Supporting Information

Biosynthesis of the Sesquiterpene Botrydial in *Botrytis cinerea*. Mechanism and Stereochemistry of the Enzymatic Formation of Presilphiperfolan-8-ol

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Materials and Methods. General materials and methods were as previously described.[1] (3*S*)-Nerolidyl diphosphate, (3*RS*)-nerolidyl diphosphate, (3*S*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate (77.6% d_1, 22.4% d_2) and (3R*S*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate (14.3% d_0, 82.1% d_1, 3.6% d_2) were prepared as previously described.[2,3] [13,13,13-$^2H_3$]FPP (**2d**) were prepared by Dr. P. C. Prabhakaran.[4] Presilphiperfolan-8β-ol (**3**) isolated from *E. staechifolium* was a gift from Drs. Robert M. Coates and Juan A. Faldos of the University of Illinois, Urbana, IL. Recombinant BcBOT2 protein was purified as previously described.[1] Preparative-scale incubation of recombinant BcBOT2 protein with deuterated FPP and NPP samples was carried out as previously described.[1] Standard GC-MS instruments and temperature programs were used as previously described.[1] Chiral capillary GC–MS spectra were recorded at 70 eV EI, operating in positive ion mode, with a HYDRODEX β-6TBDM heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin capillary column (25 m × 0.25 mm), using a temperature program of 100-160 °C, 2.3 °C min^{-1} (10 min hold) followed by 160-200 °C, 10 °C min^{-1} (2 min hold).

NMR – General. NMR spectra were obtained on Bruker Avance NMR spectrometers operating at 399.85 MHz ^{1}H frequency. Chemical shifts are referenced to C_6D_6 at room temperature.

Presilphiperfolan-8β-ol (3). NMR assignments. ^{1}H NMR (C_6D_6, 399.85 MHz) δ 2.34 (m, 1 H, H-2β), 2.19 (d, *J* = 11.4 Hz, 1 H, H-5β), 2.12 (m, 1 H, H-3β), 1.83 (m, 1 H, H-2α), 1.53 (m, 1 H, H-10α), 1.51 (m, 1 H, H-11β), 1.42 (s, 3 H, H-14), 1.33 (m, 1 H, H-7), 1.33 (m, 1 H, H-11α), 1.29 (m, 1 H, H-9), 1.23 (d, *J* = 11.4 Hz, 1 H, H-5α), 1.21 (m, 1 H, H-3α), 1.21 (s, 3 H, H-13), 1.16 (s, 3 H, H-12), 1.03 (m, 1 H, H-1), 0.91 (m, 1 H, H-10β), 0.88 (d, *J* = 6.4 Hz, 3 H, H-15); ^{13}C NMR (C_6D_6, 399.85 MHz, ppm): 96.2 (C, C-8), 56.4 (C, C-4), 52.3 (CH, C-7), 50.2 (CH, C-1), 49.4 (CH_2, C-5), 48.0 (C, C-6), 37.5 (CH, C-9), 36.4 (CH_3, C-14), 34.5 (CH_2, C-10), 34.0 (CH_2, C-3), 33.3 (CH_2, C-2), 28.1 (CH_3, C-12), 28.0 (CH_2, C-13), 27.1 (CH_2, C-11), 21.8 (CH_3, C-15). Figure S2 shows the ^{1}H NMR spectrum of the enzymatically-generated presilphiperfolan-8β-ol (**3**), which was shown to be identical with the authentic sample of **3** as described previously.[1] (Figures S3-S7 show the ^{13}C and 2D NMR spectra of the reference sample, which contained an unknown impurity that displayed a characteristic ethyl pattern with two ^{1}H signals (δ 2.43 q, δ 1.00 t) correlated with two ^{13}C signals (46.7 and 12.3 ppm)).

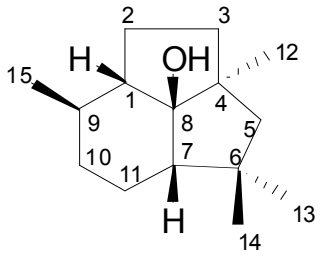

Figure S1. Presilphiperfolan-8β-ol (**3**).

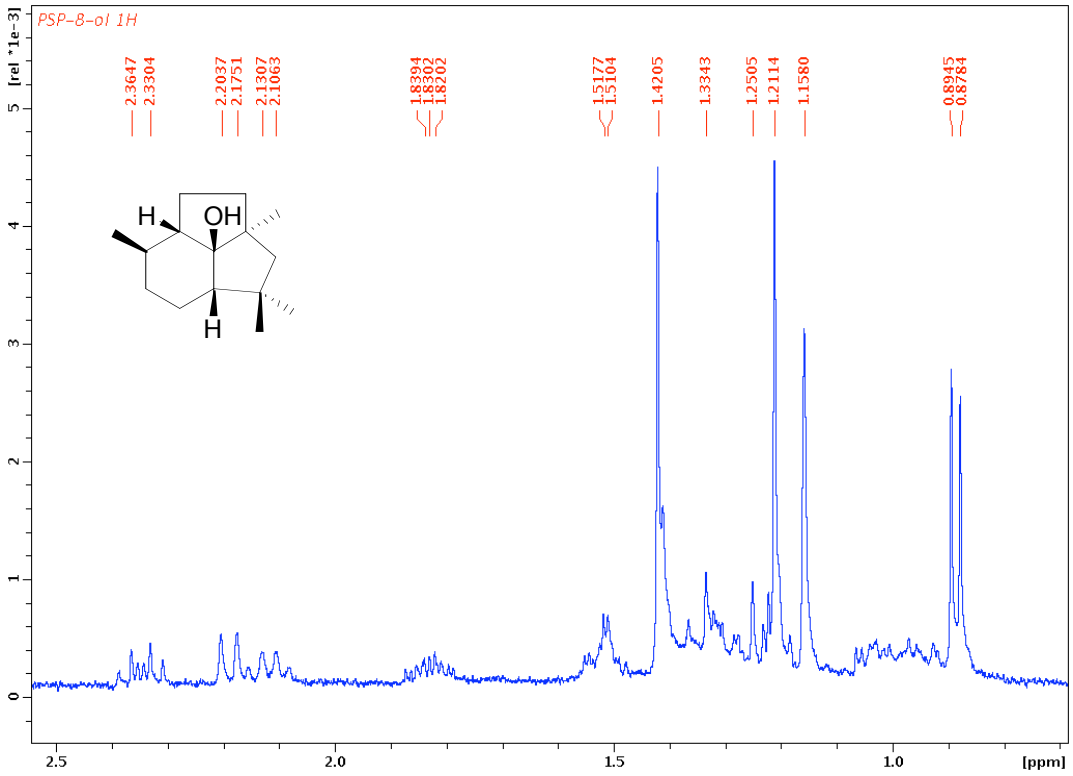

Figure S2. ^{1}H NMR (C_6D_6, 399.85 MHz) spectrum of enzymatically generated presilphiperfolan-8β-ol (**3**).

