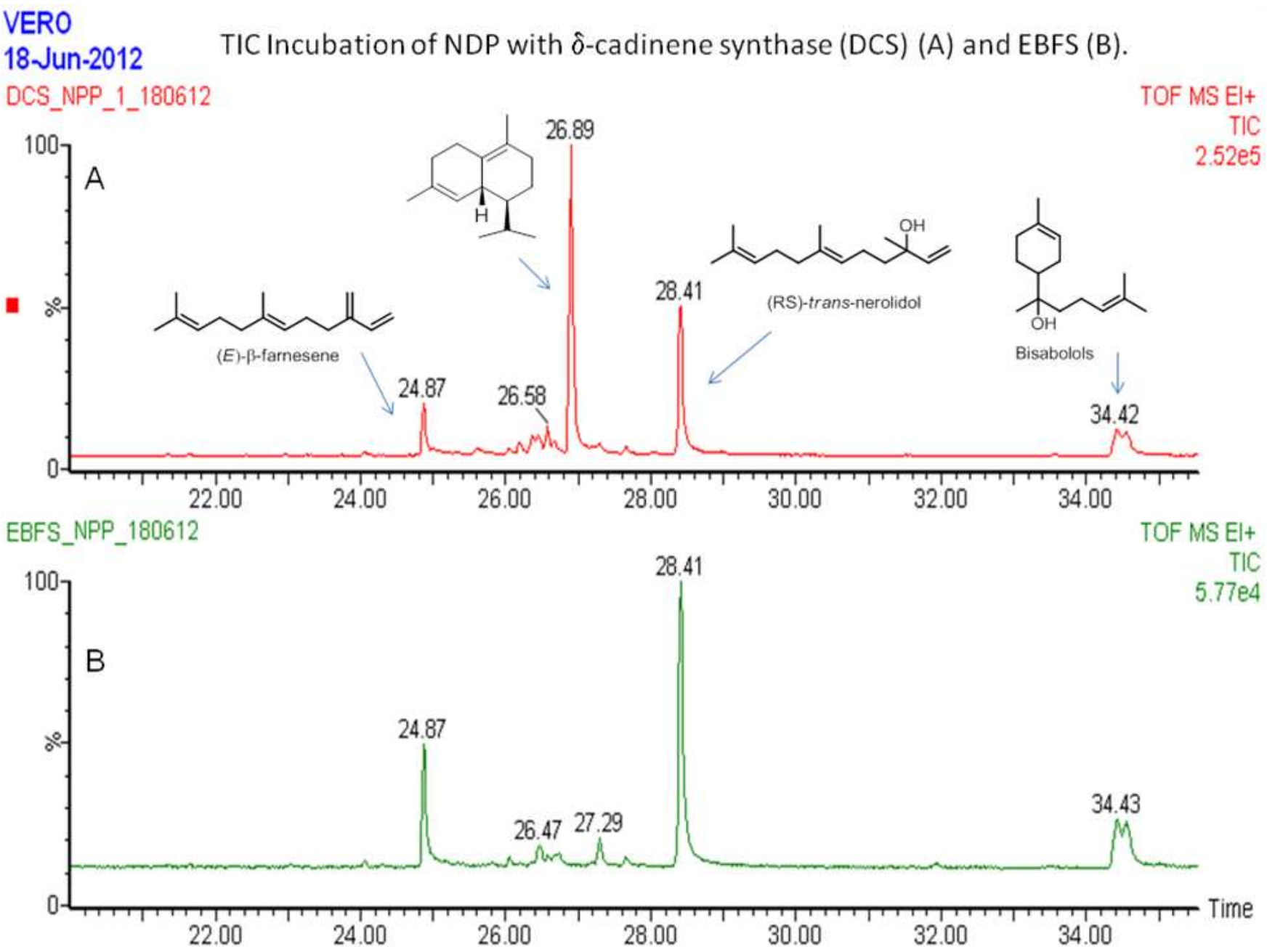
AMANG
22-Dec-2010

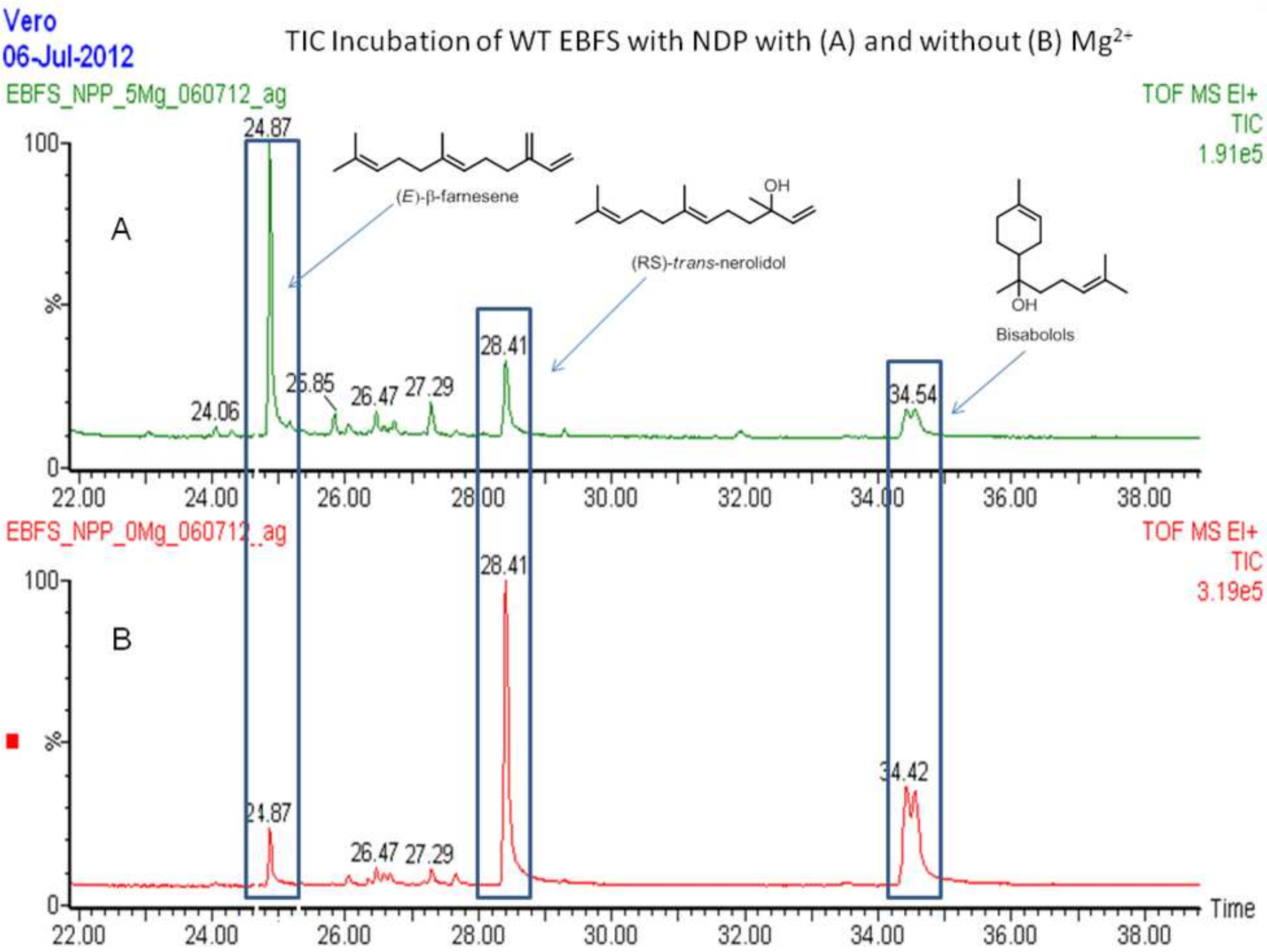
EI MS spectra of enzymatic (A) and synthetic (B) (*E*)-β-2F-farnesene

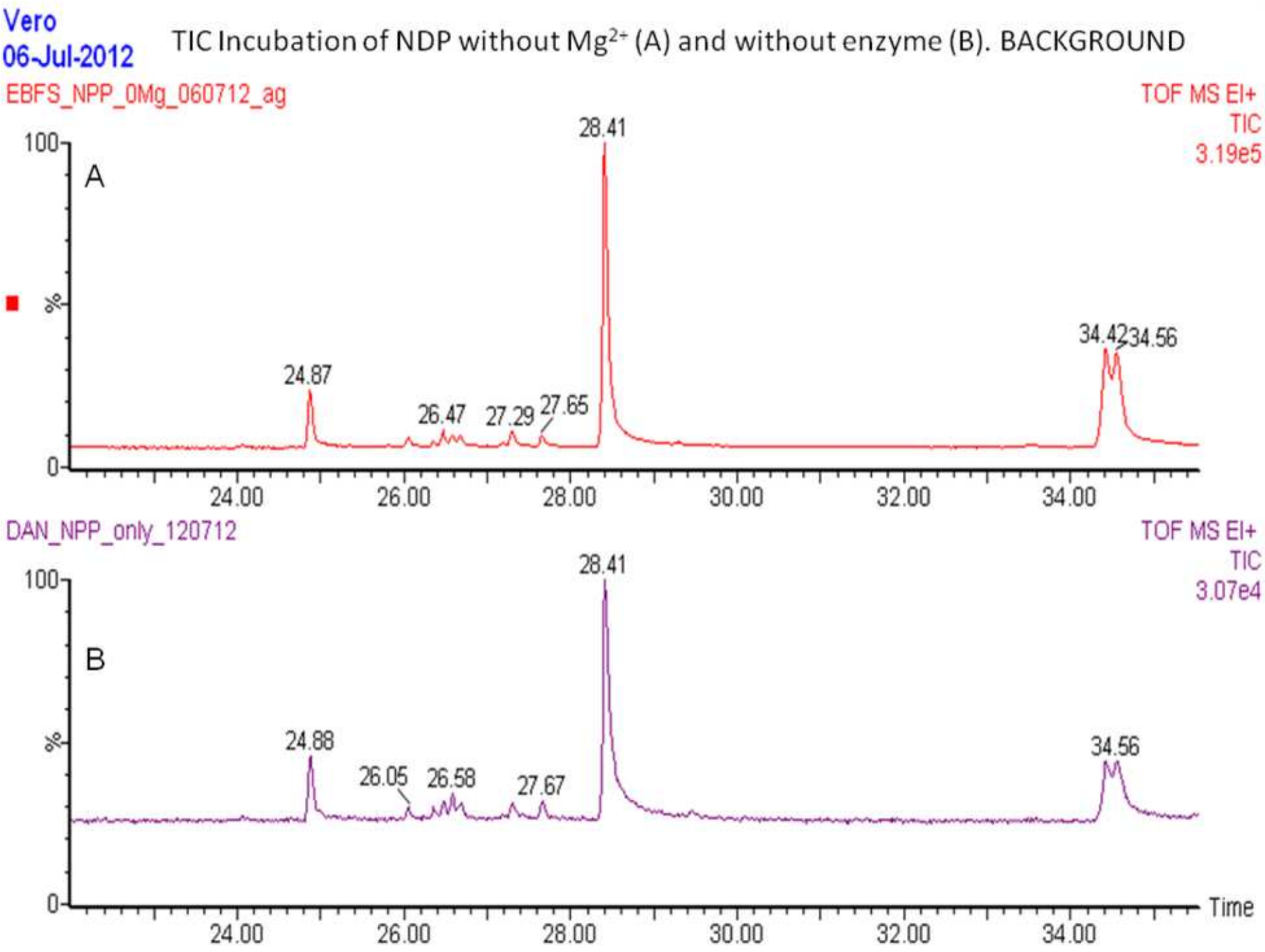


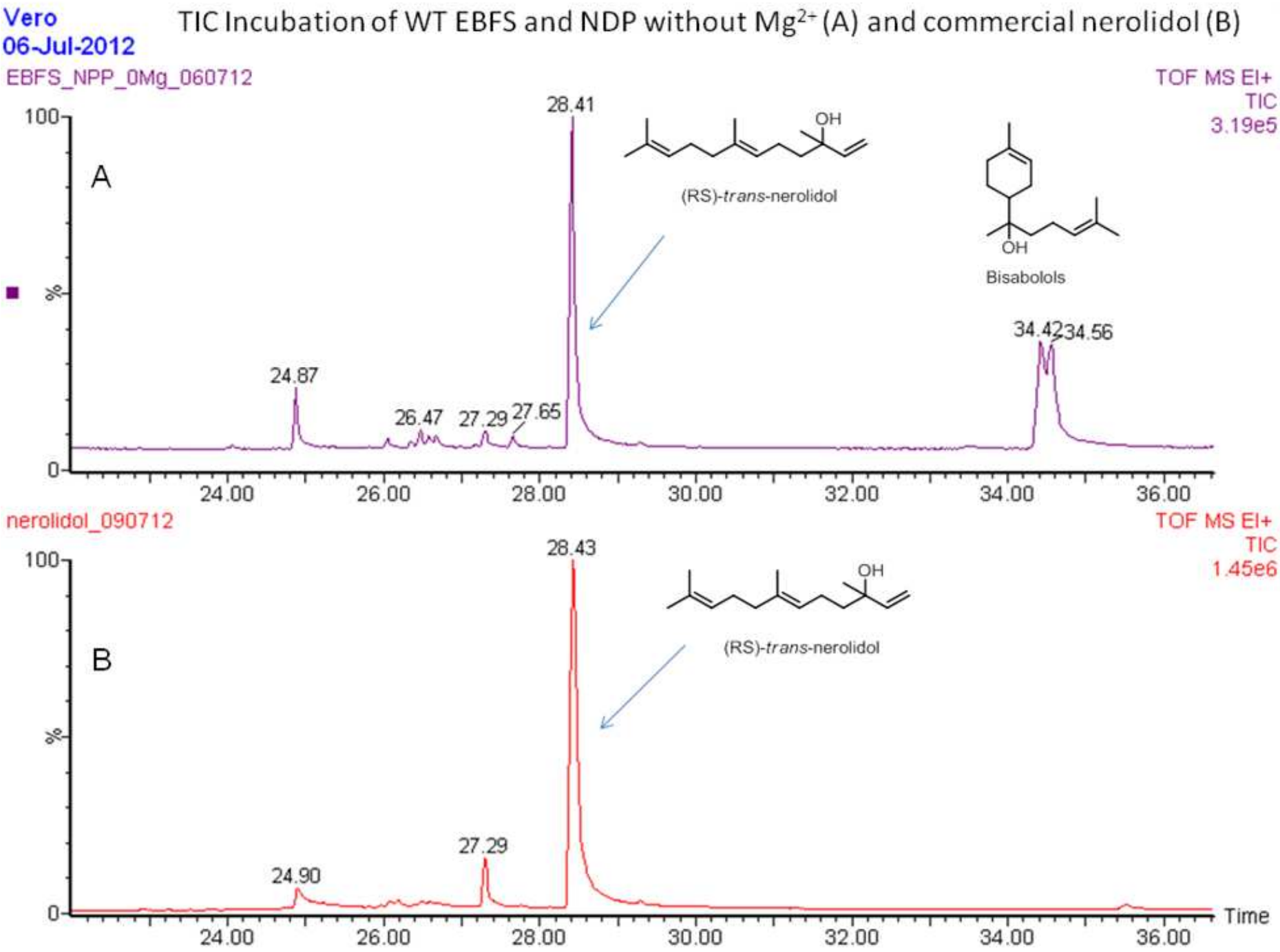

JUAN
FPP_EBFS
TIC Incubation of WT EBFS with trans (A) and cis (B) FDP.
TOF MS EI+
TIC
7.23e4
24.51
69.0840
A
OPP
100
%
0
22.00
23.00
24.00
25.00
26.00
27.00
28.00
29.00
EBF5 CFPP_2
TOF MS EI+
TIC
1.04e6
24.50
93.0523
H
OPP
B
23.43
119.0841
23.68
68.9941
25.50
68.9943
26.41
161.1320
Time

