Sodorifen biosynthesis in the rhizobacterium *Serratia plymuthica* involves methylation and cyclization of MEP-derived farnesyl pyrophosphate by a SAM-dependent C-methyltransferase.

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SUPPORTING INFORMATION

Experimental Procedures

Cultivation of *Serratia plymuthica* 4Rx13

Serratia plymuthica 4Rx13 was isolated from the rhizosphere of *Brassica napus* by Prof. Gabriele Berg, Institute of Environmental Biotechnology, University of Graz, Austria.[1] *S. plymuthica* was cultivated at 30 °C in complex media such as nutrient agar (NAII, Sifin Diagnostics GmbH, Berlin, Germany; 3.5 g/L peptone from casein, 2.5 g/L peptone from meat, 2.5 g/L peptone from gelatin, 1.5 g/L yeast extract, 5.0 g/L NaCl, 15.0 g/L agar, pH 7.2) or liquid nutrient broth (NBII, Sifin Diagnostics GmbH, Berlin, Germany). For the feeding and incorporation experiments *S. plymuthica* was cultivated in Davis Minimal Medium[2] (DMM; 7.0 g/L K_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L trisodium citrate, 0.1 g/L $MgSO_4$, 1.0 g/L $(NH_4)_2SO_4$) supplemented with either 55 mM of a single amino acids, 20 mM of amino acids in combinations, or with 55 mM of organic acids, glucose, or pyruvate as carbon source.

Comparative analysis of *Serratia plymuthica* 4Rx13 mutant and wild type VOCs

Serratia plymuthica 4Rx13 wild type, *C*-methyltransferase knockout (MT::kan), and terpene cyclase knockout (TC::kan) were grown in 6 ml NBII medium overnight at 30 °C and 170 rpm and were then transferred into a 100 ml main culture once OD_{600} = 0.5-1 was reached. The volatile compounds within the headspace of a liquid bacterial culture were trapped on Super-Q adsorbent (50 mg; Alltech, Deerfield, IL, USA) using a VOC collection system as previously described.[3] Volatiles were collected in 24 h intervals over 96 h. The trapped compounds were eluted using dichloromethane or methanol for GC-MS or LC-MS analysis respectively, or with benzene-d_6 for NMR analysis. Nonyl acetate was added as an internal standard with a concentration of 5 ng/µl for GC-MS measurements.

Heterologous expression of *Serratia plymuthica* methyltransferase and terpene cyclase

The *Serratia plymuthica* 4Rx13 genes methyl transferase (MT, SOD_c20760) and terpene cyclase (TC, SOD_c20750) were amplified by PCR to generate blunt end products using Champion™ pET directional

TOPO expression kits (Invitrogen, Thermo Fischer, Germany) according to the manufacturer's protocol. The desired PCR products were directionally cloned into the pET151/D-TOPO vector. The recombinant vectors were transformed into *E. coli* XL-blue. Positive transformants were analyzed by colony PCR and plasmids with the correct insert were isolated and sequenced. Positive clones were streaked on LB-ampicillin plates (8.0 g/L tryptone, 4.0 g/L yeast extract, 5.0 g/L NaCl, 15 g/L agar, 100 µg/ml ampicillin, pH 7) and incubated at 37 °C for 24 h. Single colonies were isolated from the plates for inoculation in 5 ml of LB-ampicillin medium and incubated at 37 °C for 24 h.

For the heterologous expression of *S. plymuthica* 4Rx13 methyl transferase and terpene cyclase, *E. coli* BL21(DE3) cells were transformed with the pET151/D-TOPO plasmid including the genes of interest. LB-ampicillin plates were incubated at 37 °C for 24 h, and single colonies of positive transformants were grown overnight at 37 °C at 170 rpm in 5 ml of LB-ampicillin medium (pre-cultures). Main cultures were prepared in 200 ml LB-ampicillin medium supplemented with 1% glucose by inoculation with 1 ml pre-culture. The cultures were grown at 37 °C and after reaching an OD_{600} of 0.8-1.0 they were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 20 °C, 170 rpm for 20 h.

Proteins were extracted and purified as previously described.[4] Briefly, crude extracts were obtained by incubating the cell pellet with lysozyme (final concentration, 1 mg mL^{-1}), sonication and centrifugation to separate cell debris from the enzyme containing soluble fraction. The overexpressed protein was purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Protein concentrations were measured using the standard Bradford assay. Protein purity was confirmed using SDS-PAGE and Western blotting (anti-His tagged antibody and anti-rabbit IgG; Sigma, Steinheim, Germany). The purified proteins were stored at -20 °C or -70 °C.

***In vitro* enzyme assays**

For functional characterization of the purified proteins double enzyme assays were performed using the methyl transferase (MT) and the terpene cyclase (TC) of *S. plymuthica* 4Rx13. Purified enzymes (20 µg each) in 50 µL of assay buffer (250 mM HEPES-KOH, 100 mM $MgCl_2$, 2.5 mM $MnCl_2$, 50% (v/v) glycerol, pH 8), 30 mM dithiothreitol (DTT), 2.3 mM of S-adenosyl methionine (SAM, Sigma-Aldrich, Steinheim, Germany), 0.06 mM of farnesyl pyrophosphate (FPP, **1a**, Echelon Biosciences, Salt Lake City, USA), and 200 µL double distilled water were incubated at 37 °C for 3.5 h. Coupled enzyme assays were performed as described above using either the *C*-methyltransferase (MT) or the terpene cyclase (TC) and after the first incubation 10 U of alkaline phosphatase (AP, Thermo Fischer Scientific) was added and additionally incubated for 1 h at 37 °C. All assay samples were overlaid with 200 µL of hexane (with nonyl acetate as internal standard at 5 ng/µL). The reaction products were extracted by vortexing for 30 s followed by centrifugation (2 min at 5.000 g). One microliter of the separated hexane phase was used for GC-MS analysis.

In vivo feeding experiments with stable isotope labelled precursors

Feeding experiments with stable isotope labelled precursors were performed using *S. plymuthica* 4Rx13 wild type or the terpene cyclase knockout mutant (TC::kan). Cultures were grown in 100 ml of Davis Minimal Medium (DMM)[2] supplemented with either 20 mM of 20% [1,4-^{13}C]-succinate, 20% [2,3-$^{13}C_2$]-succinate, or 20% [U-$^{13}C_4$]-succinate in a background of natural abundance succinate, 20 mM of 20% L-[3-^{13}C]-alanine or 20% L-[2,3-$^{13}C_2$]-alanine in a background of natural abundance L-alanine or succinate, or 20 mM of 100% L-[*S*-$^{13}CH_3$]-methionine in a background of natural abundance L-threonine and L-alanine (each at 20 mM) as major carbon sources. Cultures were incubated at 37 °C, 250 rpm and VOCs were collected on Super-Q adsorbent (50 mg, Alltech, Deerfield, IL, USA) for four consecutive 24 h intervals and eluted with 300 µl and 100 µl C_6D_6. Crude samples were analyzed using ^{1}H and ^{13}C {^{1}H} NMR spectroscopy. Maximum ^{13}C incorporation was observed in the 24 - 48 h and 48 - 72 h intervals, whereas longer incubation times resulted in increased scrambling of isotope labels and a decrease of site-specific ^{13}C enrichment.

GC-EIMS analysis of *S. plymuthica* VOCs

GC-EIMS analysis was performed using a Shimadzu GC/MS-QP500 system (Kyoto, Japan) with a CTC autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a DB5-MS column (60 m × 0.25 mm × 0.25 µm; J&W Scientific, Folsom, California, USA). Samples of 1 µL were injected at 200 °C using either a 1:25 split ratio or splitless mode. Helium was used as carrier gas at a flow rate of 1.1 mL/min. A temperature gradient starting from 35 °C for 2 min followed by increase of +10 °C/min to 280 °C within 24.5 min, followed by 15 min at 280 °C was applied. Electron ionization at 70 eV was used. Mass spectra were recorded from m/z = 40 – 280 amu. Data were analyzed using the Lab Solution software (Shimadzu, Duisburg, Germany).

HRMS analysis of *S. plymuthica* VOCs

High resolution mass measurements were performed by UHPLC-ESI-HRMS using an Ultimate 3000 series RSLC UHPLC system (Dionex, Sunnyvale, CA, USA) coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Ten microliter of the VOC containing methanol extract were loaded onto the Acclaim C18 column (150 × 2.1 mm, 2.2 mm, Dionex, Sunnyvale, CA, USA) linked to a C18 3.5 µm guard column (2.1 × 10 mm, Waters, Dublin, Ireland) with a flow rate of 300 ml min^{-1} in a binary solvent system of water and acetonitrile, both containing 0.1% (v/v) formic acid. Ionization was accomplished using a Electrospray Ionization (ESI) source in positive mode. ESI source parameters were set to 4 kV for spray voltage and 35 V for transfer capillary voltage at a capillary temperature of 275 °C. The samples were measured in the mass range of m/z 100 to 1500 using 30’000 m/Δm resolving power in the Orbitrap mass analyzer. Data were analyzed using the Xcalibur software (Thermo Fisher Scientific Inc., Waltham, MA, USA).

NMR analysis of *S. plymuthica* VOCs

NMR spectra of crude *S. plymuthica* 4Rx13 VOCs were recorded using a Bruker AMX400 instrument at the Max Planck Institute for Chemical Ecology, Jena, Germany, or a Bruker AVANCE 400 instrument at the Neuchatel Platform for Analytical Chemistry (NPAC), Neuchatel, Switzerland. Samples were prepared by eluting the volatiles trapped on Super-Q with C_6D_6 and concentrated to a total volume of 550 µl in a gentle stream of dry nitrogen. Standard ^{1}H NMR spectra were recorded by accumulating 128 scans using an acquisition time of 5 s. Standard broadband decoupled ^{13}C {^{1}H} NMR spectra were recorded by accumulating 1024 – 10k scans using an acquisition time of 1.4 s. Two-dimensional phase cycled double-quantum-filtered (*dqf*)-COSY spectra were recorded using 8192 data points in F2 and 512 increments in F1 by accumulating eight scans each, using an acquisition time of 1.5 s. Two-dimensional heteronuclear HSQC spectra were recorded using 2048 data points in F2 and 256 increments in F1 by accumulating 64 scans. Two-dimensional heteronuclear HMBC spectra were recorded using 4096 data points in F2 and 128 increments in F1 by accumulating 128 scans. Two-dimensional homonuclear H,H-NOESY spectra were recorded using 2048 data points in F2 and 256 increments in F1 by accumulating 64 scans, using a mixing time of 750 ms. For NOESY measurements the samples were degassed by bubbling argon gas through the solution for 5 min. Two-dimensional spectra were zero filled to 8k × 4k, manually phased and baseline corrected using the MNova 9.0 (MestreLab) or Topspin 3.5 software (Bruker).

