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Supporting Information

Non-canonical Biosynthesis of the Brexane-Type Bishomosesquiterpene Chlororaphen through Two Consecutive Methylation Steps in *Pseudomonas chlororaphis* O6 and *Variovorax boronicumulans* PHE5-4

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Supporting Information

Supporting figures

Figure S1: GC-EIMS chromatograms of VOCs collected from *in vitro* assays with FPP (**3a**), SAM, and heterologously expressed enzymes showing negative controls for Figure 2 (IS1 = 5 ng/µL nonyl acetate as internal standard).

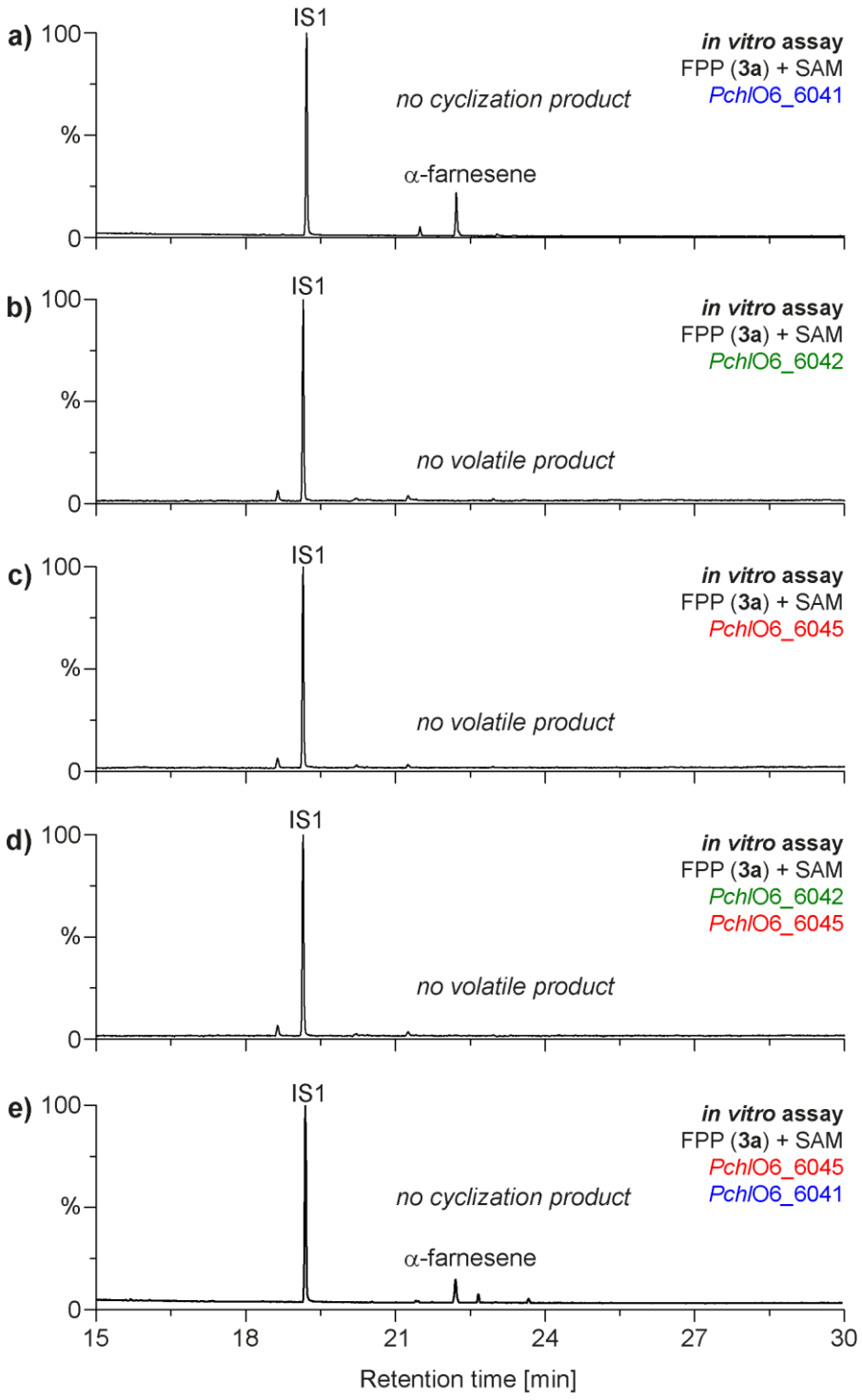


Figure S2: GC-EIMS chromatograms and mass spectra of VOCs collected from *in vitro* assays with FPP (**3a**), SAM, and heterologously expressed chlororaphen synthase (ChloS *Pchl*O6_6041 from *P. chlororaphis* O6 (IS1 = 5 ng/µL nonyl acetate as internal standard).

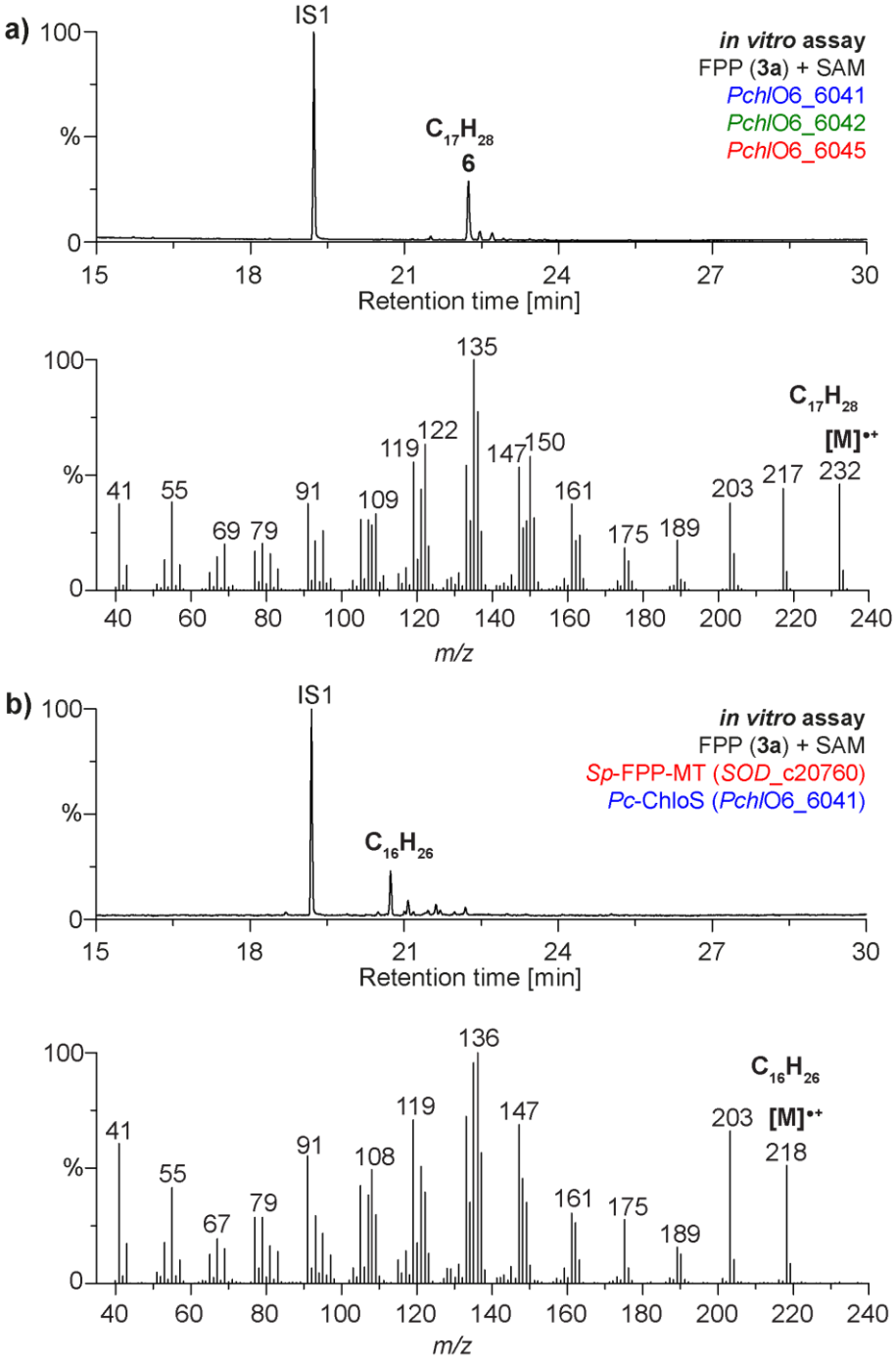


Figure S3: GC-EIMS chromatograms of VOCs collected from *in vitro* assays with heterologously expressed terpene synthase *Pchl*O6_6044 from *P. chlororaphis* O6 (IS1 = 5 ng/µL nonyl acetate as internal standard).

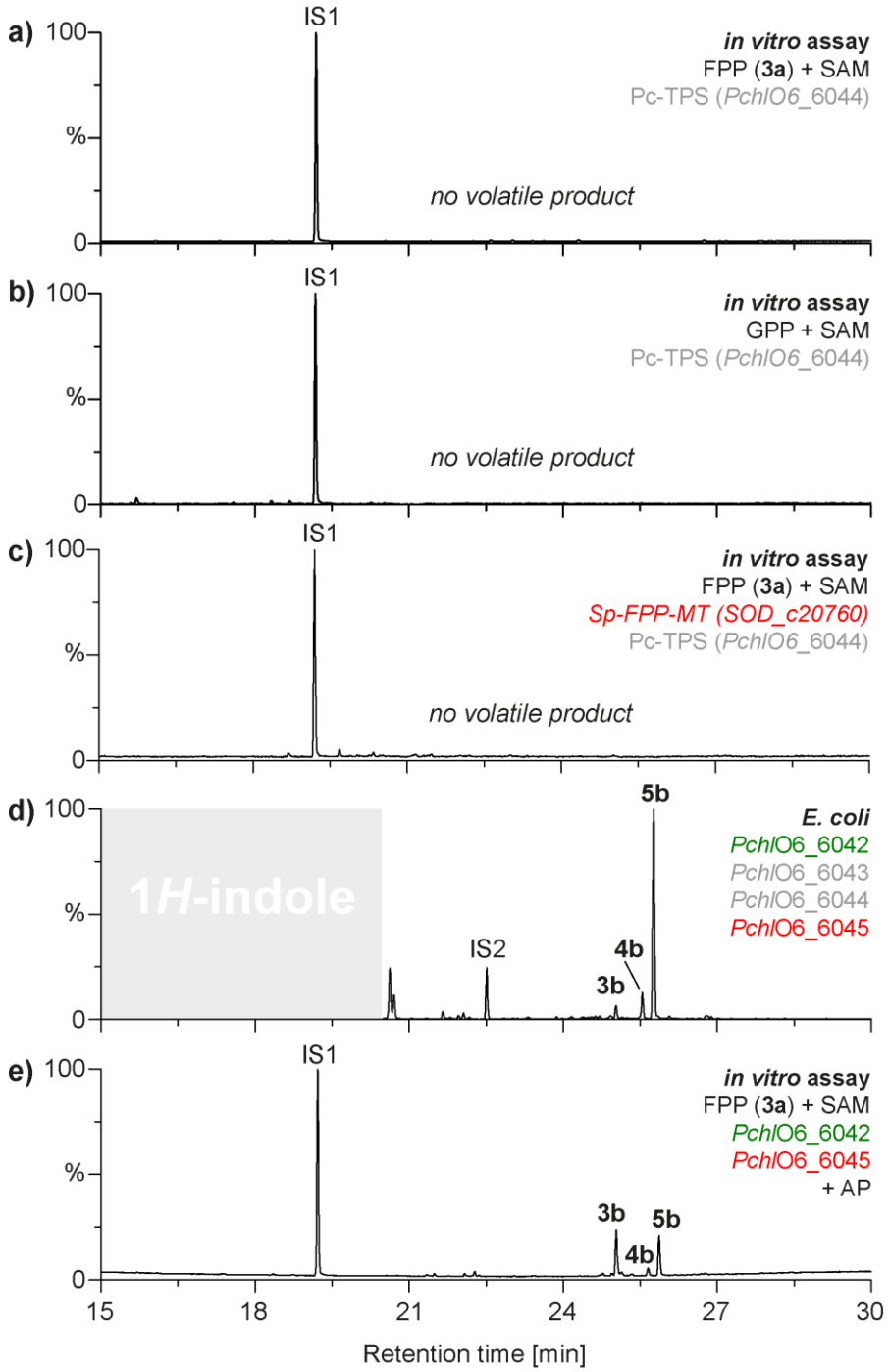


Figure S4: GC-EIMS chromatograms of VOCs collected from *in vitro* assays with heterologously expressed enzymes from *V. boronicumulans* PHE5-4 (IS1 = 5 ng/µL nonyl acetate as internal standard).

