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Supporting Information

Engineering of a Plant Isoprenyl Diphosphate Synthase for Development of Irregular Coupling Activity

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Supporting Information

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S1 Materials and Methods

S1.1 Chemicals

Isopentenyl, dimethylallyl, geranyl and neryl diphosphates were purchased as tri-ammonium salts from Echelon Biosciences, USA. The compounds were dissolved in the mixture of methanol: water 7:3 at the concentrations of 1, 5 or 10 mg/mL and stored at -20 °C.

Geraniol was obtained from Sigma-Aldrich, USA. Lavandulol and planococcol were synthesized according to the methods described in ^[1] and ^[2], respectively; the spectroscopical properties of the samples were fully coincident with those reported in the literature. For synthesis of maconelliol, in a rounded bottom flask equipped with a reflux condenser, sulphuric acid (55 mg) was added over a suspension of anhydrous magnesium sulphate (1.0 g) in anhydrous tetrahydrofuran (30 ml) at room temperature. After stirring for 30 min, 650 mg of racemic planococcol were added via syringe, and the suspension was refluxed under continuous stirring. The reaction was monitored via GC-MS. After 40 h of continuous stirring, the conversion of planococcol to maconelliol was estimated as 85 % (*via* peak integration). The suspension was cooled down to room temperature and filtered. The solution was successively washed with saturated sodium bicarbonate (2 x 10 mL), brine (2 x 10 mL), and dried with anhydrous sodium sulphate. The residue after rotary evaporation was carefully chromatographed (silica gel; hexane:Et₂O 98:2 mixture). Pure fractions were collected and concentrated to get 110 mg of maconelliol, whose spectroscopical properties were fully coincident with those described in the literature.^[3] The stock solutions of terpene alcohols were prepared in methanol at the concentration of 1 mg/mL and kept at -20 °C.

Lavandulyl diphosphate was produced from DMAPP using lavandulyl diphosphate synthase as described in S1.3.

S1.2 Enzyme expression and purification

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The sequence encoding neryl diphosphate synthase (NCBI Acc. No. NM_001247704.1) without chloroplast targeting peptide was amplified from the cDNA of tomato leaves (variety Dulcita) using primers Fw 5'-AAGACCATGGGCTCACTCAACTTAC-3' and Rv 5'-GCATGGATCCTCAATATGTGTGTCC-3', and cloned into pMAL-c5X expression vector (New England Biolabs) using NcoI/BamHI sites. For site directed mutagenesis, the Q5-SDM kit (New England Biolabs) was applied. The N88H mutation was introduced using primers Fw 5'-GCAAAGGATAAGGGTTTAGAAGTATATG-3' and Rv 5'-CCATCTCCTATGACCATCCATTATC-3'. The proteins were expressed in BL21 *E. coli* cells. The expression was induced by 0.5 mM IPTG and carried out at 30 °C overnight. The proteins were purified on the amylose resin (New England Biolabs) and stored at -20 °C in 25 % glycerol. The protein concentration was determined by the method of Bradford.

S1.3 Enzyme activity assays

The activity assays were carried out in a total volume of 100 μ L in 35 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂ and 5 mM β -mercaptoethanol at 30 °C during 1 h. Regular coupling activity was determined using IPP and DMAPP as substrates; the assays for irregular activity contained only DMAPP. To achieve different concentrations of substrates in the assays, the concentrations of stock solutions in methanol: water 7:3 were adjusted so as to add equal volume of the solvent to each assay. To determine enzyme kinetic parameters of the regular coupling reaction, the concentration of one substrate was held constant (1000 μ M), and the second substrate was added at the concentrations of 50, 100, 200, 300, 400, 600, 800, and 1000 μ M; the assay contained 10 μ g of protein. Enzyme kinetic measurements of the irregular coupling reaction with the wild type *S/NPPS* were carried out using 100 μ g of protein at DMAPP concentrations of 50, 100, 200, 300, 400, 600, 800, and 1000 μ M. For calculation of kinetic parameters of N88H mutant, 10 μ g of protein were incubated with 200, 300, 400, 600, 800, 1000, 2000, 3000, and 4000 μ M DMAPP. The reaction was stopped by adding 100 μ L of

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chloroform, and the proteins were precipitated by vigorous shaking followed by centrifugation (10 min at 16 000 g). The water phase was used for LC-MS analysis (injection volume 5 μ L). The quantity of formed products was calculated based on peak areas using a calibration line build for GPP.

S1.4 Preparative synthesis of monoterpenes

To prepare samples for NMR analysis, 60 mg of protein were incubated in 35 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$ and 5 mM β -mercaptoethanol with 1 mM DMAPP in a total volume of 9.6 mL at 30 °C overnight. The pH of the reaction mixture was adjusted by adding 1.92 mL of 0.5 M glycine-NaOH, pH 10.5, containing 5 mM $ZnSO_4$, and the products were dephosphorylated by 1200 units of calf intestinal alkaline phosphatase (Serva) during 1 h. The terpene alcohols were extracted three times in equal volumes of tert-butylmethylether. The volume of the extract was reduced under the air flow, and the mixture was separated by TLC on aluminium silica gel 60 sheets with 0.2 mm layer (ALUGRAM® Xtra SIL G/UV254, Macherey-Nagel, Germany). The solvent system consisted of hexane:acetone 4:1. For detection of terpenes, the edges of the plates were cut off, dipped into vanillin reagent (1.4 g vanillin in 40 mL methanol with 250 μ L H_2SO_4) and developed at 130 °C. The corresponding zones of silica gel were scraped from the plates, and the terpene alcohols were eluted with $CDCl_3$ (AppliChem, Germany).

S1.5 Analytical assays

LC-MS analysis was performed on 1260 Infinity HPLC system coupled to G6120B quadrupole mass spectrometry detector (Agilent). The separation was carried out on Poroshell 120 EC-C18 (3.0 x 50 mm, 2.7 μ m) column (Agilent) using the method described in ^[4]. The detection of C10 prenyl diphosphates was performed in positive single ion mode, m/z 313.

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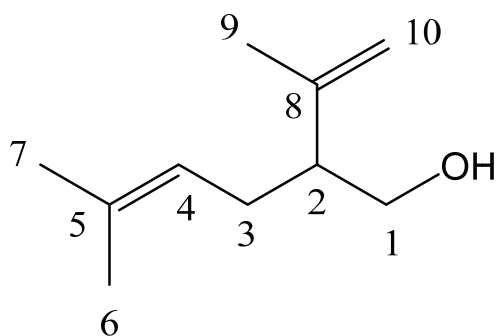
^1H and ^{13}C NMR, ^{13}C -DEPT, CLIP-COSY, NOESY, ^{13}C -HSQC and ^{13}C -HMBC spectra of lavandulol and *trans*-planococcol were recorded in CDCl_3 using the AVANCE III HD instrument (Bruker, USA) equipped with the QCI CryoProbe at 700 MHz (^1H) and 176 MHz (^{13}C).

S1.6 Modelling of 3-D protein structure

The 3-D model of *S/NPPS* without the predicted chloroplast targeting peptide (amino acids 1-53) was built on the SWISS-MODEL homology-modelling server^[5] using the crystal structure of *LiLPPS//DMASPP/isoprene/PPi/Mg²⁺* complex as a template.

S2 Tables

Table S2.1 ^1H and ^{13}C NMR data of lavandulol in CDCl_3 . Values are in ppm. The multiplicities and coupling constants (J in Hz) are in parentheses. ^1H and ^{13}C NMR signals are in agreement with the literature data of lavandulol^[6].