Supporting Figures

Figure S1. Mass spectra of (*E*,*E*)-farnesol (**1b**), pre-sodorifen (**2b**), and sodorifen (**3**).

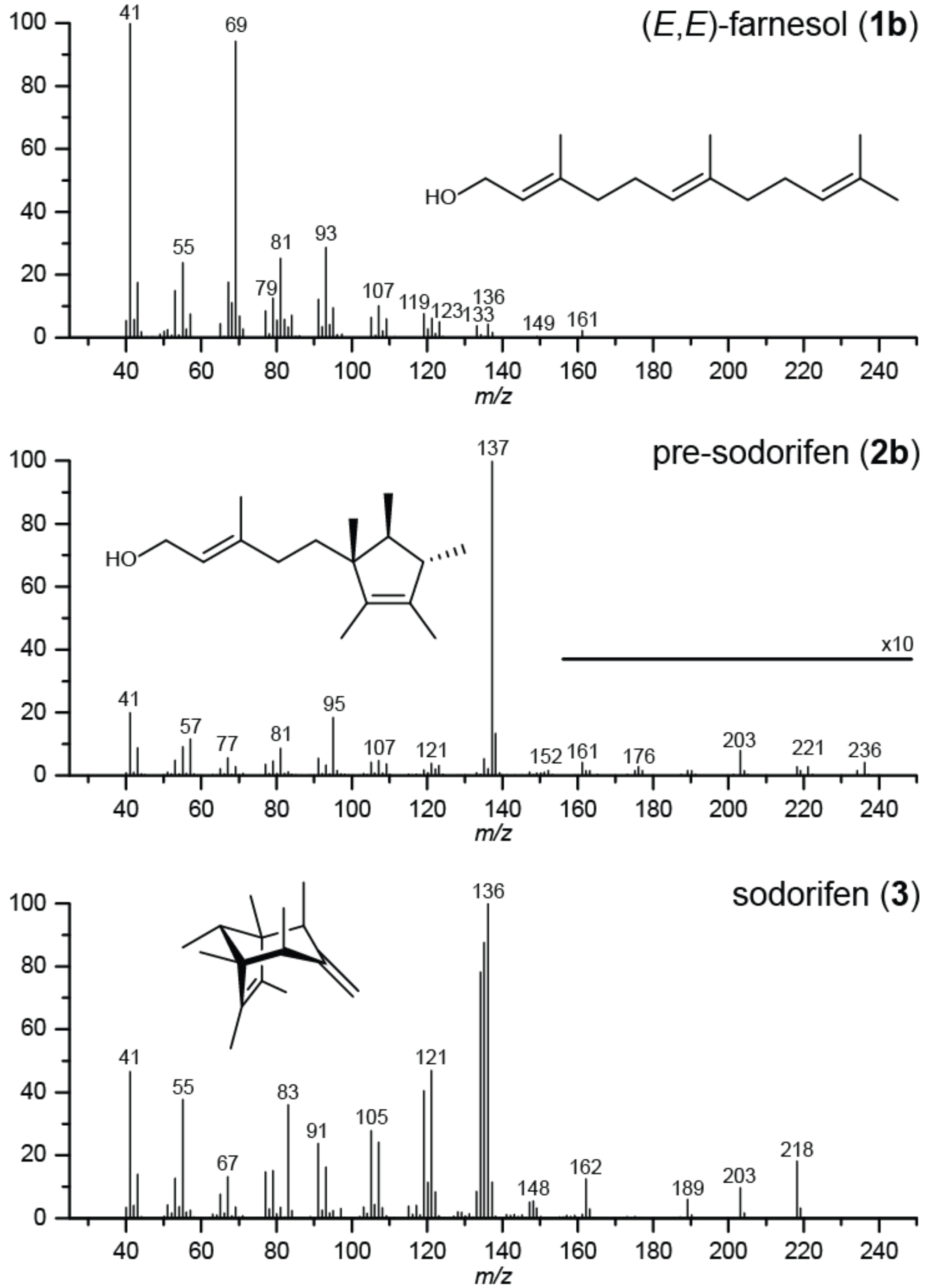

Figure S2. Mass spectrum (EI, 70 eV) of farnesylacetone (**4**) from the *Serratia plymuthica* 4Rx13 *C*-methyltransferase mutant (MT::kan).

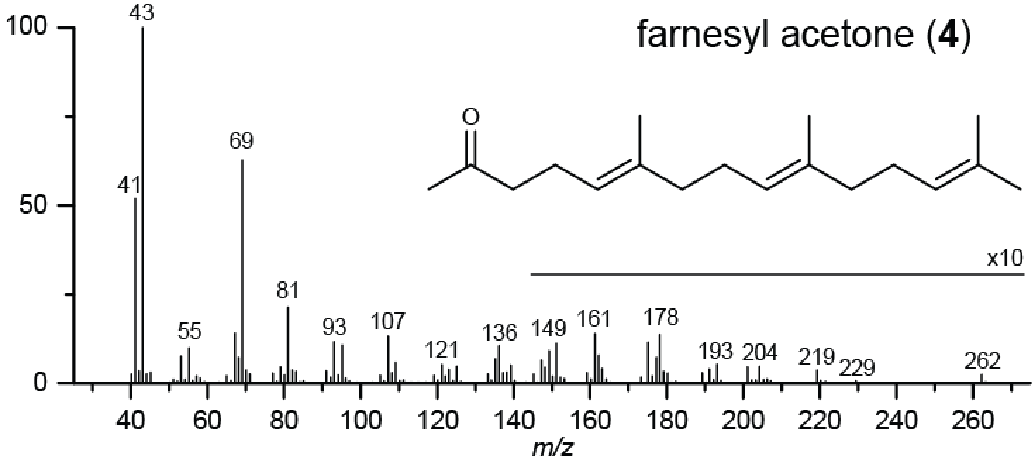

Figure S3. 400 MHz ^{1}H NMR spectrum of pre-sodorifen (**2b** in C_6D_6) from the *Serratia plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

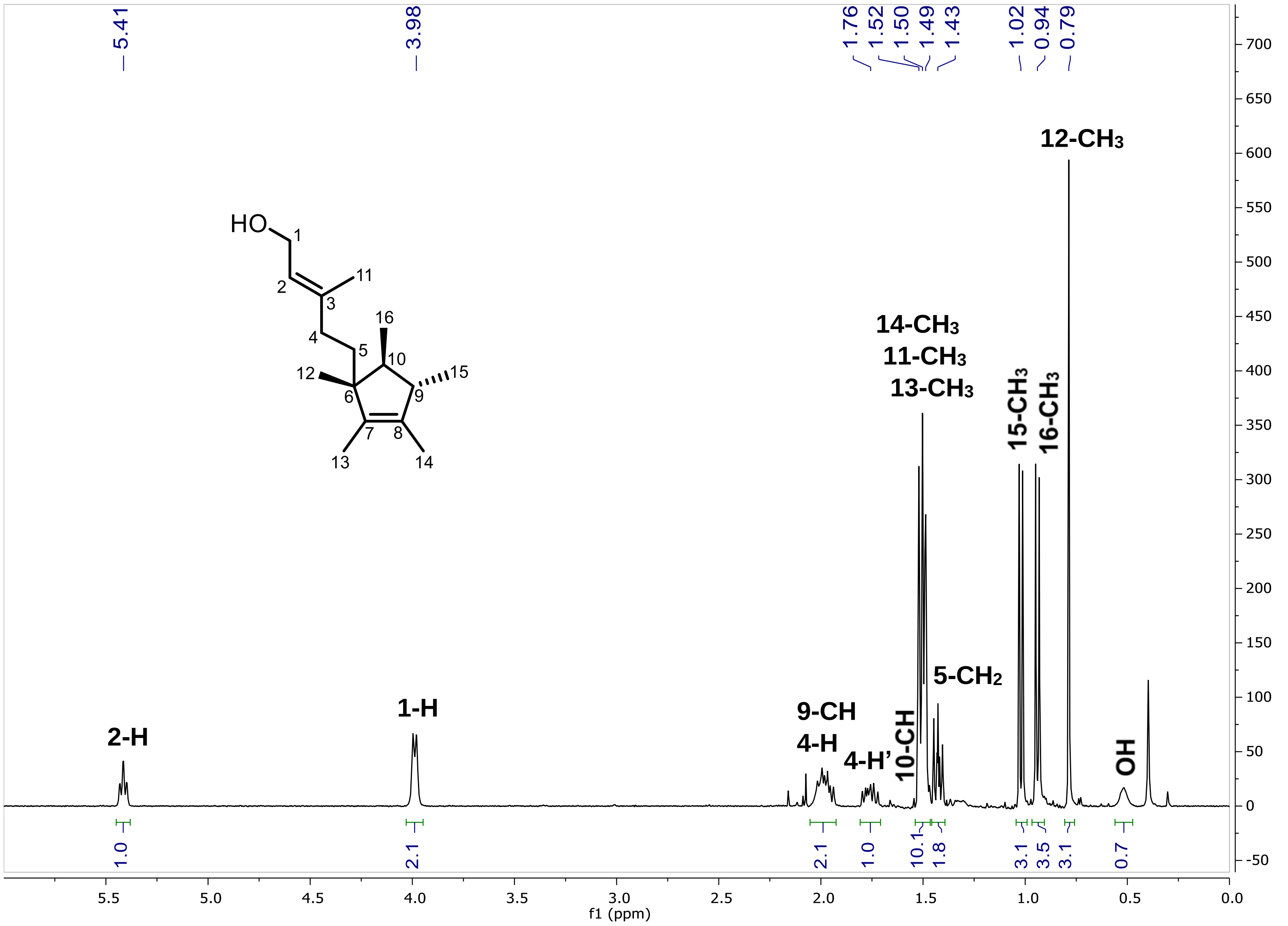

Figure S4. 100 MHz $^{13}C\ \{^{1}H\}$ NMR spectrum of pre-sodorifen (**2b** in C_6D_6) from the *Serratia plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