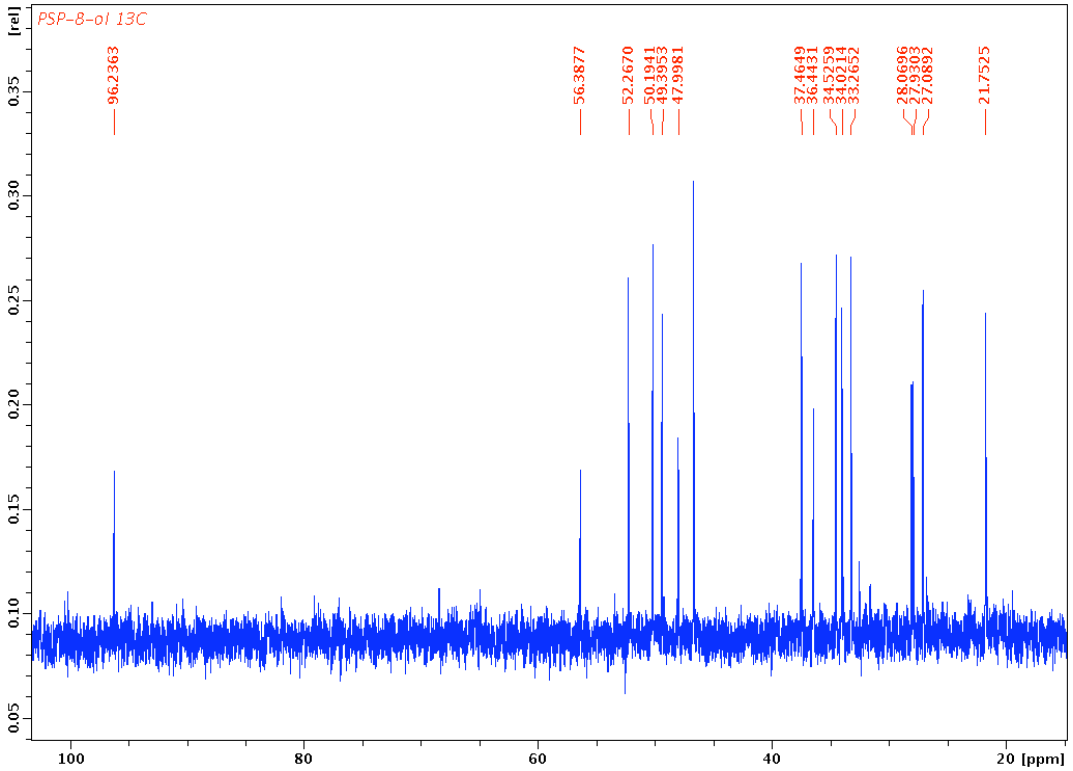

Figure S3. ^{13}C NMR (C_6D_6, 100.61 MHz) spectrum of enzymatically generated presilphiperfolan-8β-ol (**3**).

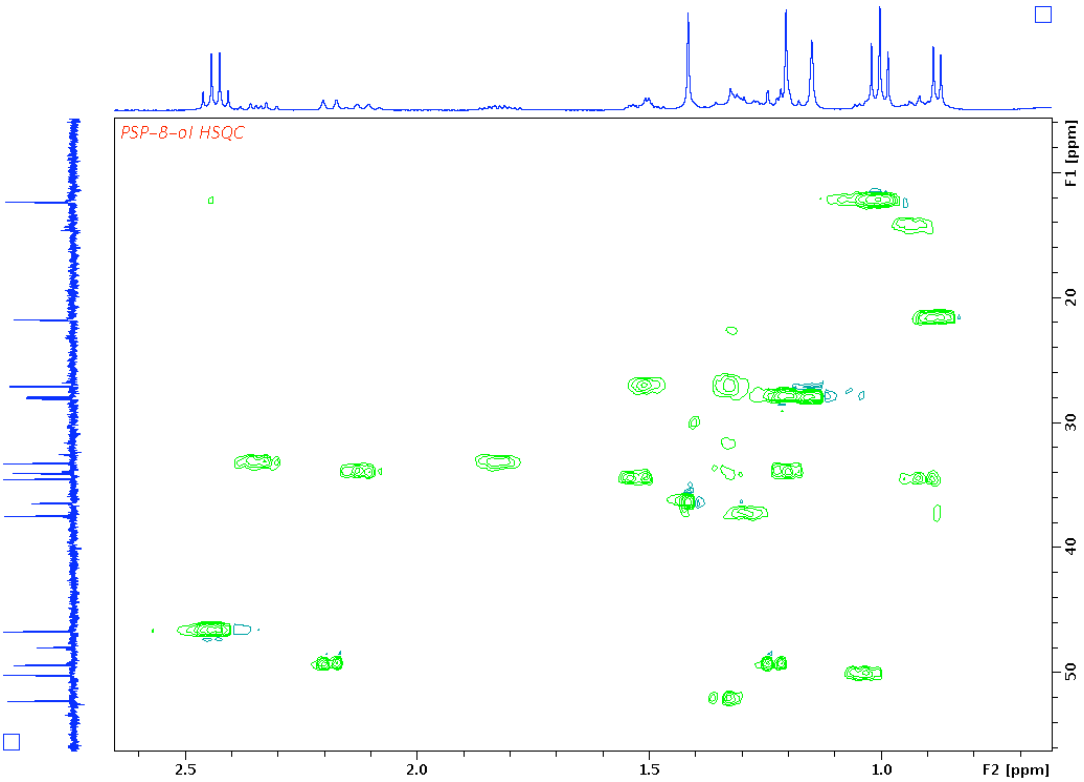

Figure S4. HSQC NMR (C_6D_6, 399.85 MHz, 100.54 MHz) spectrum of presilphiperfolan-8β-ol (**3**).