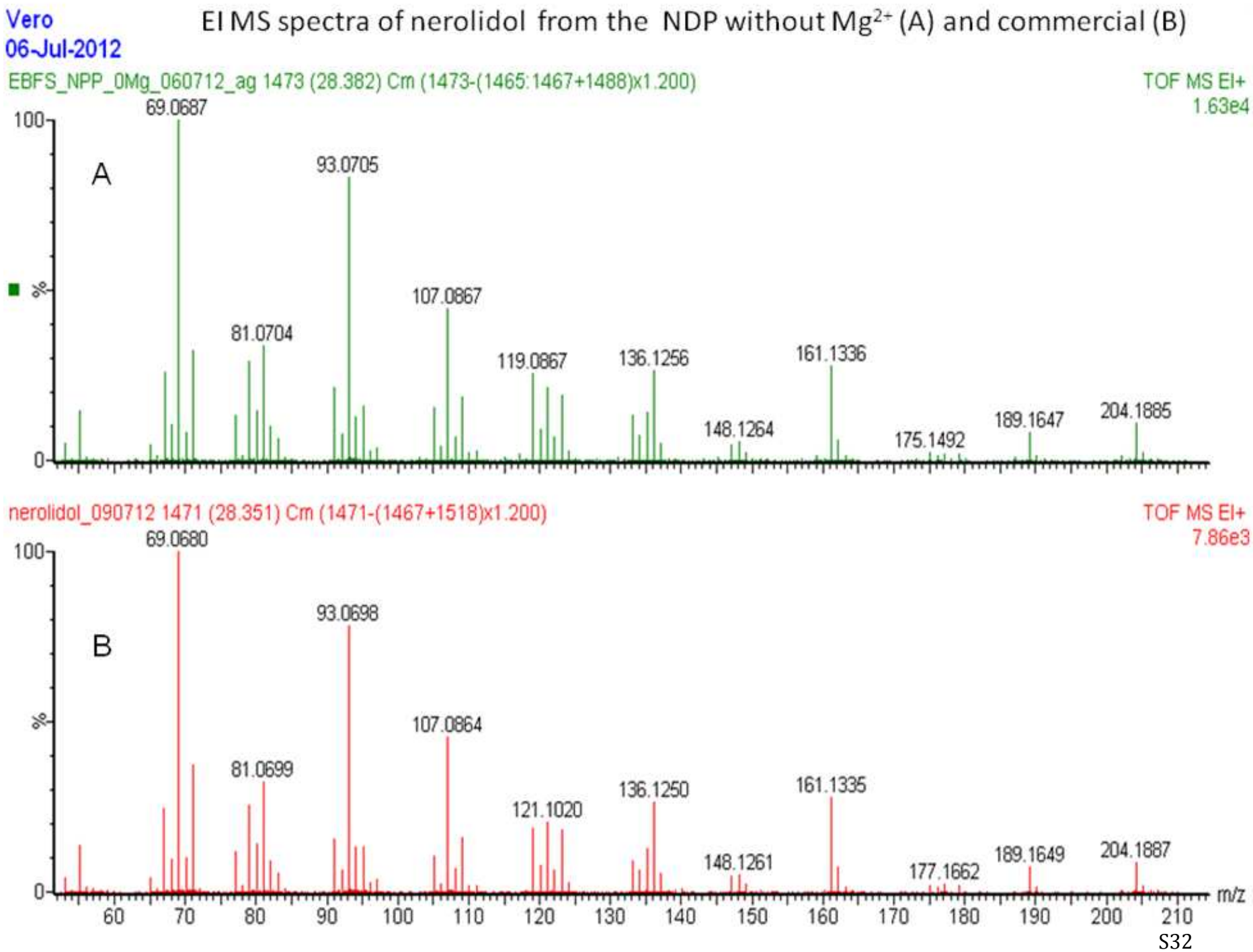

Vero
14-Jun-2012
EI MS spectra of (E)-β-farnesene from incubations with cisFDP (A) and FDP (B)
EBFS_cisFPP_
TOF MS EI+
5.14e3
A
69.0698
93.0702
133.1029
120.0944
161.1332
81.0699
107.0860
204.1880
148.1267
175.1537
189.1652
EBFS_FPP
TOF MS EI+
2.23e4
B
69.0701
93.0710
133.1027
79.0558
120.0945
161.1345
107.0871
204.1884
55.0577
148.1257
175.1516
189.1650
%
m/z

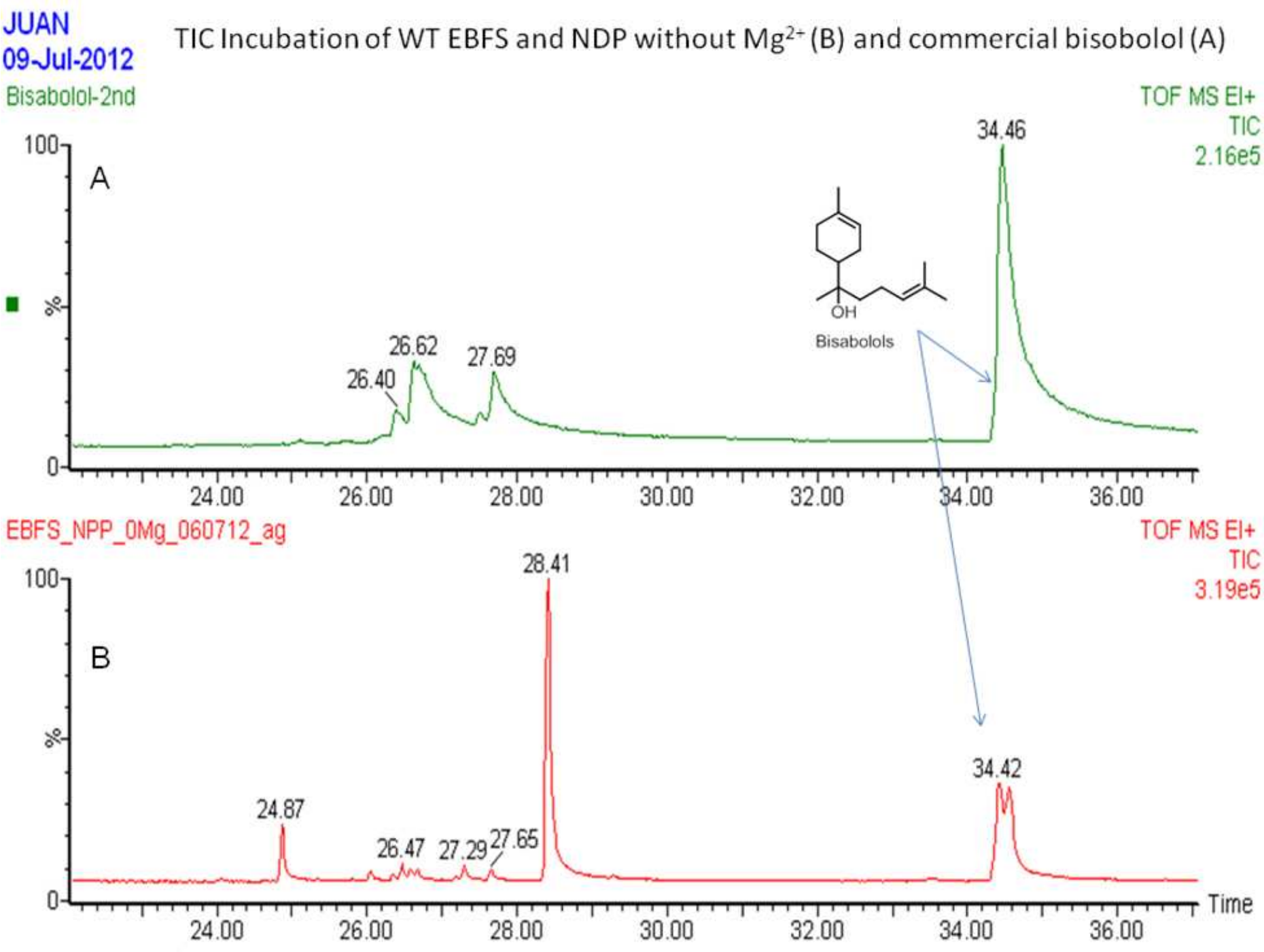

VERO
18-Jun-2012
TIC Incubation of NDP with δ-cadinene synthase (DCS) (A) and EBFS (B).
DCS_NPP_1_180612
TOF MS EI+
TIC
2.52e5
100
A
%
0
26.89
28.41
24.87
26.58
34.42
(E)-β-farnesene
(RS)-trans-nerolidol
Bisabolols
OH
H
22.00
24.00
26.00
28.00
30.00
32.00
34.00
EBFS_NPP_180612
TOF MS EI+
TIC
5.77e4
100
B
%
0
28.41
24.87
26.47
27.29
34.43
Time
22.00
24.00
26.00
28.00
30.00
32.00
34.00

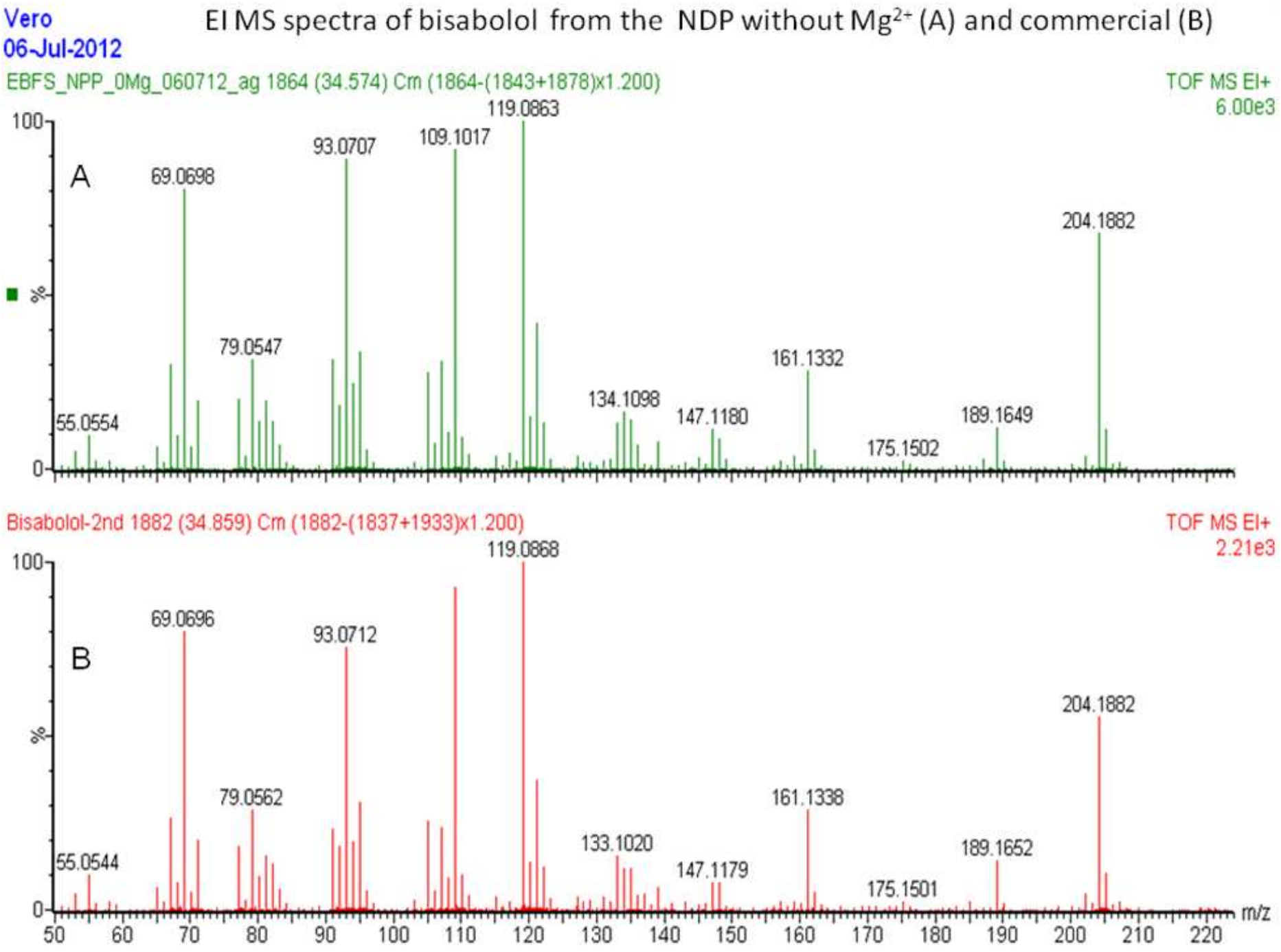

Vero
06-Jul-2012
TIC Incubation of WT EBFS with NDP with (A) and without (B) Mg2+
EBFS_NPP_5Mg_060712_ag
TOF MS EI+
TIC
1.91e5
24.87
(E)-β-farnesene
OH
(RS)-trans-nerolidol
OH
Bisabolols
A
28.41
25.85
26.47
27.29
24.06
34.54
EBFS_NPP_0Mg_060712_ag
TOF MS EI+
TIC
3.19e5
28.41
B
34.42
24.87
26.47
27.29
Time
22.00
24.00
26.00
28.00
30.00
32.00
34.00
36.00
38.00

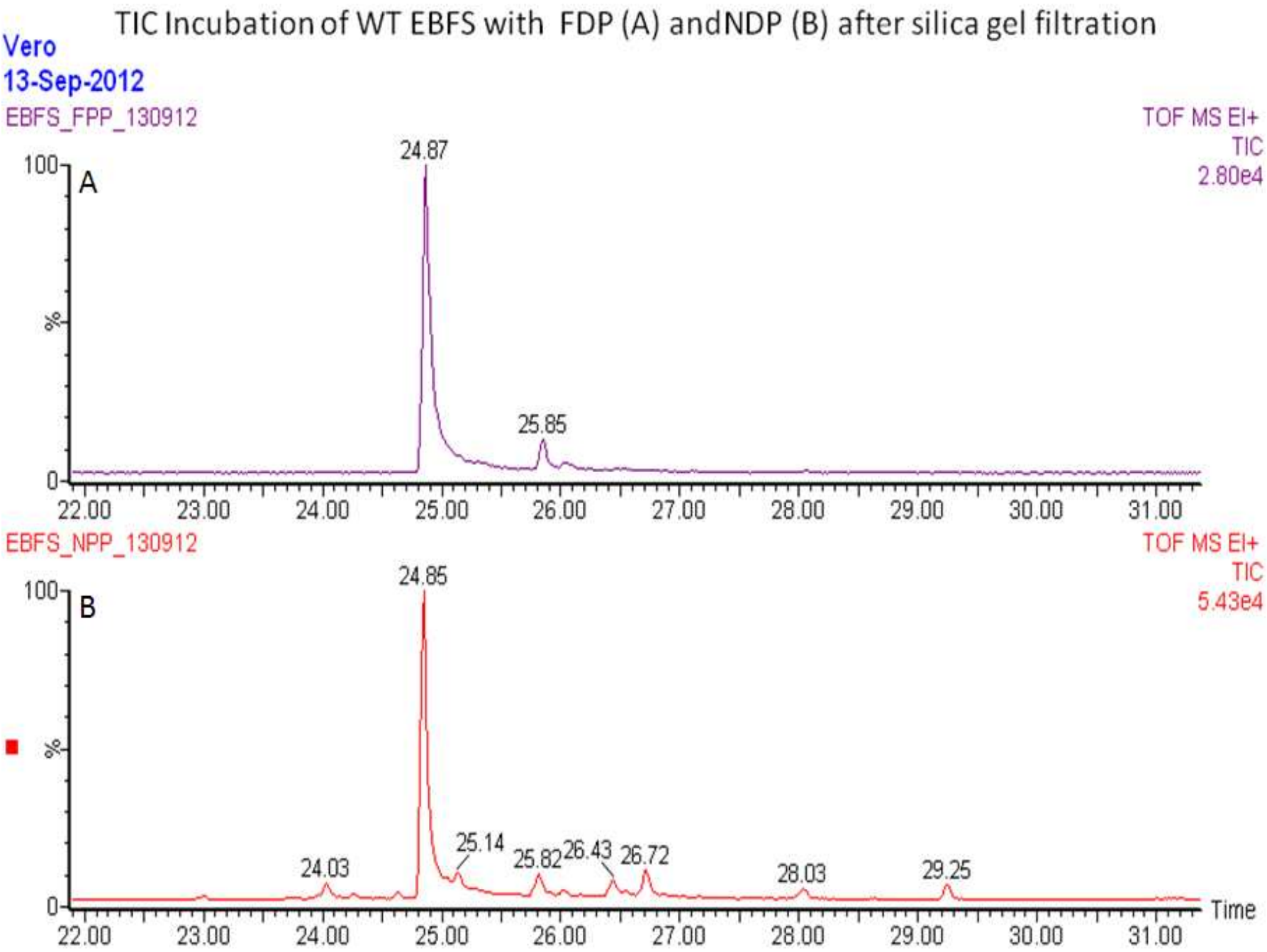

Vero
06-Jul-2012
TIC Incubation of NDP without Mg2+ (A) and without enzyme (B). BACKGROUND
EBFS_NPP_0Mg_060712_ag
TOF MS EI+
TIC
3.19e5
A
100
%
0
28.41
24.87
26.47
27.29
27.65
34.42
34.56
24.00
26.00
28.00
30.00
32.00
34.00
DAN_NPP_only_120712
TOF MS EI+
TIC
3.07e4
B
28.41
24.88
26.05
26.58
27.67
34.56
Time