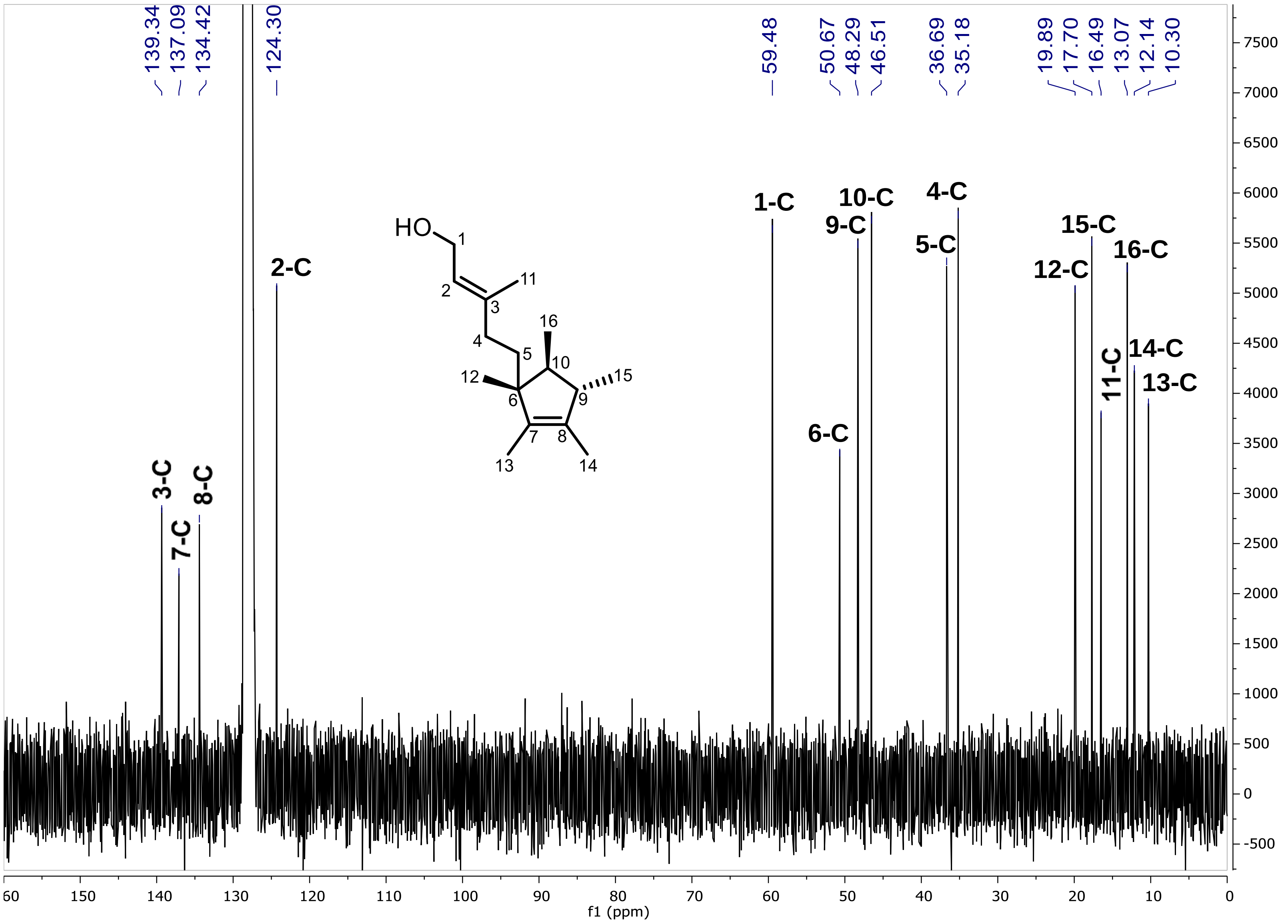

Figure S5a. 400 MHz *dqf*-COSY spectrum of pre-sodorifen (**2b** in C_6D_6) from the *Serratia plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

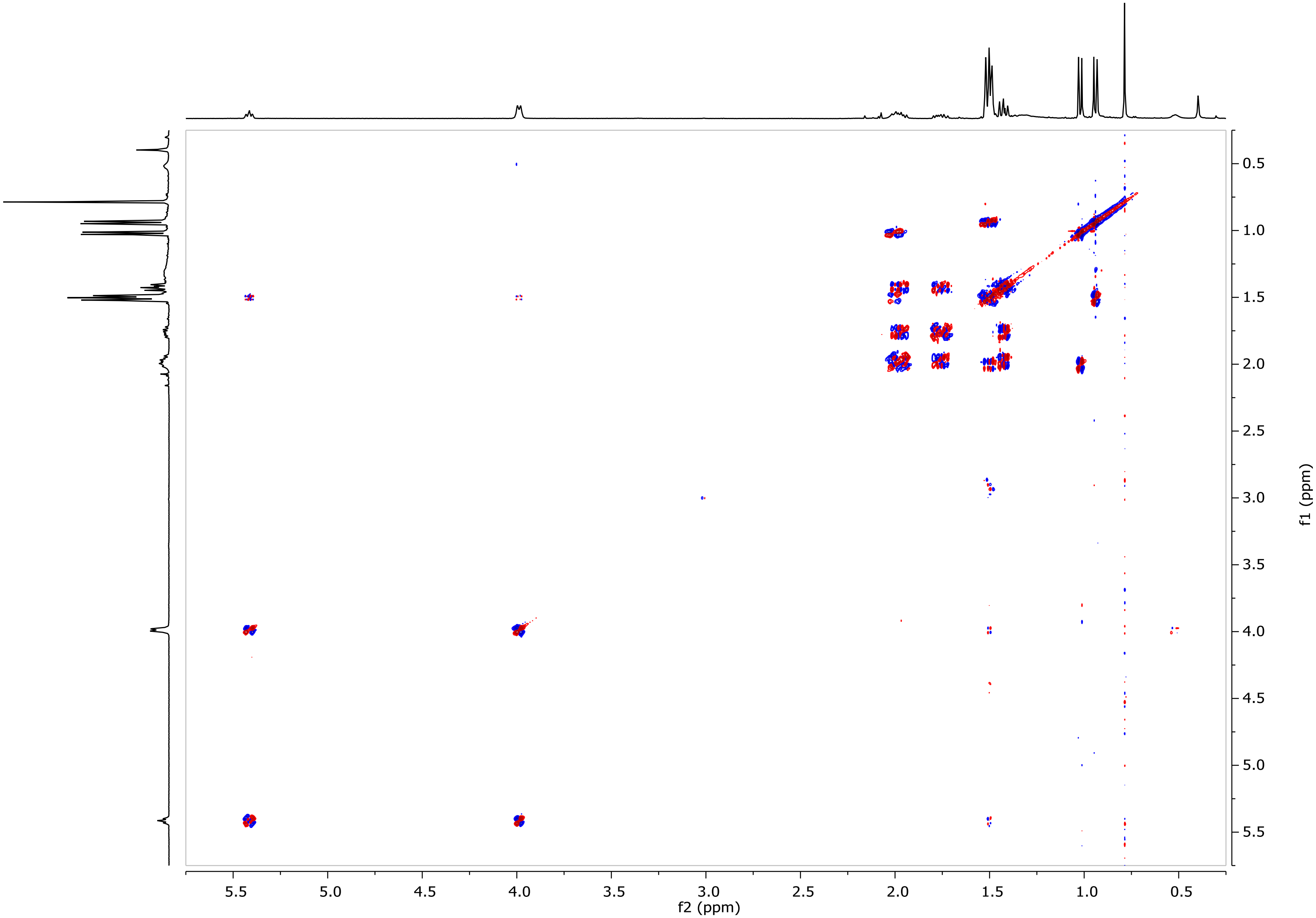

Figure S5b. Section of the 400 MHz *dqf*-COSY spectrum of pre-sodorifen (**2b** in C_6D_6) from the terpene cyclase mutant (TC::kan).