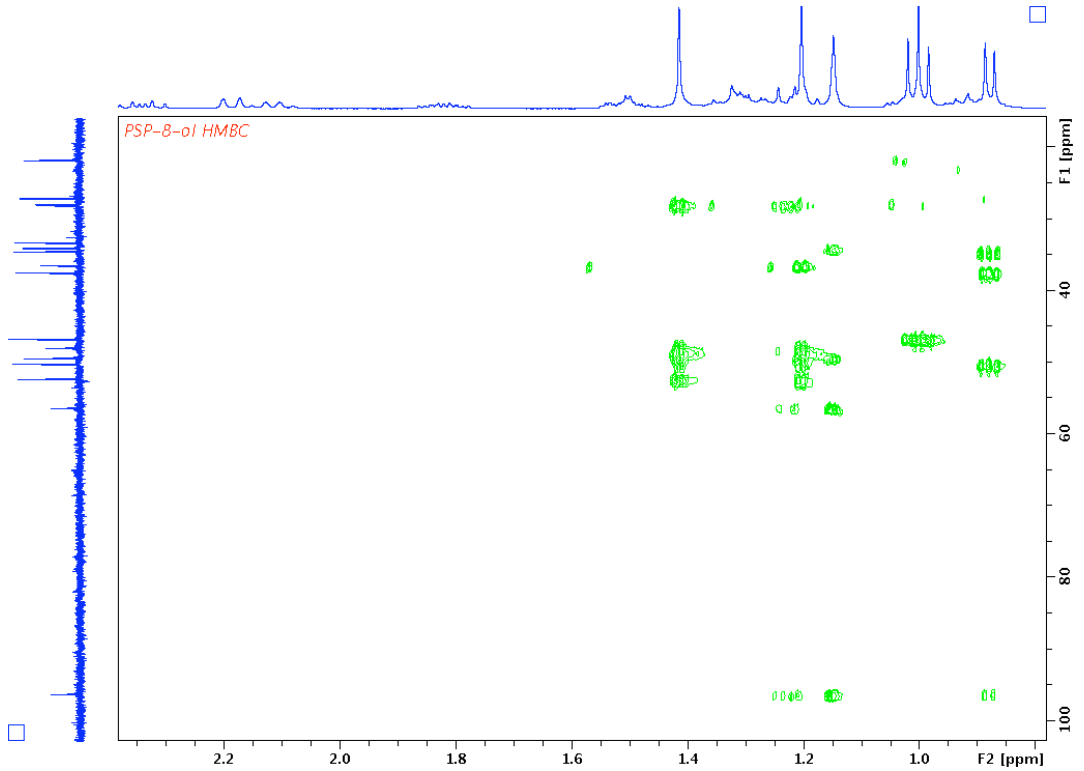

Figure S5. HMBC NMR (C_6D_6, 399.85 MHz, 100.54 MHz) spectrum of presilphiperfolan-8β-ol (**3**).

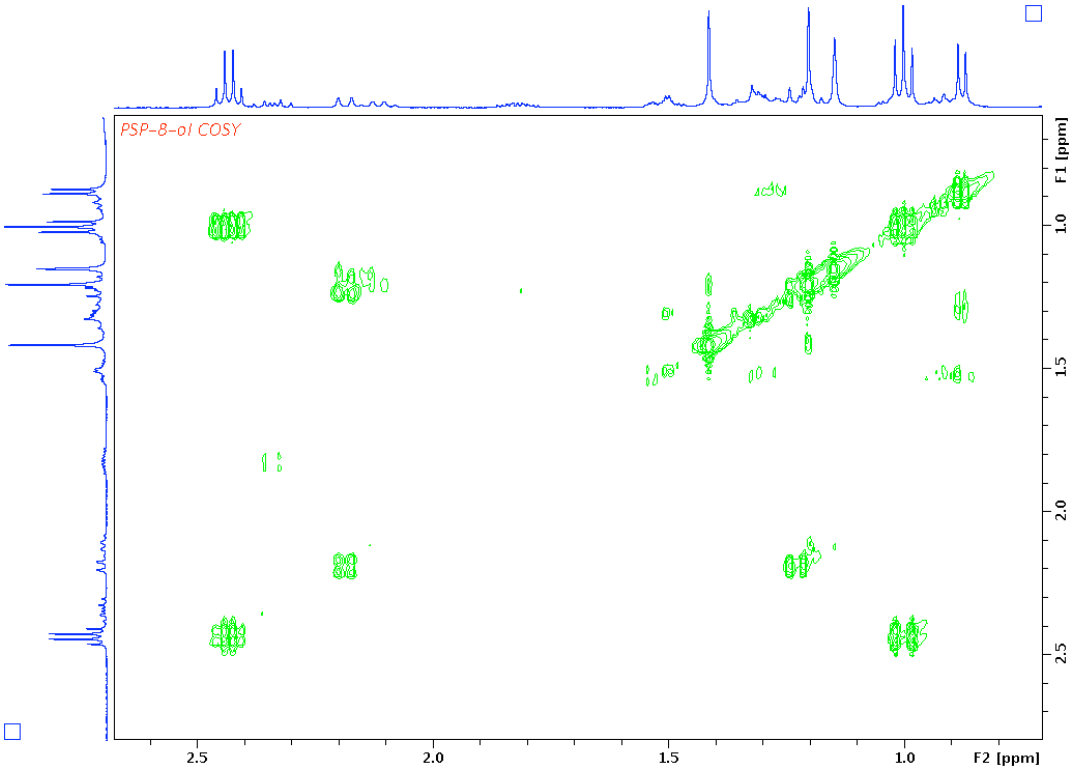

Figure S6. ^{1}H-^{1}H COSY NMR (C_6D_6, 399.85 MHz) spectrum of presilphiperfolan-8β-ol (**3**).