Position	^1H NMR	^{13}C NMR
1	3.48-3.59 (m, 2H)	63.80
2	2.28 (m, 1H)	50.12
3	2.02-2.12 (m, 2H)	28.54
4	5.1 (t, 1H, $J = 6.8$ Hz)	122.16
5	-	132.96
6	1.69 (s, 3H)	25.90
7	1.61 (s, 3H)	17.99
8	-	145.56
9	1.70 (s, 3H)	19.67
10	a) 4.82 (s, 1H); b) 4.93 (s, 1H)	113.31

S3 Figures

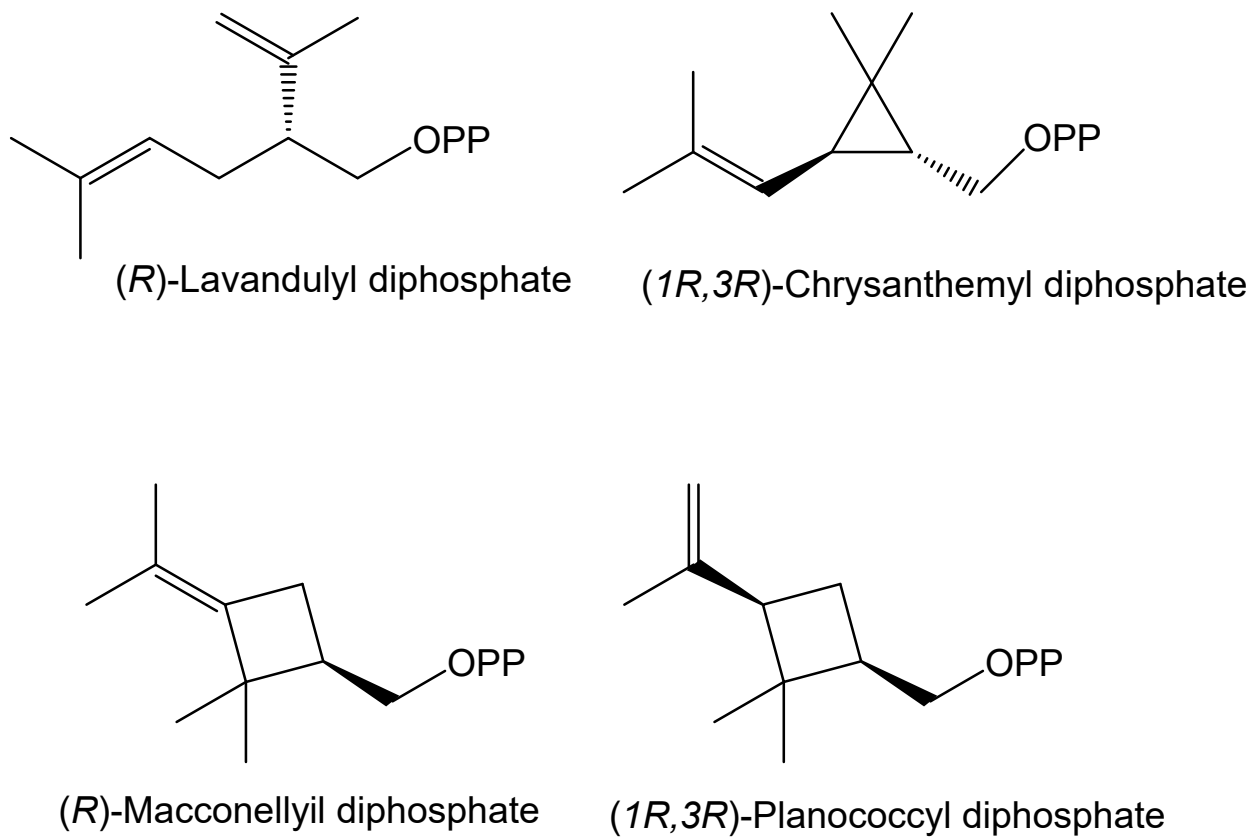


Figure S3.1 Examples of irregular monoterpene diphosphates found in nature.

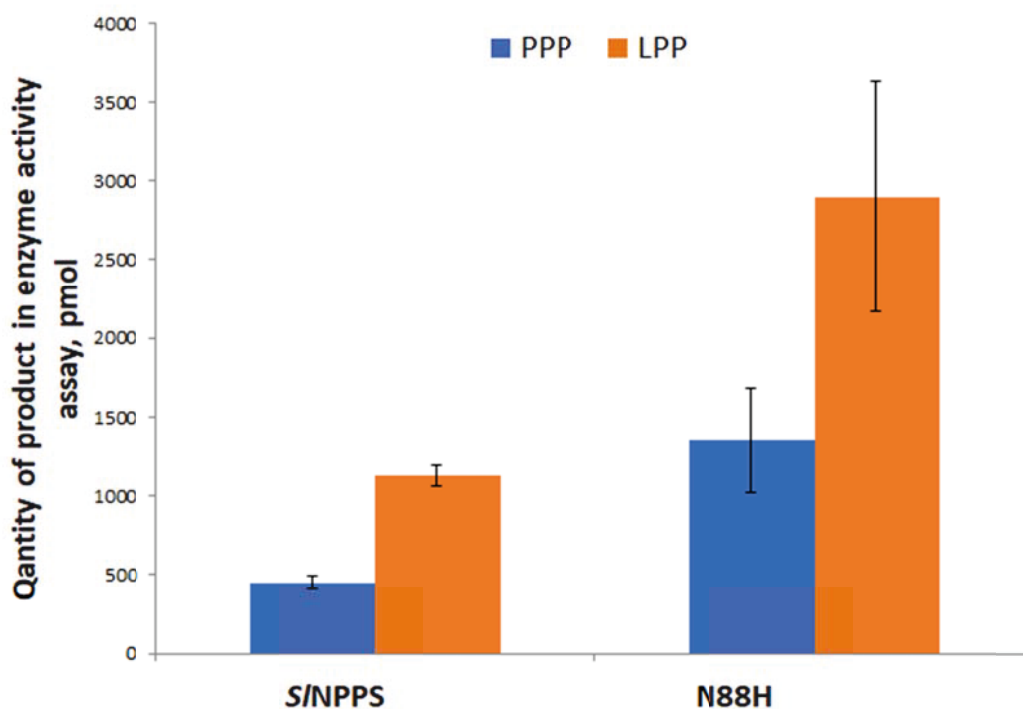


Figure S3.2 Ratio of irregular monoterpene diphosphates produced in the enzyme activity assays containing DMAPP as the only substrate with the wild-type *S/NPPS* and N88H mutant; PPP – planococcyll diphosphate, LPP – lavandulyl diphosphate. The quantity of products was calculated based on LC-MS peak areas using calibration line build for geranyl diphosphate. The bars represent the standard deviation for three and five measurements with the wild-type *S/NPPS* and N88H mutant, respectively.

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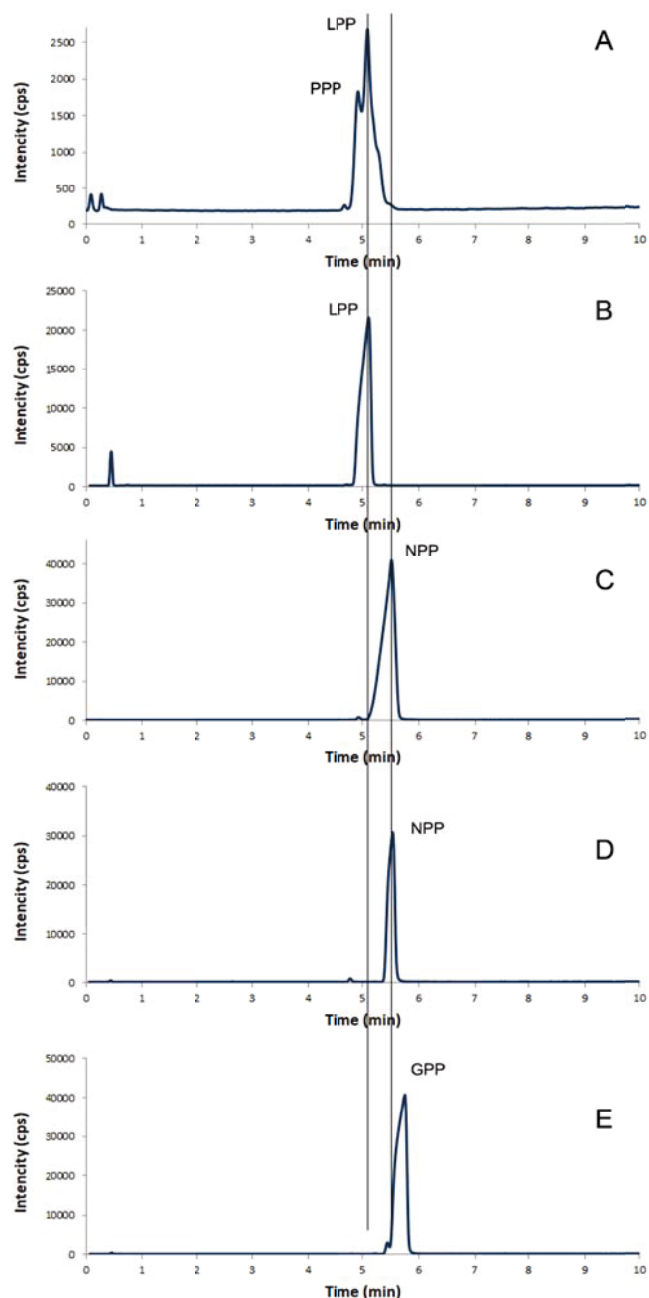


Figure S3.3 HPLC-MS analysis of monoterpene diphosphates produced by *S/NPPS*. GPP – geranyl diphosphate, NPP – neryl diphosphate, LPP – lavandulyl diphosphate, PPP – planococcyll diphosphate. A: Activity assay of *S/NPPS* with DMAPP as a substrate; B: activity assay of *L/LPPS* with DMAPP as a substrate; C: activity assay of *S/NPPS* with IPP and DMAPP as substrates; D: neryl diphosphate standard; E: geranyl diphosphate standard.