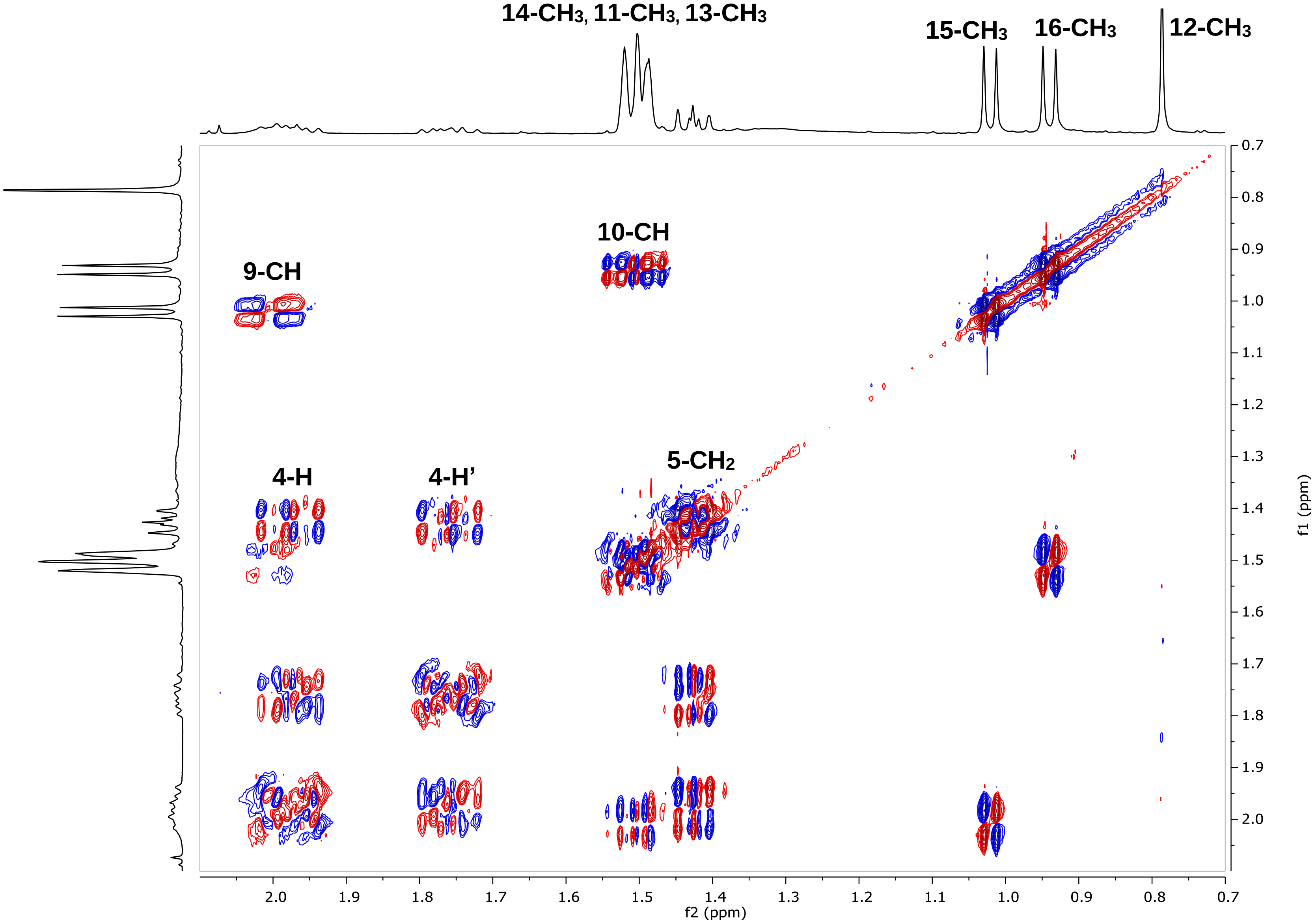

Figure S6. 400 MHz HSQC spectrum of pre-sodorifen (**2b** in C_6D_6) from the *Serratia plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

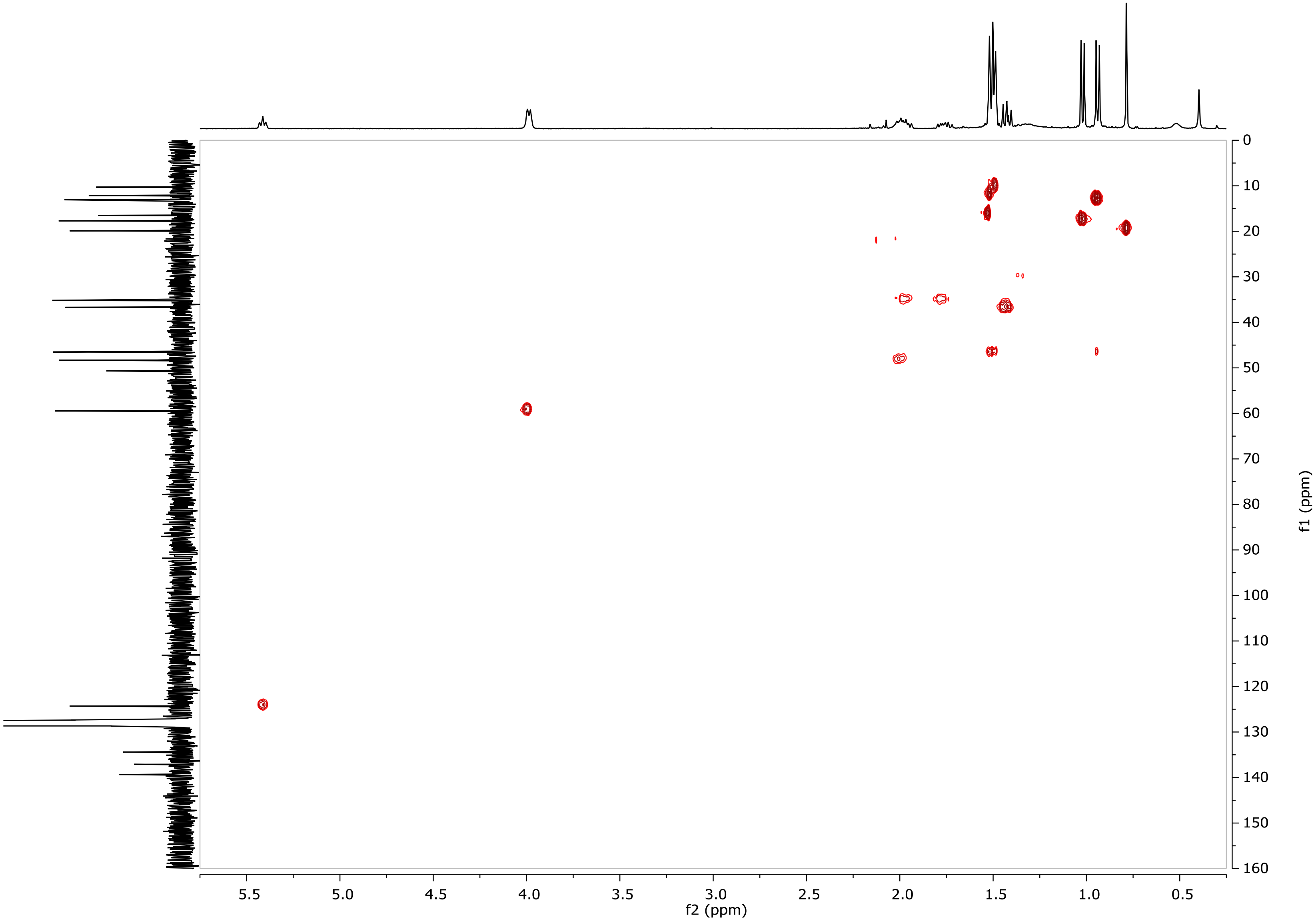

Figure S7. 400 MHz HMBC spectrum of pre-sodorifen (**2b** in C_6D_6) from the *Serratia plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

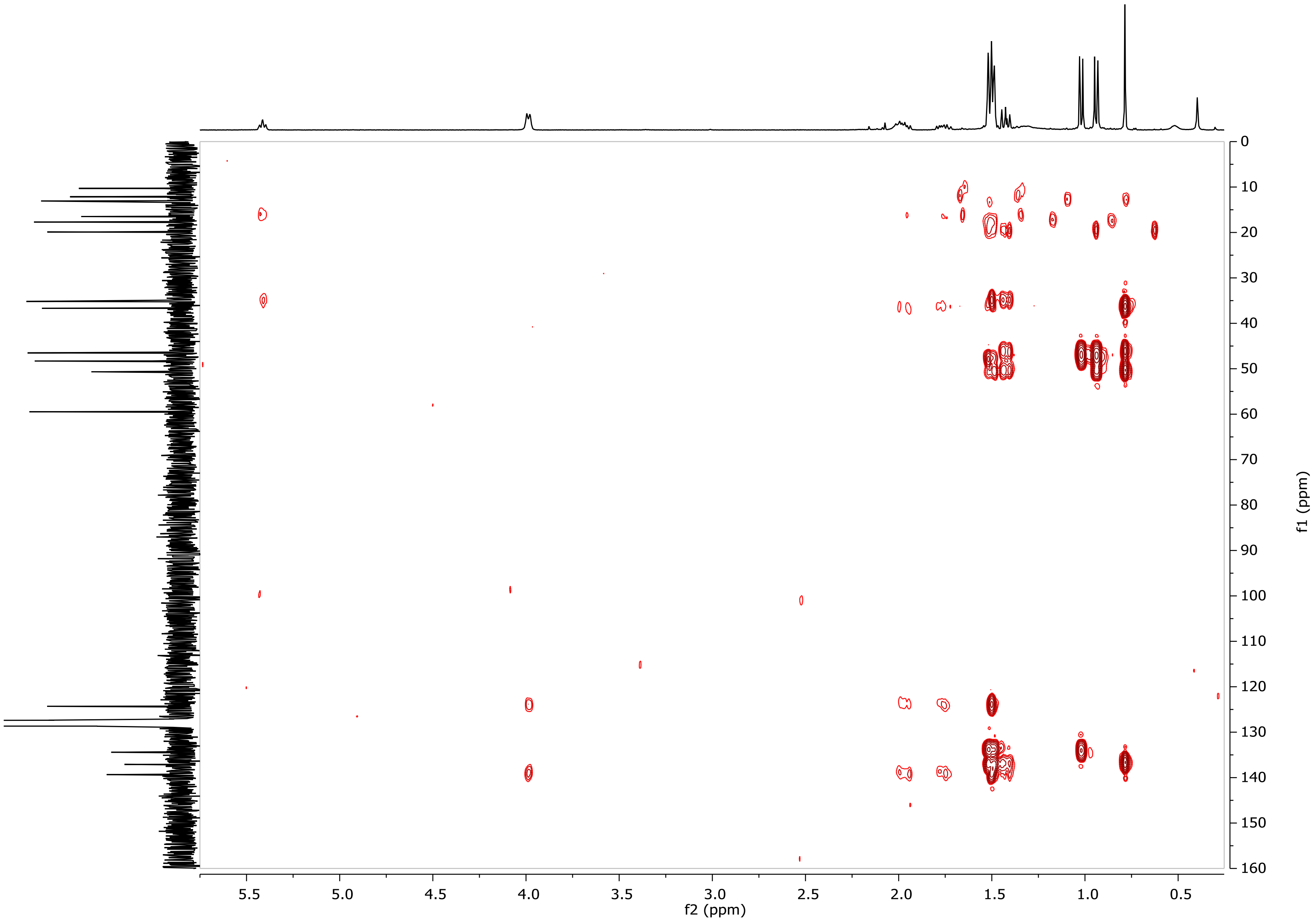

Figure S8a. 400 MHz NOESY spectrum of pre-sodorifen (**2b** in C_6D_6) from the *Serratia plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