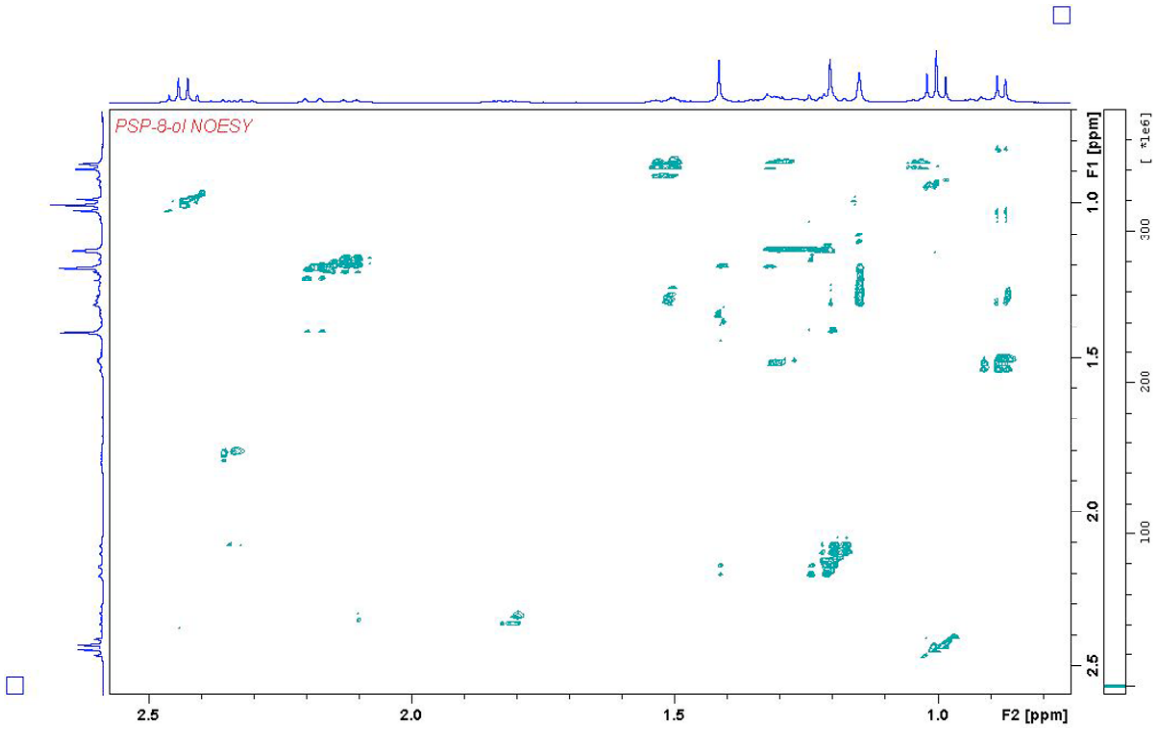

Figure S7. NOESY NMR (C_6D_6, 399.85 MHz) spectrum of presilphiperfolan-8β-ol (**3**).

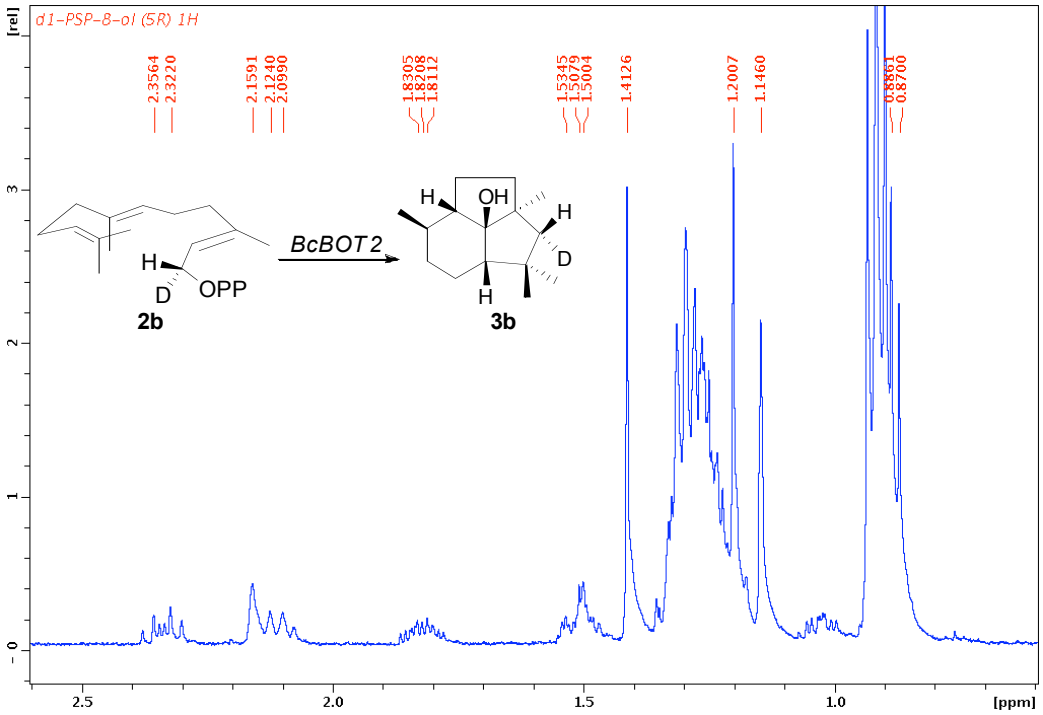

Figure S8. ^{1}H NMR (C_6D_6, 399.85 MHz) spectrum of (5*R*)-[5α-^{2}H]presilphiperfolan-8β-ol (**3b**) derived from (1*R*)-[1-^{2}H]FPP (**2b**). (The additional peaks at δ 0.9 (t) and 1.25 (m) are due to residual pentane in the 1-2 mg sample of **3b**.)