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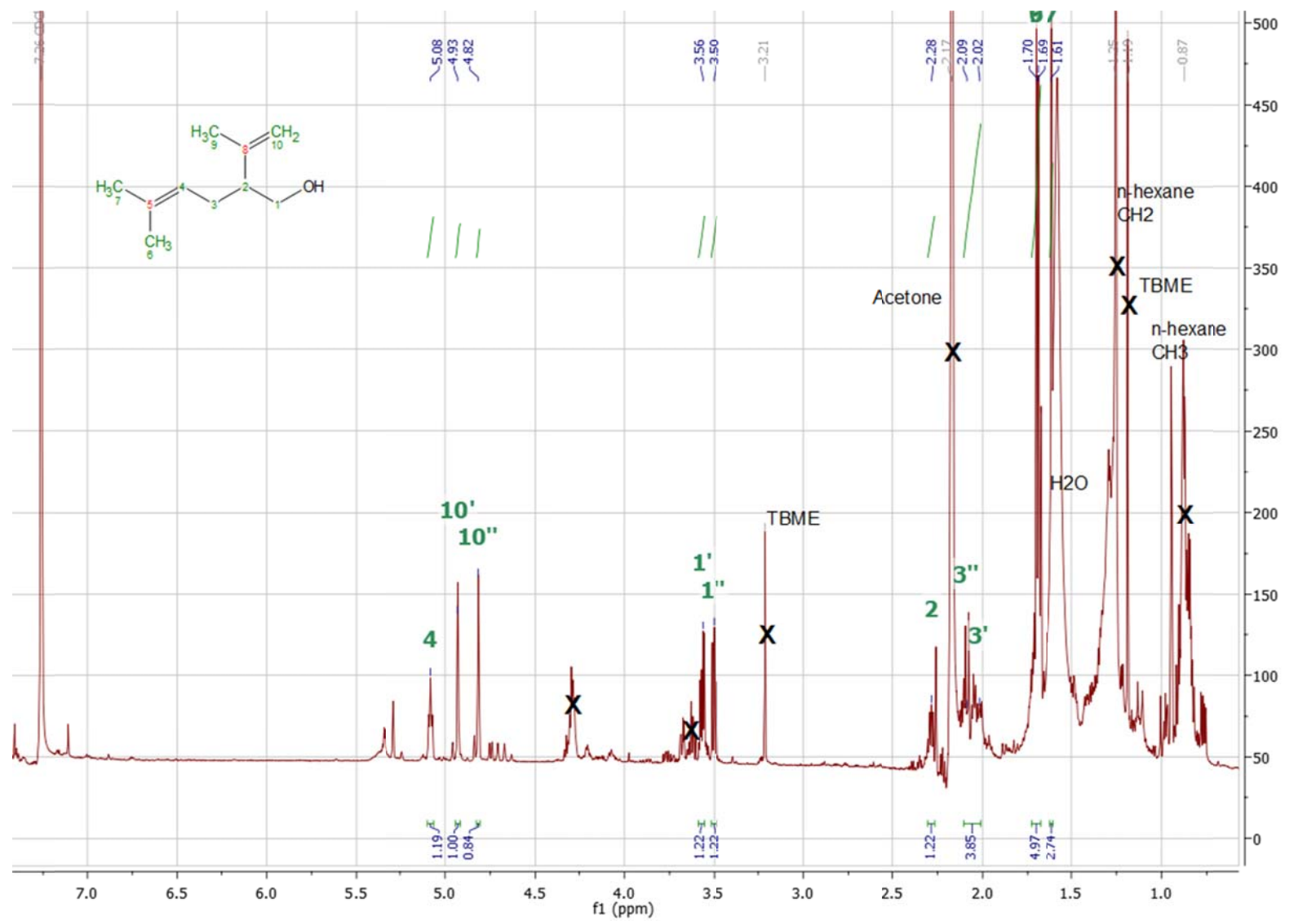


Figure S3.4 ¹H NMR spectrum of lavandulol in CDCl₃.

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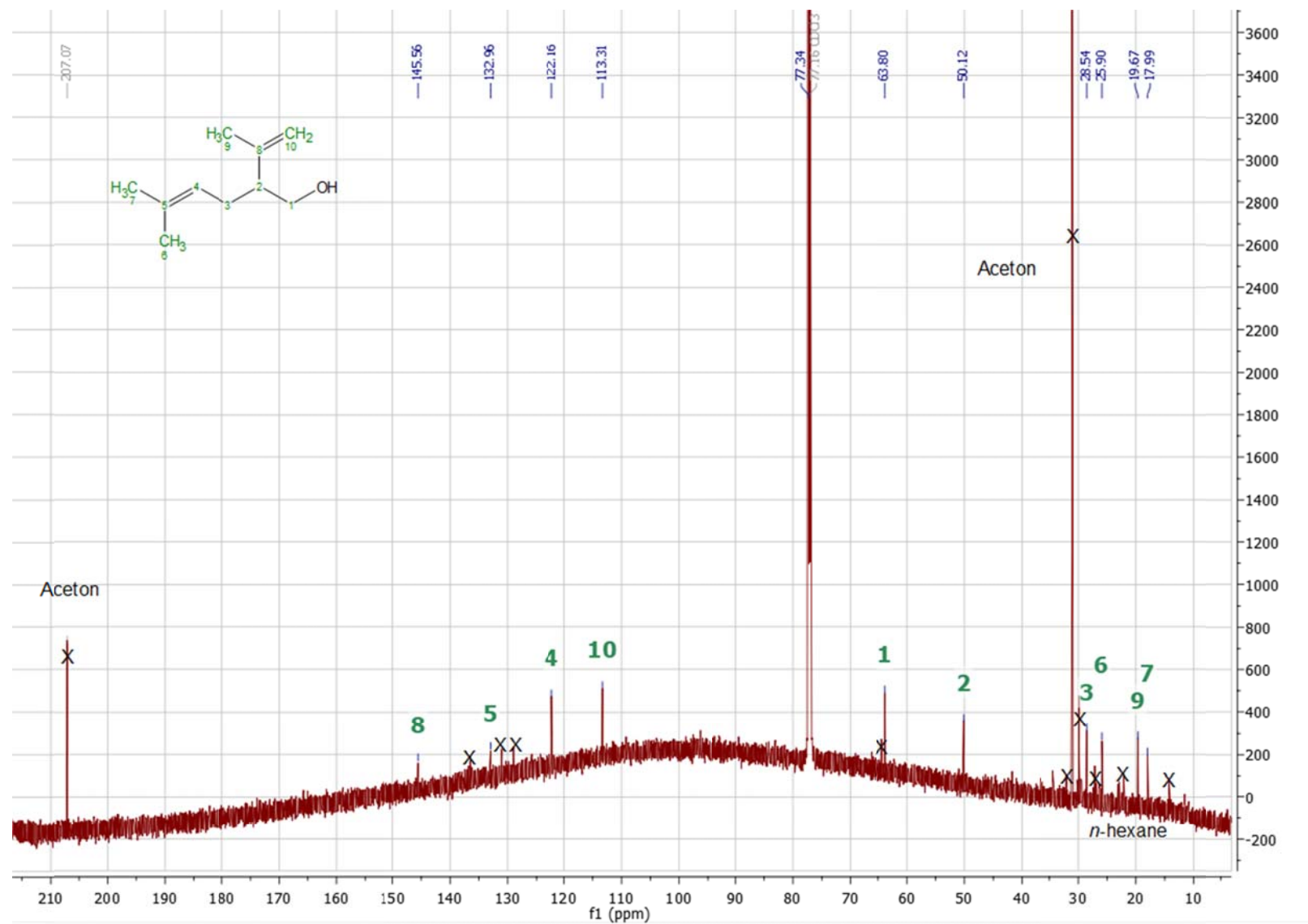


Figure S3.5 ^{13}C NMR spectrum of lavandulol in CDCl_3 .