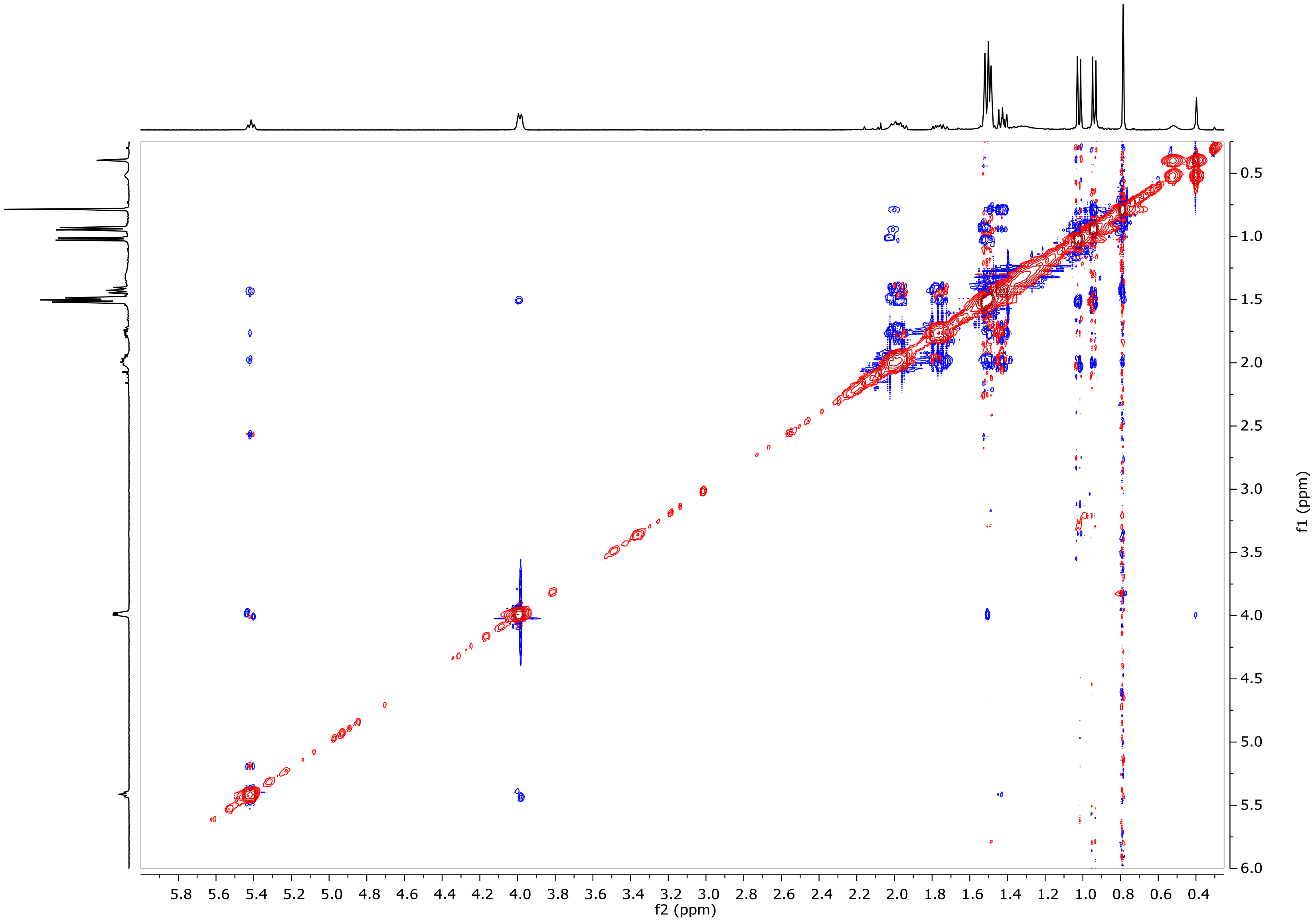

Figure S8b. Section of the 400 MHz NOESY spectrum of pre-sodorifen (**2b** in C_6D_6) showing key NOE-interactions.

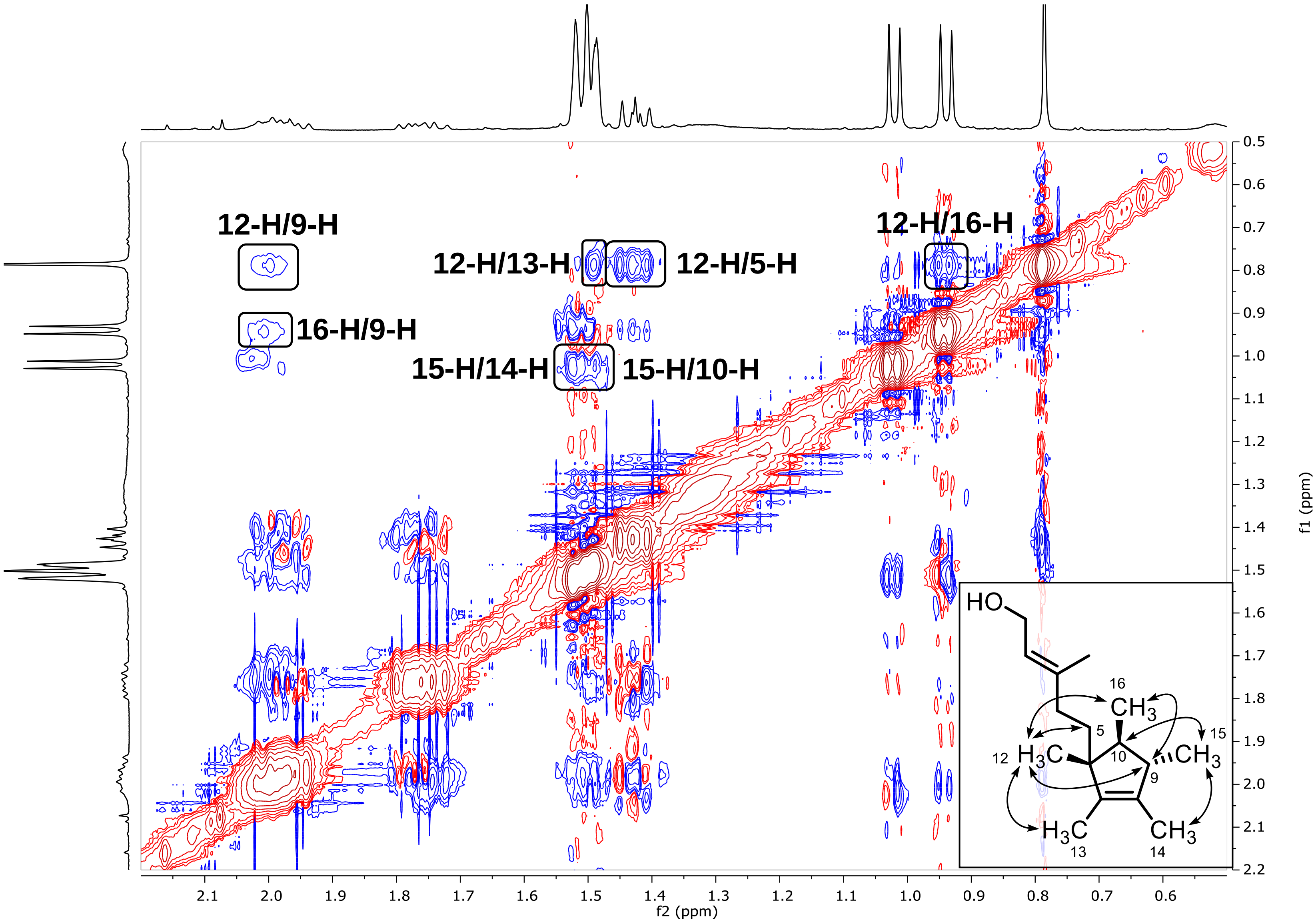

Figure S9. GC-EIMS total ion chromatograms of VOCs collected during *in vitro* enzyme assays with heterologously expressed *S. plymuthica* 4Rx13 terpene cyclase (TC) and *C*-methyltransferase (MT) (negative controls for Figure 2). (A) farnesyl pyrophosphate (FPP, **1a**) with terpene cyclase (TC); (B) FPP (**1a**) and SAM with terpene cyclase (TC); (C) FPP (**1a**) and SAM with terpene cyclase (TC) and alkaline phosphatase (AP); (D) FPP (**1a**) with *C*-methyltransferase (MT) and alkaline phosphatase (AP); (E) FPP (**1a**) and SAM with alkaline phosphatase (AP); (F) (*E*,*E*)-farnesol (**1b**) standard in nutrient agar (NA) for comparison. Farnesol (**1b**) and pre-sodorifen (**2b**) were identified based on their MS spectra (Figure S1).