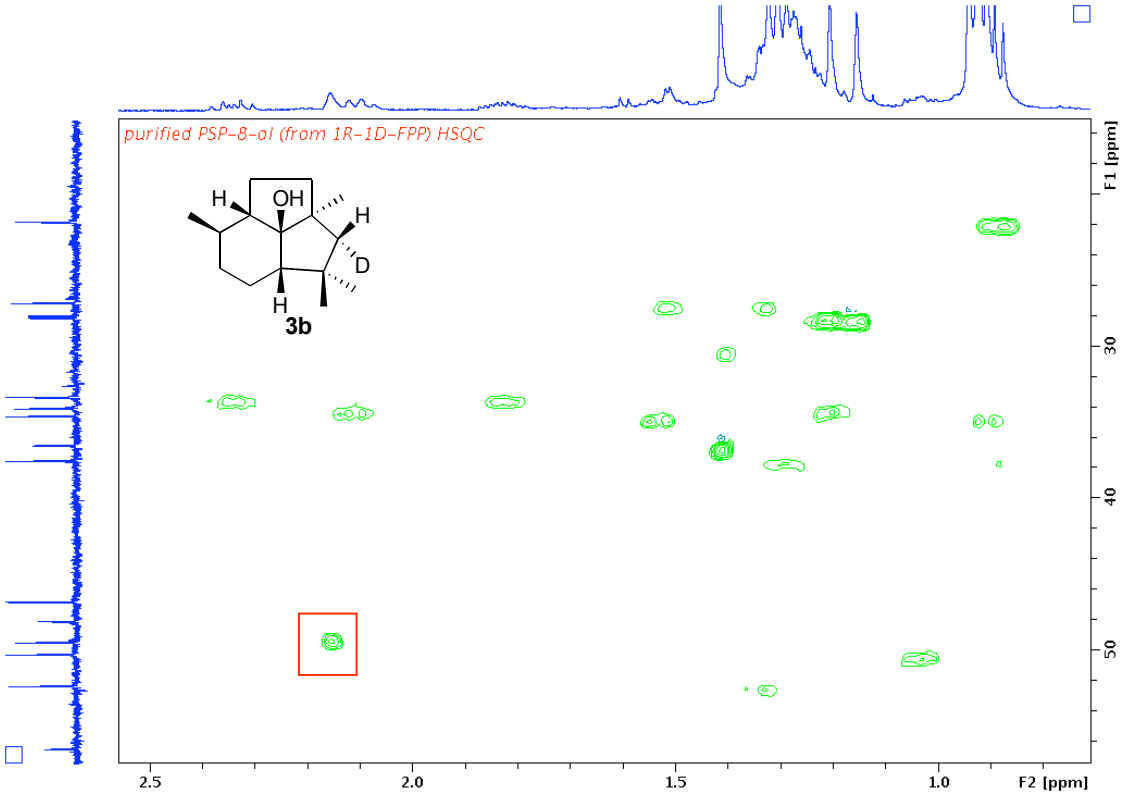

Figure S9. HSQC NMR (C_6D_6, 399.85 MHz, 100.54 MHz) spectrum of (5*R*)-[5α-^{2}H]presilphiperfolan-8β-ol (**3b**) from (1*R*)-[1-^{2}H]FPP (**2b**). The cross-peak between H-5β (δ 2.16) and C-5 (49.4 ppm) is framed in red. (The additional peaks at δ 0.9 (t) and 1.25 (m) are due to residual pentane in the 1-2 mg sample of **3b**.)

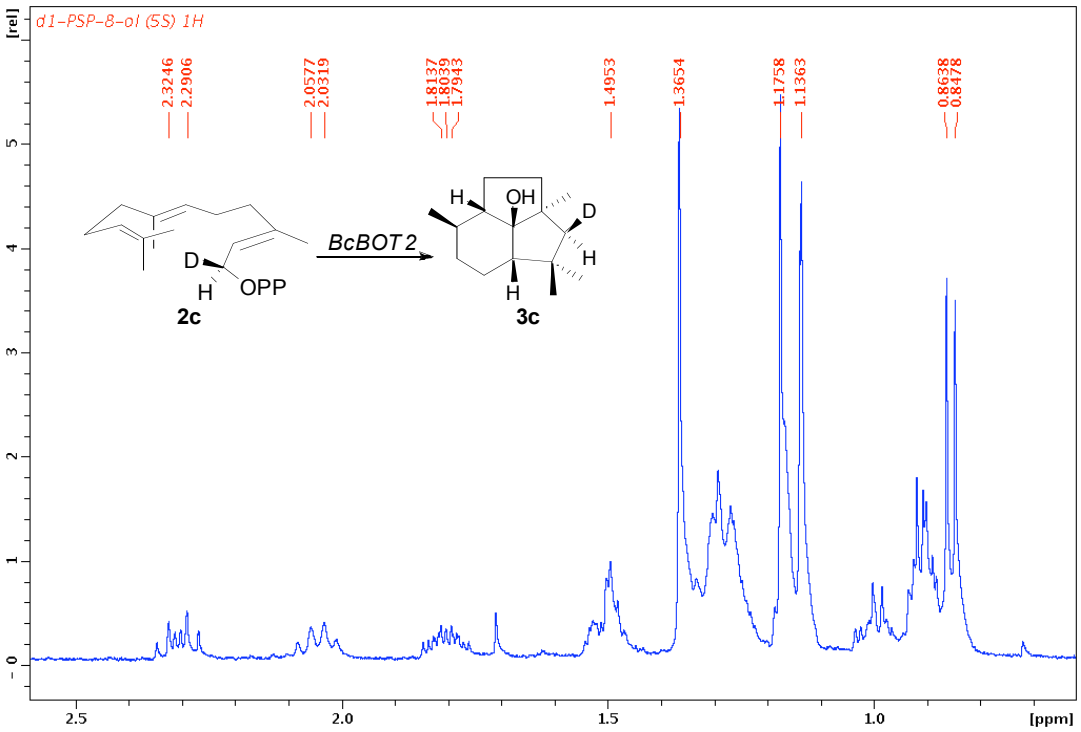

Figure S10. ^{1}H NMR (C_6D_6, 399.85 MHz) spectrum of (5*S*)-[5β-^{2}H]presilphiperfolan-8β-ol (**3c**) derived from (1*S*)-[1-^{2}H]FPP (**2c**). (The additional peaks at δ 0.9 (t) and 1.25 (m) are due to residual pentane in the 1-2 mg sample of **3c**.)