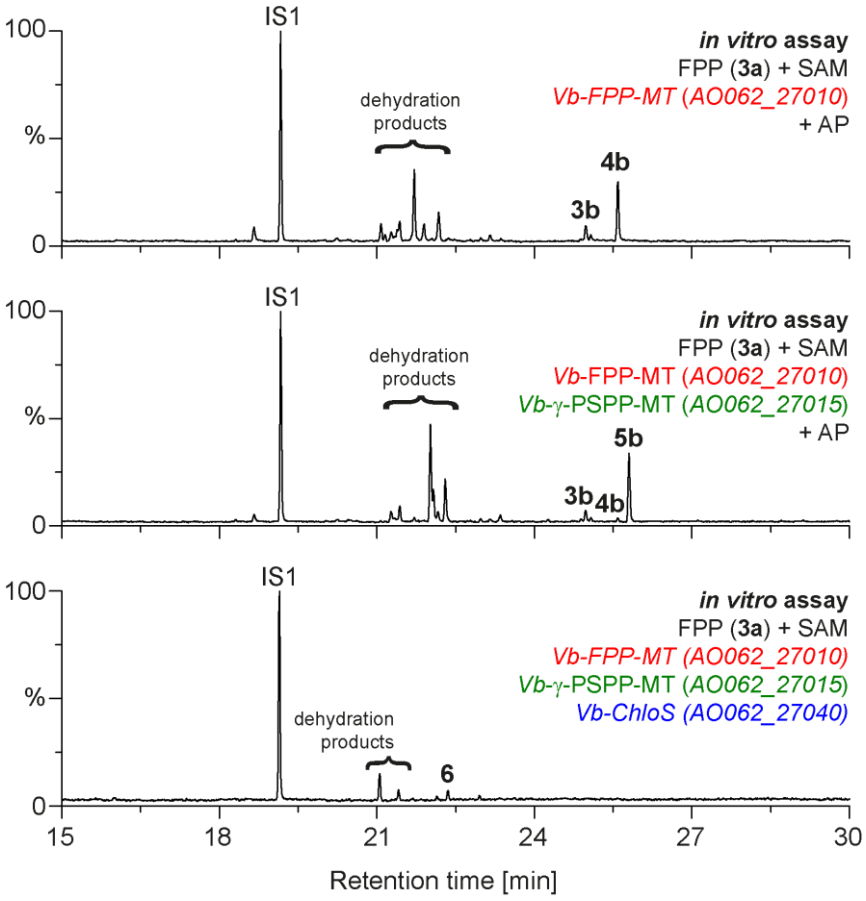


Figure S5: Structure assignment of γ-PSOH (**4b**), α-PCOH (**5b**) and chlororaphen (**6**) based on building blocks derived from H,H-correlations (*dqf*-COSY) and their linkages derived from relevant H,C-correlations (HMBC).

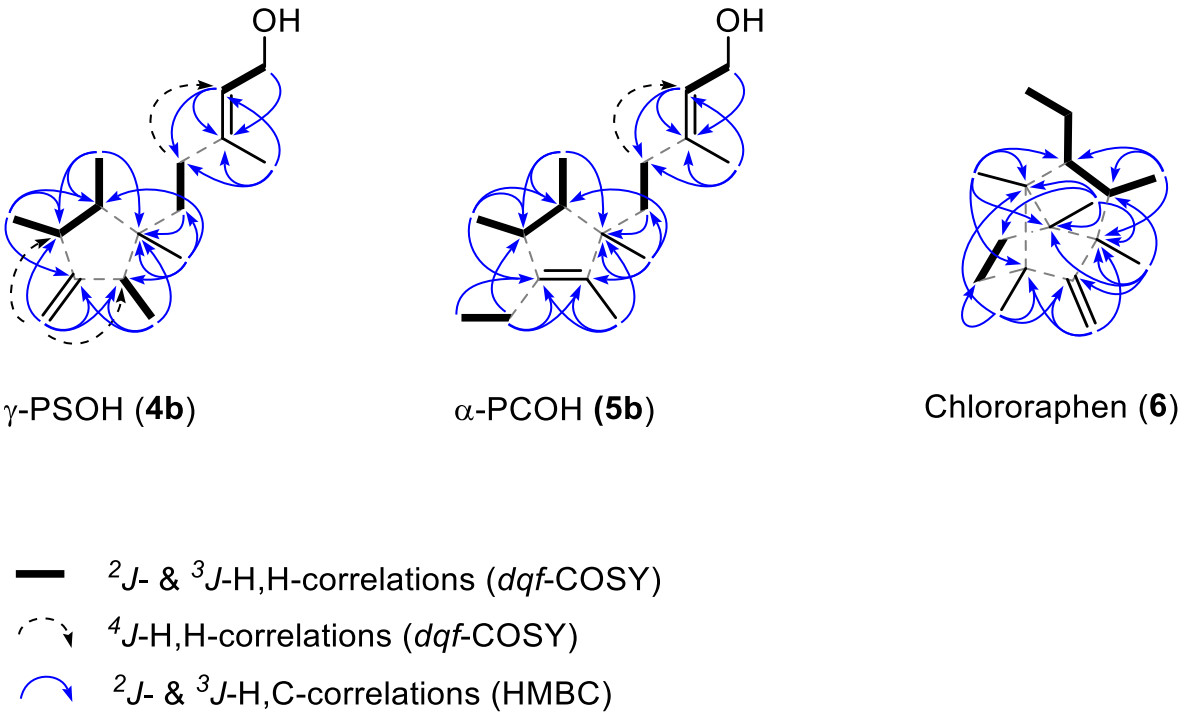


Figure S6a: relative configuration of chlororaphen (**6**, MM2 optimized model) derived from the NOESY spectrum (in C_6D_6).

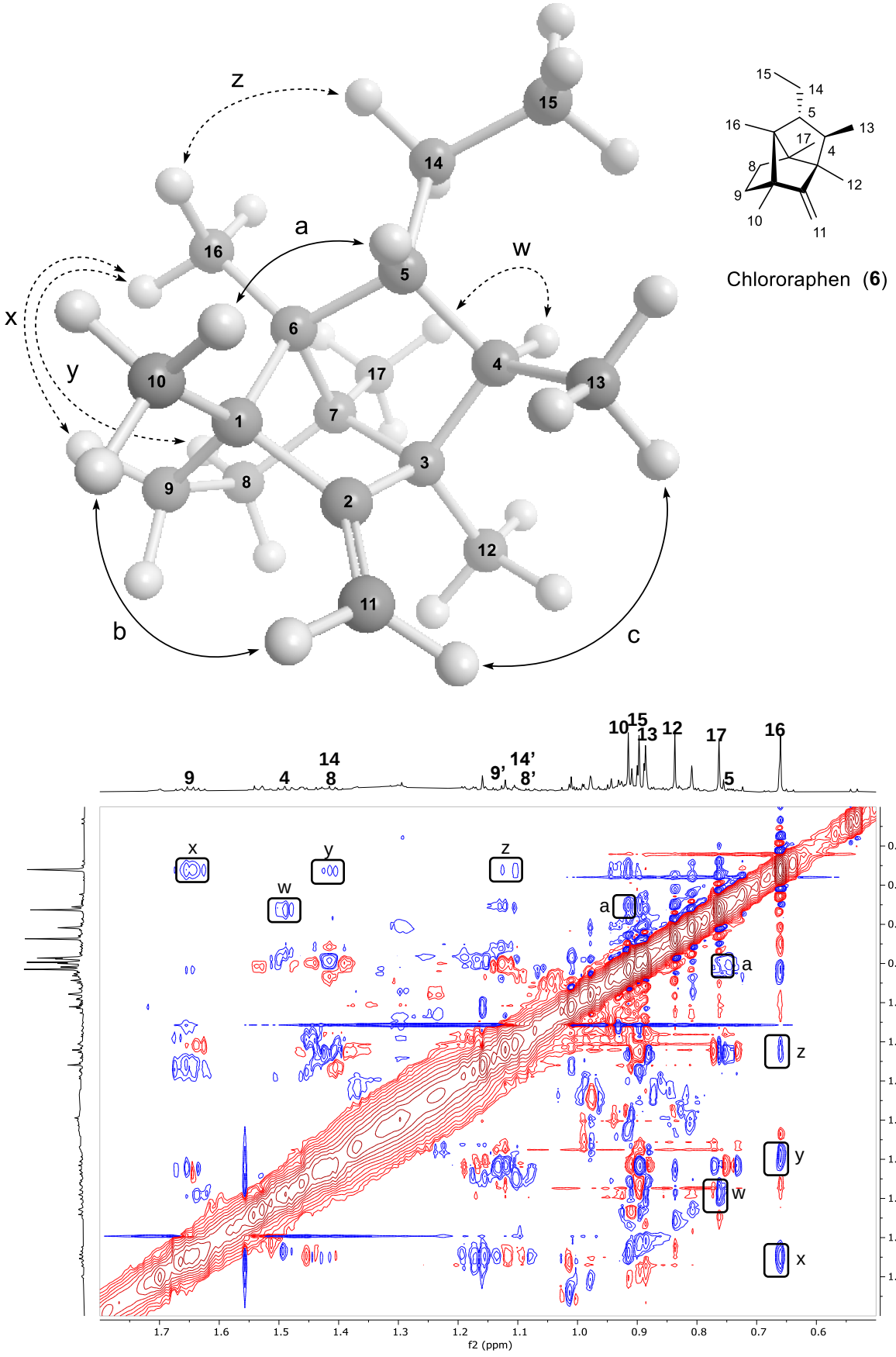


Figure S6b: NOESY correlation of the vinyl protons of chlororaphen (**6**) (in C_6D_6).

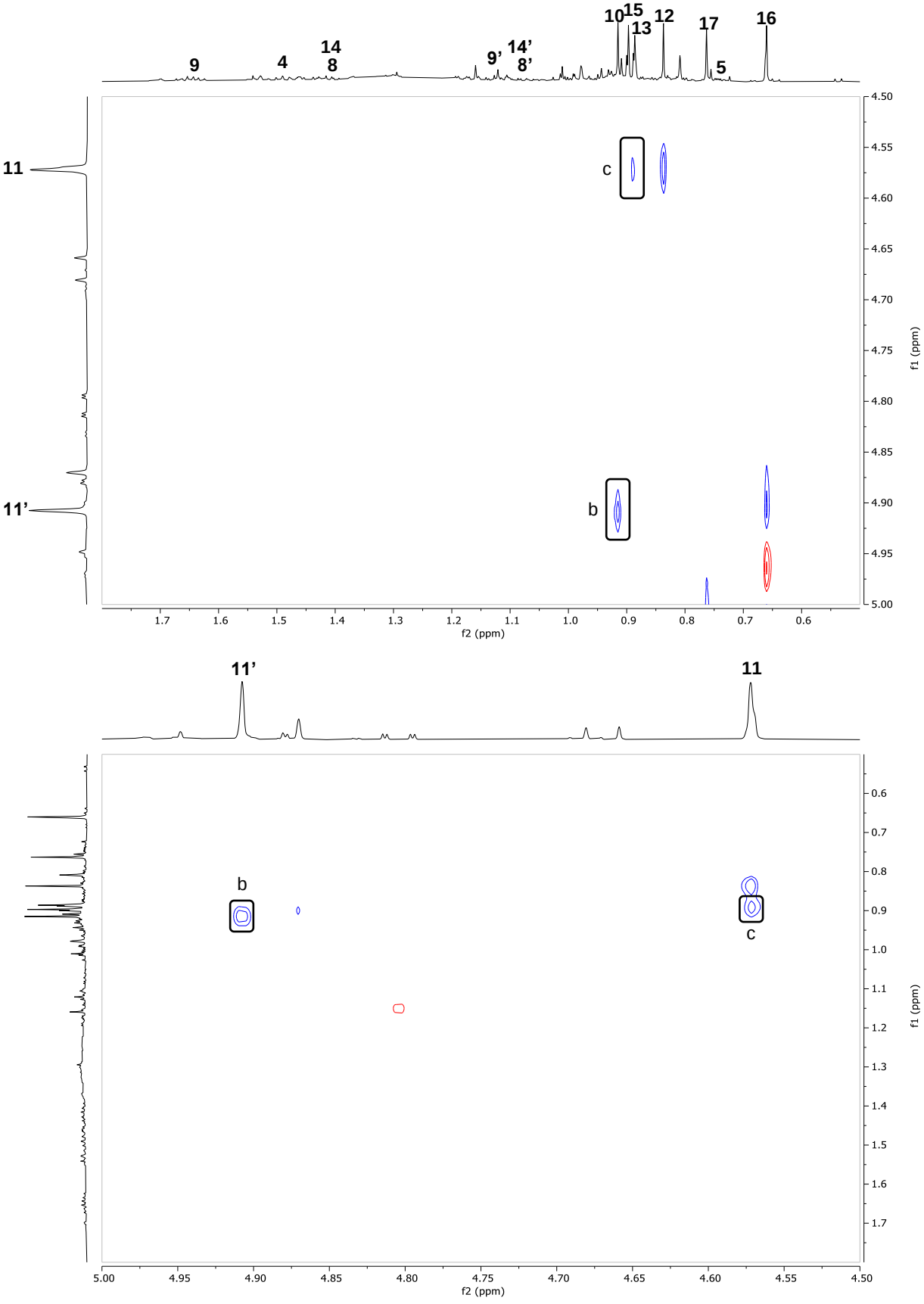


Figure S7: relative configuration of γ-PSOH (**4b**, MM2 optimized model) derived from the NOESY spectrum (for (*E*)-configured allyl alcohol moiety see Figure S9).