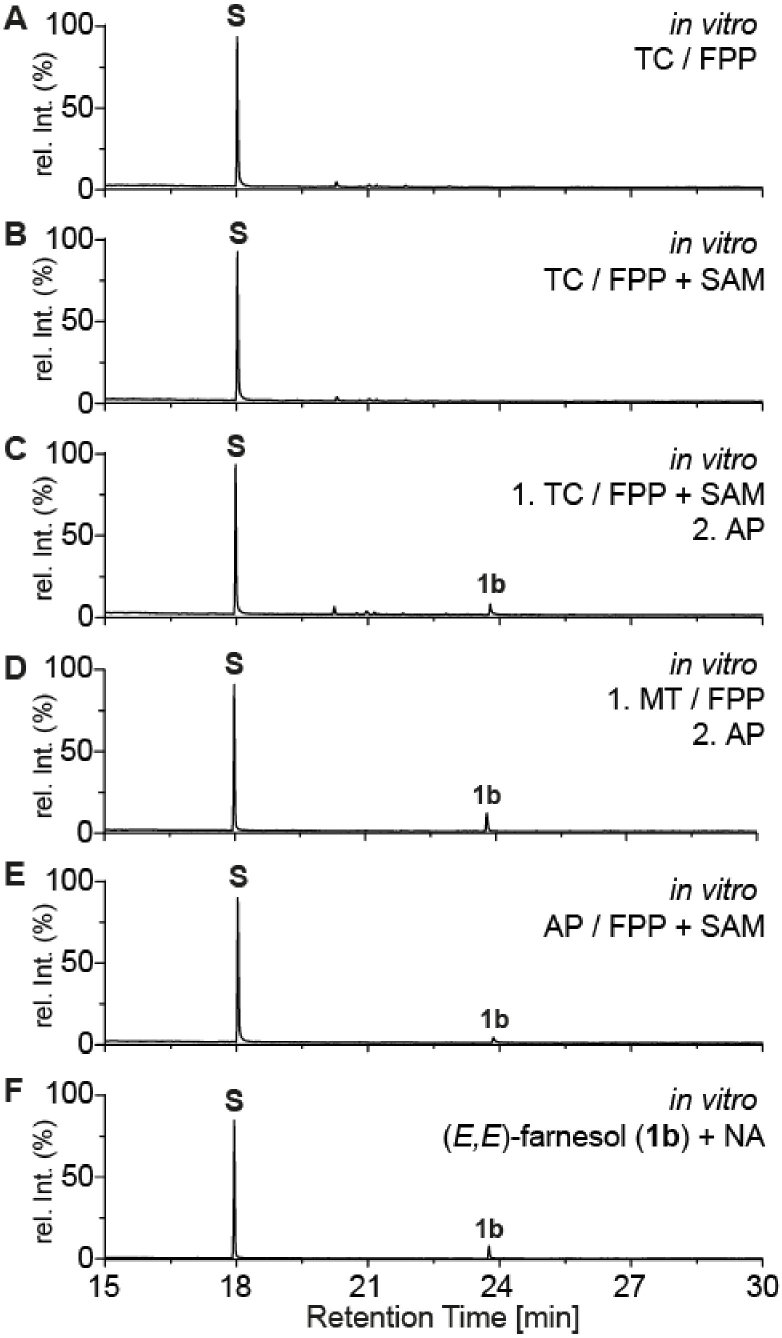

Figure S10. GC-EIMS spectra of sodorifen (**3**) from *Serratia plymuthica* 4Rx13 (A) and isotope enriched [^{13}C]-**3** from incorporation of [*S*-^{13}CH$_3$]-methionine (B). Analysis of EI-derived fragments indicates that the [^{13}C]-label is localized in the [C_6H_{11}]$^+$ fragment outside of the pentamethylcyclopentenyl moiety (C).

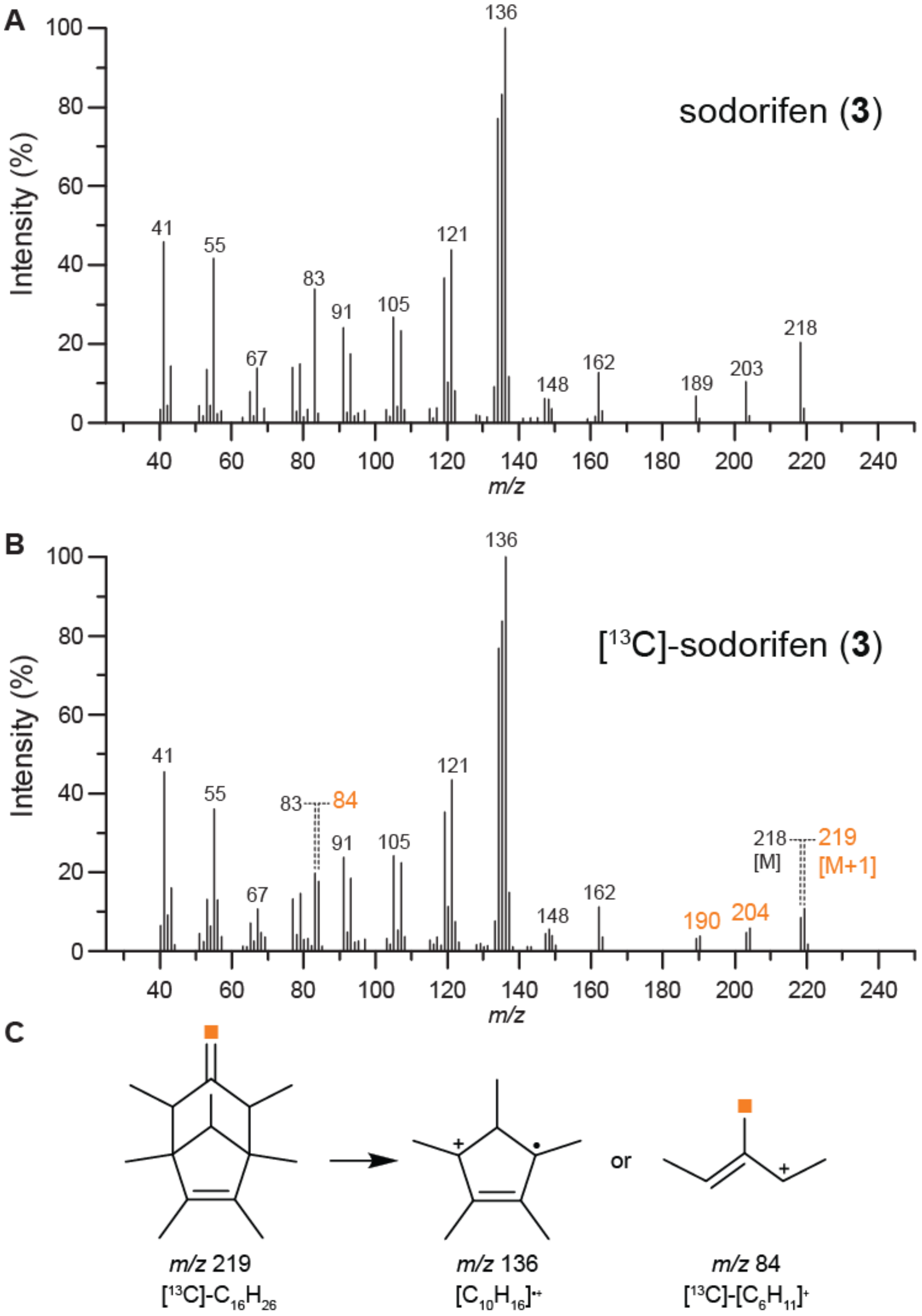

Figure S11. Relative sodorifen emission (%) of wild type *S. plymuthica* 4Rx13 grown on minimal medium supplemented with different carbon sources. Sodorifen emission was measured in the headspace of the cultures after 24 h, 48 h and 72 h using SPME. (A) amino acid combinations using 20 mM each; (B) intermediates of the citric acid cycle at 55 mM. Standard error bar with*: standard deviation, n = 3; standard error bar without *: difference to arithmetic average, n = 2; all others n = 1.

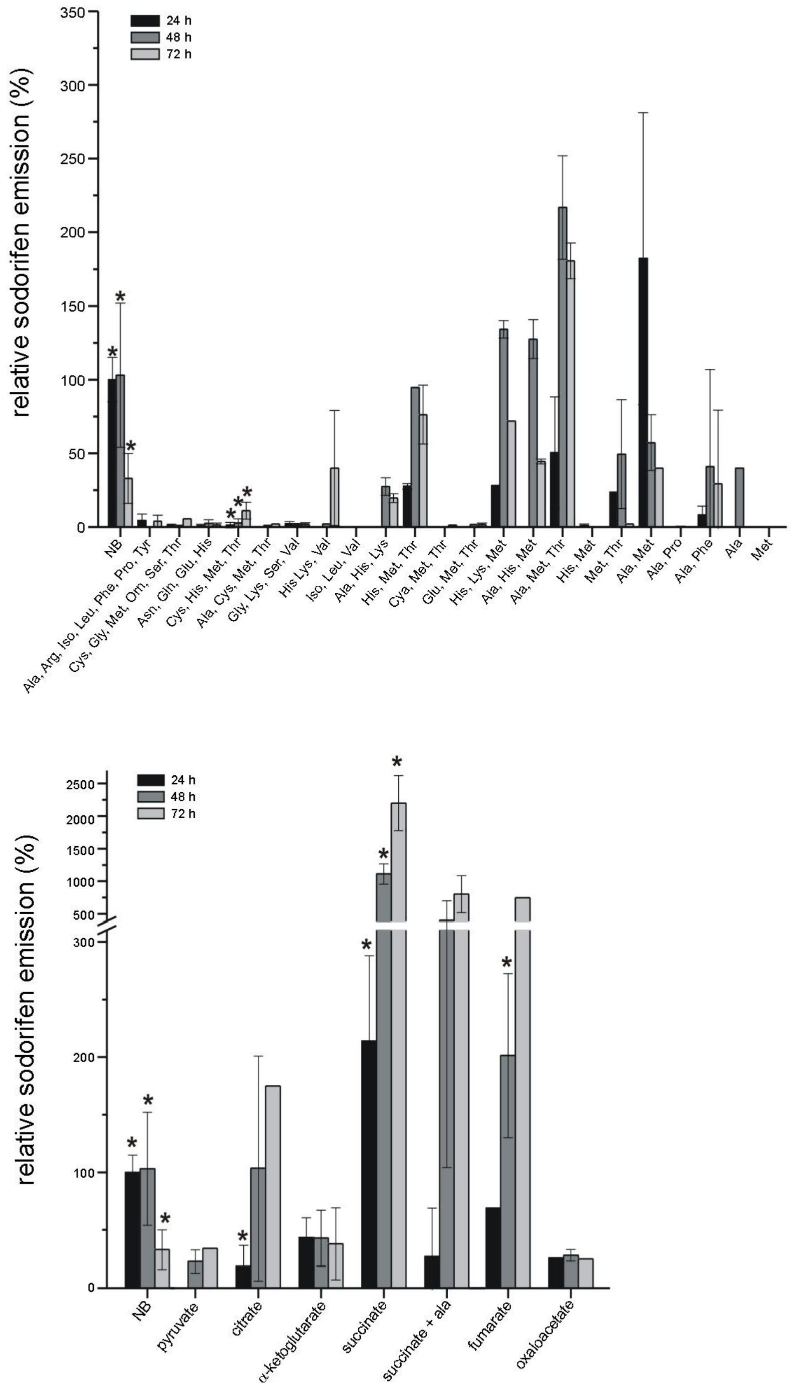

Figure S12. Partial ^{1}H NMR spectrum of pre-sodorifen (**2b**) from incorporation of 100% L-[*S*-$^{13}CH_3$]-methionine in a background of natural abundance L-alanine and L-threonine at 48 h shows 75% ^{13}C enrichment for the olefinic methyl group at position 14.