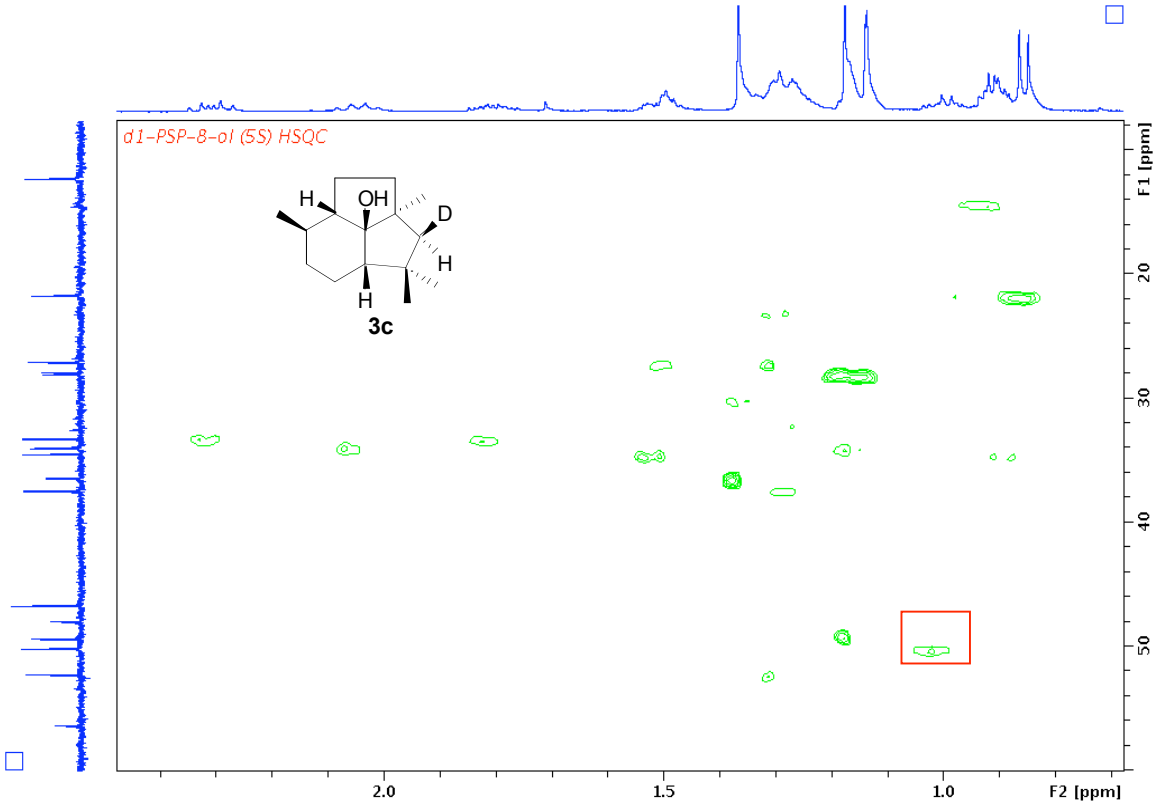

Figure S11. HSQC NMR (C_6D_6, 399.85 MHz, 100.54 MHz) spectrum of (5*S*)-[5β-^{2}H]presilphiperfolan-8β-ol (**3c**) from (1*S*)-[1-^{2}H]FPP (**2c**). The cross-peak between H-5α (δ 1.18) and C-5 (49.4 ppm) is framed in red. (The additional peaks at δ 0.9 (t) and 1.25 (m) are due to residual pentane in the 1-2 mg sample of **3c**.)

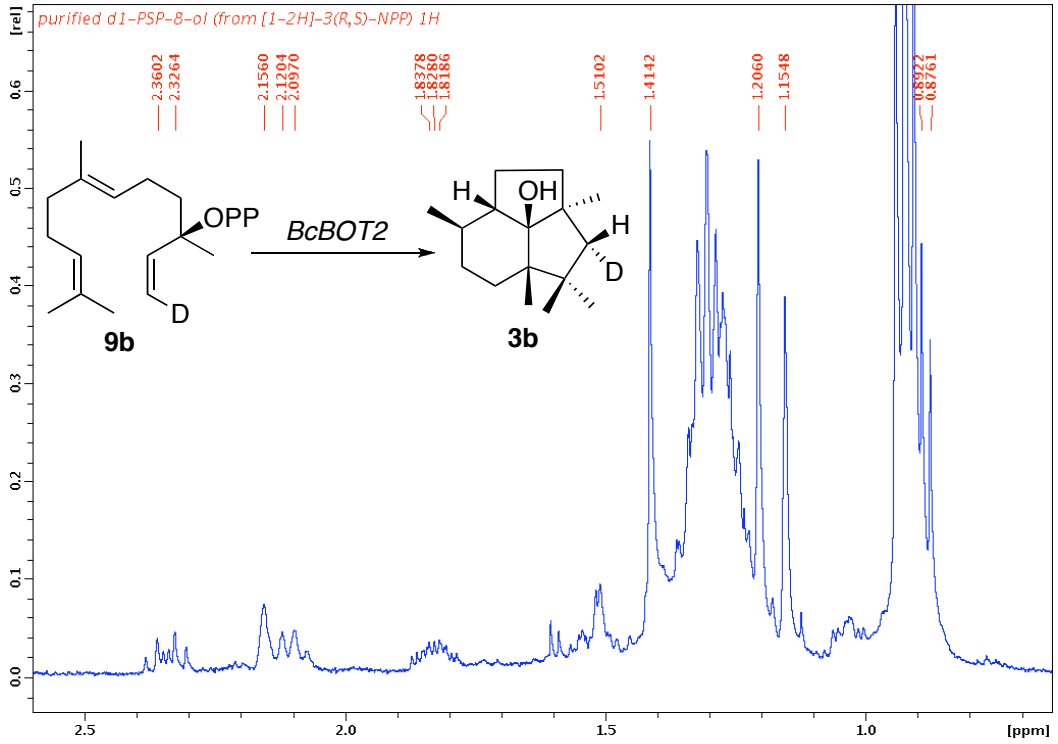

Figure S12. ^{1}H NMR (C_6D_6, 399.85 MHz) spectrum of (5*R*)-[5α-^{2}H]presilphiperfolan-8β-ol (**3b**) derived from (3*RS*)-(*Z*)-[1-^{2}H]NPP (**9b**). (The additional peaks at δ 0.9 (t) and 1.25 (m) are due to residual pentane in the 1-2 mg sample of **3b**.)