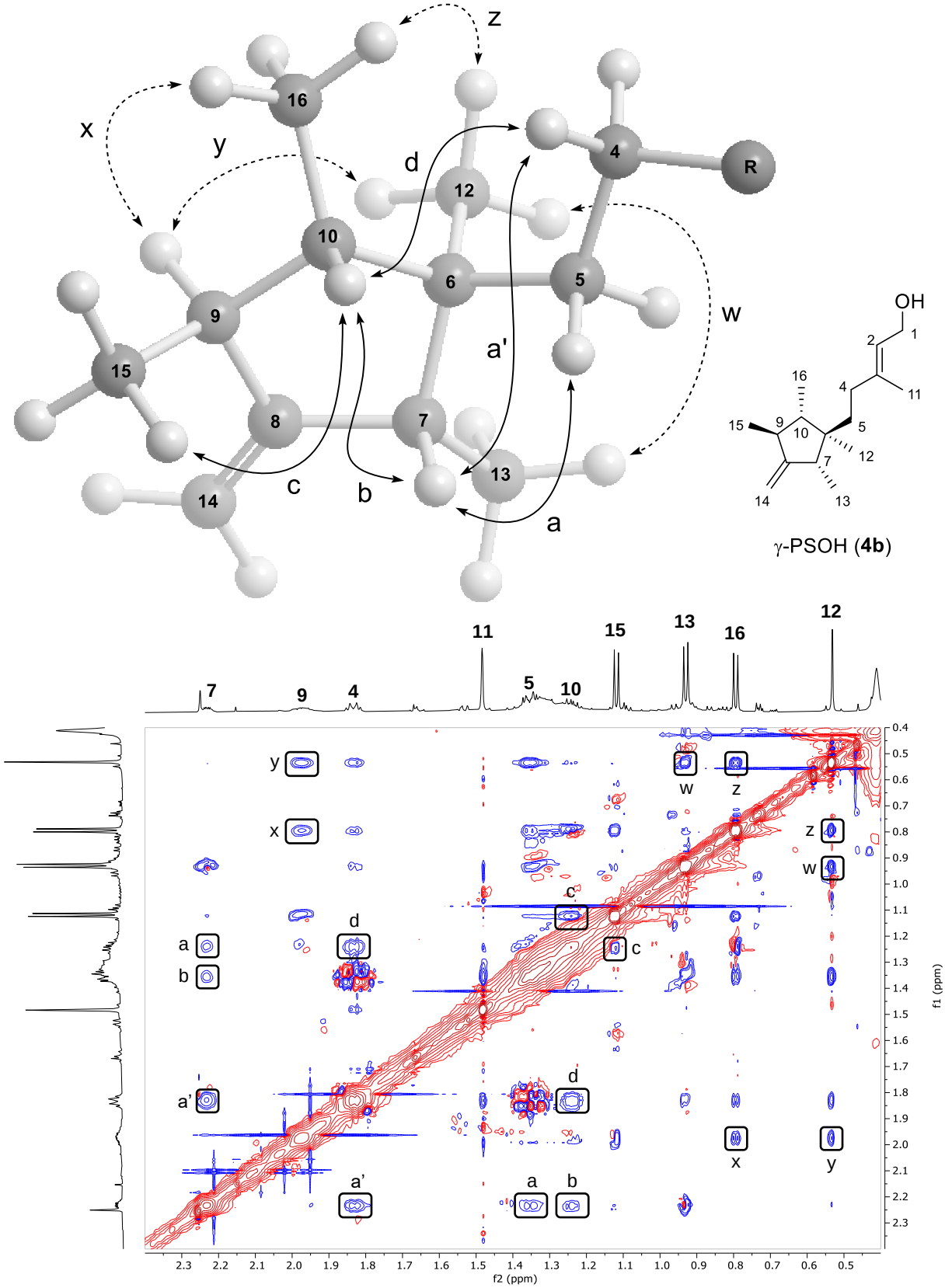


Figure S8: relative configuration of α-PCOH (**5b**, MM2 optimized model) derived from the NOESY spectrum (for (*E*)-configured allyl alcohol moiety see Figure S9).

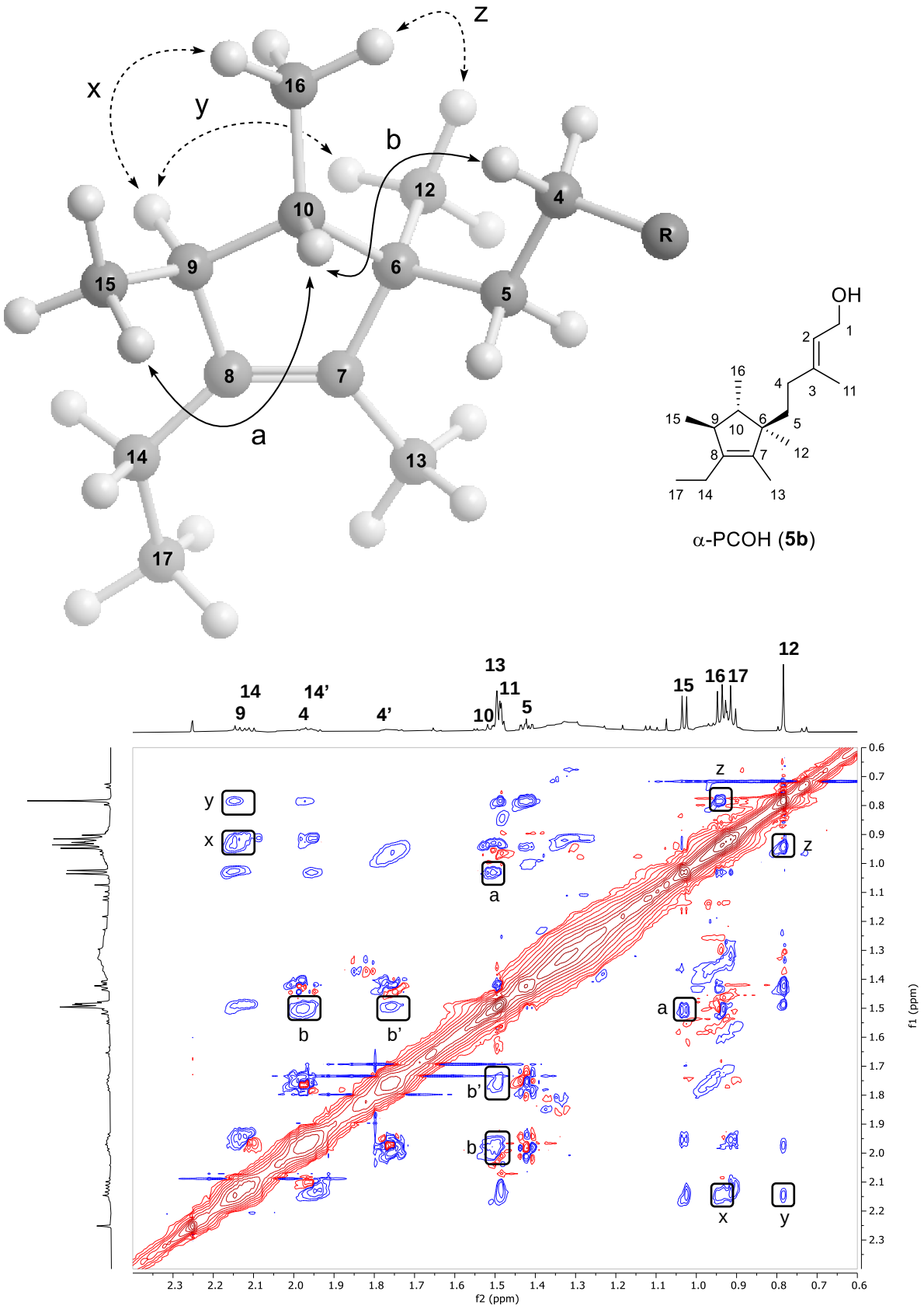


Figure S9a: (*E*)-configuration of allyl-alcohol moieties in γ-PSOH (**4b**) and α-PCOH (**5b**) as derived from their NOESY spectra.

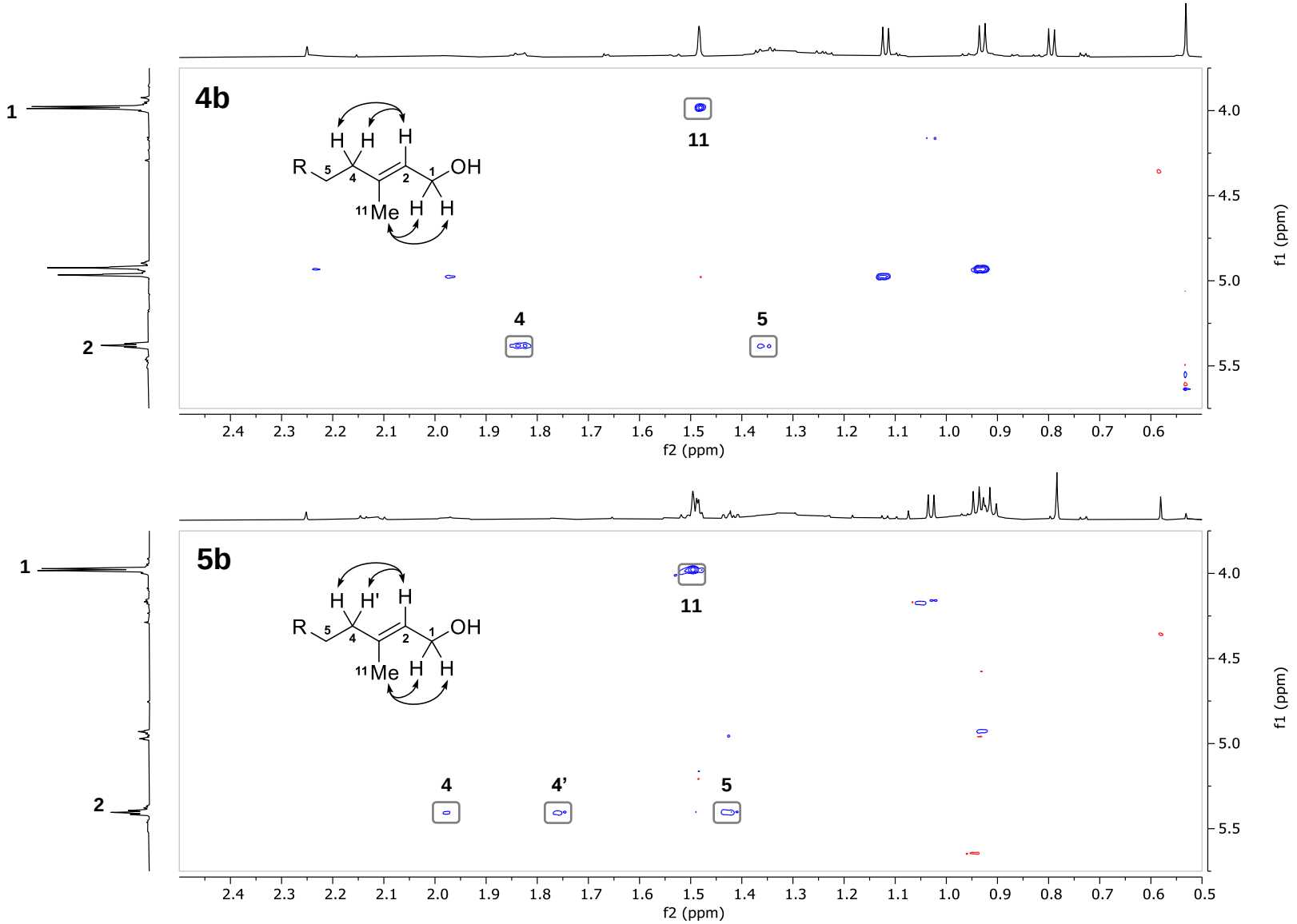


Figure S9b: (*E*)-configuration of allyl-alcohol moieties in γ-PSOH (**4b**) and α-PCOH (**5b**) as derived from their NOESY spectra.