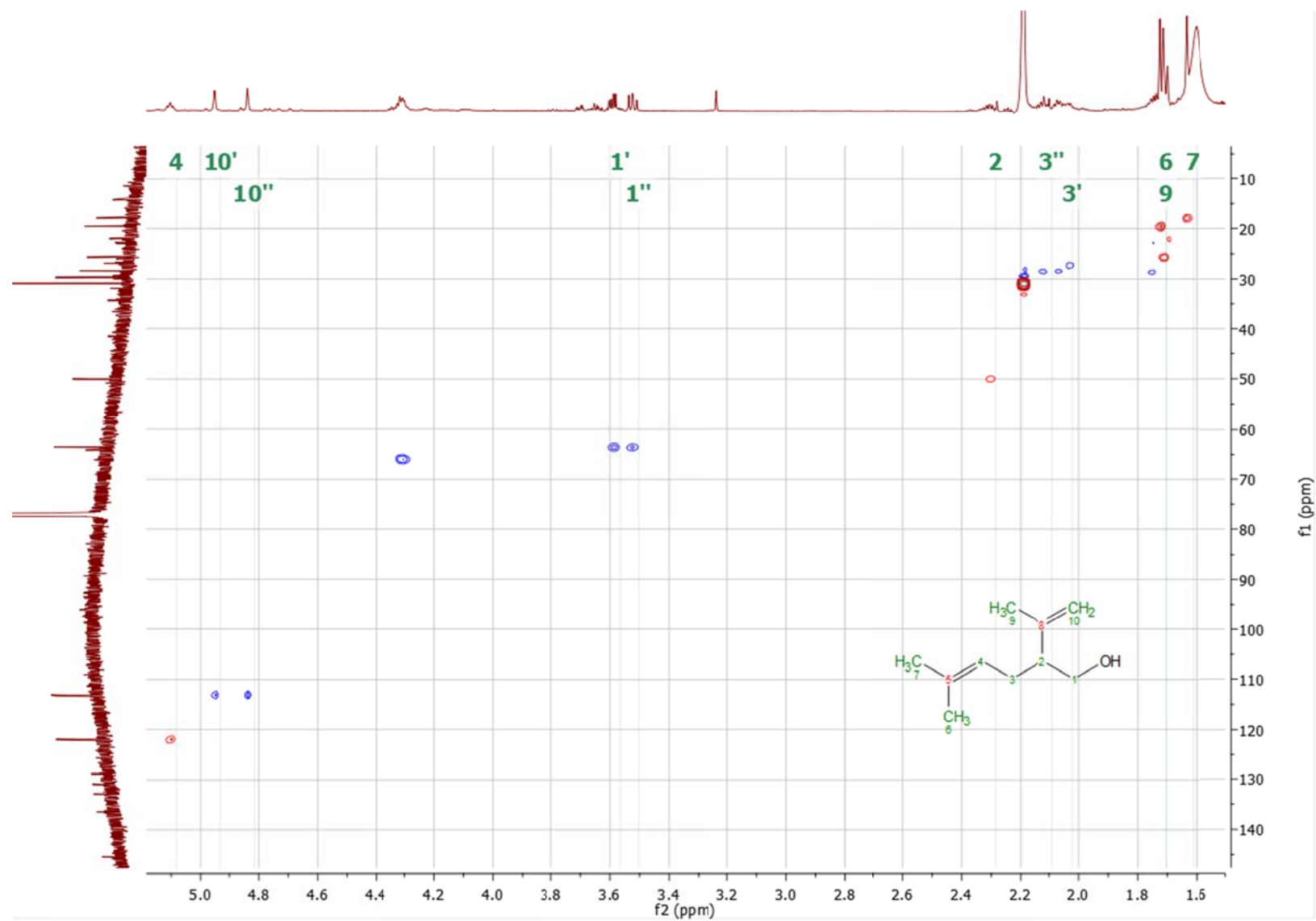


Figure S3.6 ^1H - ^{13}C HSQC spectrum of lavandulol in CDCl_3 .

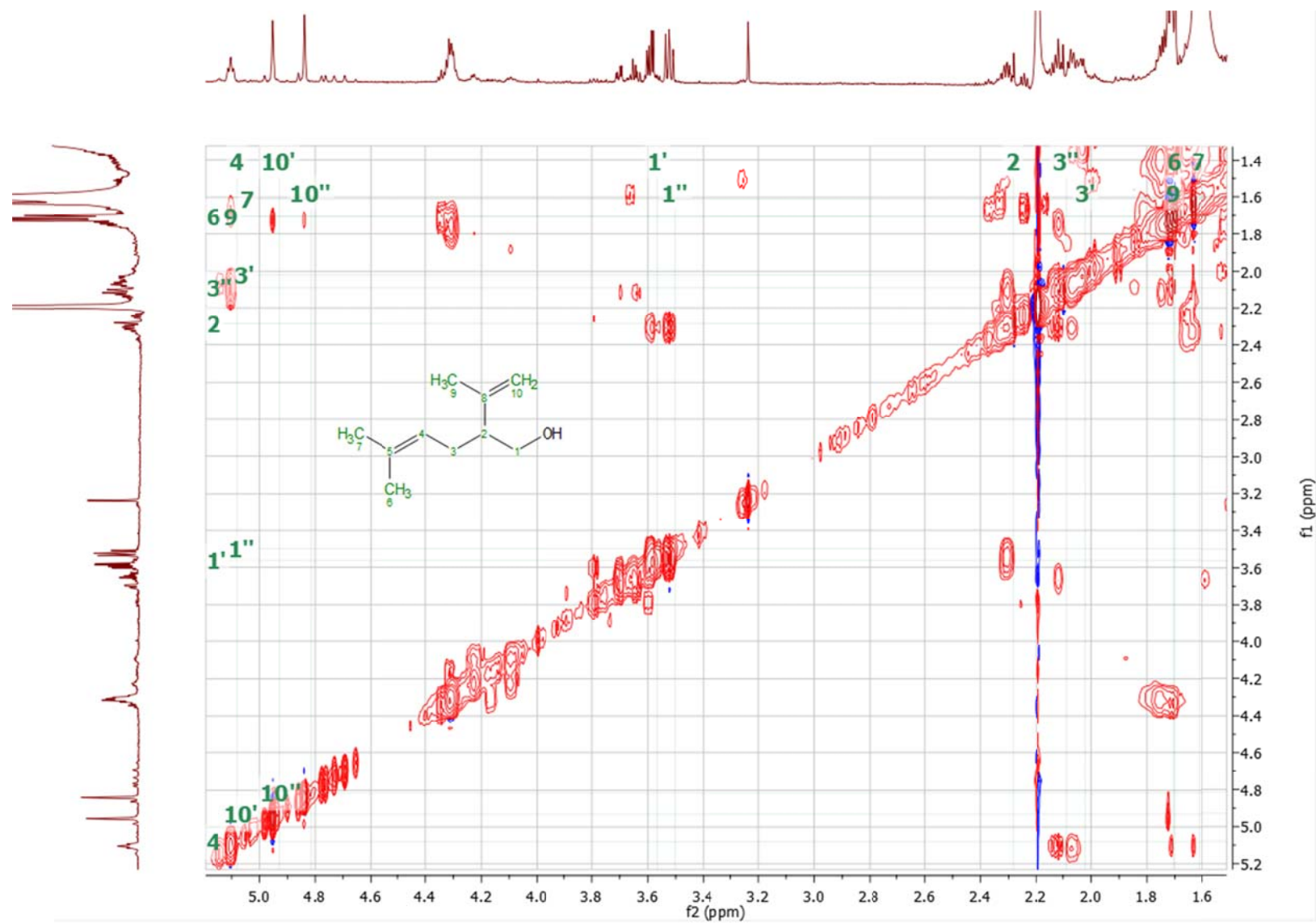


Figure S3.7 CLIP-COSY spectrum of lavandulol in CDCl_3 .