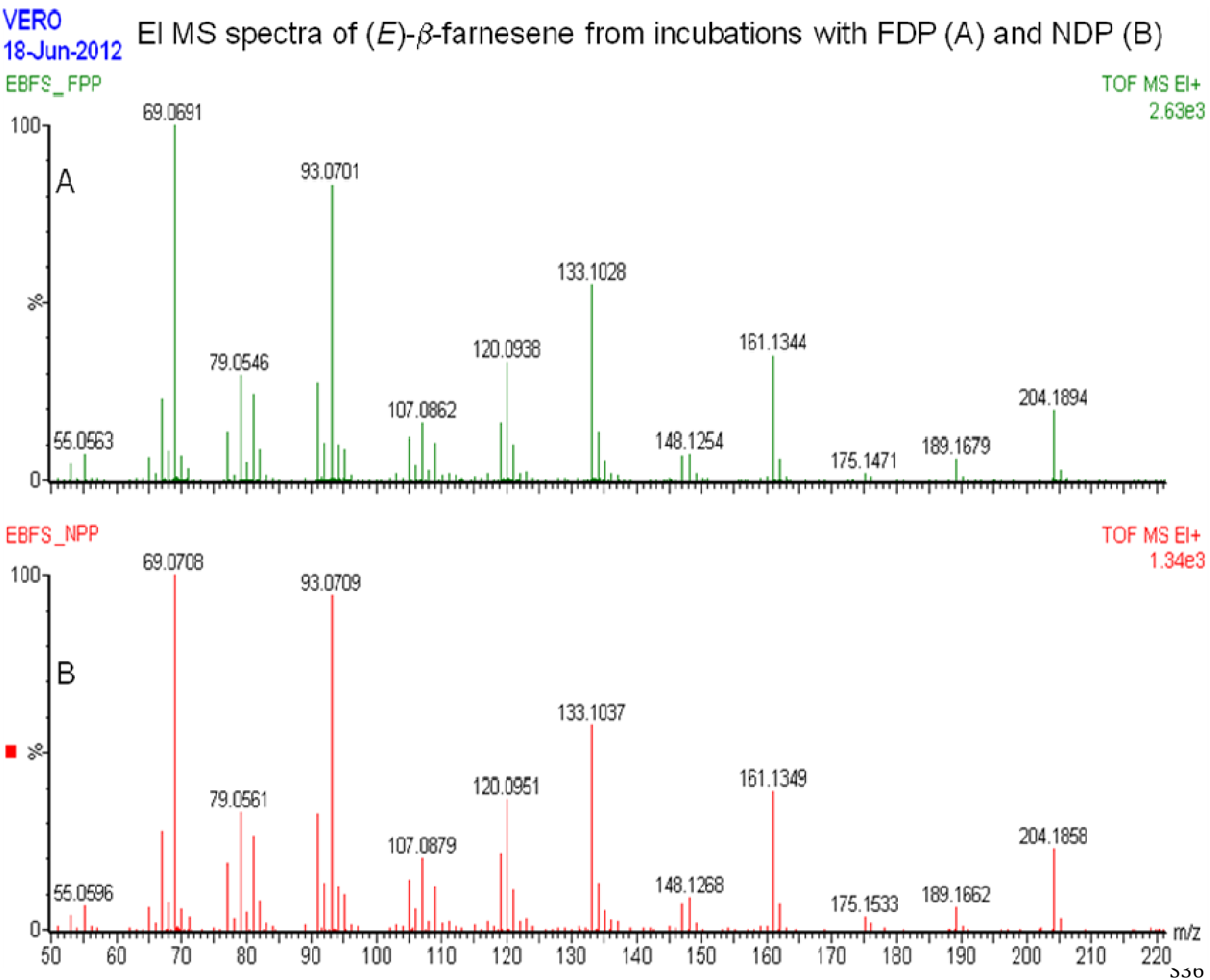

Vero
06-Jul-2012
TIC Incubation of WT EBFS and NDP without Mg2+ (A) and commercial nerolidol (B)
EBFS_NPP_0Mg_060712
TOF MS EI+
TIC
3.19e5
A
28.41
OH
(RS)-trans-nerolidol
OH
Bisabolols
34.42
34.56
24.87
26.47
27.29
27.65
100
%
0
24.00
26.00
28.00
30.00
32.00
34.00
36.00
nerolidol_090712
TOF MS EI+
TIC
1.45e6
B
28.43
OH
(RS)-trans-nerolidol
27.29
24.90
Time


Vero
06-Jul-2012
EI MS spectra of nerolidol from the NDP without Mg2+ (A) and commercial (B)
EBFS_NPP_0Mg_060712_ag 1473 (28.382) Cm (1473-(1465:1467+1488)x1.200)
TOF MS EI+
1.63e4
A
69.0687
93.0705
107.0867
81.0704
119.0867
136.1256
161.1336
148.1264
175.1492
189.1647
204.1885
nerolidol_090712 1471 (28.351) Cm (1471-(1467+1518)x1.200)
TOF MS EI+
7.86e3
B
69.0680
93.0698
107.0864
81.0699
121.1020
136.1250
161.1335
148.1261
177.1662
189.1649
204.1887
100
%
0
m/z
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210

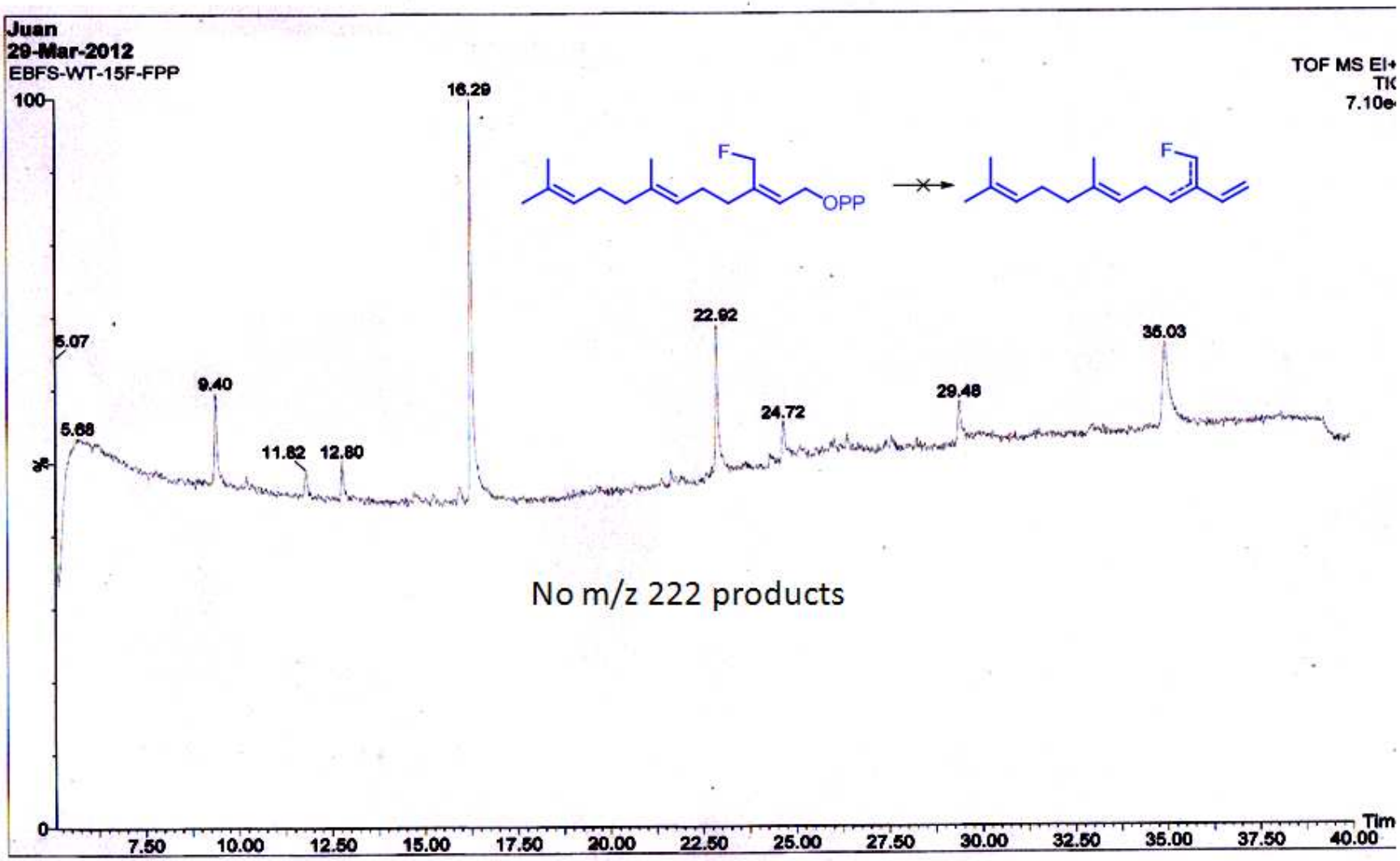

JUAN
09-Jul-2012
TIC Incubation of WT EBFS and NDP without Mg2+ (B) and commercial bisobolol (A)
Bisabolol-2nd
TOF MS EI+
TIC
2.16e5
34.46
A
100
%
0
26.40
26.62
27.69
OH
Bisabolols
24.00
26.00
28.00
30.00
32.00
34.00
36.00
EBFS_NPP_0Mg_060712_ag
TOF MS EI+
TIC
3.19e5
28.41
B
34.42
24.87
26.47
27.29
27.65
Time

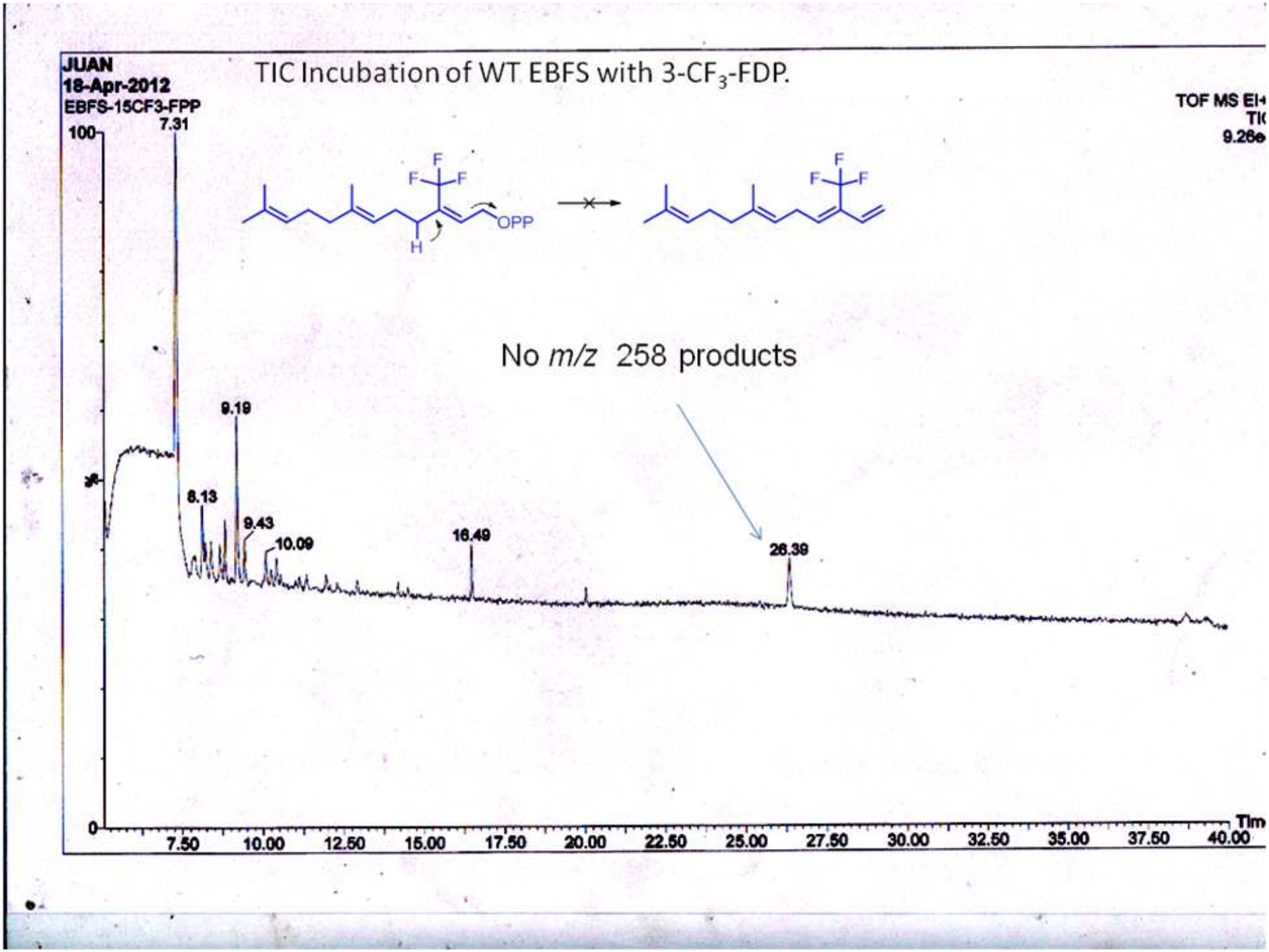

Vero
06-Jul-2012
EI MS spectra of bisabolol from the NDP without Mg2+ (A) and commercial (B)
EBFS_NPP_0Mg_060712_ag 1864 (34.574) Cm (1864-(1843+1878)x1.200)
TOF MS EI+
6.00e3
A
119.0863
109.1017
93.0707
69.0698
204.1882
79.0547
161.1332
134.1098
147.1180
189.1649
55.0554
175.1502
Bisabolol-2nd 1882 (34.859) Cm (1882-(1837+1933)x1.200)
TOF MS EI+
2.21e3
B
119.0868
69.0696
93.0712
204.1882
79.0562
161.1338
133.1020
189.1652
147.1179
55.0544
175.1501
%
m/z

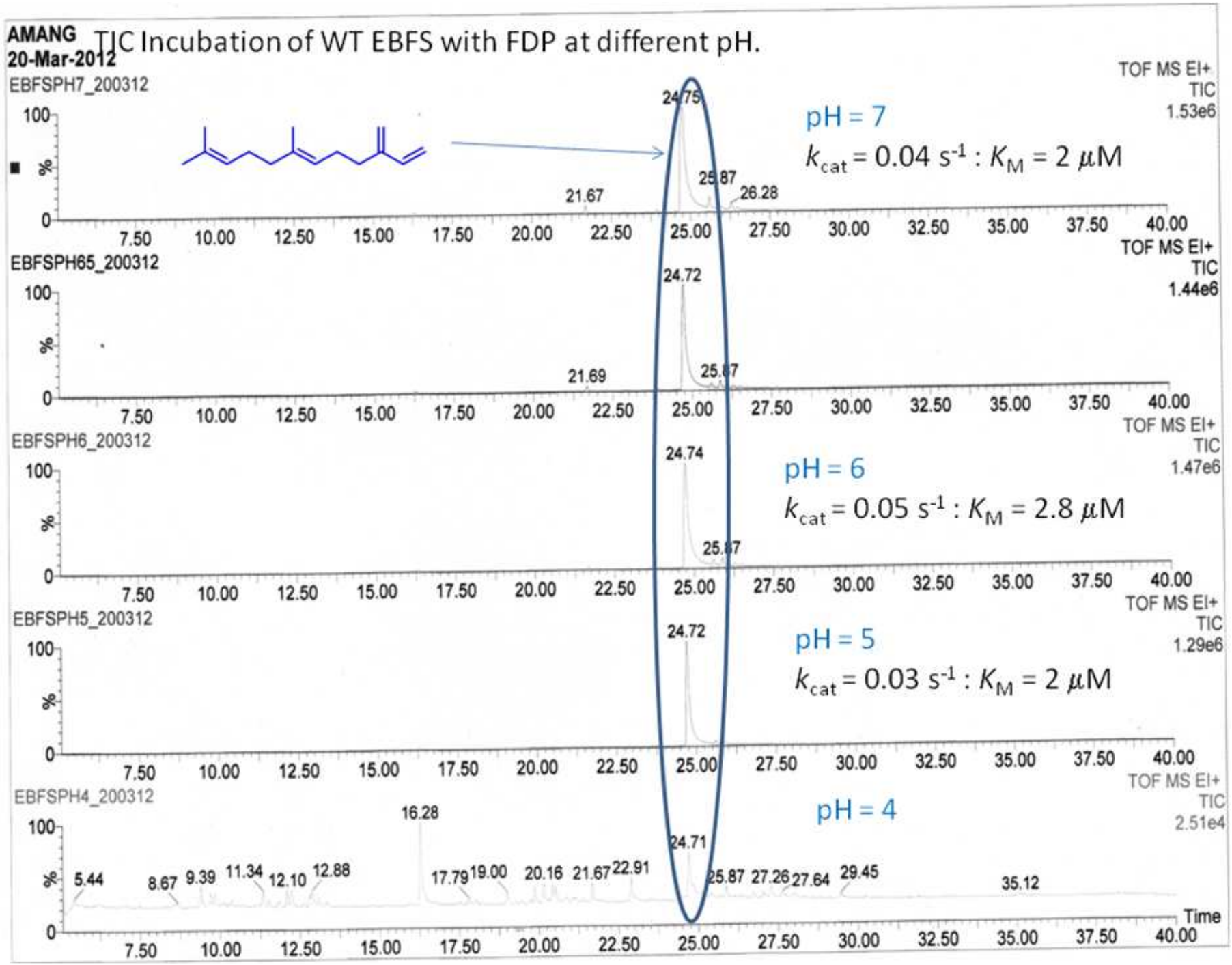

TIC Incubation of WT EBFS with FDP (A) andNDP (B) after silica gel filtration
Vero
13-Sep-2012
EBFS_FPP_130912
TOF MS EI+
TIC
2.80e4
100
A
%
0
24.87
25.85
22.00
23.00
24.00
25.00
26.00
27.00
28.00
29.00
30.00
31.00
EBFS_NPP_130912
TOF MS EI+
TIC
5.43e4
100
B
%
0
24.85
24.03
25.14
25.82
26.43
26.72
28.03
29.25
Time
22.00
23.00
24.00
25.00
26.00
27.00
28.00
29.00
30.00
31.00