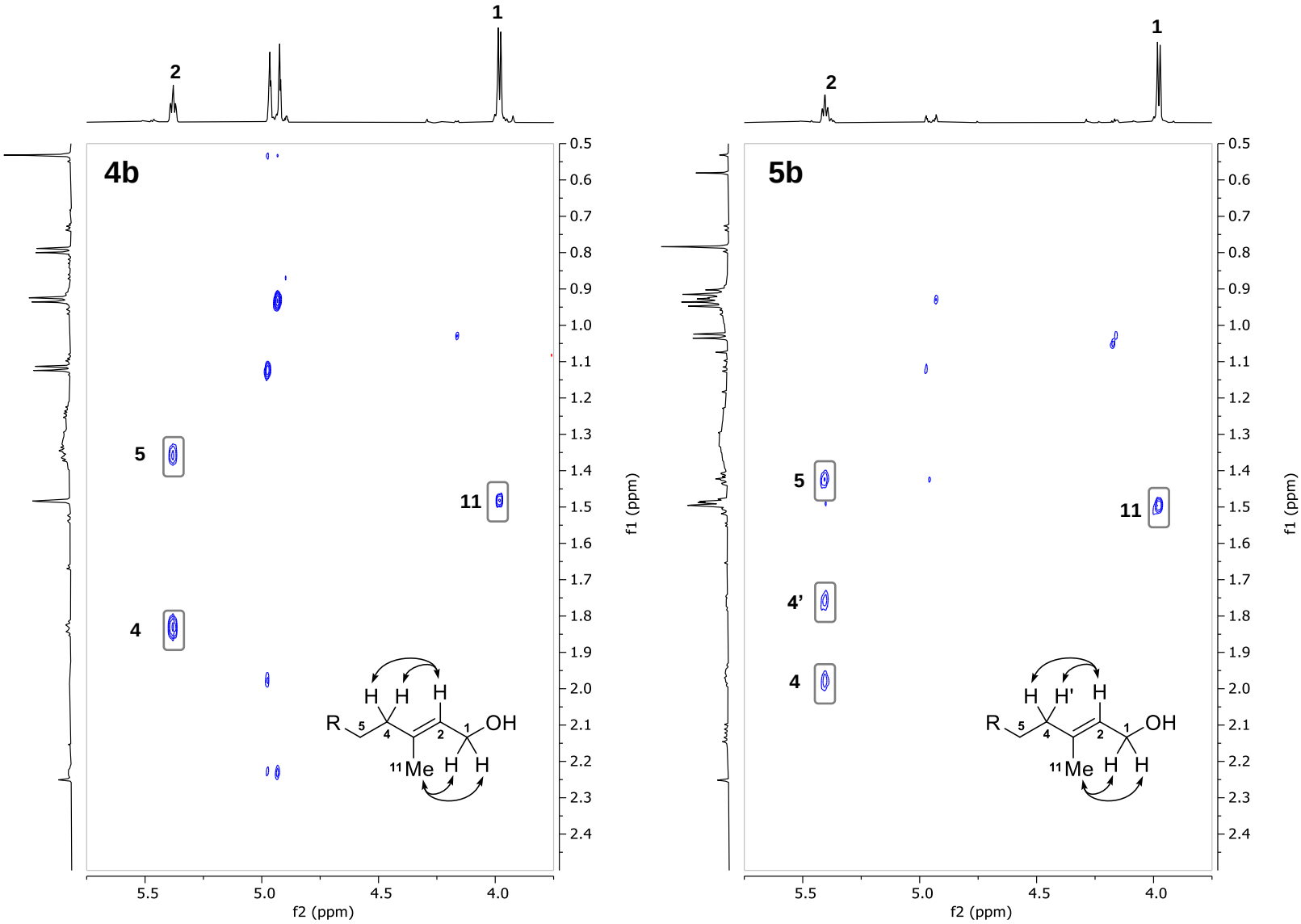


Figure S10: Amino acid alignment of farnesyl pyrophosphate methyltransferase *Pc*-FPP-MT (*Pchl*O6_6045), which converts FPP (**3a**) into γ-PSPP (**4a**) and *Pc*-γ-PSPP-MT (*Pchl*O6_6042) which converts γ-PSPP (**4a**) into α-PCPP (**5a**). Overall sequence identity was 41.25 % and sequence similarity was 57.59 %. Amino acid residues identified as part of the active site in *Sp*-FPP-MT from *S. plymuthica* 4Rx13 (which converts FPP (**3a**) into α-PSPP (**2a**))[1] are marked in grey when conserved and marked in yellow when different to *Sp*-FPP-MT. Alignment was performed using Clustal Omega with default settings.[2]

```
Pc-FPP-MT          MNQPTIDHNHPLQPVTVRHGRALPYHVIRGAEVSGYEGKVVYTYQDDPEDWRKAIGDRLM 60
Pc-γ-PSPP-MT       -----------------MQVHKSAIEVIHSSTADHYQSKIESVYADPPEEWRKVIGNEFW 43
                                    : :    .**:.: .. *:.*:  .* * **:***.**:.:

Pc-FPP-MT          FQFGVYDDPRSRPPISPDESGLRYFDRQMELAGYGRGDF--GPVKRILDVGCGWGFGLKY 118
Pc-γ-PSPP-MT       YQYGVFDEKMDPSRLPLDASGRRHMEYQFELAEQAGADLSSQSIRRAIDIGCGWGPVLSF 103
                   :*:**:*:  .   :  * ** *::: *:***  . .*:    ::* :*:*****  *.:

Pc-FPP-MT          LADRFPACPRLDGINISARQLEYCAKYHAEHRLSERINLYLCNAQDVDLLPHADEPYDLV 178
Pc-γ-PSPP-MT       LAERYPHCERIDGVNVSRPQLEYASQVISREGLAARVRLYLCNAKDIGALPDPELPYDLA 163
                   **:*:* * *:**:*:*  ****.::  :.. *: *:.******:*:. **. : ****.

Pc-FPP-MT          TVRGVISHFPNDLYERSMAKLASRLRPGATVIISDNLYNLPLEQYRSDTADDVDRLACKH 238
Pc-γ-PSPP-MT       IFRGSLFHFTPQVLQETMQSLAQRMRPGGTVVISESLYKVDLATYQSFIPDKVDRAASGH 223
                    .** : **  :: :.:* .**.*:***.**:**:.**:: *  *:*   *.*** *. *

Pc-FPP-MT          RKTPGYFRQVLEQCGLNVEDMRVLPENIDVARWFMDVKKNIETHFTPDTIPPPLEELRVM 298
Pc-γ-PSPP-MT       RKTPDSLHKALEDNGFDVIDRRITPSNEEVIRWYGLVKDNLDAHY-PDSRNPNFSELRDI 282
                   ****. :::.**: *:* * *: *.* :* **:  **.*:::*: **:  * :.*** :

Pc-FPP-MT          AVNVSVALIKDQFSTYSVIARHP    321
Pc-γ-PSPP-MT       AINFSDALRKDKASSFSFIARRR    305
                   *:*.* ** **: *::*.***:
```

Figure S11: Amino acid alignment of farnesyl pyrophosphate methyltransferases *Sp*-FPP-MT (*SOD*_c20760) and *Pc*-FPP-MT (*Pchl*O6_6045), which convert FPP (**3a**) into α-PSPP (**2a**) or γ-PSPP (**4a**), respectively. Overall sequence identity was 57.14 % and sequence similarity was 66.16 %. The amino acids identified to form the active pocket of *Sp*-FPP-MT include Y39, E42, H45, W46, F56, Q57, F58, H191, N219, V236, L239, C241, V273, W276, A303, W306,[1] most of which are conserved in *Pc*-FPP-MT (highlighted in grey), except E42D, H45D, and W306V (highlighted in yellow). The catalytic dyad of *Sp* FPP-MT is Y39, H191. Alignment was performed using Clustal Omega with default settings.[2]

```
Sp-FPP-MT     -----MSNLSEMMSPDPVYLQNLPYQVIRDSHTDSYEEKVVQTYCEEPEHWRKAIGDTLL   55
Pc-FPP-MT     MNQPTIDHNHPLQPVTVRHGRALPYHVIRGAEVSGYEGKVVYTYQDDPEDWRKAIGDRLM   60
                   :.:   :       : : ***:***.:....** *** ** ::**.******* *:

Sp-FPP-MT     FQFGVYEDAAHAPALTPTSKLKPTAGLDESSVRYFEQQLFLAGVTGSDRPAMHRILDIGC   115
Pc-FPP-MT     FQFGVYDDPRSRPPIS----------PDESGLRYFDRQMELAGYGRGDFGPVKRILDVGC   110
              ******:*    * ::           ***.:***::*: ***   .*   ::****:**

Sp-FPP-MT     GWGYILKHLAERYPECQRLDGVNVSAQQLNYCARSHAEQGLSGRINLFLCNAQDIGLLPD   175
Pc-FPP-MT     GWGFGLKYLADRFPACPRLDGINISARQLEYCAKYHAEHRLSERINLYLCNAQDVDLLPH   170
              ***: **:**:*:* * ****:*:**:**:***: ***: ** ****:******:.***.

Sp-FPP-MT     PDDLYDLVIIRGVISHFPNELYEKAMRALFTRVRPGGKVIISDNLYNVEPDRYQSDTPDI   235
Pc-FPP-MT     ADEPYDLVTVRGVISHFPNDLYERSMAKLASRLRPGATVIISDNLYNLPLEQYRSDTADD   230
               *: **** :*********:***::*  * :*:***..*********:  ::*:*** *

Sp-FPP-MT     VDRLACRHQKTPAYFSQVLEDSGFTIKDFRVLPSNVDVAHWLMDSKANIERHFP-TGVDG   294
Pc-FPP-MT     VDRLACKHRKTPGYFRQVLEQCGLNVEDMRVLPENIDVARWFMDVKKNIETHFTPDTIPP   290
              ******:*:***.** ****:.*:.::*:****.*:***:*:** * *** **    :

Sp-FPP-MT     ALEELRVLAENWSVALLKNKVSTYSVIACKK       325
Pc-FPP-MT     PLEELRVMAVNVSVALIKDQFSTYSVIARHP       321
               ******:* * ****:*::.******* :
```

Figure S12: Amino acid alignment of *S. plymuthica* 4Rx13 sodorifen synthase (*Sp*-SodS / *SOD*_c20750) and *P. chlororaphis* O6 chlororaphen synthase (*Pc*-ChloS / *Pchl*O6_6041). Overall sequence identity was 17.66 % and sequence similarity was 31.09 %. Alignment was performed using Clustal Omega with default settings.[2]

```
Sp-SodS      MNT-NDFLSPEAQP---MLTSQPVMHIVPDYNPFNRQYKVHPLKAEVEKKALDFMERYRL    56
Pc-ChloS     MNHSAQALAPEVSFYIPEMYCSVLPRIHPDYPII-------------DERNASWVREFLP    47
             **   : *:**..     : .. : :* ***  :             :::  .::..:

Sp-SodS      YWTEEQRQRLYGQDCGGIAGYVYTLAPNAEQLQLGADLAMIAFTWDDEFCDEGPTRDKP-    115
Pc-ChloS     FTDEAAQLRF--------------------------LRLHTPMWDSMIFPIGSADRLVH    80
             :  *  : *:                           * : :  **. :   * :

Sp-SodS      --------MEMADSAFRTIRALECHDIIVDKNDRYAVAMRDILQRVRQLSPDYLANQWVD    167
Pc-ChloS     TSCVTSLITAIDDMPLGRHAMFHDGEVALLEGHPFARAAQDIFGKLRQHMPAPVYRRYCQ    140
                       : *  :     :.  :: : :.. :* * :**: ::**  *  : .:: :

Sp-SodS      SVRHWFFIEIQKASNVARGIRPNLSDYVVTR-MHTGATPTFMLNTQIANGLELGPGLLFD    226
Pc-ChloS     ELQAWFESVEEEARLVAAGKVLPLDEFLELRHPNTGLLPSFPVAEFL-YDLDLTELLAQD    199
             .:: **    ::*  ** *    *.:::  *  :**  *:* :   :  .*:*   *  *

Sp-SodS      RRVNALMELARTVVNWSSDCYSYFKEAERTADGYNIIDVLMDTHNLSVEAA---MAMAFN    283
Pc-ChloS     RELQLAIRVTNEHVGLVNDILSHRKEHA-IGVTLNAMESLRIVHGHSAQEAADILCQRIR    258
             *.::  :.::.  *.  .*  *: **    .   * :: *  .*. *.: *   :.  :.

Sp-SodS      MQDRMLMR-FVELRDEVLNGPHDKGAEIYIDALEEYTIGGILWCQETQRYRFIDGTTSGR    342
Pc-ChloS     EADRARVELCEVLRHRYANRPDADRIGMYLDGLGRICAGNLRWLLESDRYVDSRGNGWD-    317
               **  :.    **..  * *. .   :*:*.* .   *.: *  *::**    *.  .

Sp-SodS      LAYTASGFTRQARGNELSEPIDIPTIAWWWQVGERADQYGRR     384
Pc-ChloS     --WTRSRLIV-----LDPEPAPAPALAQS-------------     339
               :* * :          **   *::*
```