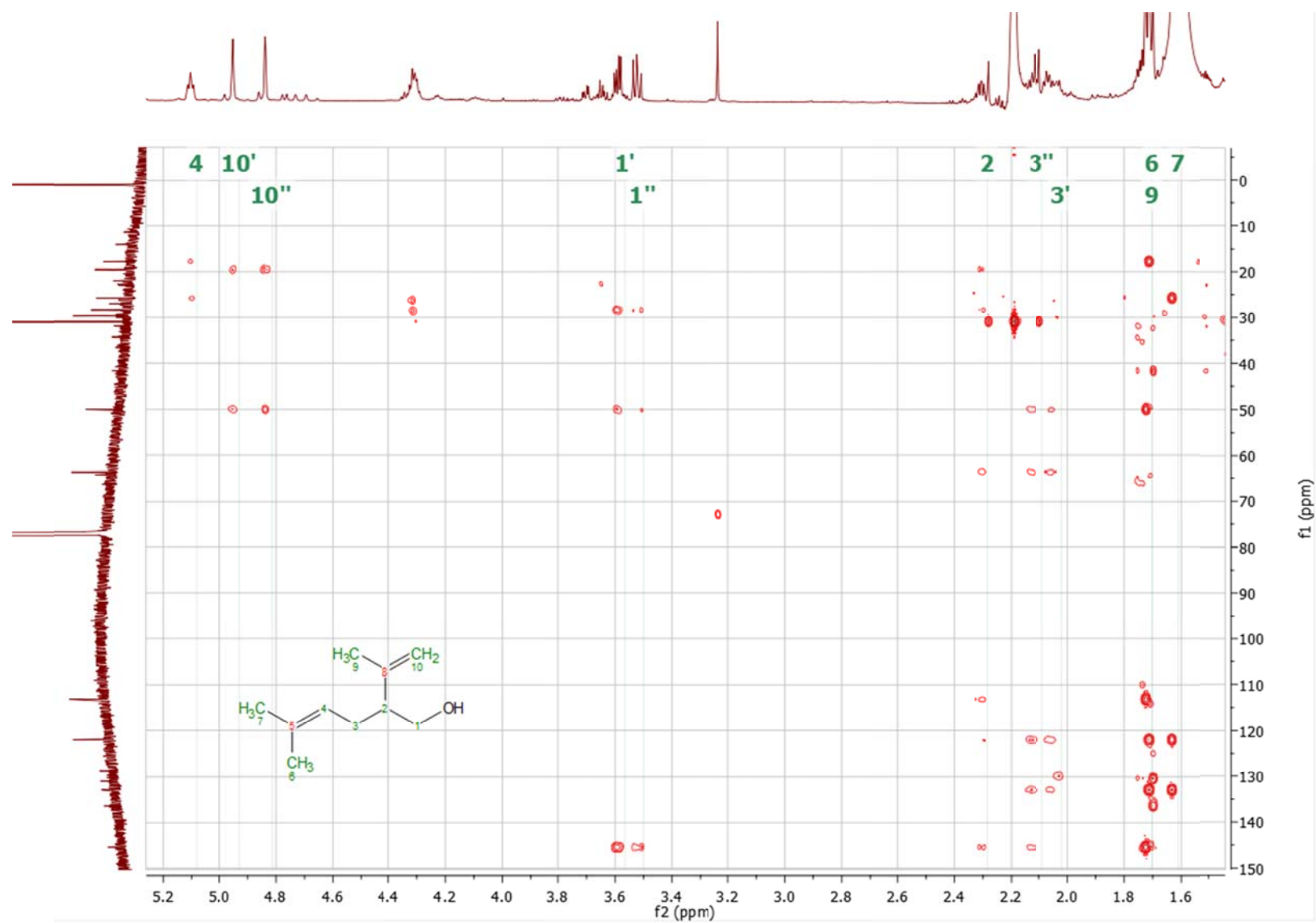


Figure S3.8 ^1H - ^{13}C HMBC spectrum of lavandulol in CDCl_3 .

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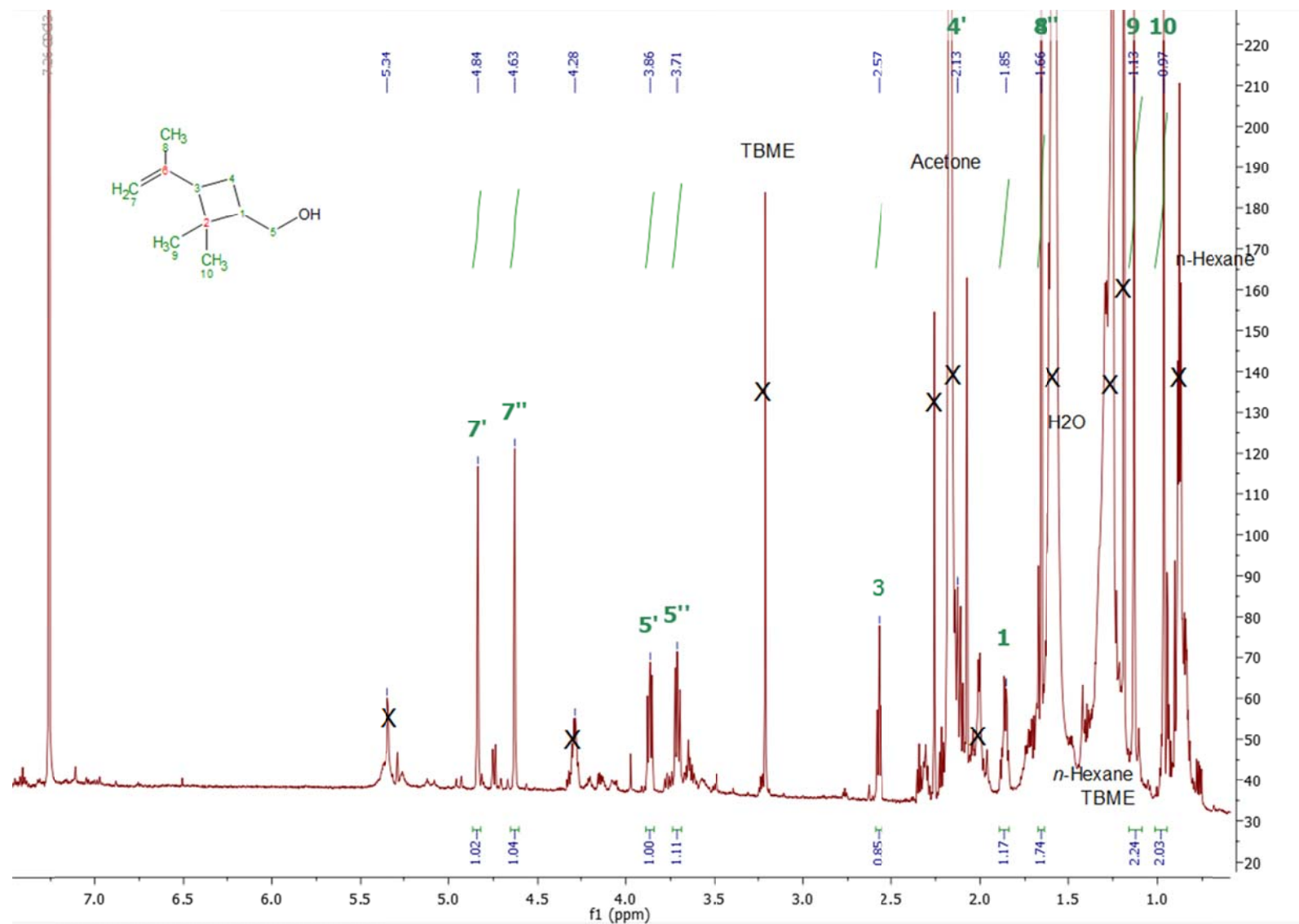


Figure S3.9 ¹H NMR spectrum of planococcol in CDCl₃.