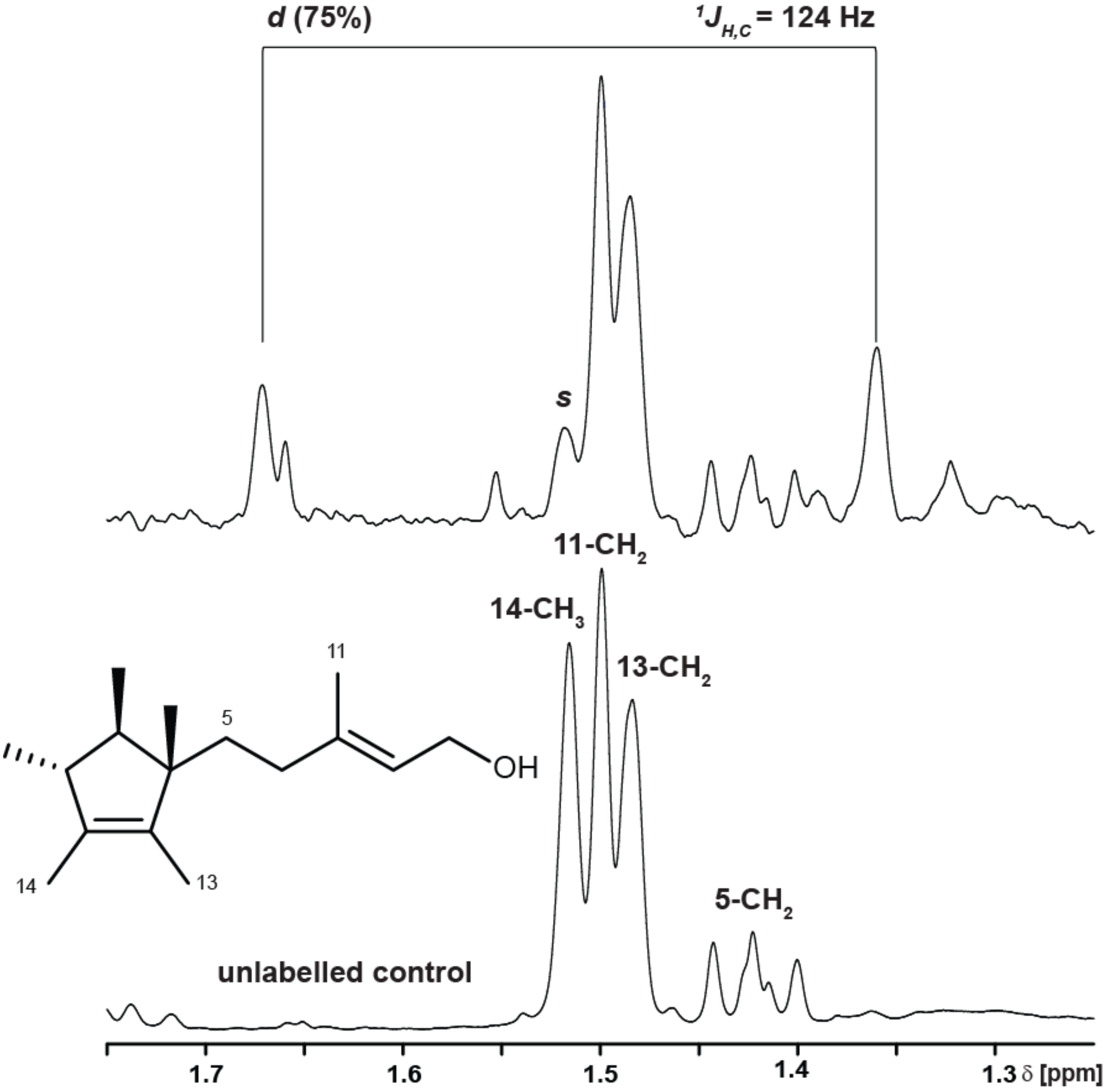

Figure S13. Partial ^{1}H NMR spectrum of sodorifen (**3**) from incorporation of 100% L-[*S*-$^{13}CH_3$]-methionine in a background of natural abundance L-alanine and L-threonine at 48 h shows 79% ^{13}C enrichment for the exocylic methylene group at position 11.

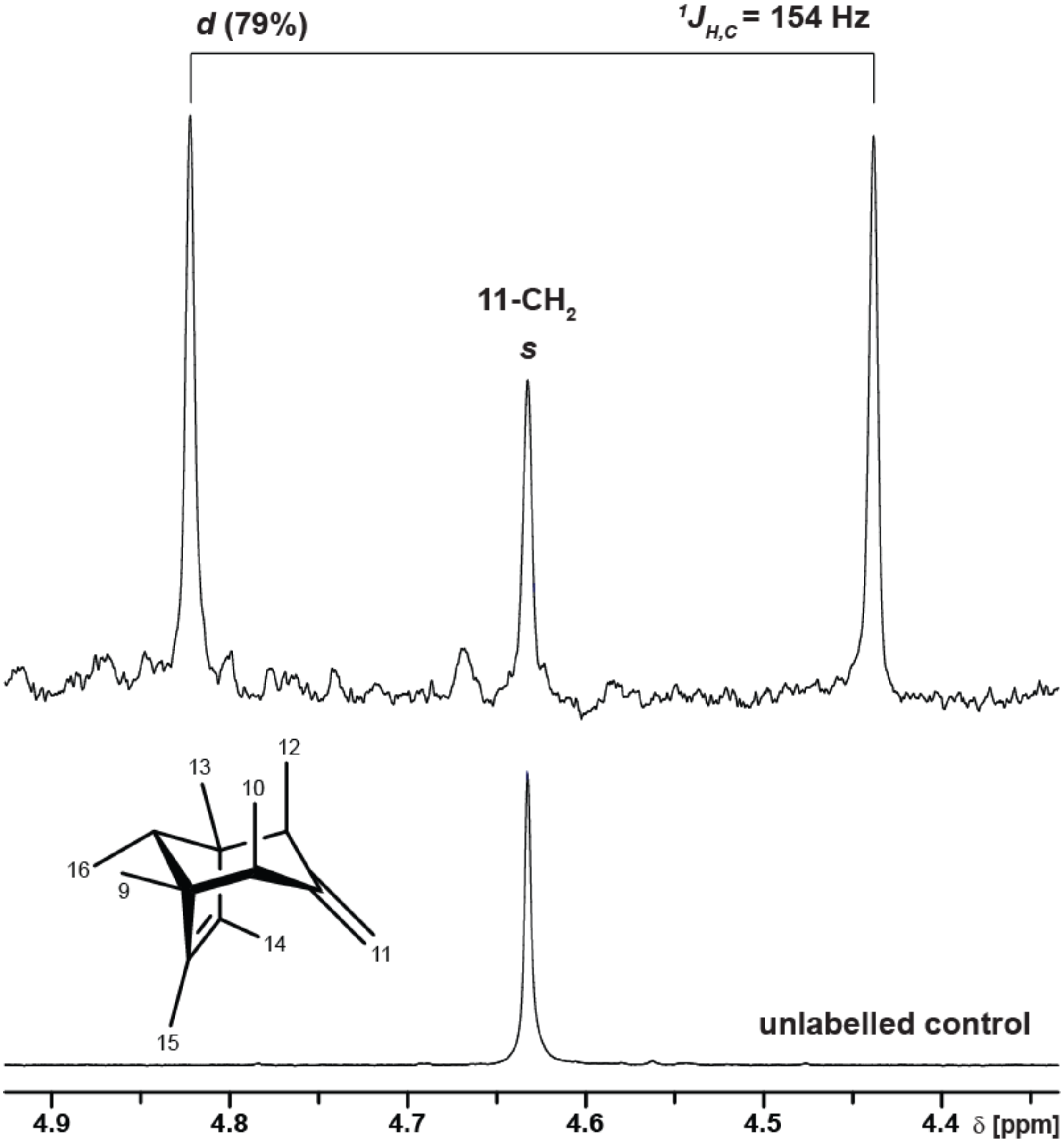

Figure S14. Partial ^{1}H NMR spectrum of sodorifen (**3**) from incorporation of 20% L-[3-^{13}C]-alanine in a background of natural abundance succinate at 48 h shows the ^{13}C enrichment of both anisochoric methyl groups at positions 9 and 13 (61% ^{13}C) and one methyl group at position 14 or 15 (31% ^{13}C).