Figure S13: Amino acid alignment of *S. plymuthica* 4Rx13 sodorifen synthase (*Sp*-SodS / *SOD*_c20750) and the inactive *P. chlororaphis* O6 TPS (*Pchl*O6_6044). Overall sequence identity was 41.96 % and sequence similarity was 54.69 %. Alignment was performed using Clustal Omega with default settings.[2] Conserved residues of the aspartate-rich motif (DDxxxDE) and the NSE triad (NxxxSxxxE), both essential elements of TPS enzymes, are marked in grey, differences are marked in yellow.

```
Sp-SODS       MNTNDFLSPEAQPMLTSQPVMHIVPDYNPFNRQYKVHPLKAEVEKKALDFMERYRLYWTE    60
PchlO6_6044   --------------METPSVIQMSASYNPFKE-SRIHPKTDEADAKATAFGERYELYWDT    45
                            : :  *::: .****:.  ::** . *.: **  * ***.***

Sp-SODS       EQRQRLYGQDCGGIAGYVYTLAPNAEQLQLGADLAMIAFTWDDEFCDEGPTRDKPMEMAD    120
PchlO6_6044   AQKDRILHQFCGGLAGYLFPDA-SEELLQIAADFSMFAFAWDDQNCDEGPERDVILELSR    104
               *::*:  * ***:***::  * . * **:.**::*:**:***: ***** **  :*::

Sp-SODS       SAFRTIRALECHDIIVDKNDRYAVAMRDILQRVRQLSPDYLANQWVDSVRHWFFIEIQKA    180
PchlO6_6044   SSFRLLRTIESLETVVYPEDNYAMALRDISLRVRKHTNTYQYLLWVDSLRNWFYMEQFKT    164
              *:** :*::*. : :*  :*.**:*:***  ***: :  *    ****:*:**::*  *:

Sp-SODS       SNVARGIRPNLSDYVVTRMHTGATPTFMLNTQIANGLELGPGLLFDRRVNALMELARTVV    240
PchlO6_6044   AHVQRGVLSNLSEYCNTRMYSGASLSYIHLNHIVAQLDFSSDMLADRRVYALTEITQVAA    224
              ::* **:  ***:*  ***::**: :::  .:*.  *::. .:* **** ** *:::...

Sp-SODS       NWSSDCYSYFKEAERTADGYNIIDVLMDTHNLSVEAAMAMAFNMQDRMLMRFVELRDEVL    300
PchlO6_6044   NWASDLFAFRKEAARSPDGNNAVNVISREYGCSMHEGFDIANRMLDRMINRFVVLRDEVL    284
              **:** ::: *** *: ** * ::*:   :. *:. .: :* .* ***: *** ******

Sp-SODS       NGPHDKGAEIYIDALEEYTIGGILWCQETQRYRFIDGTTSGRLAYTASGFTRQARGNELS    360
PchlO6_6044   KSEHHPLVPQFIDVLETYIVGCIAWCQIATRYLFVDGTDNGEAVFASPGFTQSS-SDVST    343
              :. *.  .  :**.** * :* * *** : ** *:*** .*. .::: ***:.: .:  :

Sp-SODS       EPIDIPTIAWWWQVGERADQYGRR      384
PchlO6_6044   EPLPIPSIAWWWHVGEPTGPSRLS      367
              **: **:*****:*** :.
```

Figure S14: Amino acid alignment of cyclizing farnesyl pyrophosphate methyltransferases (FPP-MT) from *P. chlororaphis* O6 (*Pchl*O6_6045) and *V. boronicumulans* PHE5-4 (*AO062*_27010), which convert FPP (**3a**) into γ-PSPP (**4a**). Overall sequence identity was 69.47 % and sequence similarity was 78.50 %. Amino acid residues identified as part of the active site in *Sp*-FPP-MT from *S. plymuthica* 4Rx13 (which converts FPP (**3a**) into α-PSPP (**2a**)) [(1)] are marked in grey when conserved and marked in yellow when different. Alignment was performed using Clustal Omega with default settings.[(2)]

```
Pc-FPP-MT       MNQPTIDHNHPLQPVTVRHGRALPYHVIRGAEVSGYEGKVVYTYQDDPEDWRKAIGDRLM   60
Vb-FPP-MT       MSTAT--------ARRIDAPAPPSLQVIRGAEANPYHAKVEYTYQDDPEDWRKAIGEDLM   52
                *.  *           :         :******.. *.:** ***************: **

Pc-FPP-MT       FQFGVYDDPRSRPPISPDESGLRYFDRQMELAGYGRGDFGPVKRILDVGCGWGFGLKYLA  120
Vb-FPP-MT       FQFGVYDDPRSVPPISLNESGLRYFDQQMEIAGLDQPPPFPIRRILDVGCGWGFGLRYLA  112
                *********** **** :********:***:** .:     *::**************:***

Pc-FPP-MT       DRFPACPRLDGINISARQLEYCAKYHAEHRLSERINLYLCNAQDVDLLPHADEPYDLVTV  180
Vb-FPP-MT       EHFPQCRQLDGINISETQLAHCARYHAQQHLSDRVNLYLCDAQDVALLPNPDTPYDLVTI  172
                ::** * :*******  ** :**:***:::**:*:*****:**** ***: * ******:

Pc-FPP-MT       RGVISHFPNDLYERSMAKLASRLRPGATVIISDNLYNLPLEQYRSDTADDVDRLACKHRK  240
Vb-FPP-MT       RGVISHFPNDLYERSMKQVAARMRPGAKVVISDNLYNIPLDNYRSDIEDEVDRLACKHRK  232
                **************** ::*:*:****.*:********:**::****  *:**********

Pc-FPP-MT       TPGYFRQVLEQCGLNVEDMRVLPENIDVARWFMDVKKNIETHFTPDTIPPPLEELRVMAV  300
Vb-FPP-MT       TPAYFRRVLEESGFVVEDMRVLPRNIDVARWFTDVKENIEKNFTPDTIPPPLEELRVMAV  292
                **.***:***:.*: ********.******** ***:***.:******************

Pc-FPP-MT       NVSVALIKDQFSTYSVIARHP      321
Vb-FPP-MT       SLSVALIKNKFSTYSVIARRL      313
                .:******::*********:
```

Figure S15: Amino acid alignment of γ-presodorifen pyrophosphate methyltransferases (γ-PSPP-MT) from *P. chlororaphis* O6 (*Pchl*O6_6042) and *V. boronicumulans* PHE5-4 (*AO062*_27015), which convert γ-PSPP (**4a**) into α-PCPP (**5a**). Overall sequence identity was 73.29 % and sequence similarity was 82.74 %. Alignment was performed using Clustal Omega with default settings.[2]

```
Pc-γ-PSPP-MT     --MQVHKSAIEVIHSSTADHYQSKIESVYADPPEEWRKVIGNEFWYQYGVFDEKMDPSRL   58
Vb-γ-PSPP-MT     MQASAQAVGIDVIHSRSLDHYQDKIEQVYADTPDQWRKAIGNELWYQYGVFDEKMDPHDL   60
                    ..:  .*:**** : ****.***.**** *::***.****:*************  *

Pc-γ-PSPP-MT     PLDASGRRHMEYQFELAEQAGADLSSQSIRRAIDIGCGWGPVLSFLAERYPHCERIDGVN  118
Vb-γ-PSPP-MT     PLDASGRRHMEHQFELAEQAGADLSPATVRRAIDIGCGWGPVLKFIAQRYPQCARIDGVN  120
                 ***********:*************  ::***************.*:*:***:* ******

Pc-γ-PSPP-MT     VSRPQLEYASQVISREGLAARVRLYLCNAKDIGALPDPELPYDLAIFRGSLFHFTPQVLQ  178
Vb-γ-PSPP-MT     VSRPQLECARELIAHEGLADRVRLYQCNAKDIGELPDAQVPYDLAILRGSLIHFTPEVLE  180
                 ******* * ::*::**** ***** ******* *** ::******:****:****:**:

Pc-γ-PSPP-MT     ETMQSLAQRMRPGGTVVISESLYKVDLATYQSFIPDKVDRAASGHRKTPDSLHKALEDNG  238
Vb-γ-PSPP-MT     QAMASLSARMRTGGTVIISESLYKVDLATYKSHIPDKVDRAASGYRKTPDGVKQVLERHG  240
                 ::* **: *** ****:**************:*.***********:*****.::::.** :*

Pc-γ-PSPP-MT     FDVIDRRITPSNEEVIRWYGLVKDNLDAHYPDSRNPNFSELRDIAINFSDALRKDKASSF  298
Vb-γ-PSPP-MT     FAVLDQRVLPSNAEVIRWYGLVKDNLDAHHPTSKTATFTELRDIAVSFSDALLKDKASSF  300
                 * *:*:*: *** ****************:* *:. .*:******:.***** *******

Pc-γ-PSPP-MT     SFIARRR  305
Vb-γ-PSPP-MT     SFIARRT  307
                 ******
```

Figure S16: Amino acid alignment of the chlororaphen synthases (ChloS) from *P. chlororaphis* O6 (*Pchl*O6_6041) and *V. boronicumulans* PHE5-4 (*AO062*_27040), which convert α-PCPP (**5a**) into chlororaphen (**6**). Overall sequence identity was 73.16 % and sequence similarity was 80.83 %. Alignment was performed using Clustal Omega with default settings.[2]

```
Pc-ChloS        MNHSAQALAPEVSFYIPEMYCSVLPRIHPDYPIIDERNASWVREFLPFTDEAAQLRFLRL   60
Vb-ChloS        MNYPDRIVFPEVSFHIPEMFCSVPPKIHTDYPVIDERNAAWGREFLPFSDEATRLKFLRL   60
                **:  : : *****:****:*** *:** ***:******:* ******:***::*:*****

Pc-ChloS        HTPMWDSMIFPIGSADRLVHTSCVTSLITAIDDMPLGRHAMFHDGEVALLEGHPFARAAQ   120
Vb-ChloS        HLPMWDCLLFPIGSADRIFLTSCVTGLILAIDDMPLGRHAVCGDGDVALLEGHPLARAAA   120
                * ****.::********:. *****.** ***********:  **:********:****

Pc-ChloS        DIFGKLRQHMPAPVYRRYCQELQAWFESVEEEARLVAAGKVLPLDEFLELRHPNTGLLPS   180
Vb-ChloS        DIFGRLRQSMSPQVYRRYCLEWKAWFDSVEVEAGLVAEGKVLPYDEFLALRHPNTGLLPY   180
                ****:*** *   ****** * :***:*** ** *** ***** **** **********

Pc-ChloS        FPVAEFLYDLDLTELLAQDRELQLAIRVTNEHVGLVNDILSHRKEHAIGVTLNAMESLRI   240
Vb-ChloS        FPVSEFIYDLDLTELLAQDADLQRAILATNEHVALVNDLLSHHKEHAVGVTLNAMESLRM   240
                ***:**:************ :** ** .*****.****:***:****:***********:

Pc-ChloS        VHGHSAQEAADILCQRIREADRARVELCEVLRHRYANRPDADRIGMYLDGLGRICAGNLR   300
Vb-ChloS        AHGRSPQEAADILCQRIREADRTRVDACELLRRRYAHRPDADRFGLYLDSFGLMCAGNLR   300
                .**:* *****************:**: **:**:***:******:*:***.:* :******

Pc-ChloS        WLLESDRYVDSRGNGWDWTRSRLIVLDPEPAPAPALAQS       339
Vb-ChloS        WILENDRYVDSRGNGWDWTRSRHVVLDRP----------       329
                *:**.***************** :***
```

Experimental Procedures

Heterologous gene expression in *E. coli*

The *Pseudomonas chlororaphis* O6 methyltransferase (*Pc*-γ-PSPP-MT, *Pchl*O6_*6042*; *Pc*-FPP-MT, *Pchl*O6_*6045*) and terpene synthase genes (*Pc*-TPS1 (*Pc*-ChloS), *Pchl*O6_*6041*; *Pc*-TPS2, *Pchl*O6_*6044*) were obtained from genomic DNA. The *Variovorax boronicumulans* PHE5-4 methyltransferase (*Vb*-FPP-MT, *AO062_27010*; *Vb*-γ-PSPP-MT, *AO062_27015*) and terpene synthase gene (*Vb*-TPS (*Vb*-ChloS), *AO062_27040*) were codon-optimized for *E. coli* and synthesized by GENEWIZ Germany GmbH (Leipzig, Germany). Amplification of all genes and linearization of the expression vector was carried out with the Q5® High-Fidelity DNA Polymerase (New England Biolabs GmbH, Frankfurt am Main, Germany) according to the manufacturer's instructions using specific primers (Table S1). All genes were directionally cloned into the pE-SUMOstar Kan vector (LifeSensors, Malvern, PA, USA) by Gibson assembly (NEBuilder® HiFi DNA Assembly, New England Biolabs, Frankfurt am Main, Germany). Recombinant vectors were transformed into *E. coli* XL1-blue. Plasmids were checked for correct insertion by colony PCR using T7 primers, reisolated, sequenced, and finally transformed into *E. coli* BL21(DE3). For heterologous expression, positive transformants of *E. coli* BL21(DE3) were grown overnight at 37 °C in 10 mL LB liquid medium (8.0 g/L tryptone, 4.0 g/L yeast extract, 5.0 g/L NaCl) supplemented with 50 µg/mL kanamycin. Main cultures were prepared in 200 mL LB liquid medium supplemented with kanamycin (50 µg/mL) and 3 mM glucose and inoculated with 2 mL from the overnight cultures. Cultures were incubated at 37 °C and 170 rpm until an OD_{600} of 0.8-1.0 was reached followed by induction of gene expression by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Finally, cultures were incubated for 20 h at 20 °C and 170 rpm before cells were harvested by centrifugation (10.000 xg, 15 min, 4 °C). Cell lysis and purification of the respective proteins was performed as previously described.[3,4] Protein purity was confirmed by SDS-PAGE and the isolated proteins were stored at -70 °C.