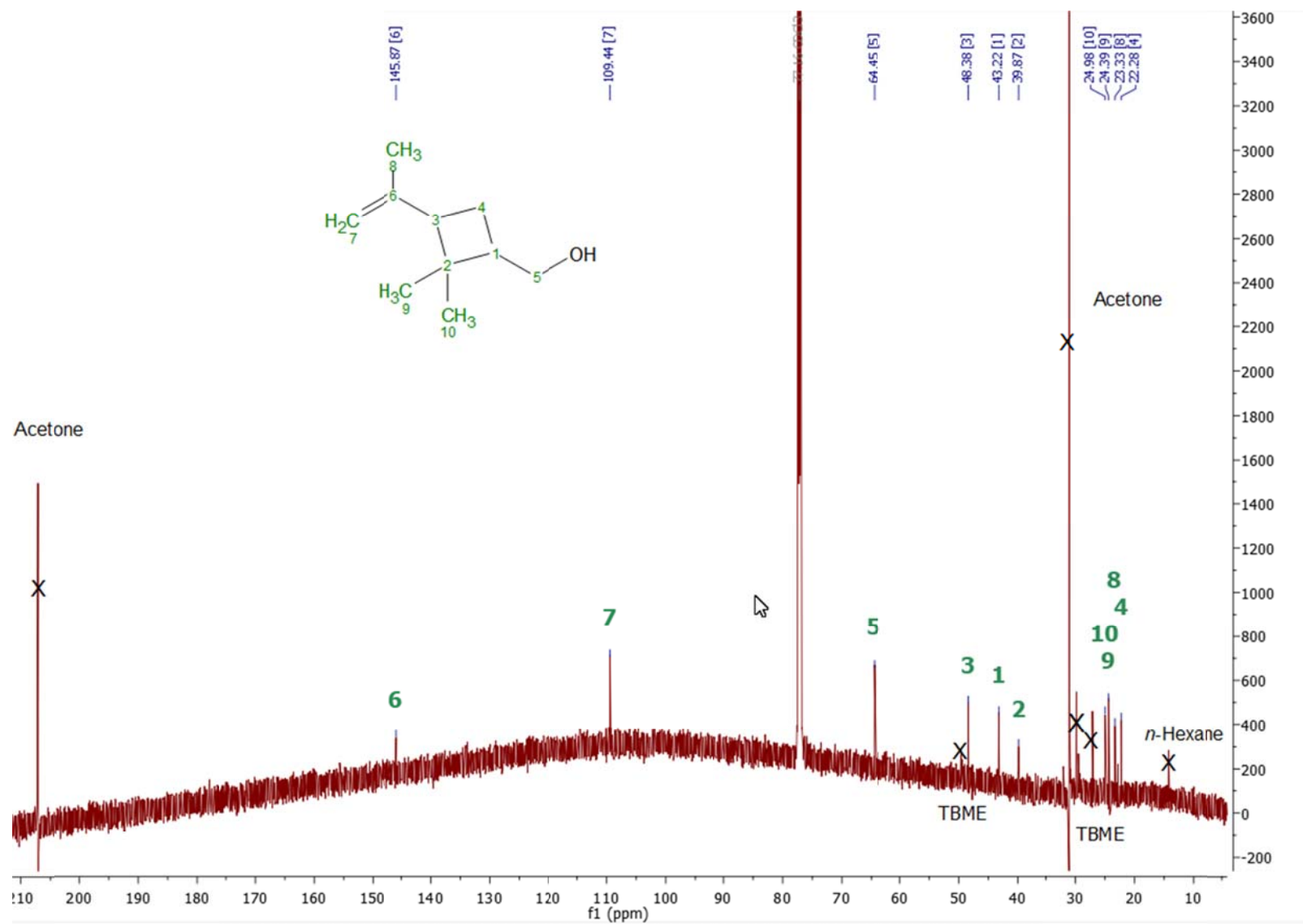


Figure S3.10 ^{13}C NMR spectrum of planococcol in CDCl_3 .

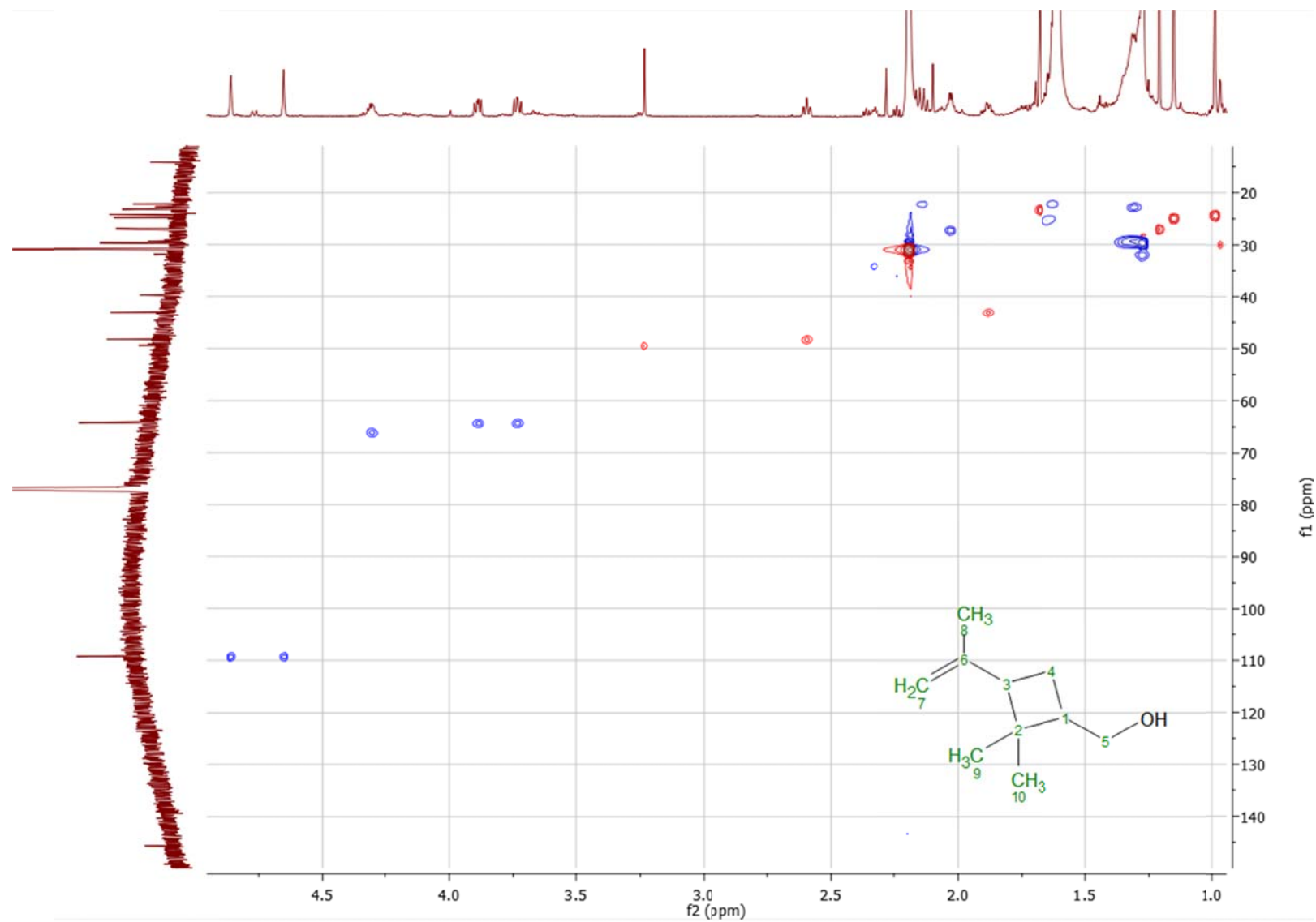


Figure S3.11 ^1H - ^{13}C HSQC spectrum of planococcol in CDCl_3 .