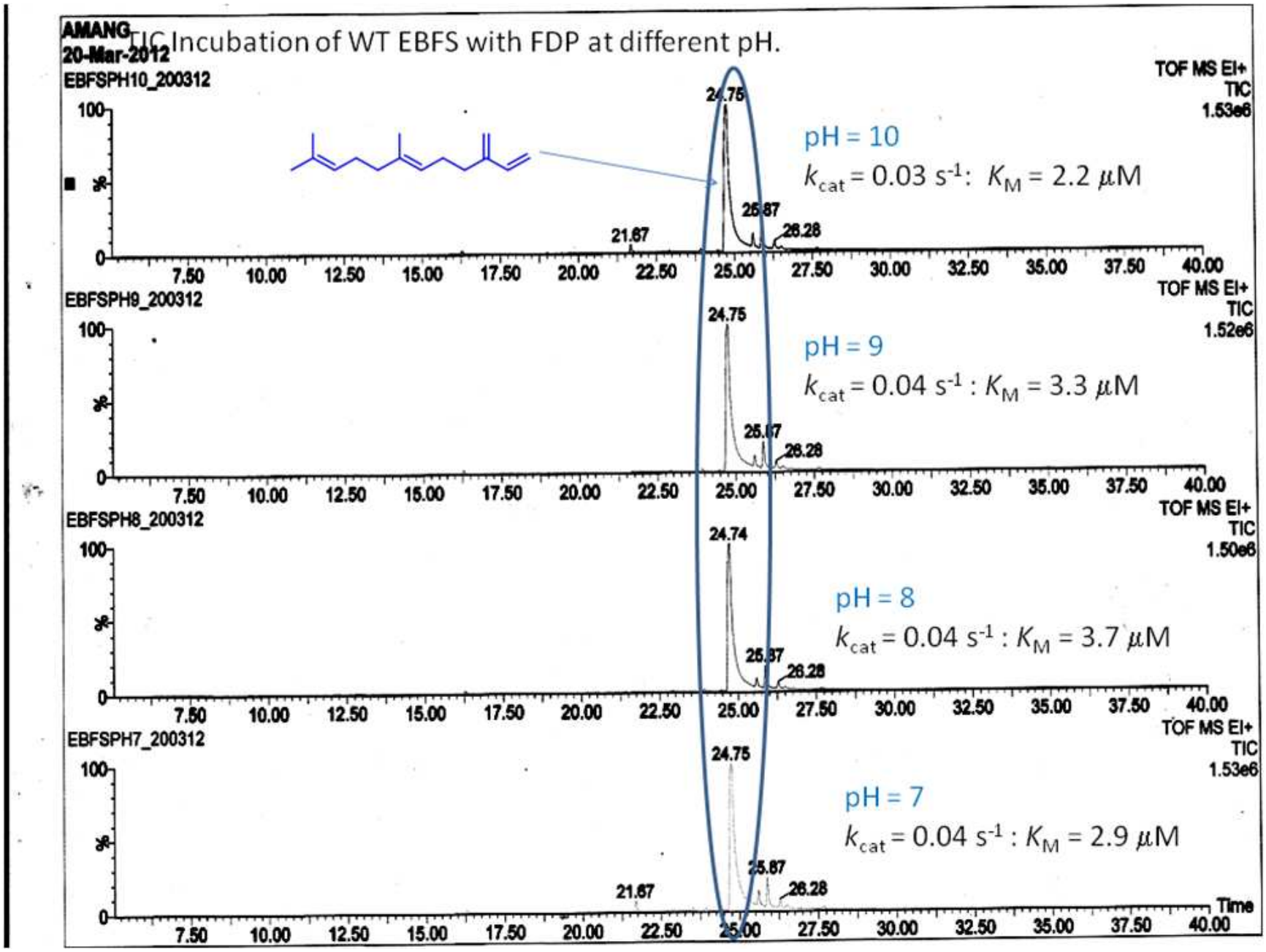

VERO
18-Jun-2012
EI MS spectra of (E)-β-farnesene from incubations with FDP (A) and NDP (B)
EBFS_FPP
TOF MS EI+
2.63e3
A
69.0691
93.0701
133.1028
79.0546
120.0938
161.1344
107.0862
204.1894
55.0563
148.1254
175.1471
189.1679
EBFS_NPP
TOF MS EI+
1.34e3
B
69.0708
93.0709
133.1037
79.0561
120.0951
161.1349
107.0879
204.1858
55.0596
148.1268
175.1533
189.1662
100
%
0
m/z
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220

TIC incubation of WT EBFS and $3CH_2F$-FDP

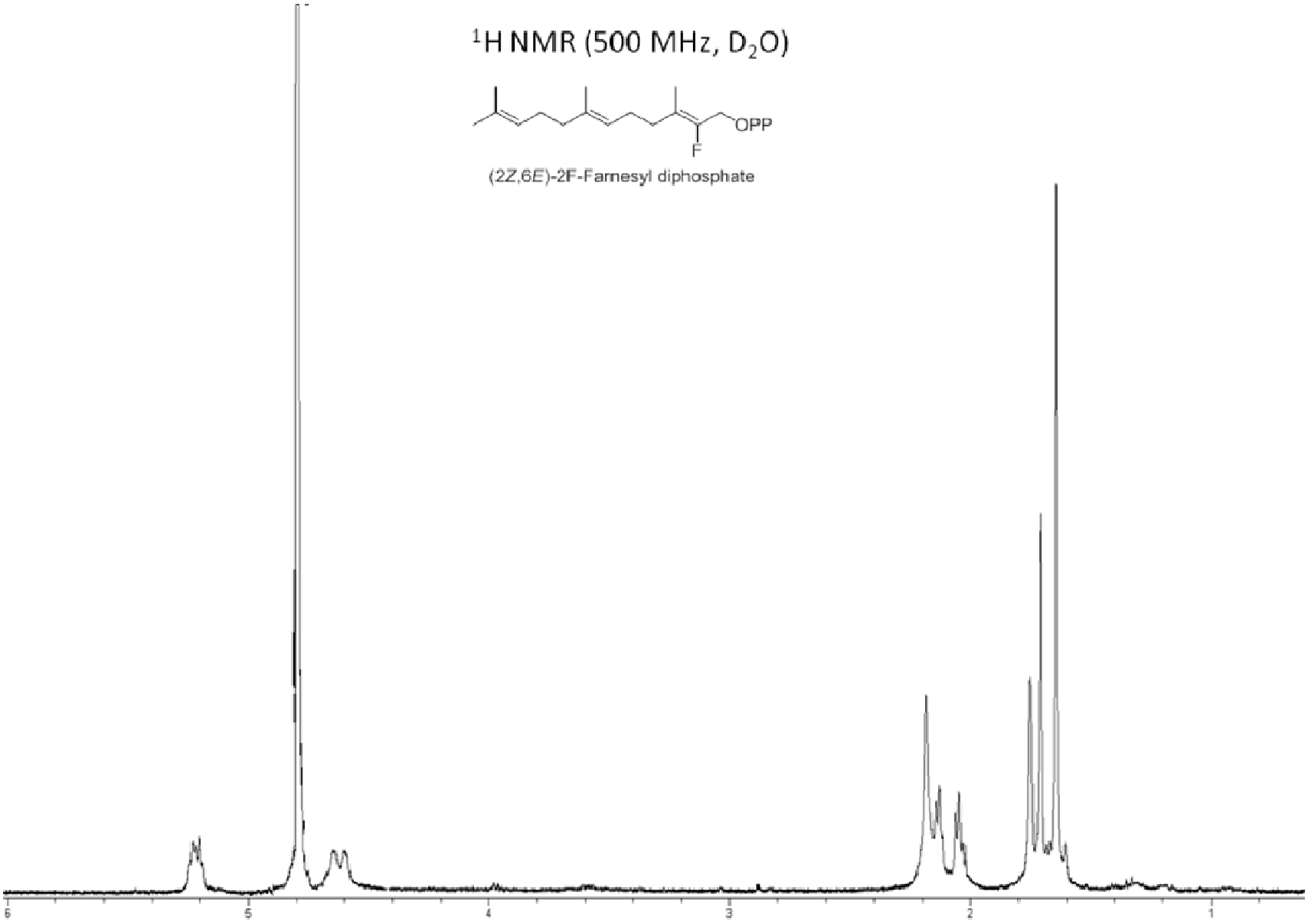



JUAN
18-Apr-2012
EBFS-15CF3-FPP
TIC Incubation of WT EBFS with 3-CF3-FDP.
TOF MS EI+
100
7.31
9.19
8.13
9.43
10.09
16.49
26.39
No m/z 258 products
OPP
H
F
F
F
0
7.50
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
30.00
32.50
35.00
37.50
40.00

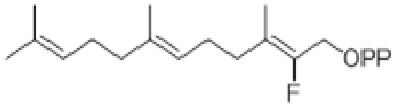

AMANG
20-Mar-2012
TIC Incubation of WT EBFS with FDP at different pH.
EBFSPH7_200312
TOF MS EI+
TIC
1.53e6
24.75
25.87
26.28
21.67
pH = 7
k_{cat} = 0.04 s^{-1} : K_M = 2 μM
EBFSPH65_200312
TOF MS EI+
TIC
1.44e6
24.72
21.69
25.87
EBFSPH6_200312
TOF MS EI+
TIC
1.47e6
24.74
25.87
pH = 6
k_{cat} = 0.05 s^{-1} : K_M = 2.8 μM
EBFSPH5_200312
TOF MS EI+
TIC
1.29e6
24.72
pH = 5
k_{cat} = 0.03 s^{-1} : K_M = 2 μM
EBFSPH4_200312
TOF MS EI+
TIC
2.51e4
16.28
24.71
5.44
8.67
9.39
11.34
12.10
12.88
17.79
19.00
20.16
21.67
22.91
25.87
27.26
27.64
29.45
35.12
pH = 4
Time
100
%
0
7.50
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
30.00
32.50
35.00
37.50
40.00


AMANG
20-Mar-2012
TIC Incubation of WT EBFS with FDP at different pH.
EBFSPH10_200312
TOF MS EI+
TIC
1.53e6
24.75
21.67
25.87
26.28
pH = 10
k_{cat} = 0.03 s^{-1}: K_M = 2.2 μM
EBFSPH9_200312
TOF MS EI+
TIC
1.52e6
24.75
25.87
26.28
pH = 9
k_{cat} = 0.04 s^{-1} : K_M = 3.3 μM
EBFSPH8_200312
TOF MS EI+
TIC
1.50e6
24.74
25.87
26.28
pH = 8
k_{cat} = 0.04 s^{-1} : K_M = 3.7 μM
EBFSPH7_200312
TOF MS EI+
TIC
1.53e6
24.75
21.67
25.87
26.28
pH = 7
k_{cat} = 0.04 s^{-1} : K_M = 2.9 μM
%
100
0
7.50
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
30.00
32.50
35.00
37.50
40.00
Time

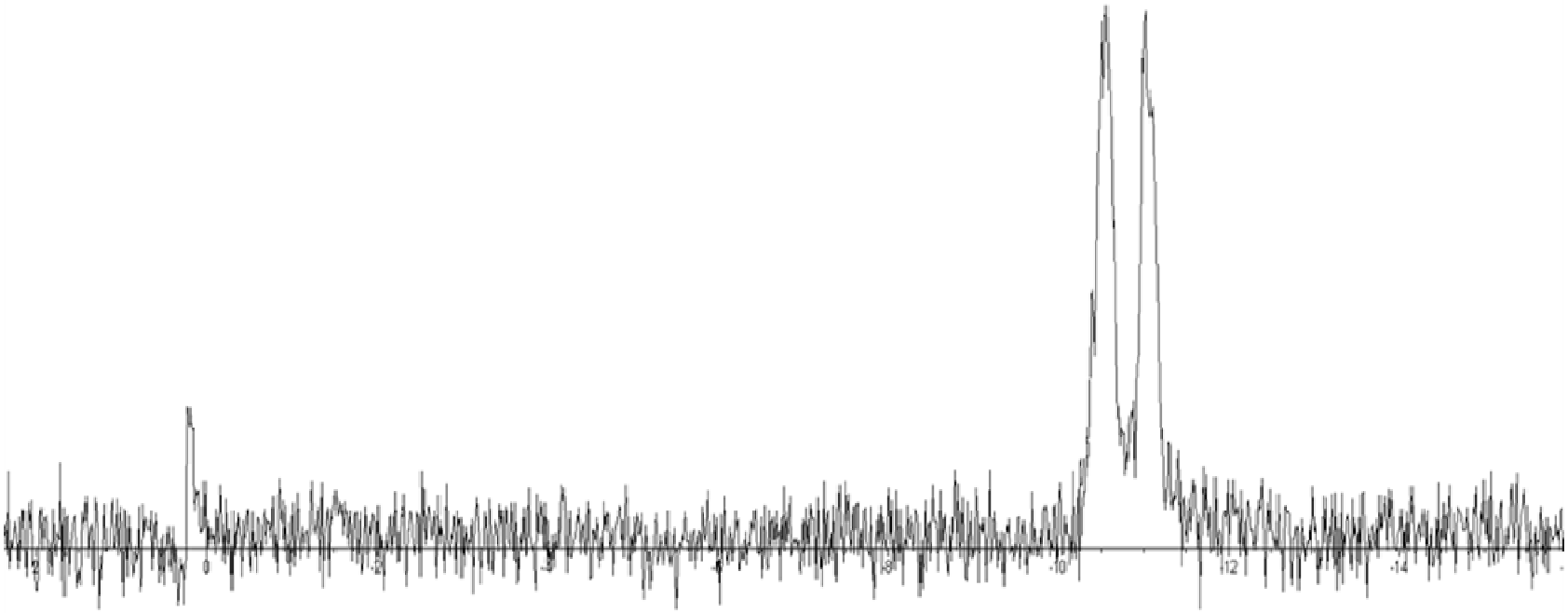
11. Collection of NMRS



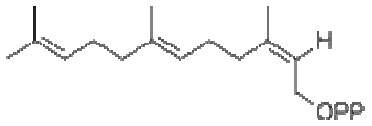
^{31}P NMR (202 MHz, D_2O)



(2Z,6*E*)-2F-Farnesyl diphosphate

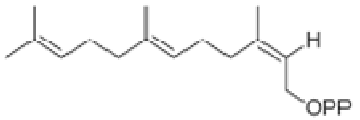


^{1}H NMR (500 MHz, D_2O)



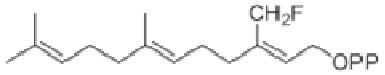
(2Z,6*E*)-Farnesyl diphosphate

^{31}P NMR (202 MHz, D_2O)