Table S1: Sequences of primers used in this study. Small letters indicate overhangs complementary to the cloning site of the vector pE-SUMOstar Kan. Capital letters represent parts of the primers binding to the target gene.

name	sequence (5‘ → 3‘)	function
T7_fwd	TAATACGACTCACTATAGGG	verification of correct insertion
T7_rev	CCGCTGAGCAATAACTAGC	
SUMO_fwd	GATCCGGCTGCTAACAAAG	amplification of pE-SUMOstar Kan
SUMO_rev	ACCTCCAATCTGTTCGCG	
6045_fwd	accgcgaacagattggaggtAACCAGCCCACCATCGATC	amplification of *Pc*-FPP-MT
6045_rev	gctttgttagcagccggatcTCAGGGGTGTCGCGCAAT	
6042_fwd	accgcgaacagattggaggtCAGGTACACAAGAGTGCGATTGAGGTC	amplification of *Pc*-γPSPP-MT
6042_rev	gctttgttagcagccggatcCTAGCGCCTGCGCGCGAT	
6041_fwd	accgcgaacagattggaggtAACCATTCTGCACAGGCCCTC	amplification of *Pc*-TPS1 (*Pc*-ChloS)
6041_rev	gctttgttagcagccggatcTCAGCTCTGCGCGAGGGC	
6044_fwd	accgcgaacagattggaggtGAAACGCCTTCAGTCATTC	amplification of *Pc*-TPS2
6044_rev	gctttgttagcagccggatcTCATGACAATCGGGAAGG	
Vb_27010_fwd	accgcgaacagattggaggtAGTACCGCGACCGCGCGC	amplification of *Vb*-FPP-MT
Vb_27010_rev	gctttgttagcagccggatcTTACAGGCGGCGCGCAATCAC	
Vb_27015_fwd	accgcgaacagattggaggtCAAGCGAGCGCGCAAGCG	amplification of *Vb*-γ-PSPP-MT
Vb_27015_rev	gctttgttagcagccggatcTTAGGTGCGGCGCGCAATAAAG	
Vb_27040_fwd	accgcgaacagattggaggtAACTATCCGGATCGCATTGTG	amplification of *Vb*-ChloS
Vb_27040_rev	gctttgttagcagccggatcTTACGGGCGATCCAGCAC	
6041-6045_fwd	accgcgaacagattggaggtATGAACCAGCCCACCATCGATC	amplification of *PchlO6_6041-6045*
6041-6045_rev	gctttgttagcagccggatcTCAGCTCTGCGCGAGGGC	
6042-6045_fwd	accgcgaacagattggaggtATGAACCAGCCCACCATCGATCATAAC	amplification of *PchlO6_6042-6045*
6042-6045_rev	gctttgttagcagccggatcCTAGCGCCTGCGCGCGAT	

In vitro enzyme assays

To functionally characterize the enzymatic activities of the purified proteins, different enzyme assays were performed *in vitro*. Investigation of methyltransferase activities was performed in coupled enzyme assays containing 20 µg of purified methyltransferase enzyme, 20 µL TRIS buffer (50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 100 µg/mL BSA, pH 7.9), 30 mM dithiothreitol, 2.3 mM of *S*-adenosyl methionine (Merck Sigma-Aldrich, Darmstadt, Germany), 0.06 mM of farnesyl pyrophosphate (FPP, **3a**, Echelon Biosciences, Salt Lake City, USA), and double distilled water (ad 190 µL). All constituents were mixed and incubated at 37 °C for 3 h 30 min. Afterwards, 10 U of shrimp alkaline phosphatase (New England Biolabs, Frankfurt am Main, Germany) was added and incubation continued for 1 h at 37 °C. Extraction of reaction products was conducted by addition of 200 µL hexane (containing 5 ng/µL nonyl acetate as internal standard) followed by 30 s vigorous shaking and centrifugation (2 min, 4000 g). Finally, the hexane phase was collected, and reaction products analyzed by GC-EIMS.

Combined methyltransferase (MT) and terpene synthase (TPS) activities were investigated in double (MT + TPS) and triple enzyme assays (FPP-MT + γ-PSPP-MT + TPS), respectively. 20 µg of each investigated enzyme were added to 50 µL HEPES buffer (250 mM HEPES–KOH, 100 mM $MgCl_2$, 2.5 mM $MnCl_2$, 50% (*v/v*) glycerol, pH 8), 30 mM dithiothreitol, 2.3 mM of *S*-adenosyl methionine (Merck Sigma-Aldrich, Darmstadt, Germany), 0.06 mM of farnesyl pyrophosphate (FPP, **3a**, Echelon Biosciences, Salt Lake City, USA), and double distilled water (ad 200 µL). After incubation for 3 h 30 min at 37 °C, reaction products were extracted with hexane as described above. To evaluate terpene synthase activity alone, single enzyme assays were prepared according to the double and triple enzyme assays without addition of purified methyltransferases.

Pathway cloning in *E. coli*

Different parts of the chlororaphen biosynthetic operon were cloned into pE-SUMOstar Kan and subsequently expressed in *E. coli* followed by collection of the produced volatile compounds. For γ-PSOH (**4b**) accumulation pE-SUMOstar Kan + *Pchl*O6_*6045* was used. α-PCOH (**5b**) synthesis was achieved by simultaneous cloning of the genes *Pchl*O6_*6042-Pchl*O6_*6045* into pE-SUMOstar Kan. Finally, chlororaphen (**6**) synthesis was achieved by direct cloning of the genes *Pchl*O6_*6041-Pchl*O6_*6045* in pE-SUMOstar Kan. Cloning was performed via Gibson Assembly as described above and generated plasmids were checked for correct insertion via colony PCR and sequencing.

Collection of volatiles

Heterologous production of γ-presodorifenol (γ-PSOH, **4b**), α-prechlororaphenol (α-PCOH, **5b**), and chlororaphen (**6**) was performed in *E. coli* BL21(DE3) as a production host. For this purpose, overnight and main cultures were performed as described above. Following induction with 1 mM IPTG cultures were split and 100 mL each were transferred into a VOC collection system as previously described [5] and incubated at 30 °C and 250 rpm. Volatiles collected on Porapak Q (80-100 mesh, Sigma-Aldrich, St. Louis, Missouri, USA) were eluted with C_6D_6 (99.96 % D, Deutero GmbH, Kastellaun, Germany), after 24 h (for chlororaphen (**6**) sampling) and 48 h (for sampling of **4b** and **5b**), respectively. Eluted samples were pooled for NMR analysis, and an aliquot was treated with *cis*-nerolidol as internal standard (final concentration 5 ng/µL) and analyzed by GC-EIMS.

GC-EIMS analysis

Hexane extracts of the enzyme assays and volatile compound eluates were analyzed by GC-EIMS using a GCMS-QP2010 Se system with a AOC-5000 PAL autosampler (Shimadzu Deutschland GmbH, Duisburg, Germany) equipped with a DB5-MS column (60 m x 0.25 mm x 0.25 µm; J&W Scientific, Folsom, California, USA). Samples of 1 µL were injected at 250 °C using either splitless mode for enzyme assays or 1:10 split ratio for volatile eluates. Helium was used as carrier gas at a flow rate of 1.1 mL/min. A temperature gradient was applied by starting from 35 °C for 2 min followed by an increase of 10 °C/min to 280 °C within 24.5 min, followed by 15 min at 280 °C. Electron ionization at 70 eV was used. Mass spectra were obtained using the scan mode (with *m/z* 40–280). Data analysis was performed with the Lab Solutions software (Shimadzu Deutschland GmbH, Duisburg, Germany). Kovats retention indices for the new compounds are shown in table S2.

Table S2: Kovats retention indices for γ-presodorifenol (γ-PSOH, **4b**), α-prechlororaphenol (α-PCOH, **5b**), and chlororaphen (**6**).

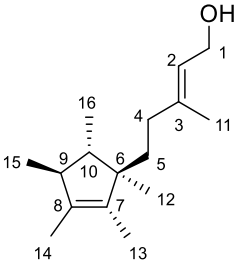
#	name	KI
4b	γ-PSOH	1773
5b	α-PCOH	1791
6	chlororaphen	1506

NMR spectroscopy

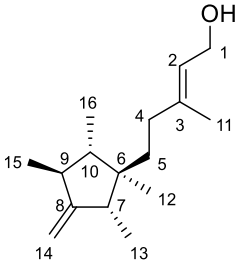
VOCs collected on Porapak Q were eluted with C_6D_6 (99.96% D) and the resulting solutions were concentrated to a total volume of 0.5 mL using a stream of nitrogen. NMR spectra of crude VOC mixtures were recorded using a 600 MHz Bruker Avance Neo Ascend instrument equipped with a 5 mm broad band inverse (BBI) probe. ^{1}H NMR, ^{13}C NMR, *dqf*-COSY, HSQC, HMBC, NOESY. High resolution *dqf*-COSY spectra were recorded using phase cycling for coherence selection with a time domain of 8192 data points in F2 and 512 increments in F1. Heteronuclear HSQC and HMBC spectra were recorded using non-uniform sampling with a sampling rate of 50% by acquiring 512 scans. NOESY spectra (SW 6 ppm, 2048 in F2, 512 in F1, NS 32 were recorded using a mixing time of 0.7 s. NMR data of **4b**, **5b**, and **6** were recorded in C_6D_6 in the presence of approximately 100 equivalents of 1*H*-indole. 2D spectra were manually phased, zero-filled, using the MestreNova 14.2 software.

Table S3: NMR data of chlororaphen (**6**) and the hydrolysis products of its monocyclic biosynthetic intermediates γ-presodorifenol (**4b**) and α-prechlororaphenol (**5b**) from *P. chlororaphis* O6, along with α-presodorifenol (**2b**) from *S. plymuthica* 4Rx13 for comparison.[3] (chemical shift (δ) in [ppm], multiplicity, coupling constant (*J*) in [Hz], in C_6D_6 with 99.96% D, **4b**, **5b**, **6** recorded in the presence of approximately 100 equivalents of 1*H*-indole).

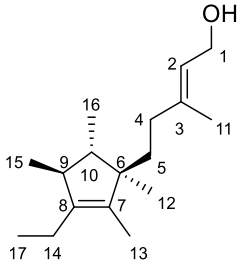
	α-Presodorifenol		**γ-Presodorifenol**		**α-Prechlororaphenol**		**Chlororaphen**	
	α-PSOH (2b)		**γ-PSOH (4b)**		**α-PCOH (5b)**		**(6)**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**
1	3.99 *d* 6.5	59.5	3.98 *d* 6.7	59.1	3.98 *d* 6.7	59.1	-	55.9
2	5.41 *t* 6.6	124.3	5.38 *tq* 6.6, 1.8	123.9	5.40 *t* 6.8	123.9	-	163.6
3	-	139.3	-	138.5	-	139.1	-	57.1
4	1.94 *m* 1.76 *m*	35.1	1.82 *m*	33.6	1.96 *m* 1.76 *m*	34.6	1.49 *dq* 7.6, 6.9	45.0
5	1.42 *m*	36.7	1.32 *m*	35.2	1.42 *m*	35.9	0.75 *ddd* 10.0, 7.5, 5.5	47.5
6	-	50.7	-	44.4	-	50.2	-	57.6
7	-	137.1	2.23 *m*	44.6	-	136.5	-	55.6
8	-	134.4	-	160.9	-	140.0	1.43 *ddd* 13.1, 9.4, 7.5 1.10 *m*	29.1
9	2.01 *m*	48.3	1.97 *m*	43.0	2.13 *m*	45.2	1.64 *m* 1.18 *m*	39.0
10	1.51 *m*	46.5	1.22 *dq* 6.9, 10.3	46.5	1.49 *m*	45.9	0.92 *s*	12.8
11	1.50 *s*	16.5	1.48 *s*	15.9	1.49 *s*	15.9	4.91 *s* 4.57 *s*	100.4
12	0.79 *s*	19.9	0.53 *s*	14.9	0.78 *s*	19.5	0.84 *s*	11.2
13	1.49 *s*	10.3	0.93 *d* 6.8	11.4	1.50 *s*	9.5	0.891 *d* 6.8	14.6
14	1.52 *s*	12.1	4.97 *t* 2.7 4.93 *t* 2.7	103.6	2.10 *dq* 15.4, 7.6 1.94 *dq* 15.6, 7.5	19.1	1.42 *m* 1.13 *m*	26.3
15	1.02 *d* 6.8	17.7	1.12 *d* 6.9	19.1	1.03 *d* 7.6	16.9	0.896 *t* 7.2	13.8
16	0.94 *d* 7.0	13.1	0.79 *d* 6.8	11.7	0.94 *d* 7.0	12.4	0.66 *s*	9.1
17	-	-	-	-	0.92 *t* 7.5	14.0	0.76 *s*	14.1



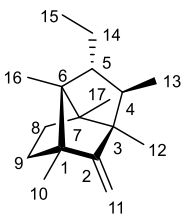
α-PSOH (**2b**)



γ-PSOH (**4b**)



α-PCOH (**5b**)



Chlororaphen (**6**)

Figure S17: ^{1}H NMR spectrum of VOCs from *E. coli* expressing *Pc*-FPP-MT (*Pchl*O6_6045) (in C_6D_6).