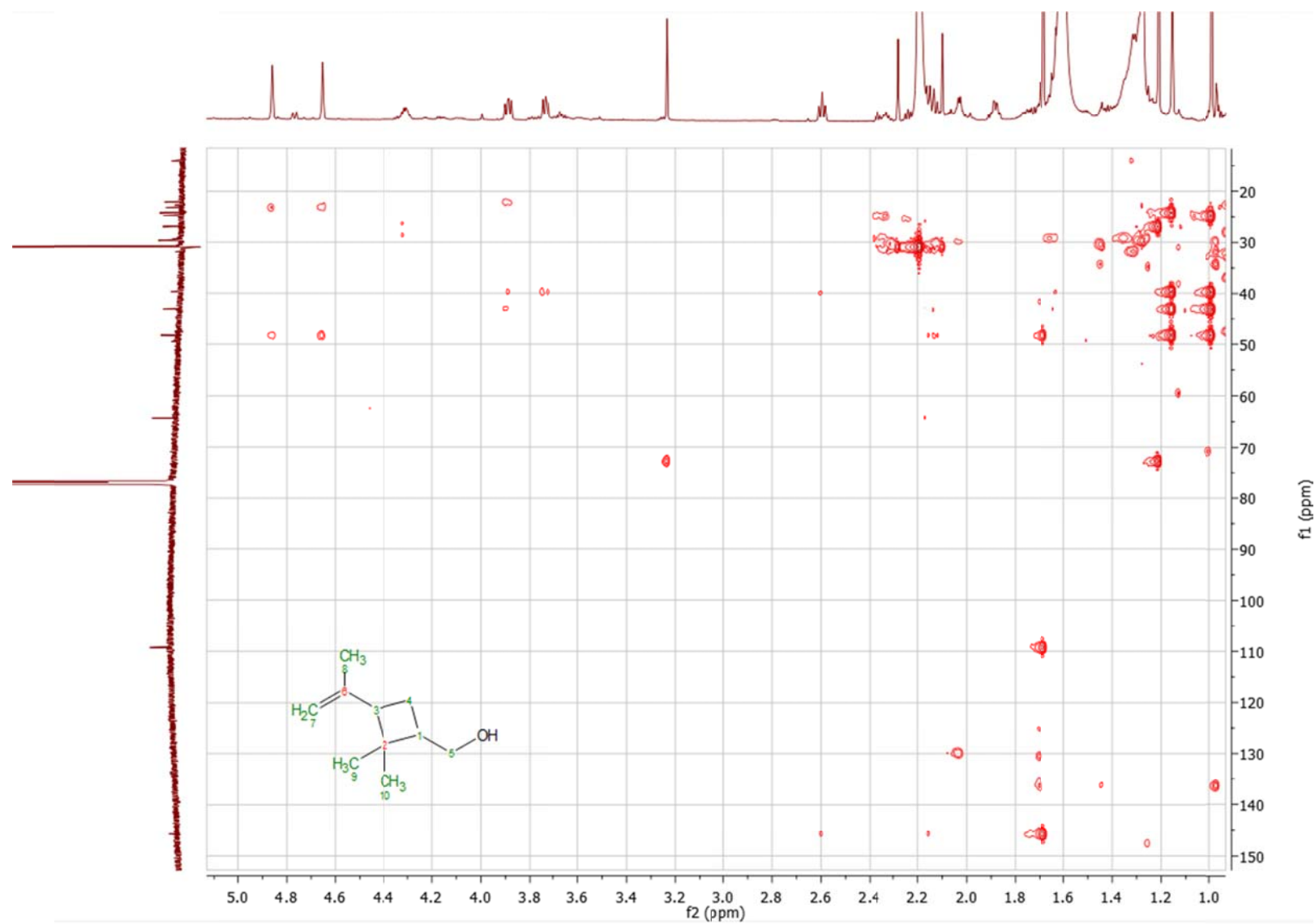


Figure S3.12 ^1H - ^{13}C HMBC spectrum of planococcol in CDCl_3 .

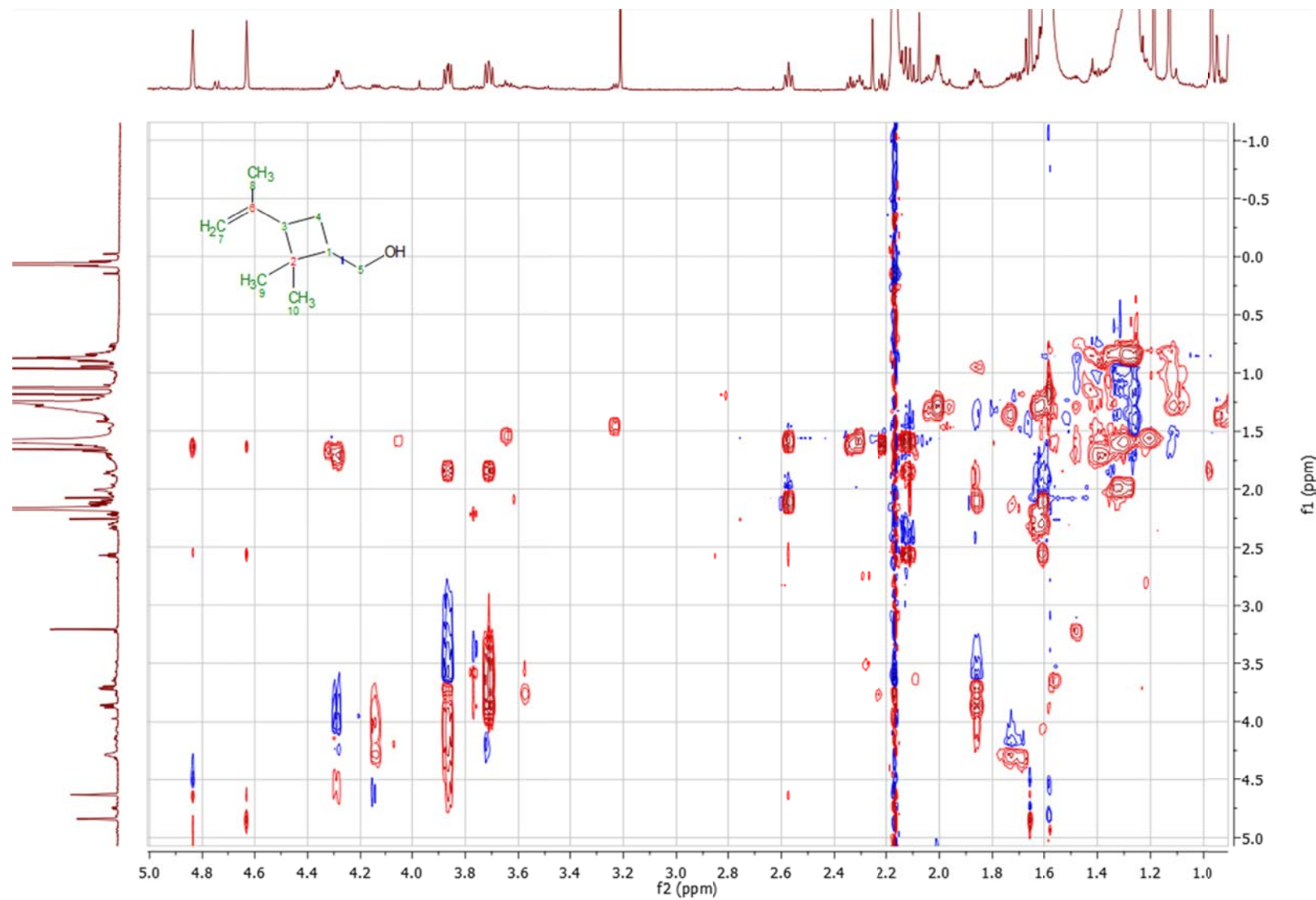


Figure S3.13 CLIP-COSY spectrum of planococcol in CDCl₃.