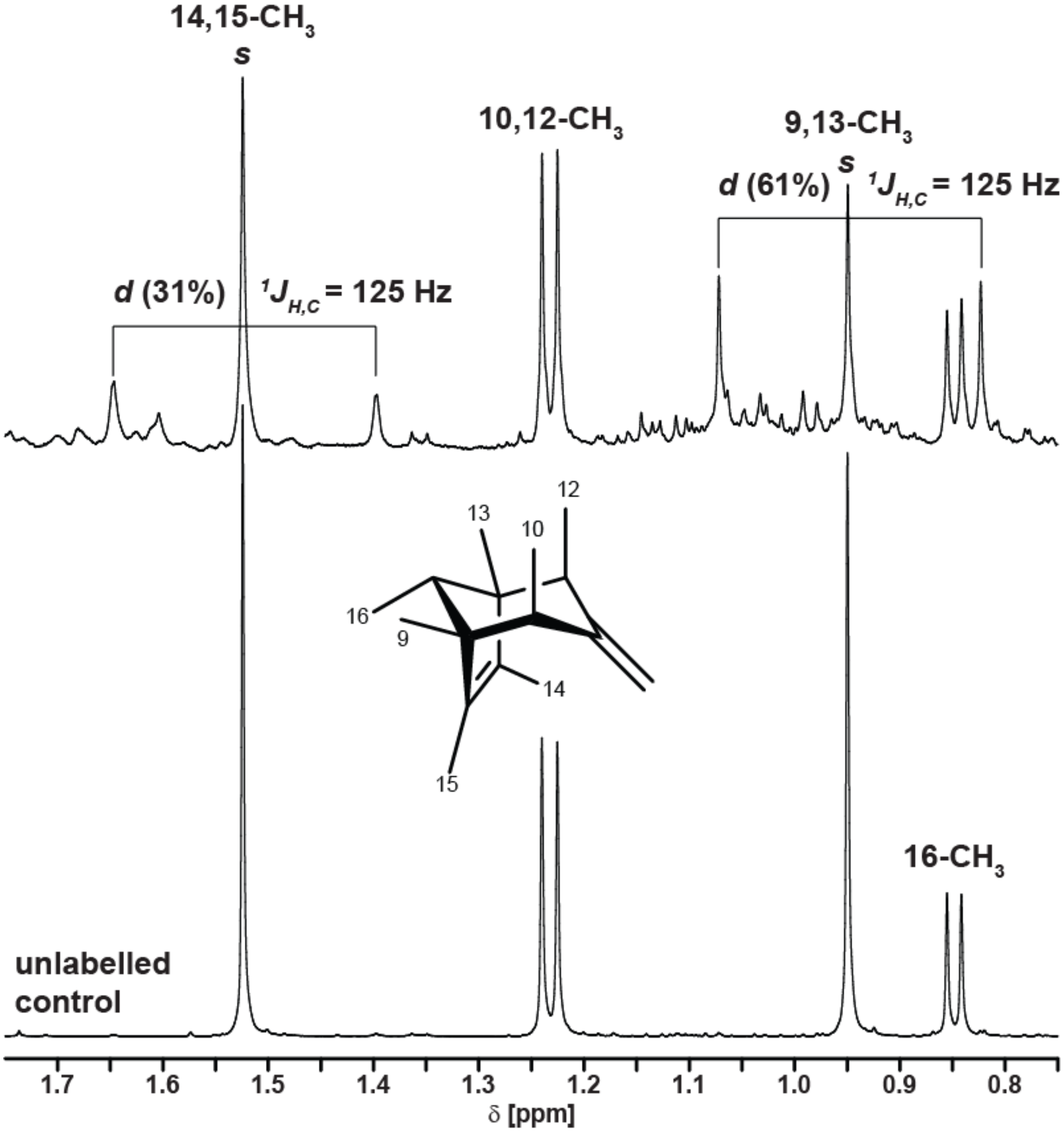

Figure S15. Alternative pathway for the *C*-methyltransferase catalyzed biosynthesis of pre-sodorifen pyrophosphate (**2a**) from FPP (**1a**) based on a key 1,3-H-shift.

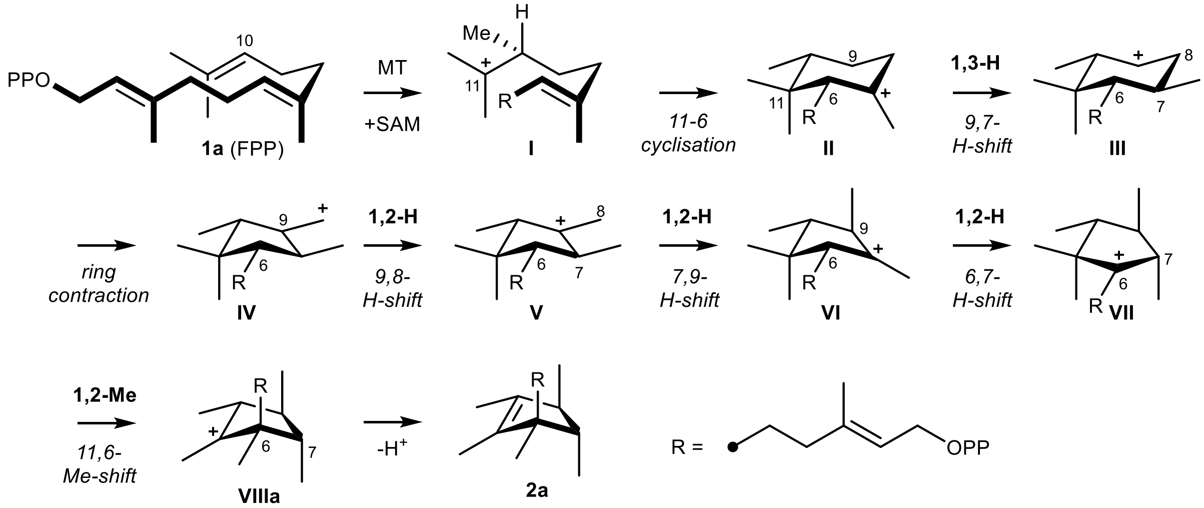

Figure S16. Alternative pathway for the *C*-methyltransferase catalyzed biosynthesis of pre-sodorifen pyrophosphate (**2a**) from FPP (**1a**) based on a series of multiple 1,2-H and 1,2-Me-shifts.

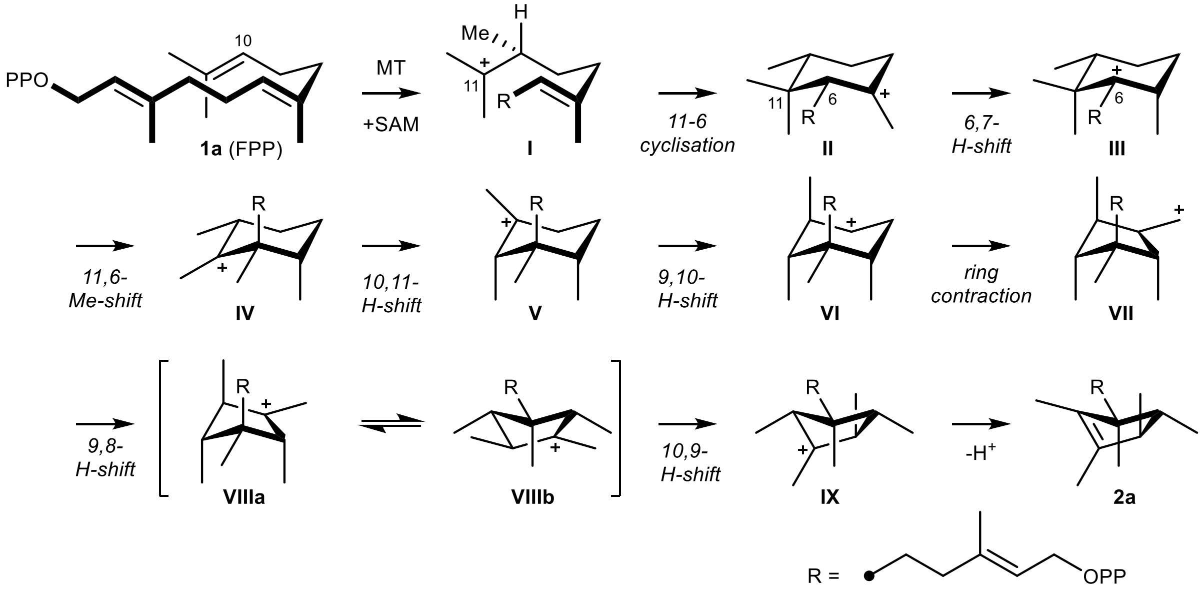

Table S1: NMR data (400 MHz, C_6D_6) of pre-sodorifen (**2b**) from the *S. plymuthica* 4Rx13 terpene cyclase mutant (TC::kan).

Position	δ_C	δ_H, mult (J, Hz)	HMBC	NOESY
1	59.5	3.99 *d* (6.5)	C-2, C-3	H-11
2	124.3	5.41 *t* (6.6)	C-4	H-4a, H-4b, H-5
3	139.3	-	-	-
4a	35.1	1.76 *m*	C-2, C-3, C5	H-2
4b		1.94 *m*	-	H-2
5	36.7	1.42 *m*	C-4, C-6, C-10	H-2, H-12
6	50.7	-	-	-
7	137.1	-	-	-
8	134.4	-	-	-
9	48.3	2.01 *m*	-	H-12, H-16
10	46.5	1.51 *m*	-	H-15
11	16.5	1.50 *s*	C-2, C-3, C-4	H-1
12	19.9	0.79 *s*	C-5, C-6, C-7, C-10	H-5, H-9, H-13, H-16
13	10.3	1.49 *s*	C-8, C-6	H-12
14	12.1	1.52 *s*	C-7, C-8, C-9	H-15
15	17.7	1.02 *d* (6.8)	C-8, C-9, C-10	H-10, H-14
16	13.1	0.94 *d* (7.0)	C-6, C-9, C-10	H-9, H-12

Using C_6D_5H as int. standard at ^{13}C 128.06 and ^{1}H 7.16 ppm. ^{1}H NMR shifts from ^{1}H NMR and *dqf*-COSY, ^{13}C NMR shifts from ^{13}C {^{1}H} NMR with assignments from HSQC and HMBC experiments

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