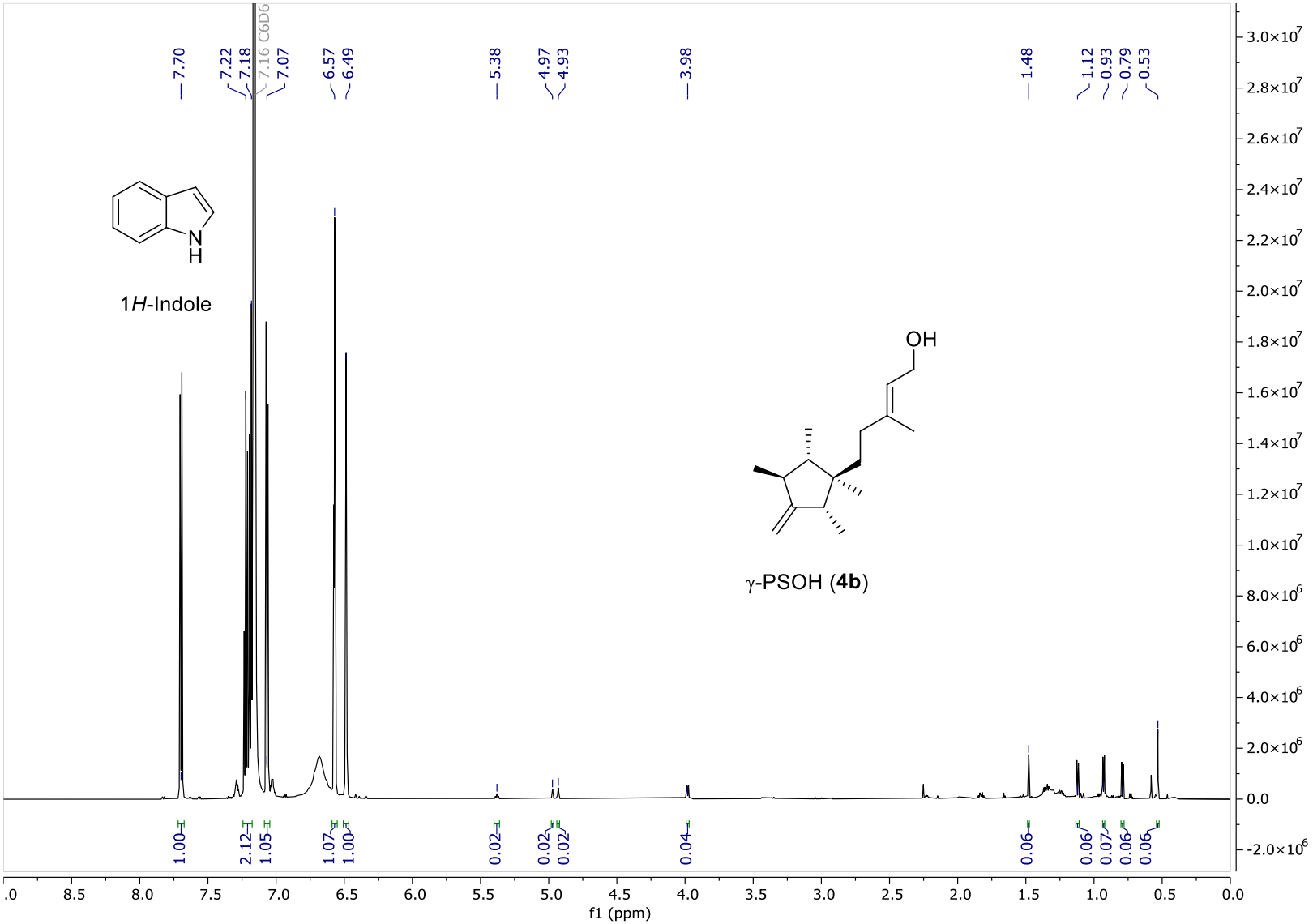


Figure S18: ^{1}H NMR spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

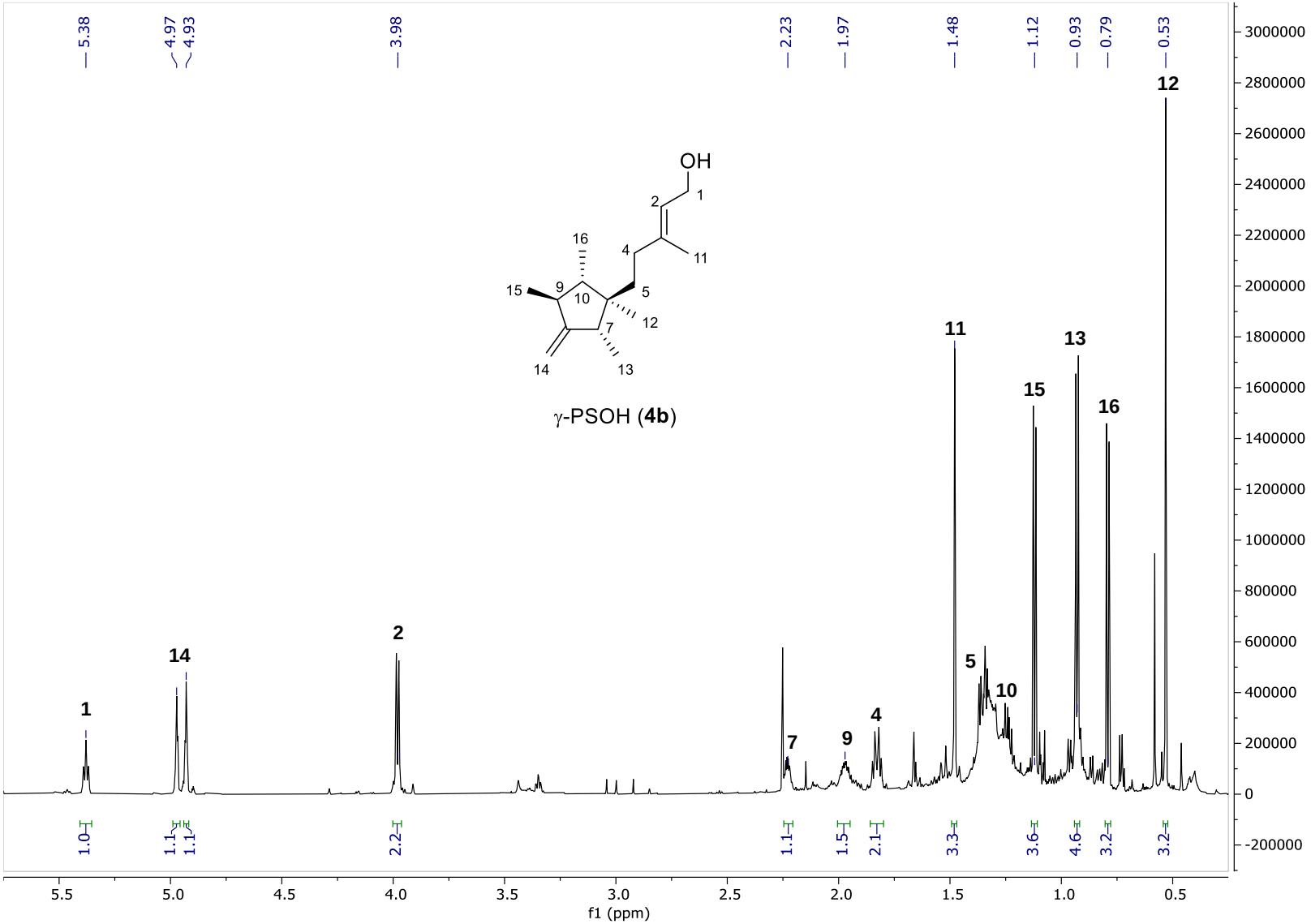


Figure S19: *dqf*-COSY spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

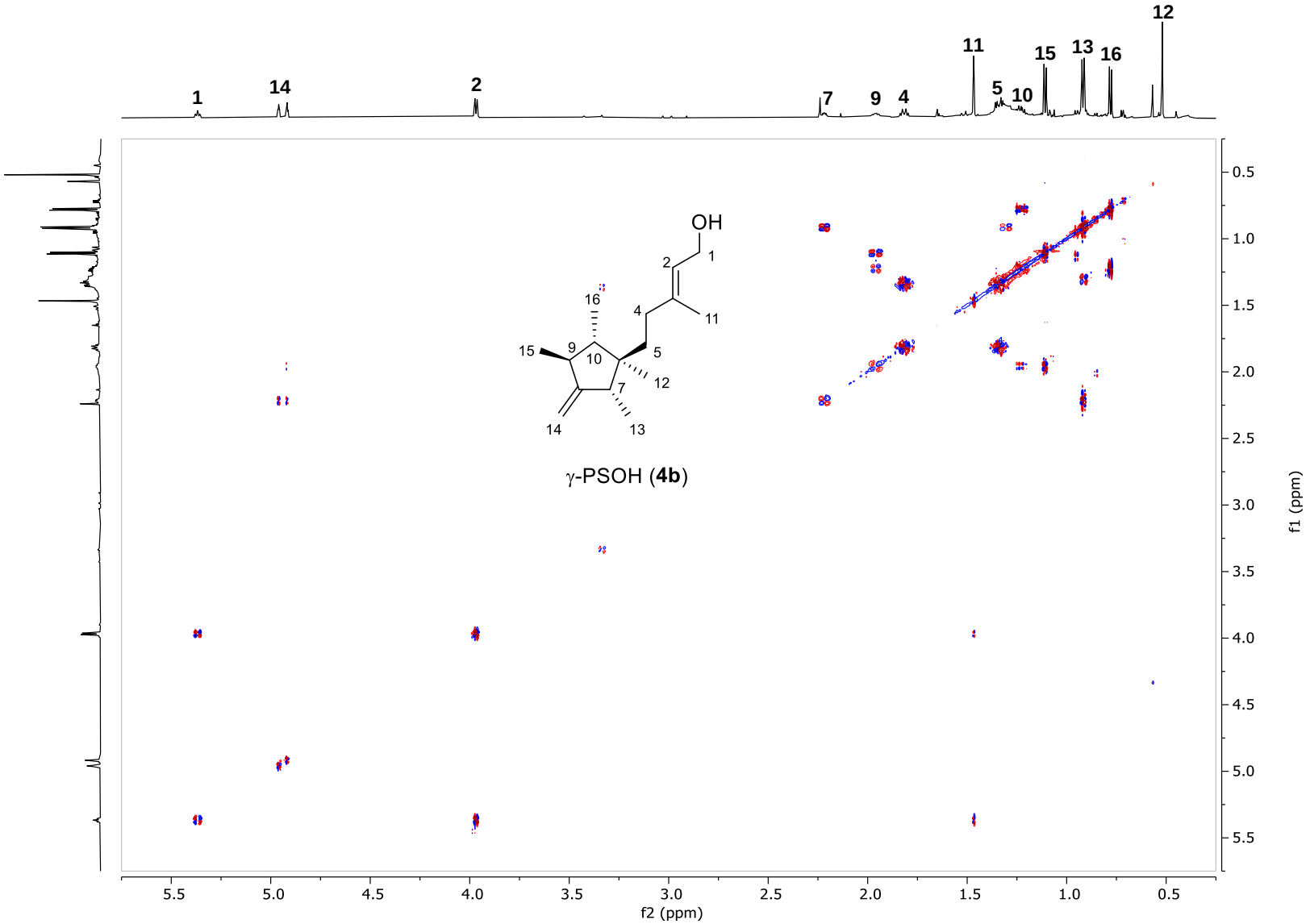


Figure S20: *dqf*-COSY spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

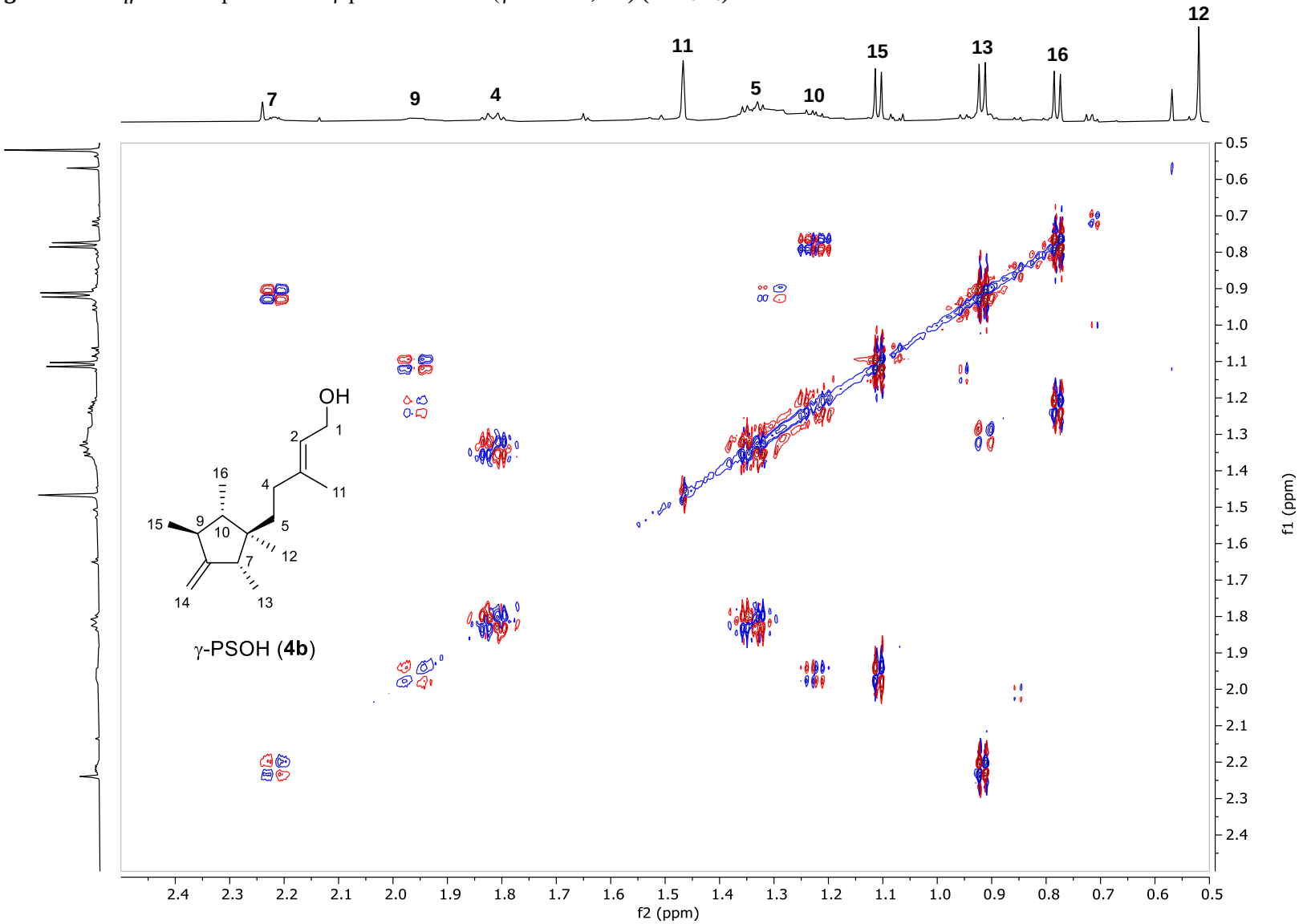