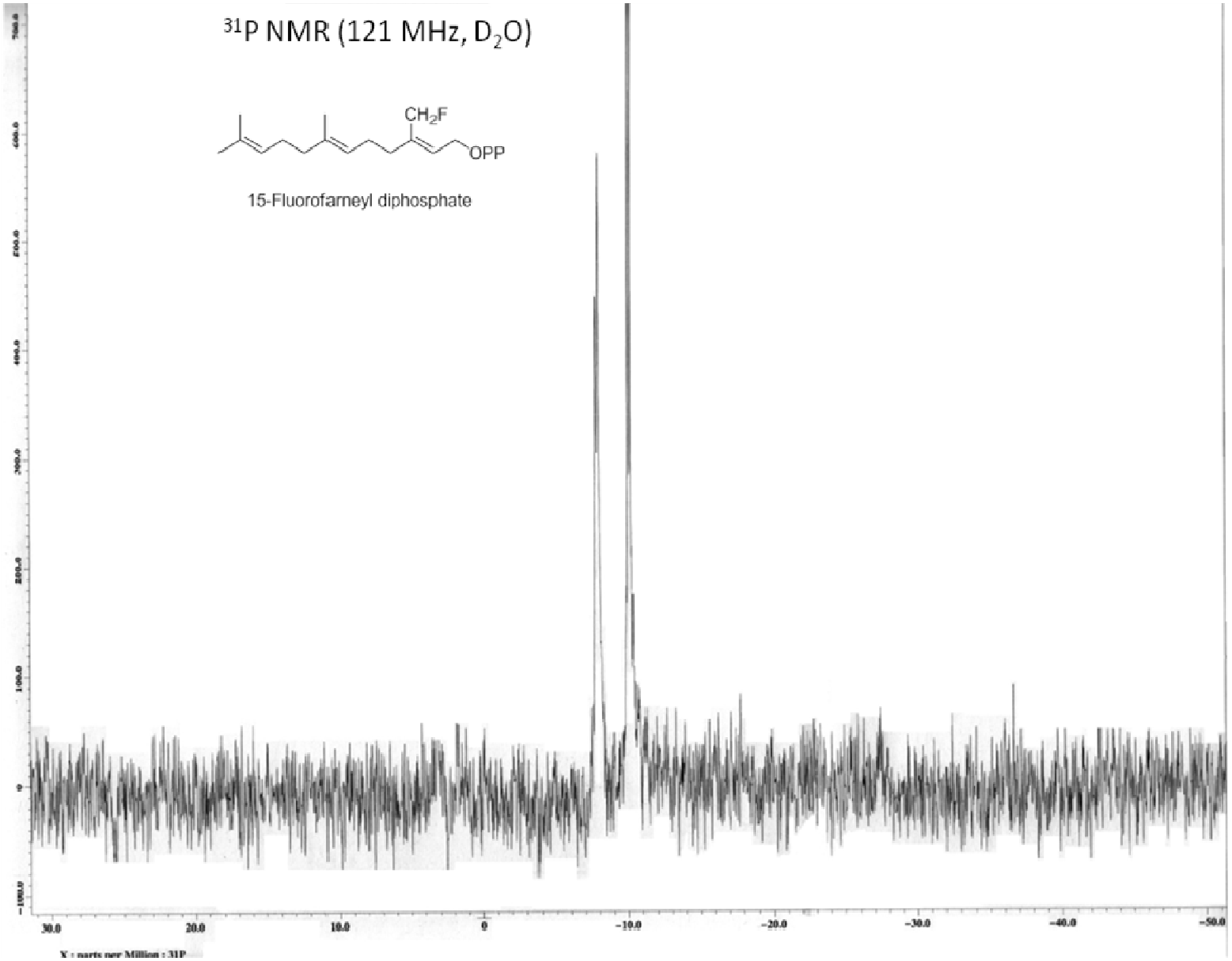


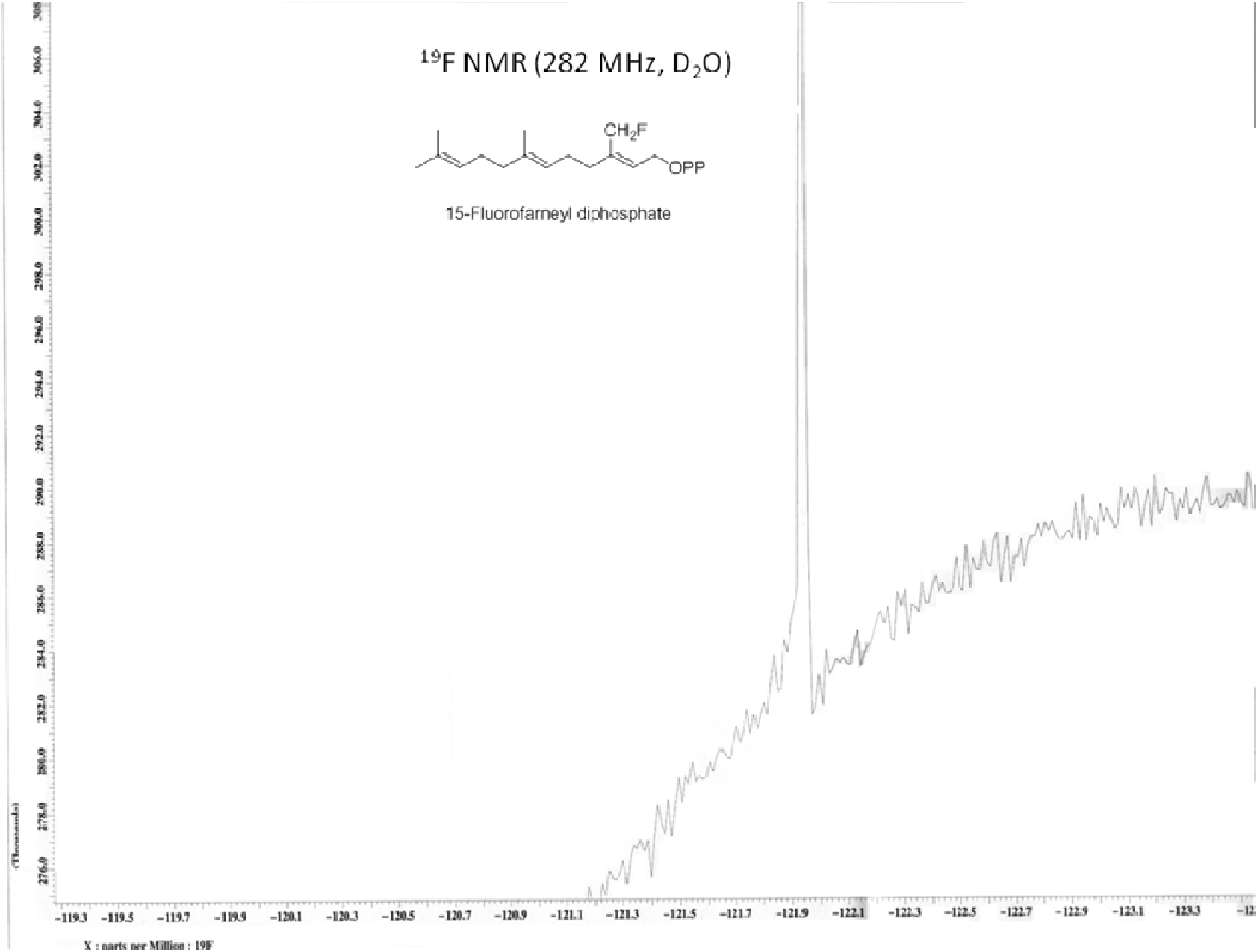
(2*Z*,6*E*)-Farnesyl diphosphate

^{1}H NMR (400 MHz, D_2O)

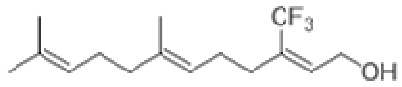


15-Fluorofarneyl diphosphate

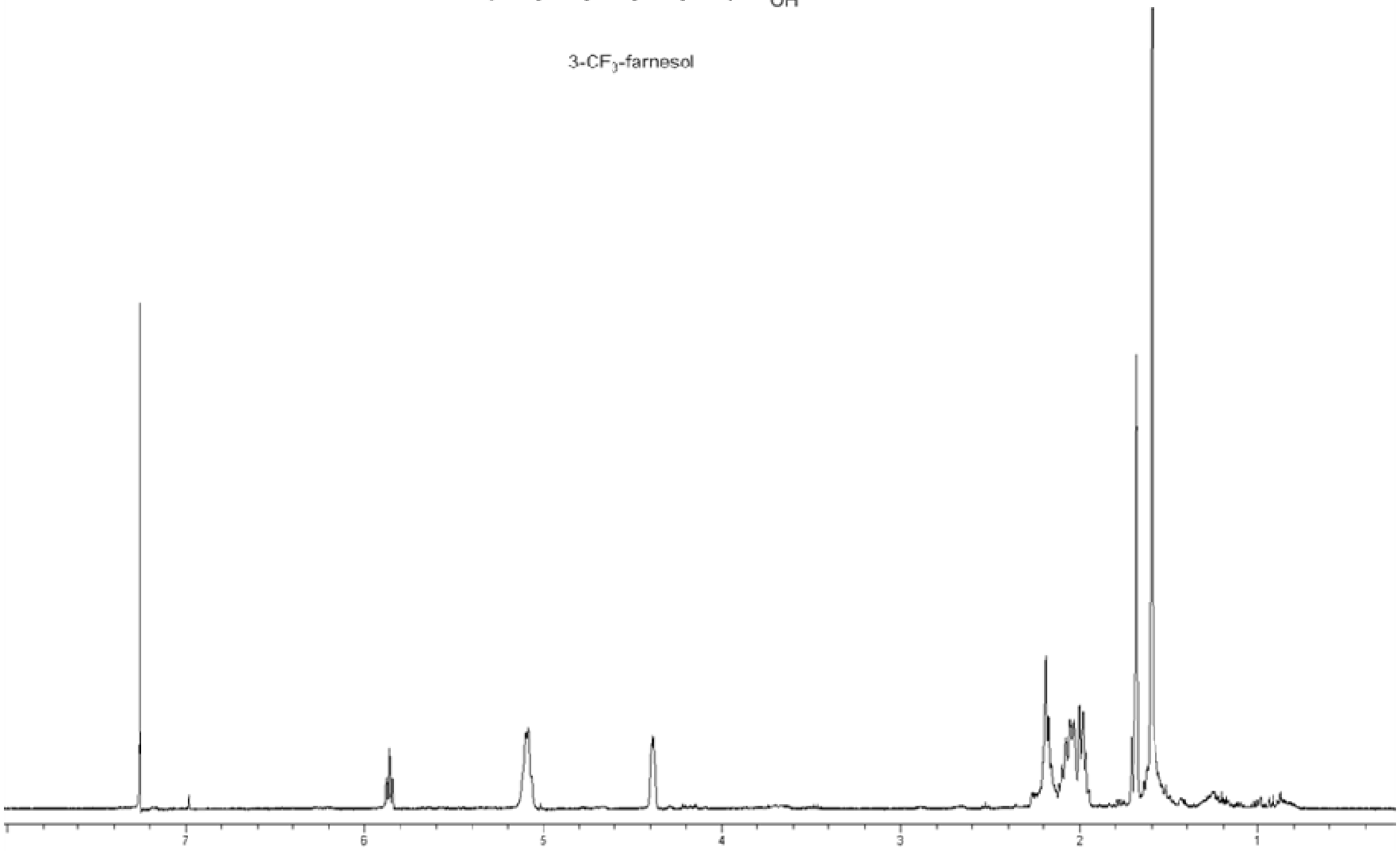

31P NMR (121 MHz, D2O)
CH2F
OPP
15-Fluorofarneyl diphosphate
30.0
20.0
10.0
0
-10.0
-20.0
-30.0
-40.0
-50.0

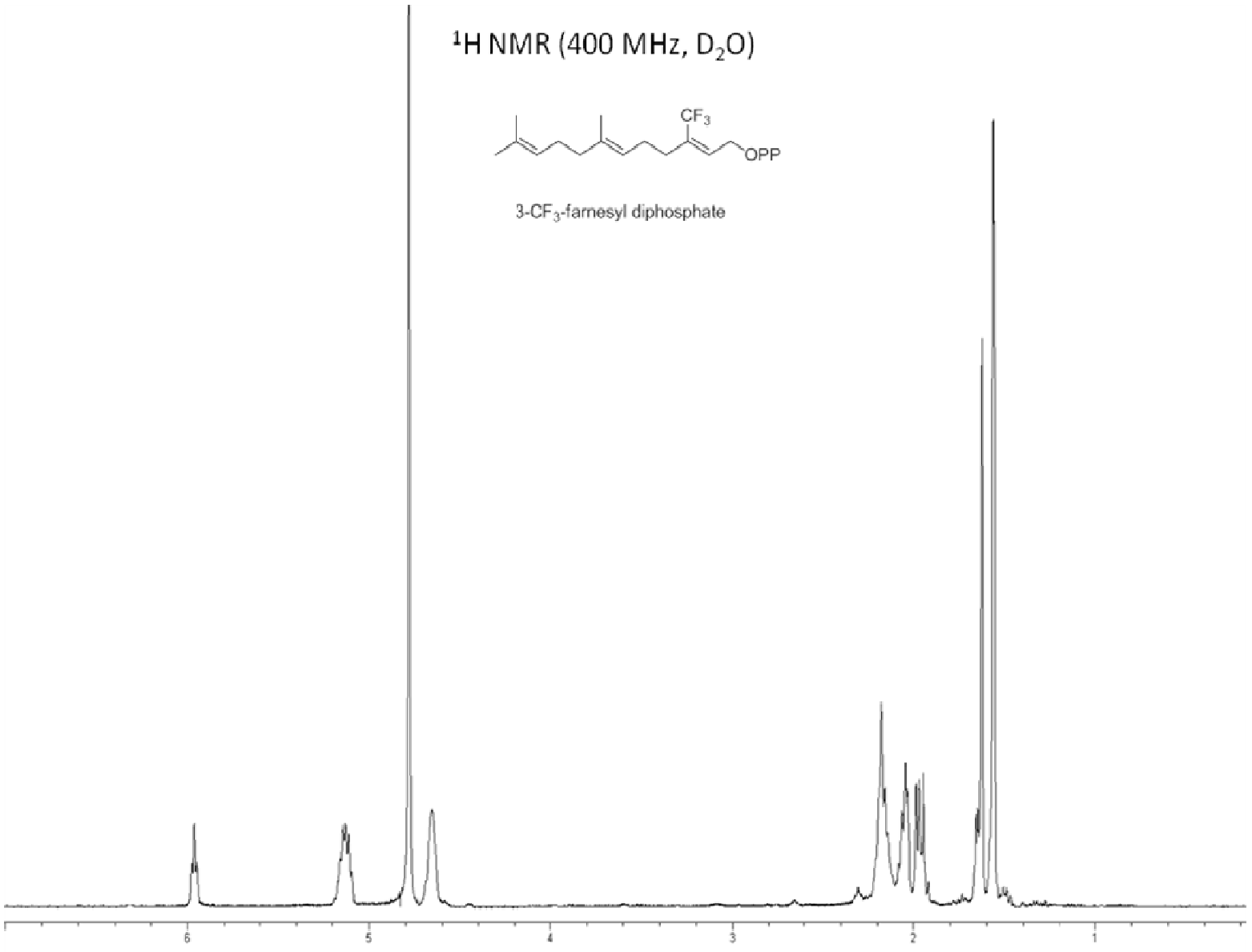

^{19}F NMR (282 MHz, D_2O)
CH_2F
OPP
15-Fluorofarnesyl diphosphate
-119.3 -119.5 -119.7 -119.9 -120.1 -120.3 -120.5 -120.7 -120.9 -121.1 -121.3 -121.5 -121.7 -121.9 -122.1 -122.3 -122.5 -122.7 -122.9 -123.1 -123.3
X : parts per Million : 19F

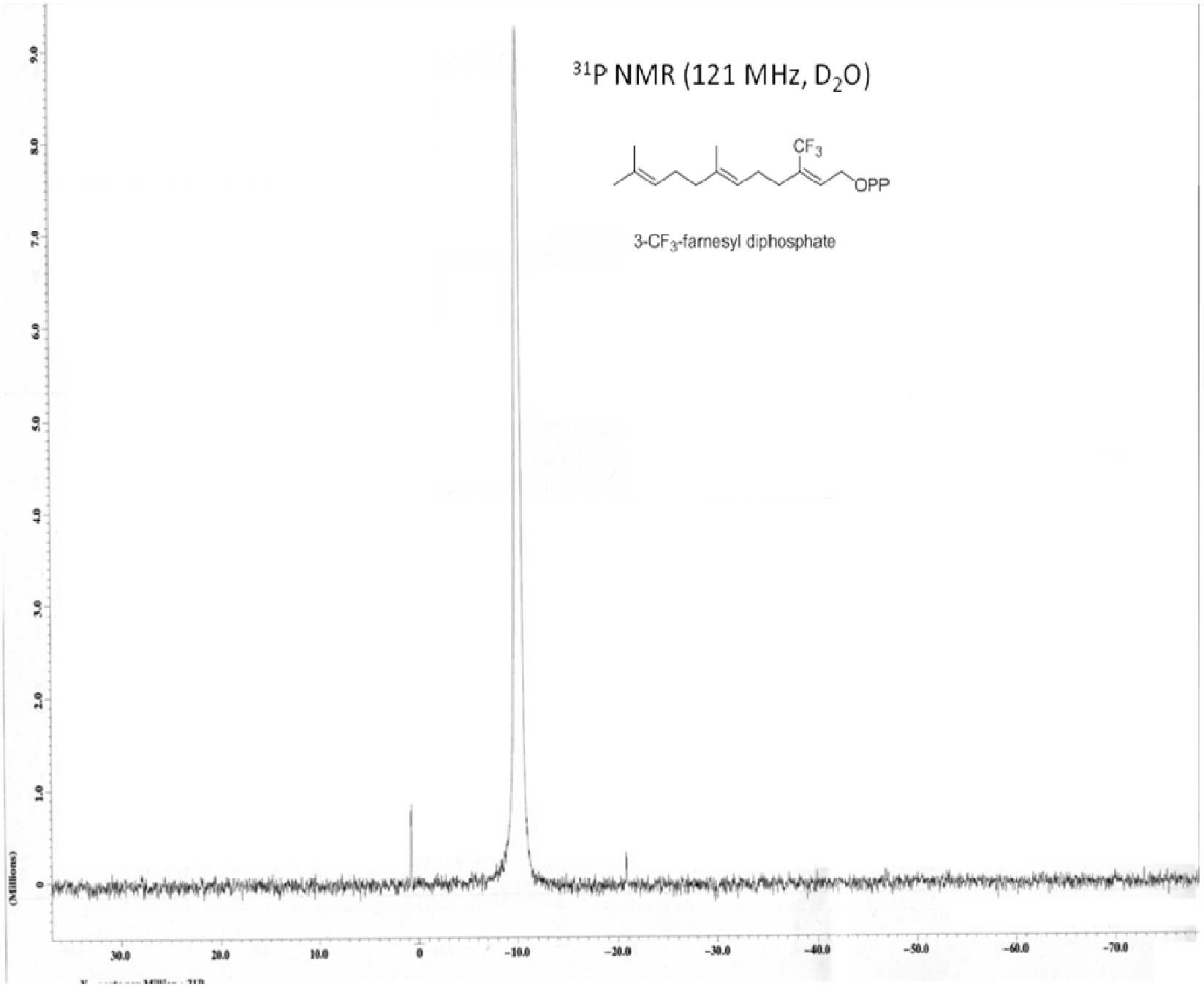
^{1}H NMR (400 MHz, $CDCl_3$)