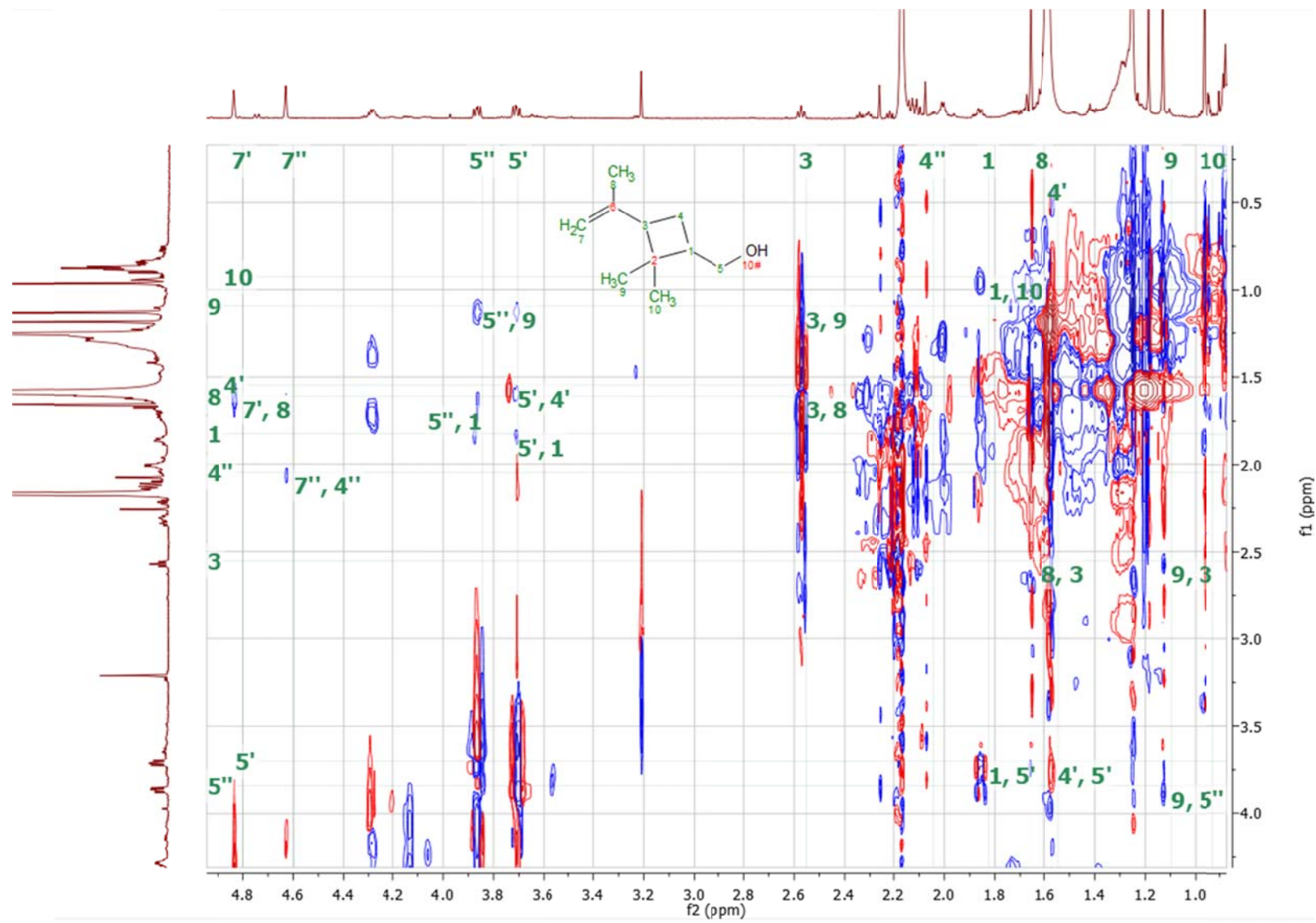


Figure S3.14 NOESY spectrum of planococcol in CDCl₃.

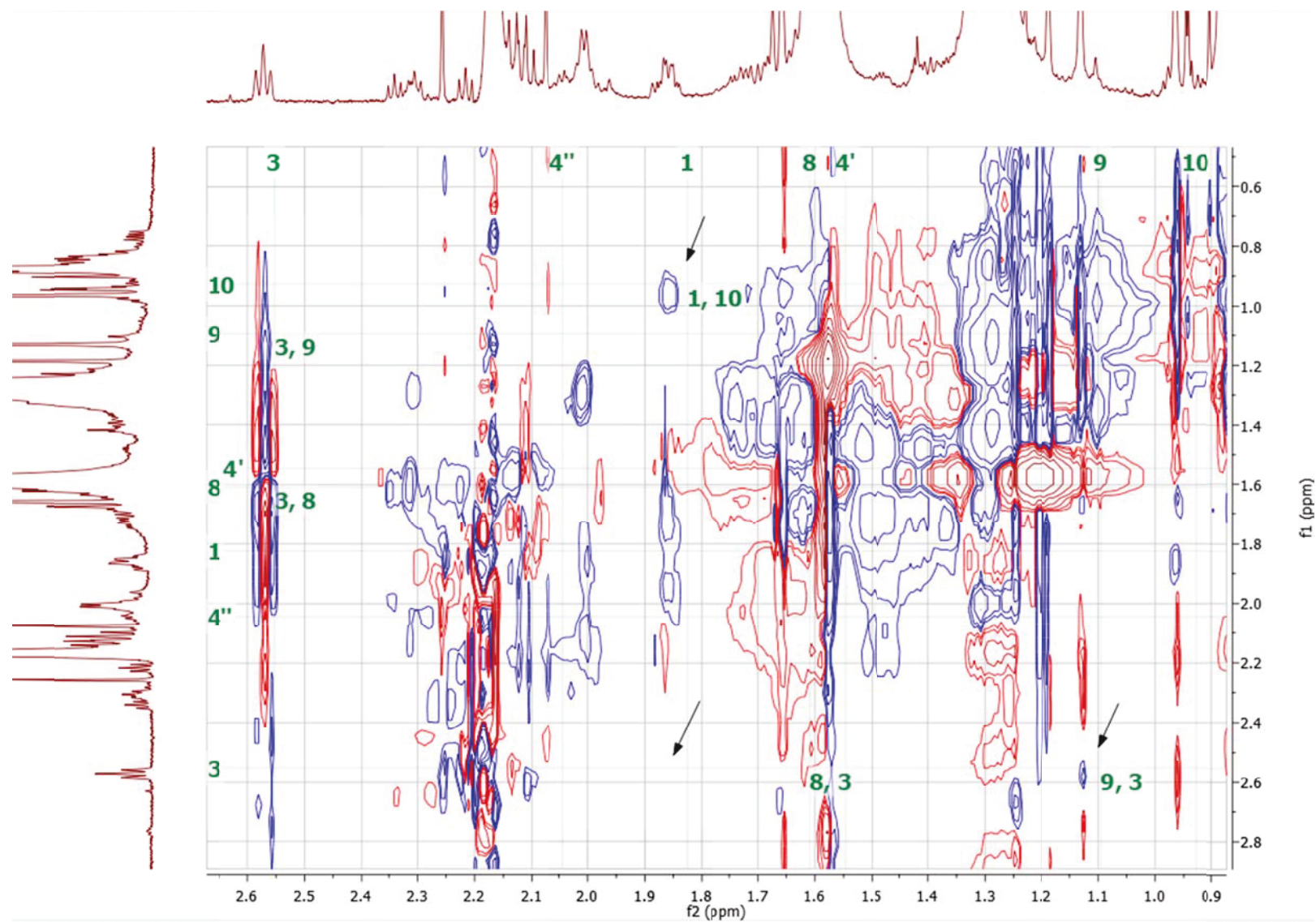


Figure S3.15 Fragment of NOESY spectrum of planococcol in CDCl₃. The selected proton-proton interactions and the potential position of NOE signal calculated for *cis* configuration of the molecule are marked with arrows.

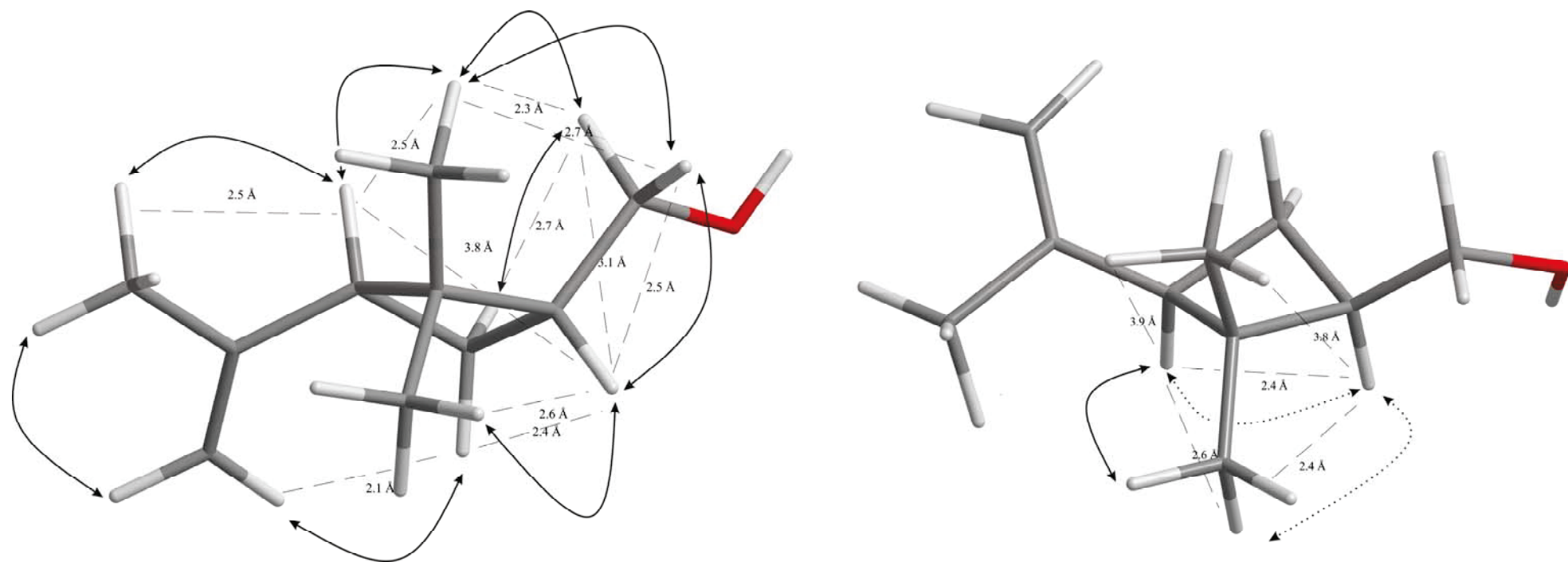


Figure S3.16 3D models of *trans*- (left) and *cis*-planococcol (right); selected NOE-interactions are marked with solid arrows (experimentally proved) or dotted arrows (theoretically calculated in *cis*-configuration), and selected interproton distances are marked with dashed lines.

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