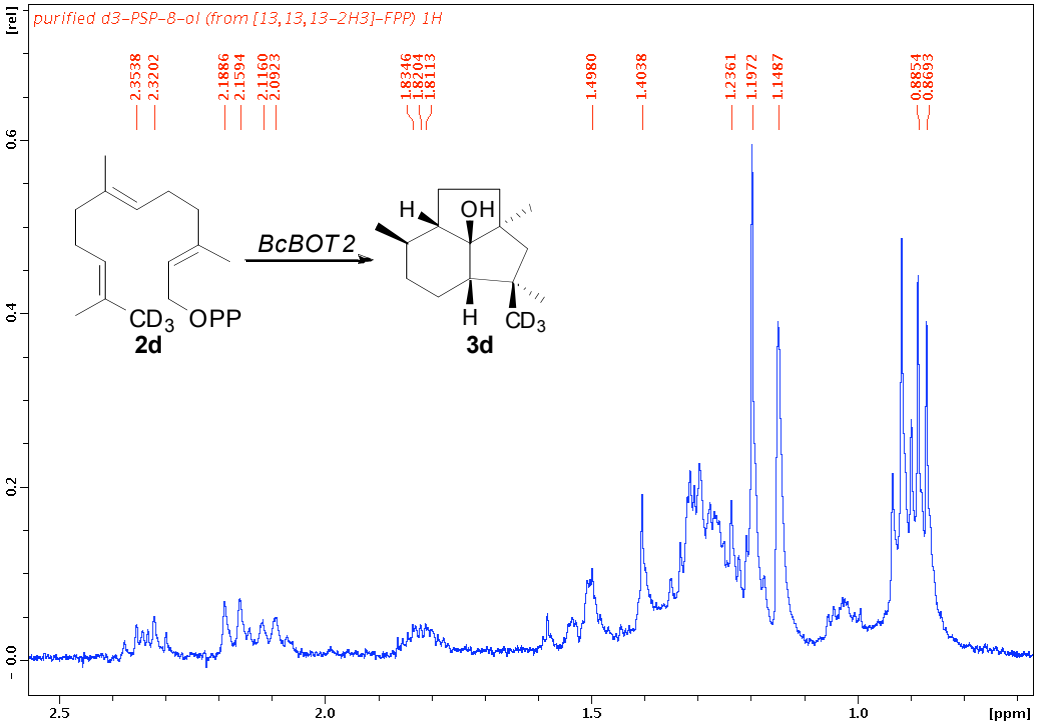

Figure S13. ^{1}H NMR (C_6D_6, 399.85 MHz) spectrum of [14,14,14-$^{2}H_3$]presilphiperfolan-8β-ol (**3d**) derived from [13,13,13-$^{2}H_3$]FPP (**2d**). (The additional peaks at δ 0.9 (t) and 1.25 (m) are due to residual pentane in the 1-2 mg sample of **2d**.)

Competitive incubation of (3*S*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate and (3*RS*)-nerolidyl diphosphate with BcBOT2. A mixture of (3*S*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate (77.6% d_1, 22.4% d_2; 10 mM, 12 μL) and (3*RS*)-nerolidyl diphosphate solution (10 mM, 12 μL) was incubated with purified recombinant BcBOT2 (1 μM) in 4 mL of assay buffer [50 mM PIPES, 5 mM $MgCl_2$, 100 mM NaCl, 5 mM of β-mercaptoethanol, pH 7.0] at 30 °C for 5 h. Meanwhile, an identical NPP mixture was hydrolyzed with excess apyrase and phosphatase[5] followed by extraction with diethyl ether, and the concentrated ether extract was analyzed by chiral GC–MS. The precise ratio of (3*R*)- to (3*S*)-NPP in the mixture was determined to be 1:1.82 (3*R*)-*trans*-nerolidol (t_R 32.54 min) to (3*S*)-*trans*-nerolidol (t_R 33.05 min), corresponding to a calculated molar ratio of (3*S*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate and (3*RS*)-nerolidyl diphosphate mixture of 1: 1.48. The BcBOT2 incubation mixture was extracted with HPLC-grade dichloromethane and the deuterium content of the resulting presilphiperfolan-8β-ol was analyzed by GC-MS/SIM of the *m*/*z* 222, 223, and 224 peaks to give a d_0:d_1 ratio of 95:5. After correction for the ratio of enantiomers in the NPP mixture and the isotopic enrichment of the (3*S*)-(1*Z*)-[1-^{2}H]NPP, the stereochemical preference for (3*R*)-NPP over (3*S*)-NPP was calculated to be 9.46:1.

Competitive incubation of (3*S*)-nerolidyl diphosphate and (3*RS*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate with BcBOT2. A mixture of (3*S*)-nerolidyl diphosphate (10 mM, 12 μL) and (3*RS*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate (14.3% d_0, 82.1% d_1, 3.6% d_2; 10 mM, 12 μL) was incubated with purified recombinant BcBOT2 (1μM) in 4 mL of assay buffer at 30 °C for 5 h. Parallel phosphatase/apyrase hydrolysis and GC–MS analysis was carried out as described above and gave an overall (3*R*)- to (3*S*)-NPP ratio of 1:7.11 and a calculated molar ratio of (3*RS*)-(1*Z*)-[1-^{2}H]nerolidyl diphosphate to (3*S*)-nerolidyl diphosphate of 1: 6.04. GC–MS/SIM analysis of the resulting **3** gave a d_0:d_1 ratio of 1:1.35. After correction for the enantiomeric enrichment and the deuterium content of the original NPP mixture, the stereochemical preference for (3*R*)-NPP over (3*S*)-NPP was calculated to be 11.94:1.