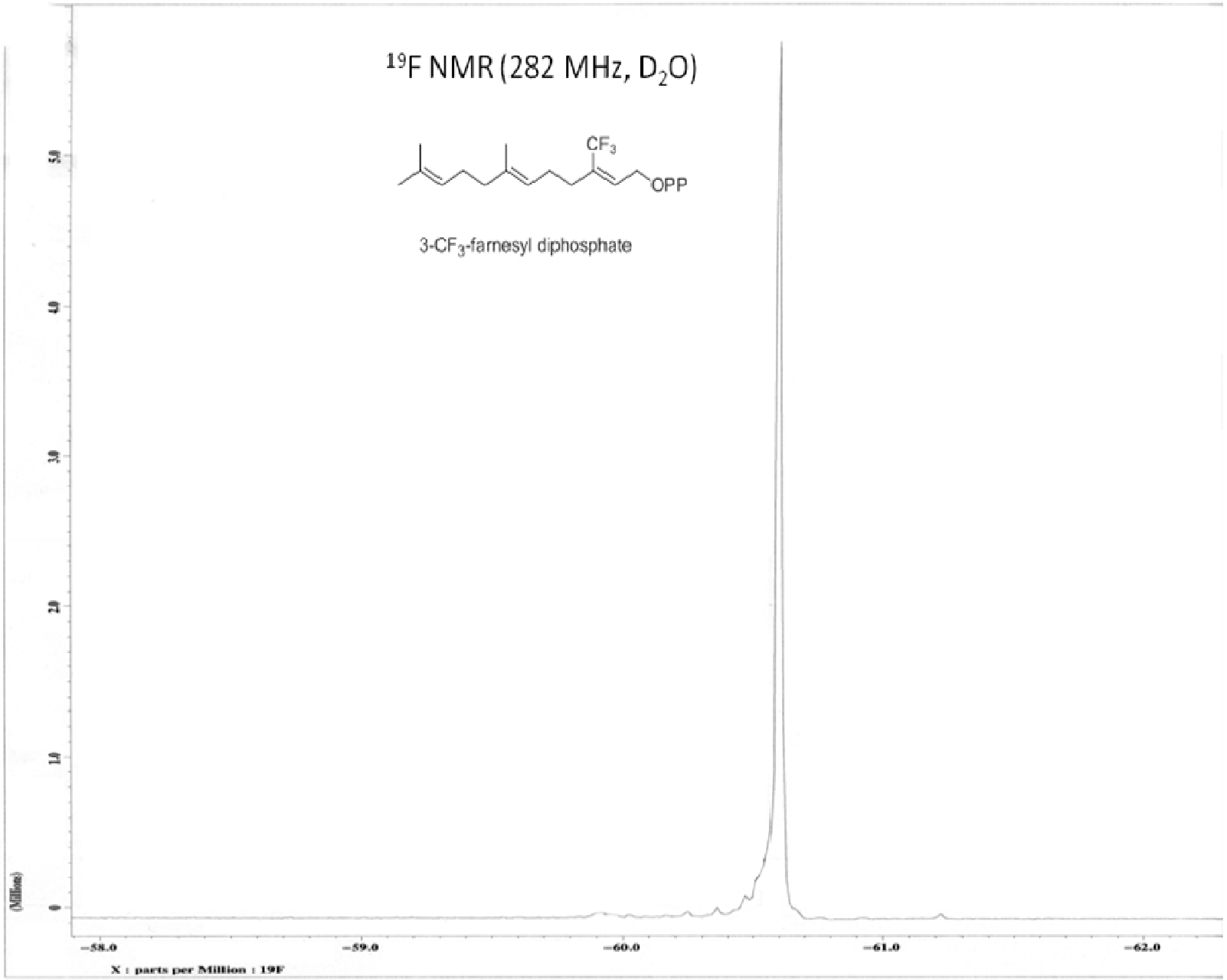


3-CF_3-farnesol

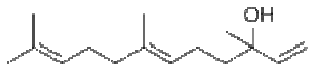



^{1}H NMR (400 MHz, D_2O)
CF_3
OPP
3-CF_3-farnesyl diphosphate
6
5
4
3
2
1

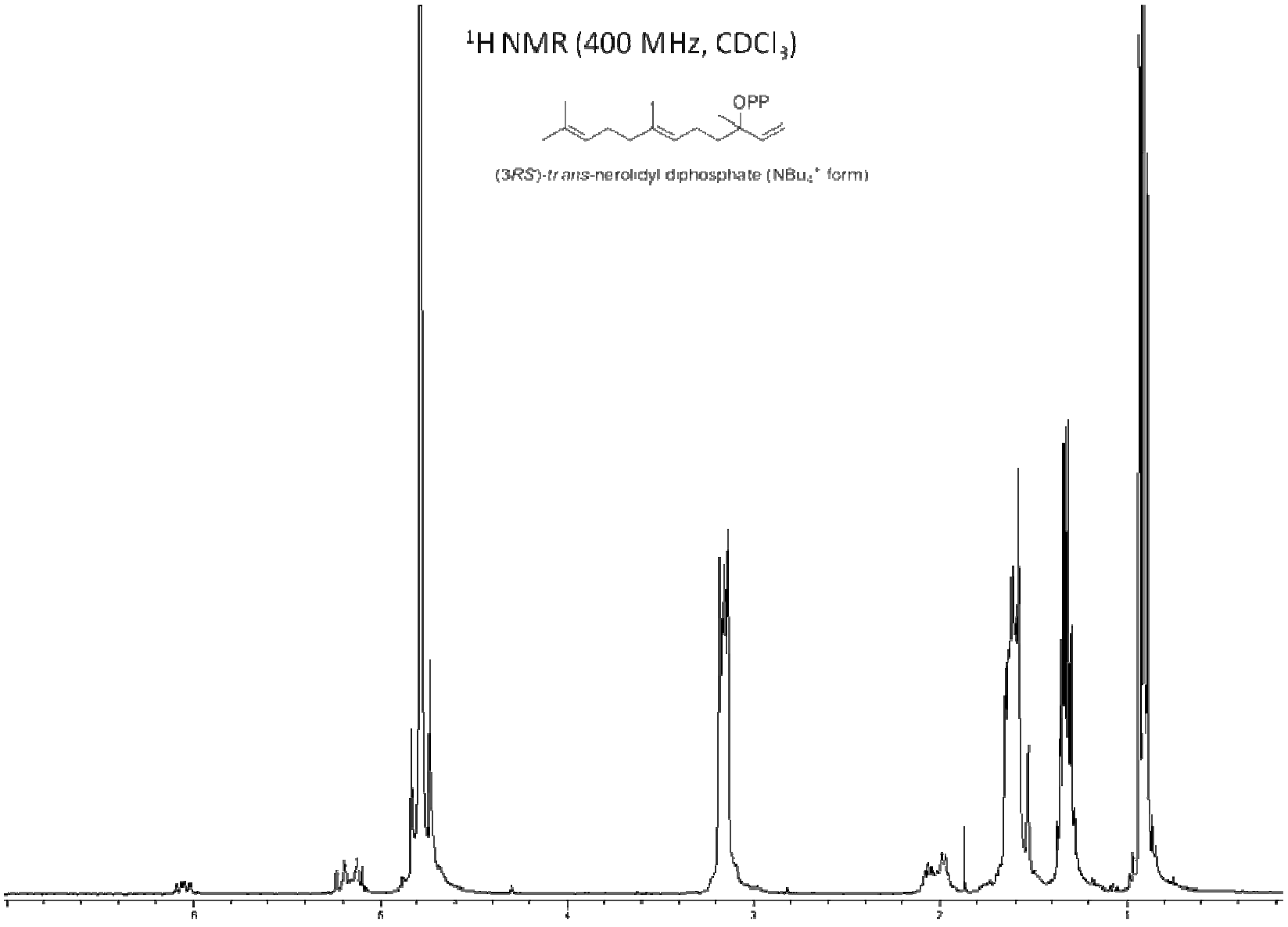

^{31}P NMR (121 MHz, D_2O)
CF_3
OPP
3-CF_3-farnesyl diphosphate
(Millions)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
30.0
20.0
10.0
0
-10.0
-20.0
-30.0
-40.0
-50.0
-60.0
-70.0

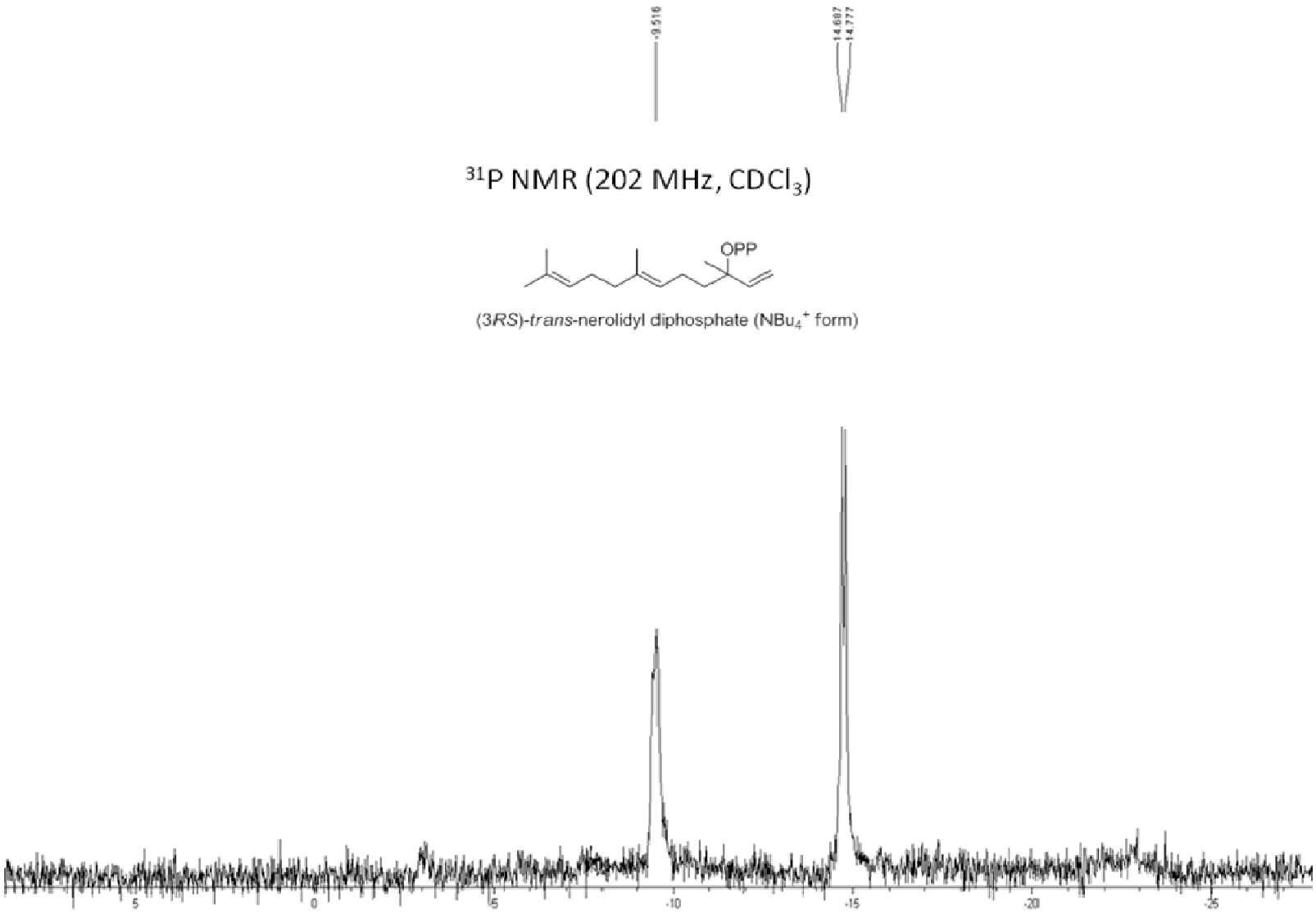

19F NMR (282 MHz, D2O)
CF3
OPP
3-CF3-farnesyl diphosphate
(Millions)
-58.0
-59.0
-60.0
-61.0
-62.0
X : parts per Million : 19F

^{1}H NMR (400 MHz, $CDCl_3$)



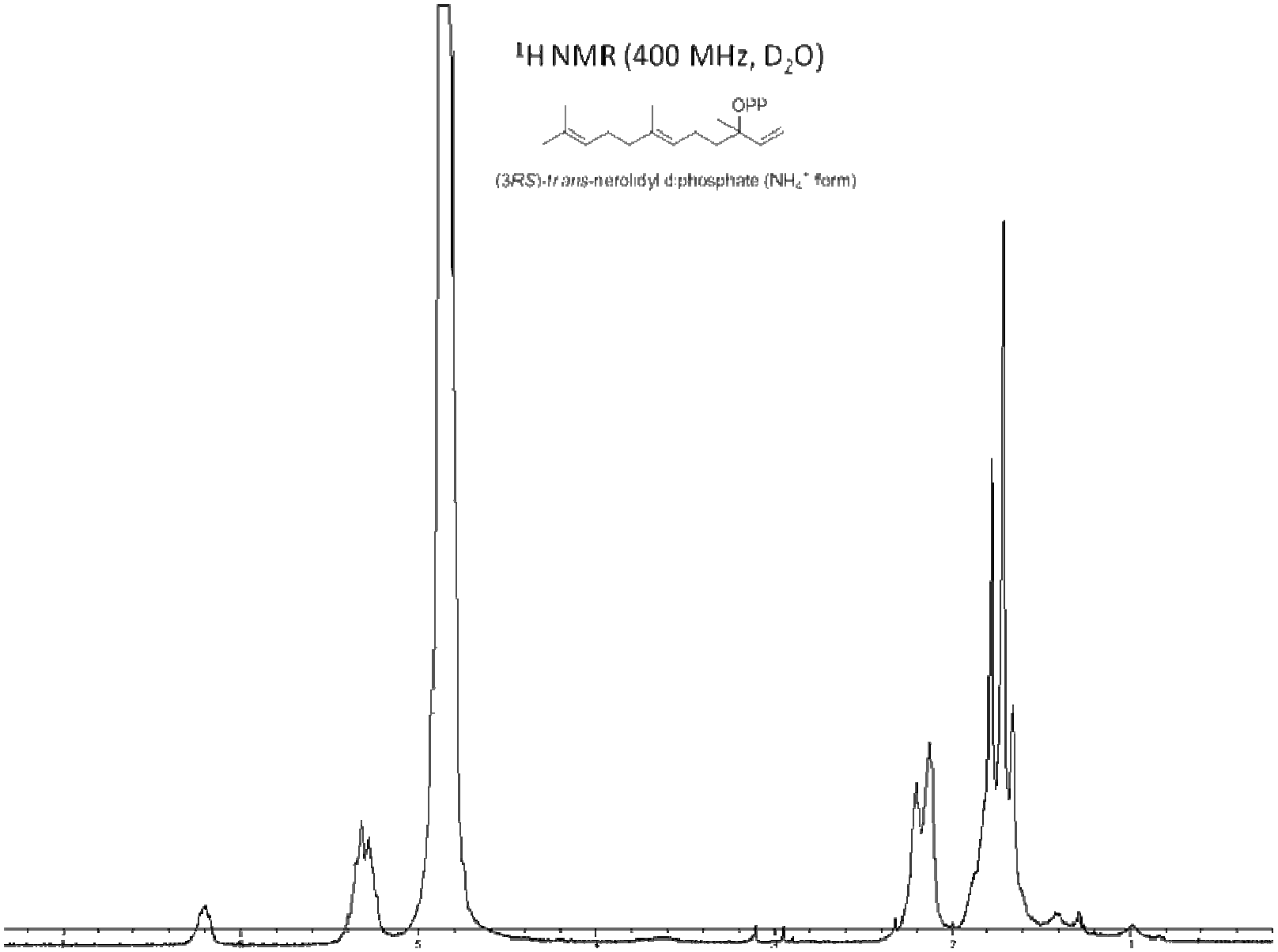
(3*RS*)-*trans*-nerolidol

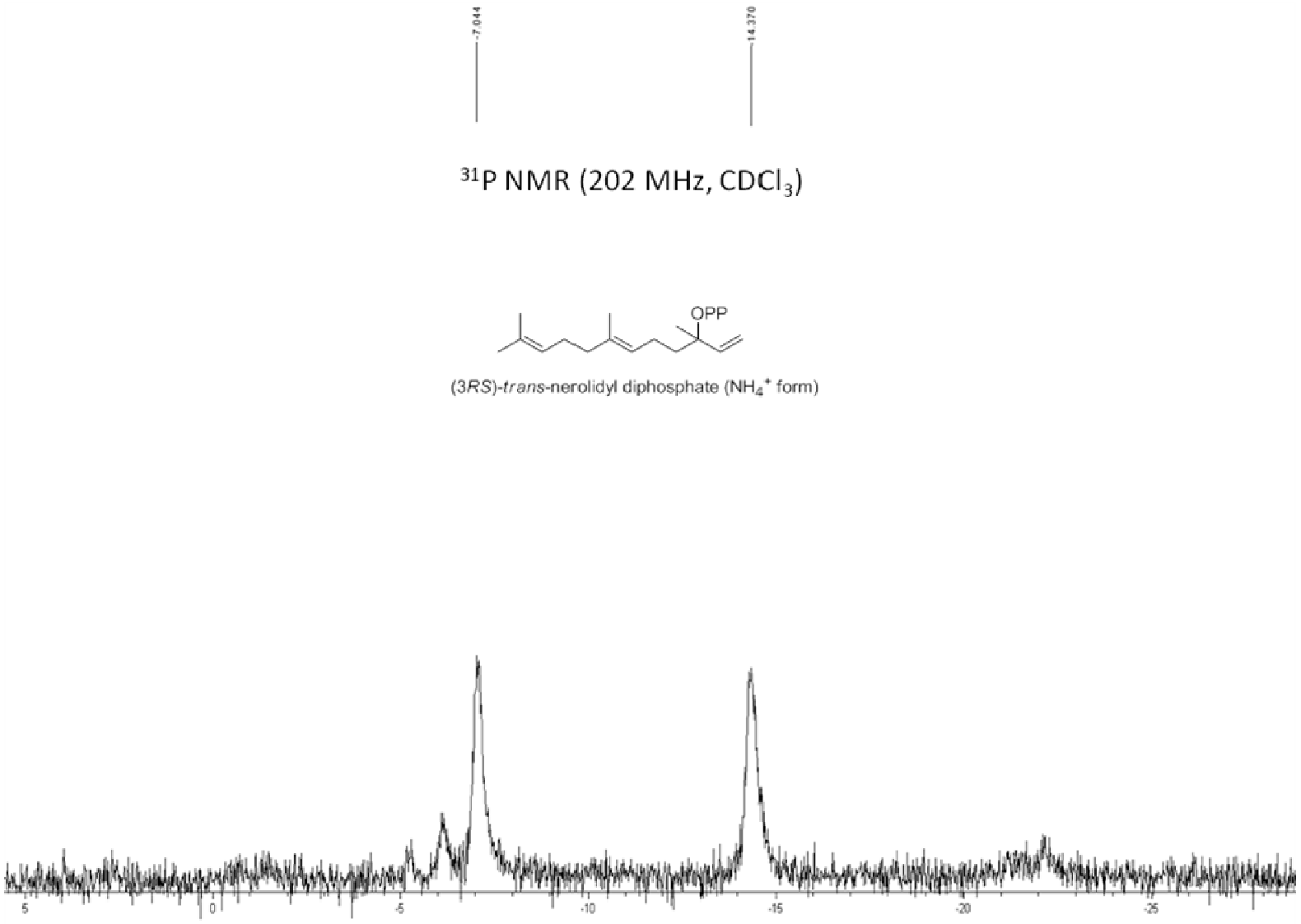

^{1}H NMR (400 MHz, $CDCl_3$)
OPP
(3*RS*)-*trans*-nerolidyl diphosphate (NBu_4^+ form)



^{31}P NMR (202 MHz, $CDCl_3$)

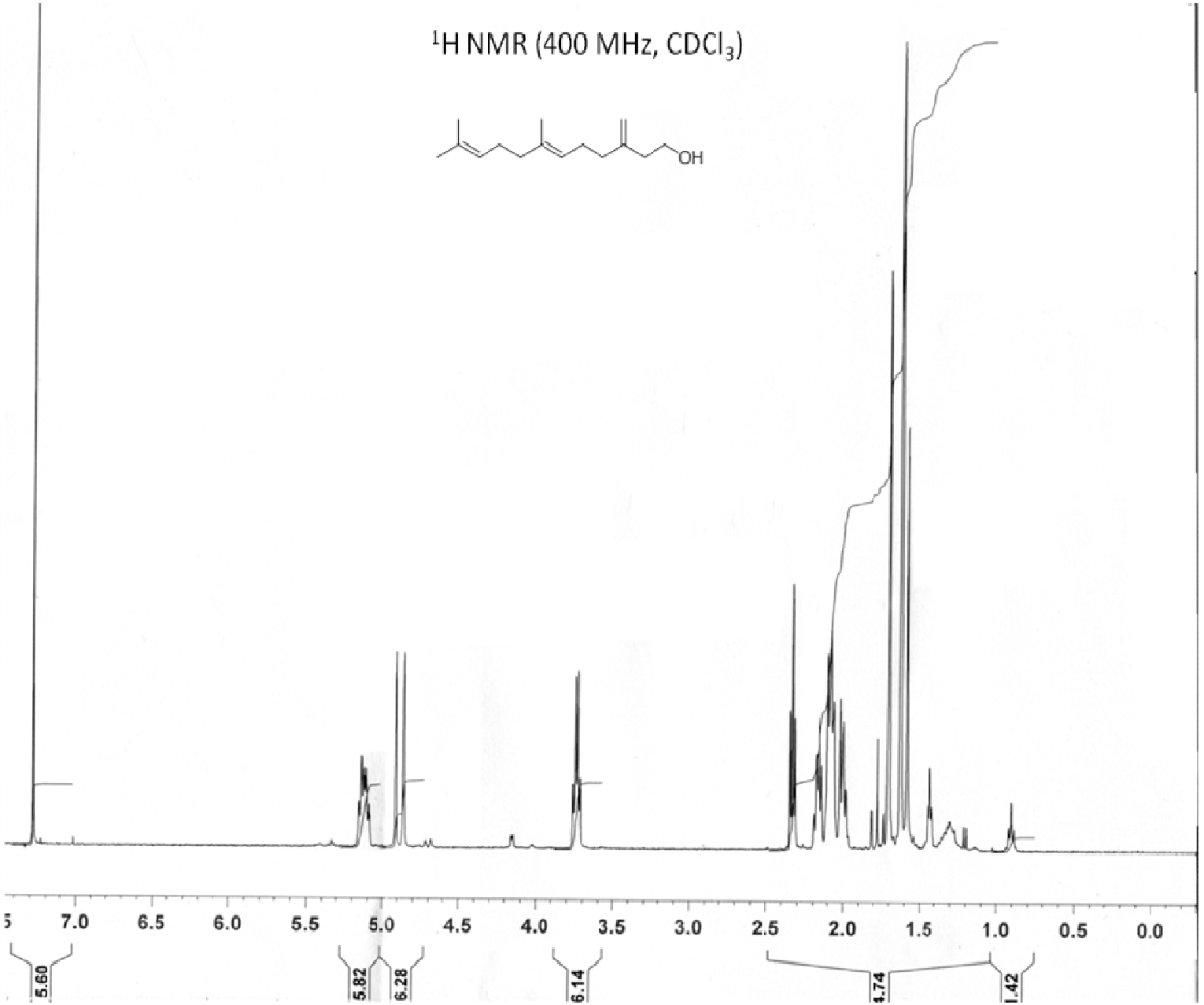
(3*RS*)-*trans*-nerolidyl diphosphate (NBu_4^+ form)

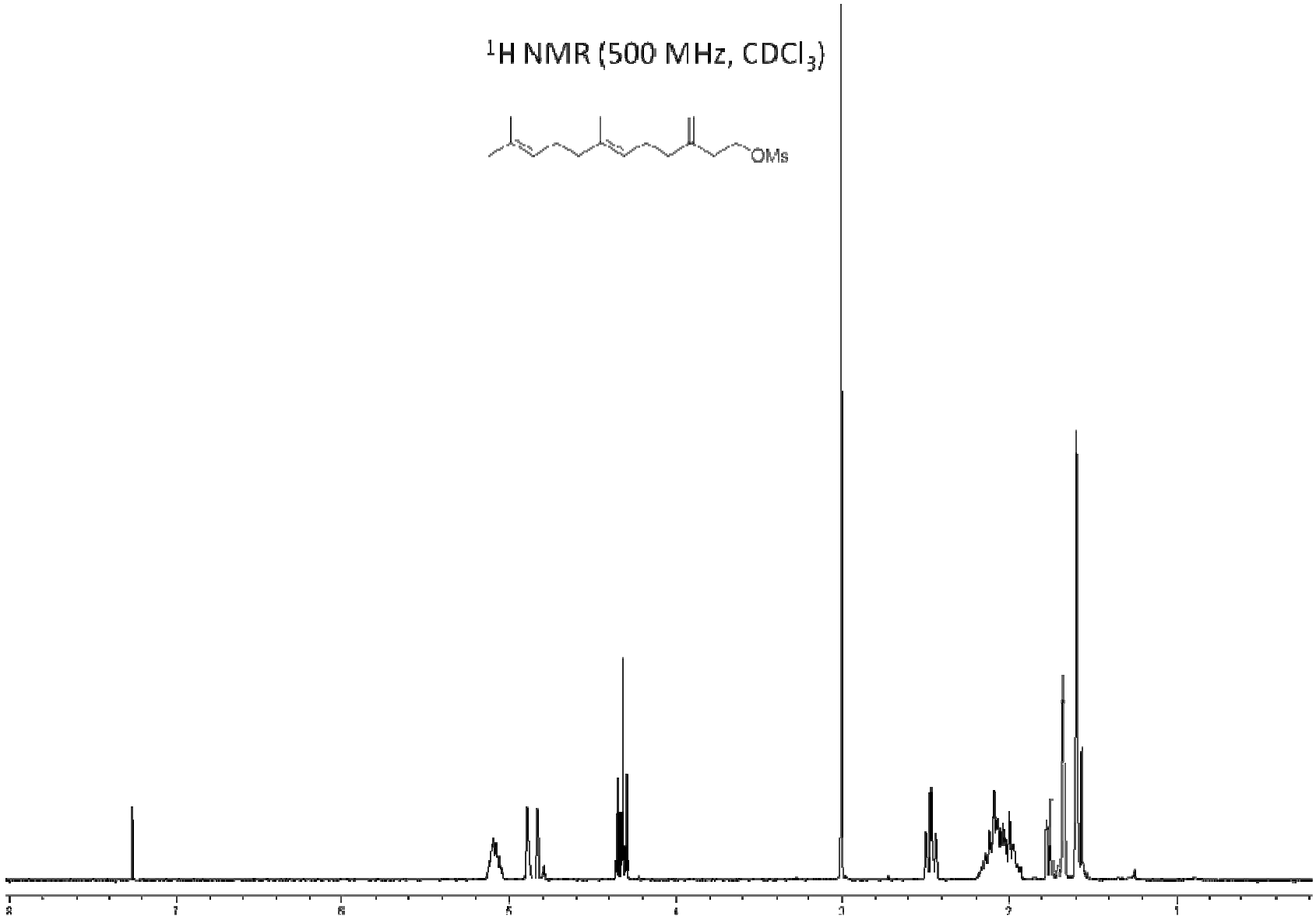

^{1}H NMR (400 MHz, D_2O)
OPP
(3*RS*)-*trans*-nerolidyl diphosphate (NH_4^+ form)



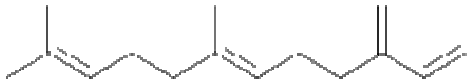
^{31}P NMR (202 MHz, $CDCl_3$)

(3*RS*)-*trans*-nerolidyl diphosphate (NH_4^+ form)

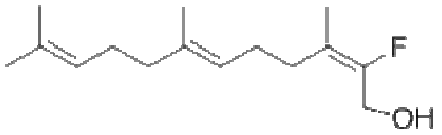

^{1}H NMR (400 MHz, $CDCl_3$)
OH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5.60
5.82
6.28
6.14
1.74
1.42


^{1}H NMR (500 MHz, $CDCl_3$)
OMs

^{1}H NMR (500 MHz, $CDCl_3$) of synthetic (*E*)-*β*-farnesene

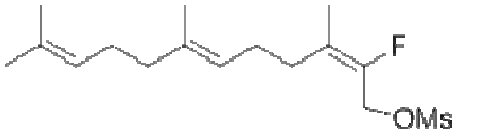


^{1}H NMR (500 MHz, $CDCl_3$)



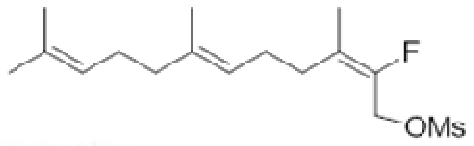
(2*E*,6*E*)-2F-Farnesol

^{1}H NMR (500 MHz, $CDCl_3$)



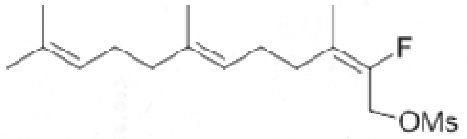
(2*E*.6*E*)-2F-Farnesyl methanesulfonate

^{13}C NMR (125 MHz, $CDCl_3$)



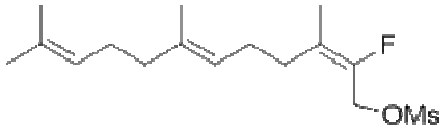
(2*E*,6*E*)-2F-Farnesyl methanesulfonate

^{13}C NMR (125 MHz, $CDCl_3$). 150-122 ppm Expansion

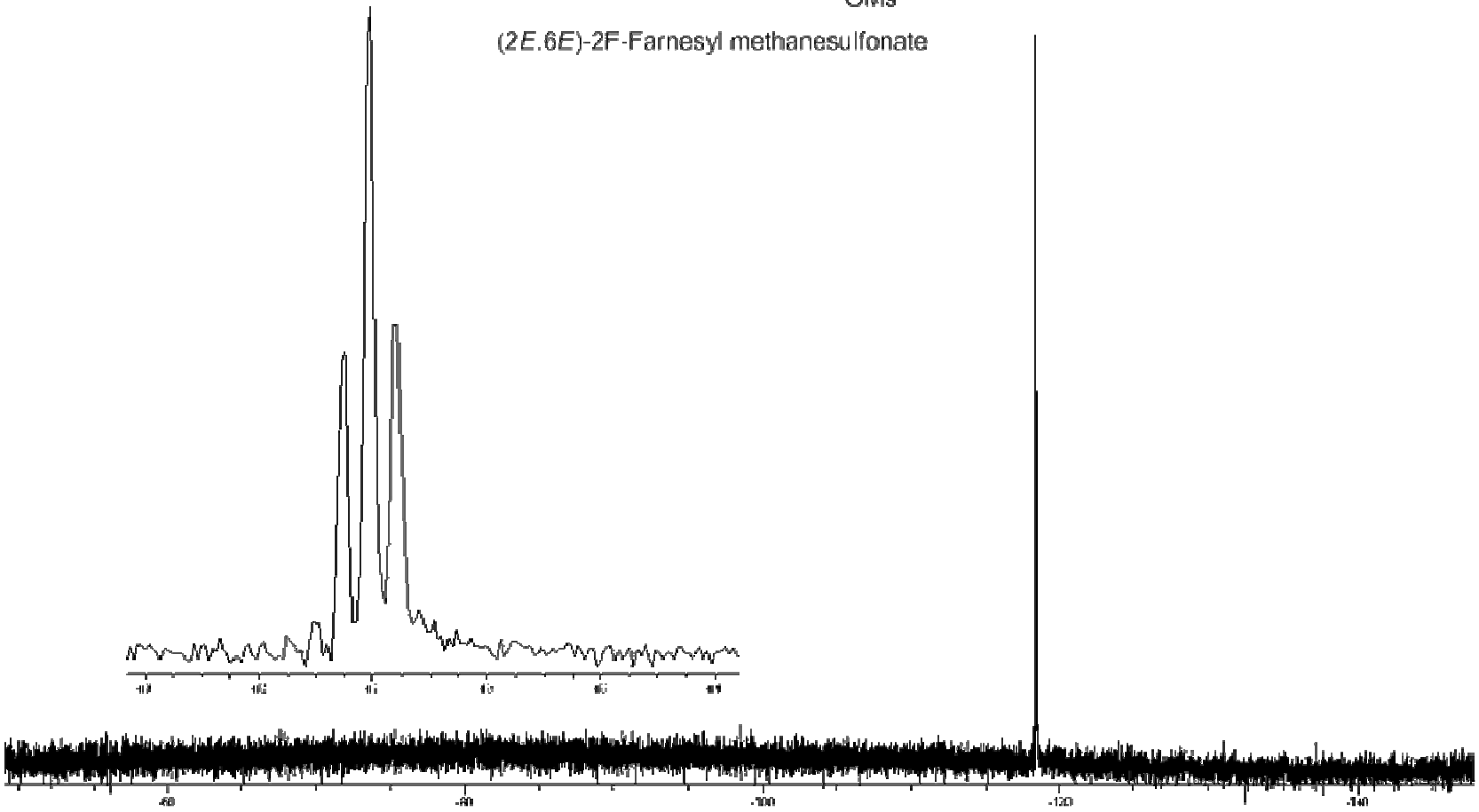


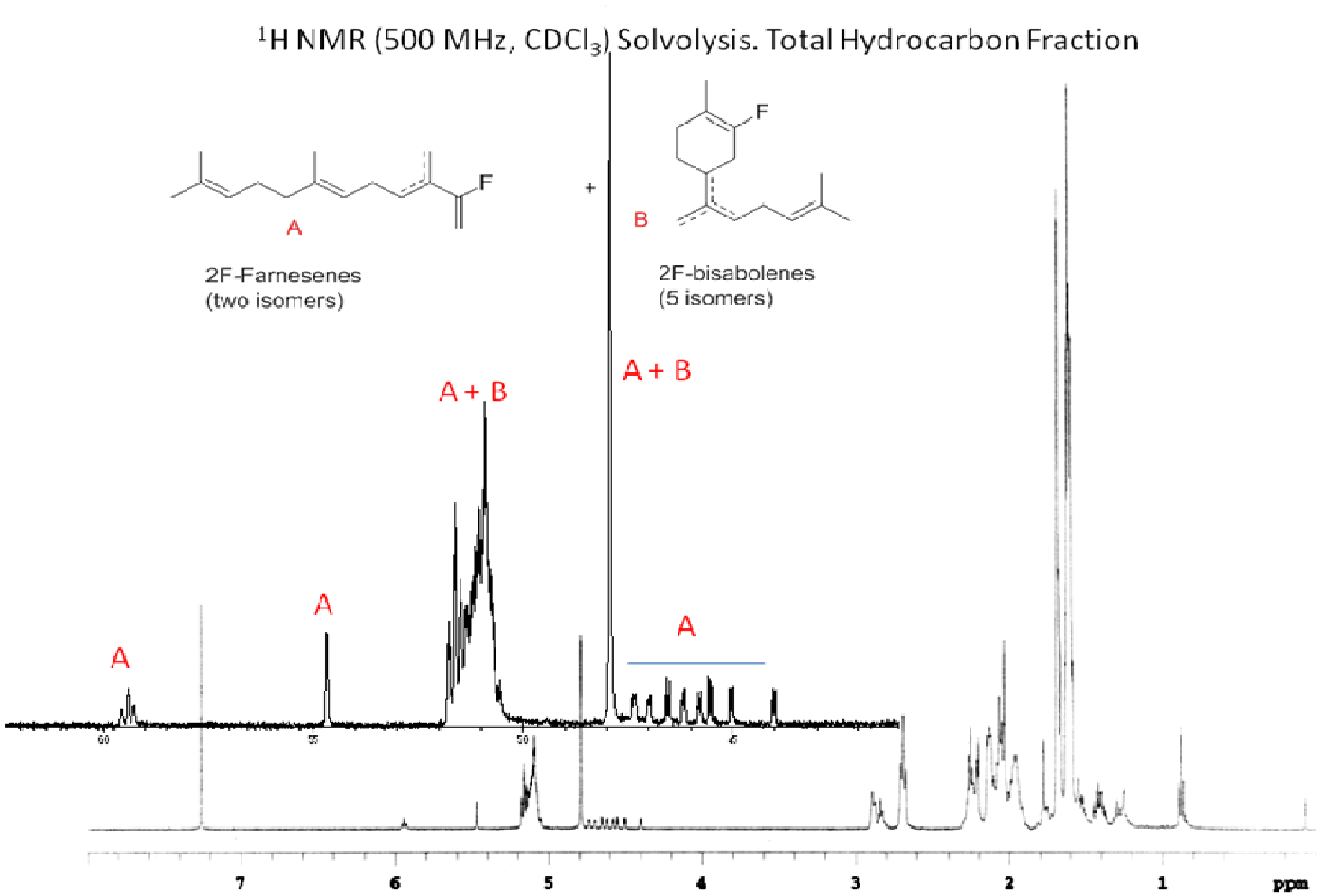
(2*E*,6*E*)-2F-Farnesyl methanesulfonate