Incubation of (3*RS*)-(1*Z*)-nerolidyl diphosphate (NPP) with BcBOT2 and GC analysis. Purified recombinant BcBOT2 (1μM) was incubated with 60μM of (3*RS*)-(1*Z*)-NPP in 4 mL of assay buffer, overlaid with 4 mL HPLC-grade pentane, at 30 °C for 5 h. The reaction mixture was extracted with HPLC-grade dichloromethane, and the combined organic extract was dried, concentrated, and analyzed by GC-MS (Figure S14). The analysis of dichloromethane-extractable products revealed the formation of β-farnesene (t_R 8.84 min, *m/z* 204, 40.2%) and presilphiperfolan-8β-ol (**3**) (t_R 9.83 min, *m/z* 222, 28.5%) as two major products. A trace amount of nerolidol (t_R 9.52min, *m/z* 222, 7.1%) and one unknown sesquiterpene (r.t. 9.16, *m/z* 204, 7.2%), possibly α-bisabolene as identified by comparison of mass spectra with known standards in the MassFinder 3.0 Database (http://www.massfinder.com), was also detectable.

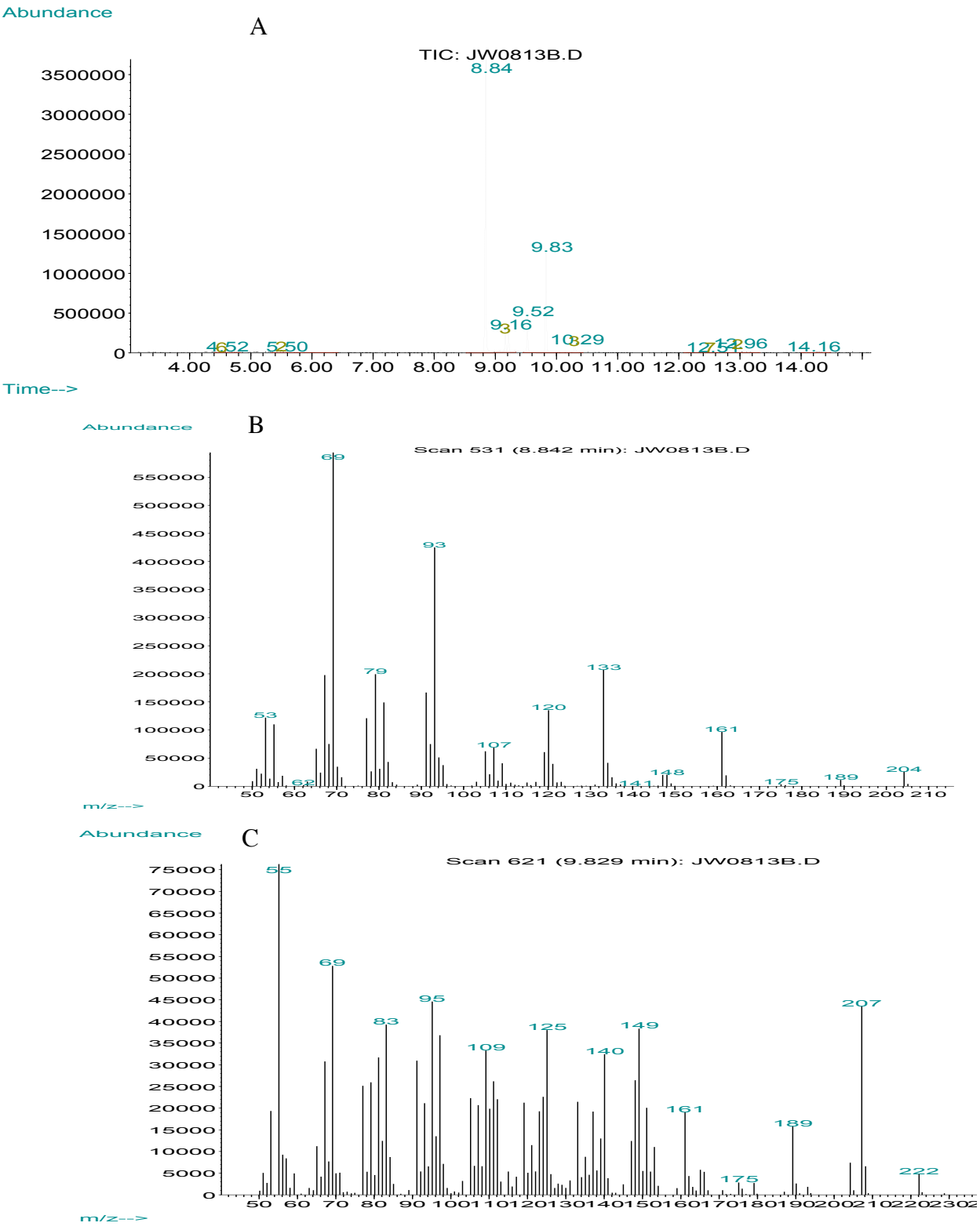

Figure S14. GC–MS spectra of enzymatic products from incubation of (3*RS*)-(1*Z*)-nerolidyl diphosphate with BcBOT2. A) GC/TIC; B) MS of β-farnesene, t_R 8.84 min; C) MS of presilphiperfolan-8β-ol (**3**), t_R 9.83 min.

References

(1) Pinedo, C.; Wang, C.-M.; Pradier, J.-M.; Dalmais, B.; Choquer, M.; Pêcheur, P. L.; Morgant, G.; Collado, I. G.; Cane, D. E.; Viaud, M. *ACS. Chem. Biol.* **2008**, *3*, 791-801.

(2) Cane, D. E.; Iyengar, R.; Shiao, M.-S. *J. Am. Chem. Soc.* **1981**, *103*, 914-931.

(3) Cane, D. E.; Ha, H. J. *J. Am. Chem. Soc.* **1988**, *110*, 6865-6870.

(4) Cane, D. E.; Prabhakaran, P. C.; Oliver, J. S.; McIlwaine, D. B. *J. Am. Chem. Soc.* **1990**, *112*, 3209-3210.

(5) Croteau. R., and Cane, D. E. *Methods in Enzymology* **1985**, *110*, 383-405.