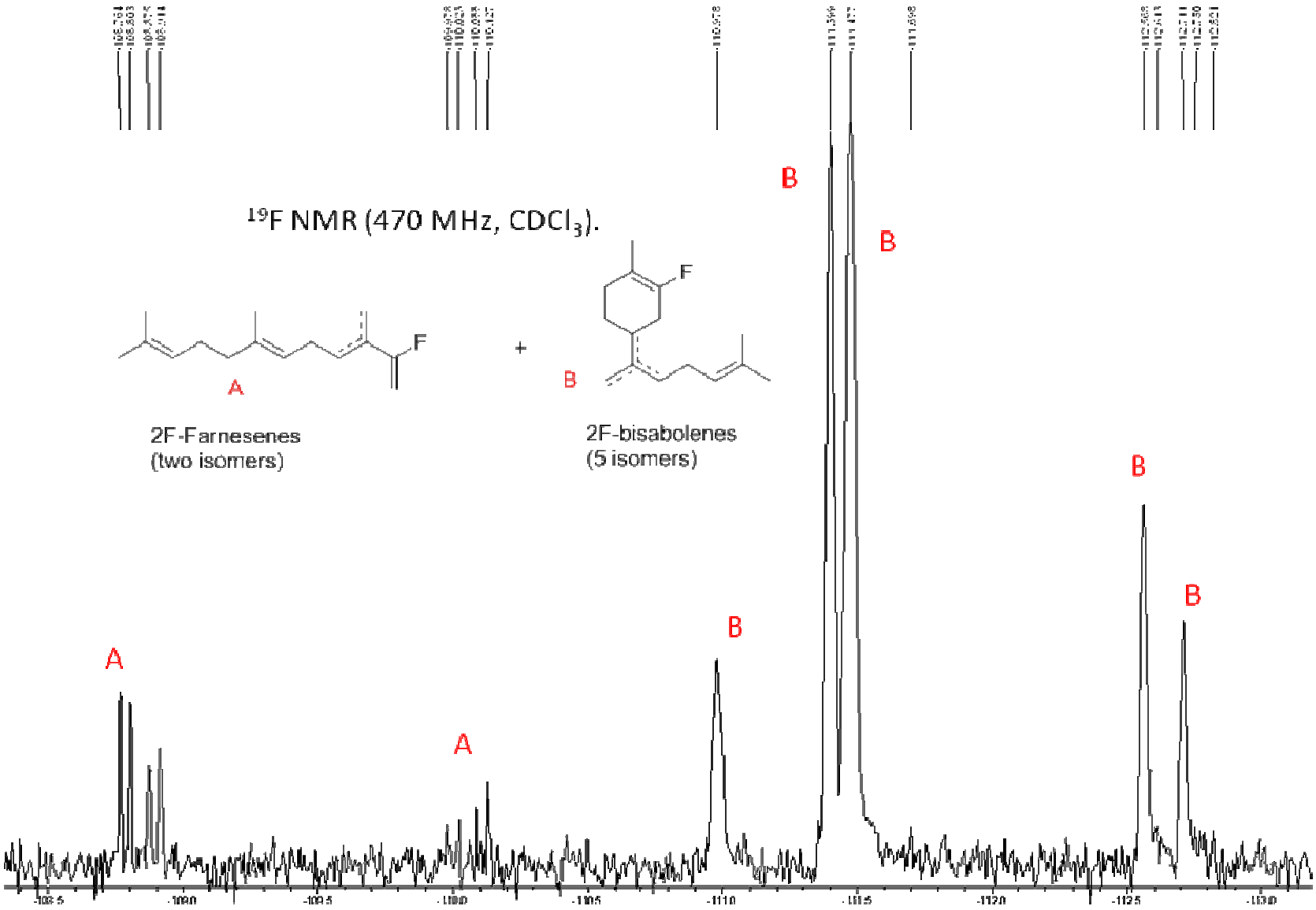
^{19}F NMR (470 MHz, $CDCl_3$).



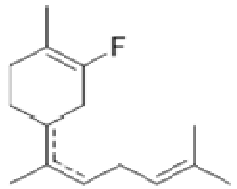
(2*E*.6*E*)-2F-Farnesyl methanesulfonate




^{1}H NMR (500 MHz, $CDCl_3$) Solvolysis. Total Hydrocarbon Fraction
F
A
+
B
F
2F-Farnesenes
(two isomers)
2F-bisabolenes
(5 isomers)
A + B
A + B
A
A
A
7
6
5
4
3
2
1
ppm

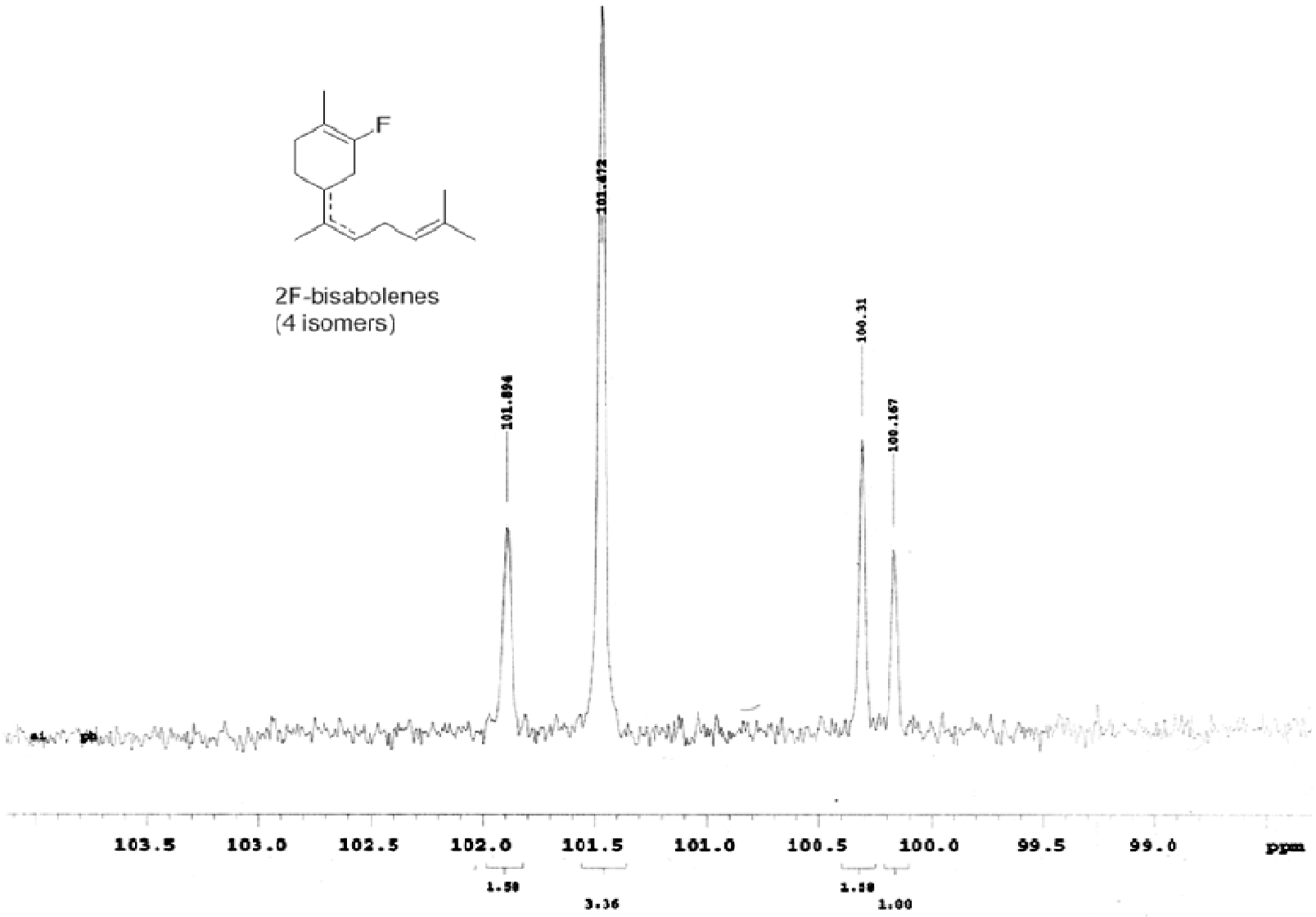

^{19}F NMR (470 MHz, $CDCl_3$).
F
A
+
F
B
2F-Farnesenes
(two isomers)
2F-bisabolenes
(5 isomers)
A
A
B
B
B
B
B

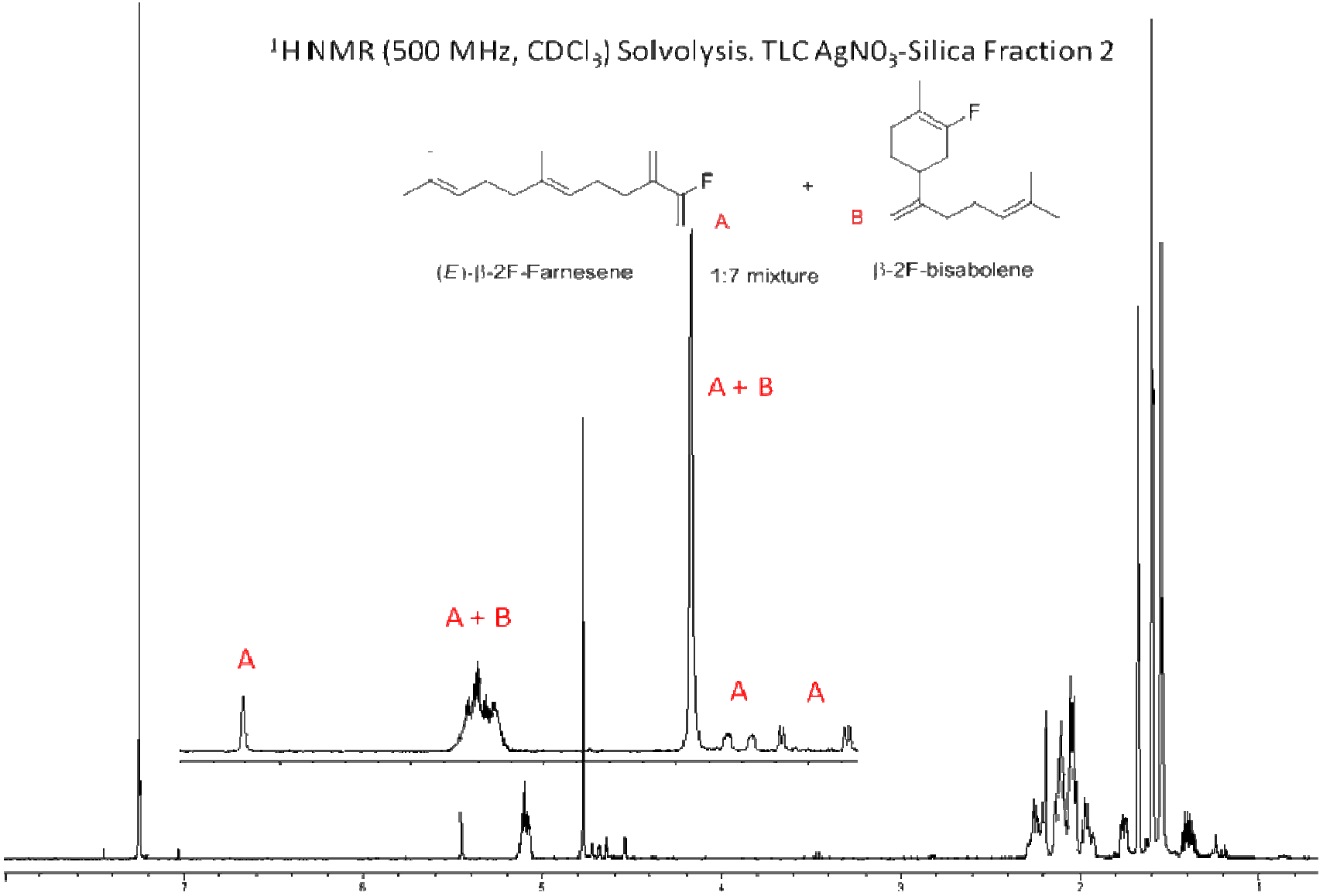
^{1}H NMR (500 MHz, $CDCl_3$) Solvolysis. TLC $AgNO_3$-Silica Fraction 1



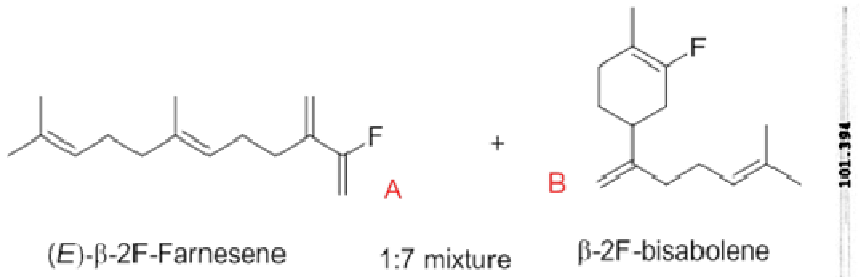
2F-bisabolene
(4 isomers)

^{19}F NMR (470 MHz, $CDCl_3$). Solvolysis. TLC $AgNO_3$-Silica Fraction 1




^{1}H NMR (500 MHz, $CDCl_3$) Solvolysis. TLC $AgNO_3$-Silica Fraction 2
F
A
+
B
F
(E)-β-2F-Farnesene
1:7 mixture
β-2F-bisabolene
A + B
A + B
A
A
A

^{19}F NMR (470 MHz, $CDCl_3$). Solvolysis. TLC $AgNO_3$-Silica Fraction 2



(*E*)-β-2F-Farnesene 1:7 mixture β-2F-bisabolene

12. References

1. Deligeorgopoulou, A.; Allemann, R. K.; *Biochemistry* **2003**, *42*, 7741-7747.
2. Woodside, A. B.; Huang, Z.; Poulter, C. D. *Org. Synth. Coll. Vol*. **1993**, *8*, 616-620.
3. Faraldos. J. A; Zhao, Y.; O'Maille, P. E.; Noel, J. P.; Coates, R. M. *ChemBioChem* **2007**, *8*, 1826-1833.
4. Bradford, M. *Anal. Biochem.* **1976**, *72*, 248-254.
5. Still, W. C.; Kahn, M.; Mitra, A., *J. Org. Chem.* **1978**, *43*, 2923-2925.
6. Faraldos, J. A.; O'Maille, P. E.; Dellas, N.; Noel, J. P.; Coates, R. M., *J. Am. Chem. Soc.* **2010**, *132* (12), 4281-4289.
7. Shishova, E. Y.; Yu, F.; Miller, D. J.; Faraldos, J. A.; Zhao, Y.; Coates, R. M.; Allemann, R. K.; Cane, D. E.; Christianson, D. W. *J. Biol. Chem.* **2008**, *283*, 15431-15439.
8. Dolence J. M.; Poulter C. D. *Tetrahedron* **1996**, *52*, 119-130.
9. (a) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D., *J. Org. Chem.* **1986**, *51*, 4768-79. (b) Woodside, A. B.; Huang, Z.; Poulter, C. D. *Org. Synth. Coll. Vol.* **1993**, *8*, 616-620.
10. (a) Cane, D. E.; Iyengar, R.; Shiao, M.-S. *J. Am. Chem. Soc.* **1981,** *103*, 914-931. (b) Cane, D. E.; Chiu, H.-T. Liang, P.-H.; Anderson, K. S. *Biochemistry* **1997**, *36*, 8332-8339.
11. (a) Danilov, L. L.; Mal'tsev, S. D.; Shibaev, V. N. *Soviet J. Bioorg. Chem.* **1988**, *14*, 712-714. (b) Cramer, F.; Rittersdorf, W. R. *Tetrahedron* **1967**, *23*, 3015-3022. (c) Conforth, R. H.; Popjak, G. *Methods Enzymol*. **1969**, *15*, 385-388.
12. Karp, F.; Zhao, Y. Santhamma, B.; Assink, B.; Coates, R. M.; Croteau, R. B. *Arch. Biochem. Biophys.* **2007**, *468*, 140-146.
13. Keller, R. K.; Thompson, R. *J. Chromatogr.* **1993**, *645,* 161-167.
14. Davisson, V. J.; Zabriskie, T. M.; Poulter, C. D. *Bioorg. Chem.* **1986**, *14*, 46-54.
15. Croteau, R. Arch. Biochem. Biophys. **1986**, *251*, 777-782.
16. Cane, D. E.; Ha, H.-J. *J. Am. Chem. Soc.* **1988,** *110*, 6865-6870.
17. Cuvigny, T.; Julia, M.; Rolando, C. *J. Chem. Soc., Chem. Commun*. **1984**, 8.
18. Satterwhite, D. M.; Wheeler, C. J.; Croteau, R. *J. Biol. Chem.* **1985**, *260*, 13901-13908.
19. Poppe, L.; Novak, L.; Szantay, C. *Synth. Commun.* **1987**, *17*, 173-179.
20. Yong, K. H.; Lotoski, J. A.; Chong, J. M. *J. Org. Chem*. **2001**, *66*, 8248-8251.
21. Faraldos, J. A.; Gonzalez, V.; Senske, M.; Allemann, R. K. *Org. Biol. Chem.* **2011**, *9*, 6920-6923
22. (a) Poulter C. D.; King, C.-H. R. *J. Am. Chem. Soc.* **1982,** *104*, 1422-1424. (b) Bunton, C. A.; Hachey, D. L.; Leresche, J.-P. *J. Org. Chem*. **1972**, *37*, 4036-4039.
23. Caruthers, J. M; Kang, I.; Rynkiewicz, M. J.; Cane, D. E.; Christianson, D. W. *J. Biol. Chem.* **2000**, *275*, 25533-25539.
24. Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658-666
25 Crock, J.; Wildung, M.; Croteau, R. *Proc. Natl. Acad. Sci.* **1997,** *94,* 12833-12838.
26. Prosser, I. A.; Adams, R. J.; Beale, M. H.; Hawkins, N. D.; Phillips, A. L.; Pickett, J. A.; Field, L. M. *Phytochemisty* **2006**, *67*, 1564-1571.