Figure S21: NOESY spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

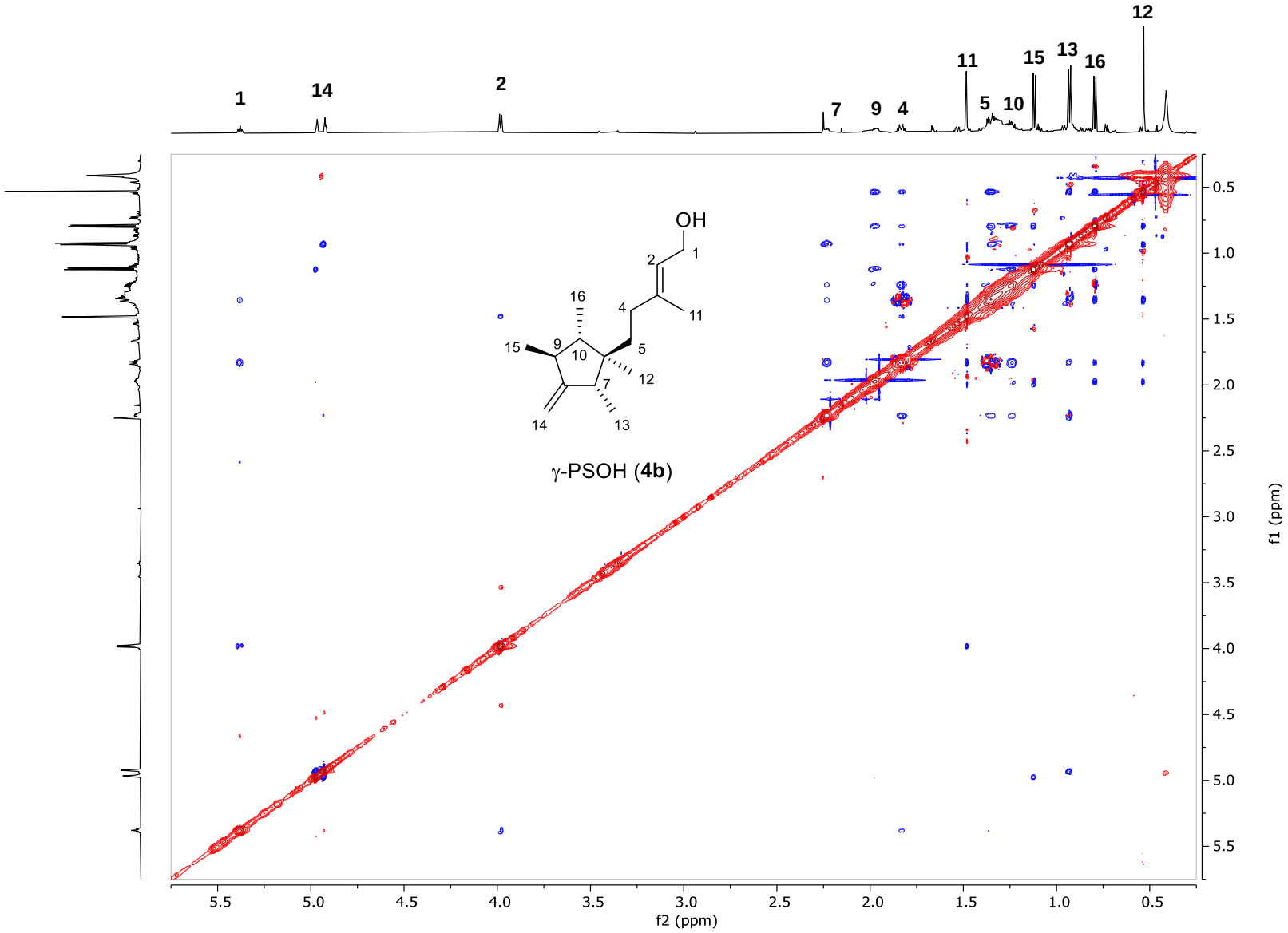


Figure S22: NOESY spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

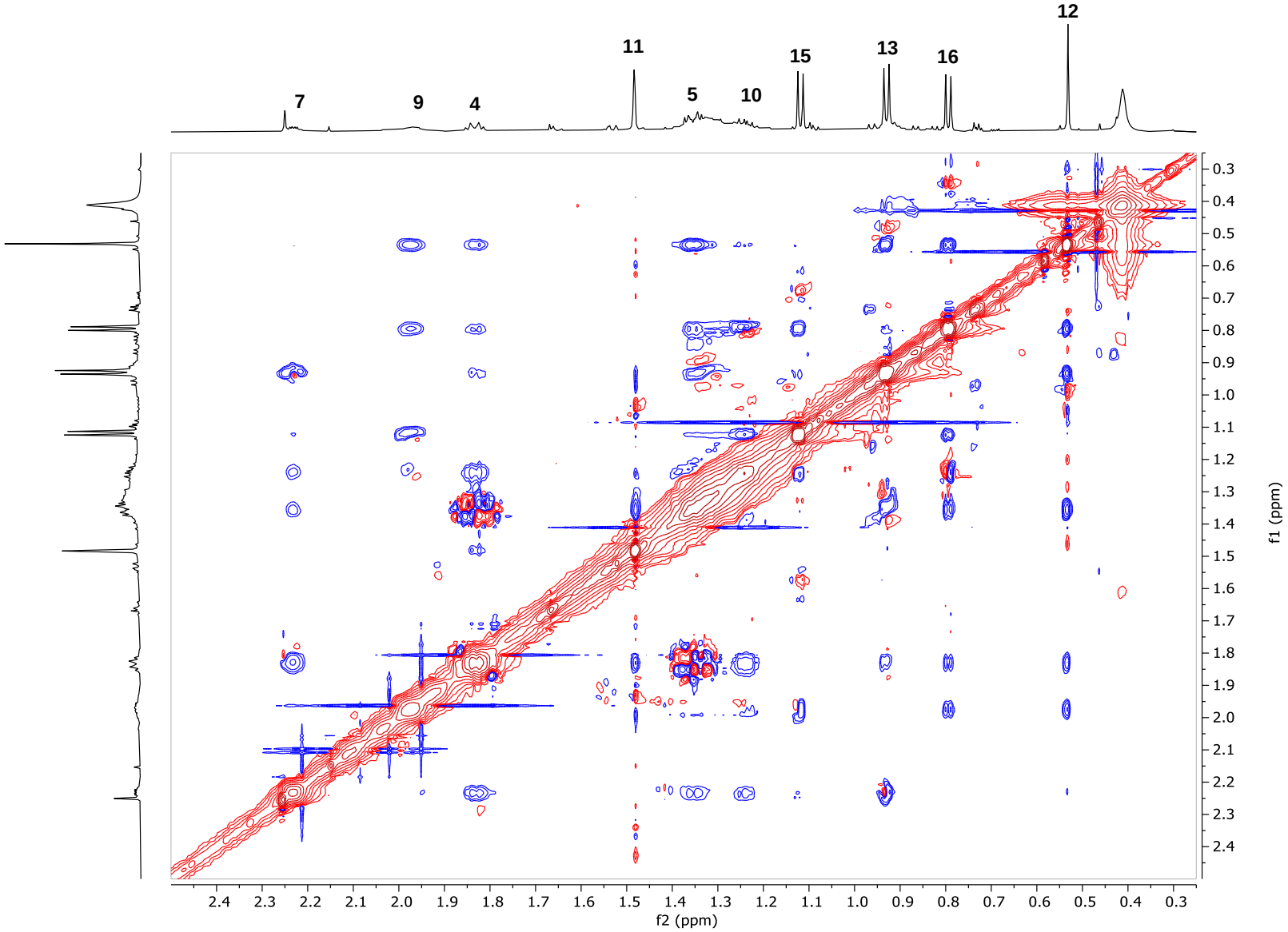


Figure S23: HSQC spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

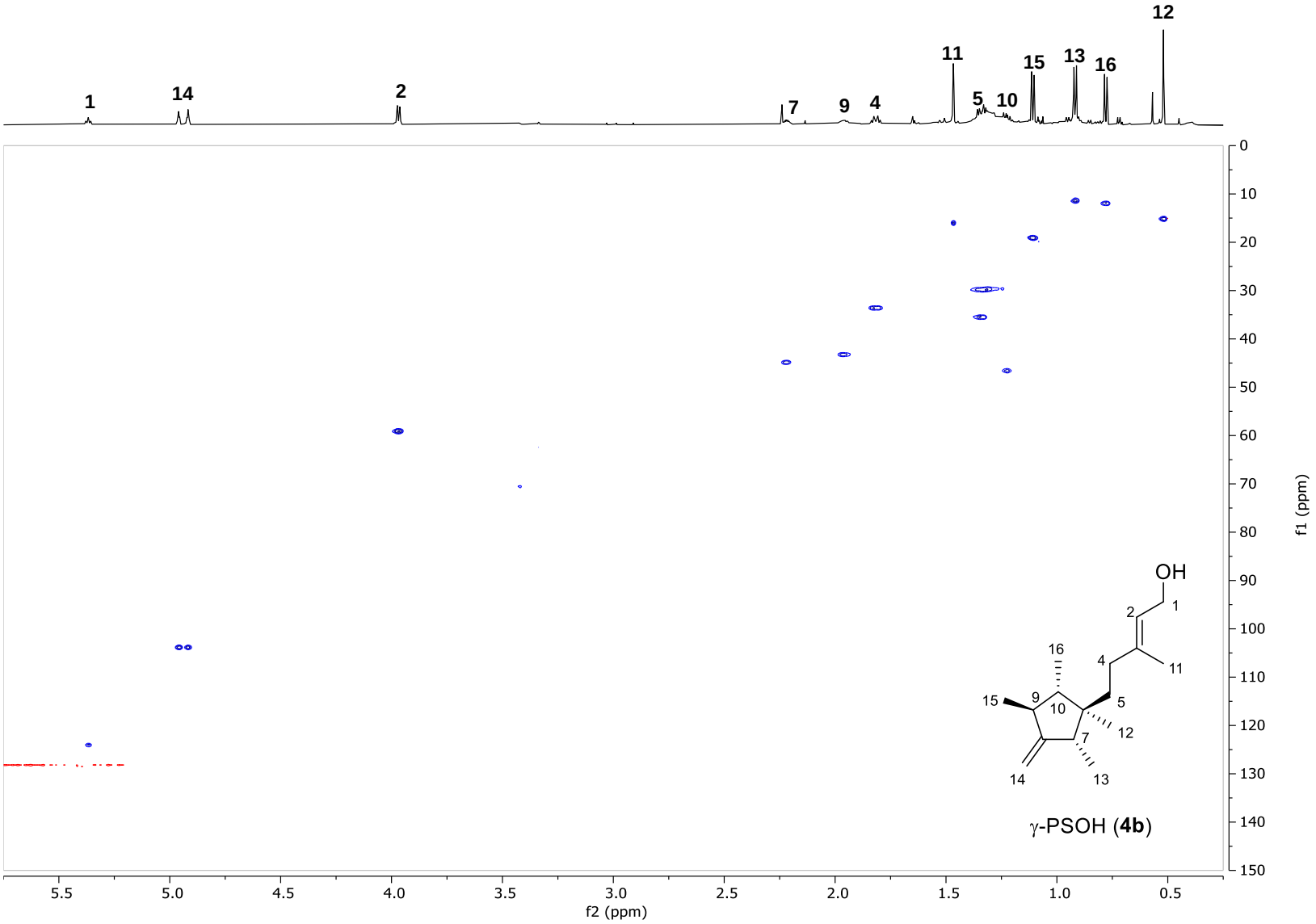


Figure S24: HMBC spectrum of γ-presodorifenol (γ-PSPOH, **4b**) (in C_6D_6).

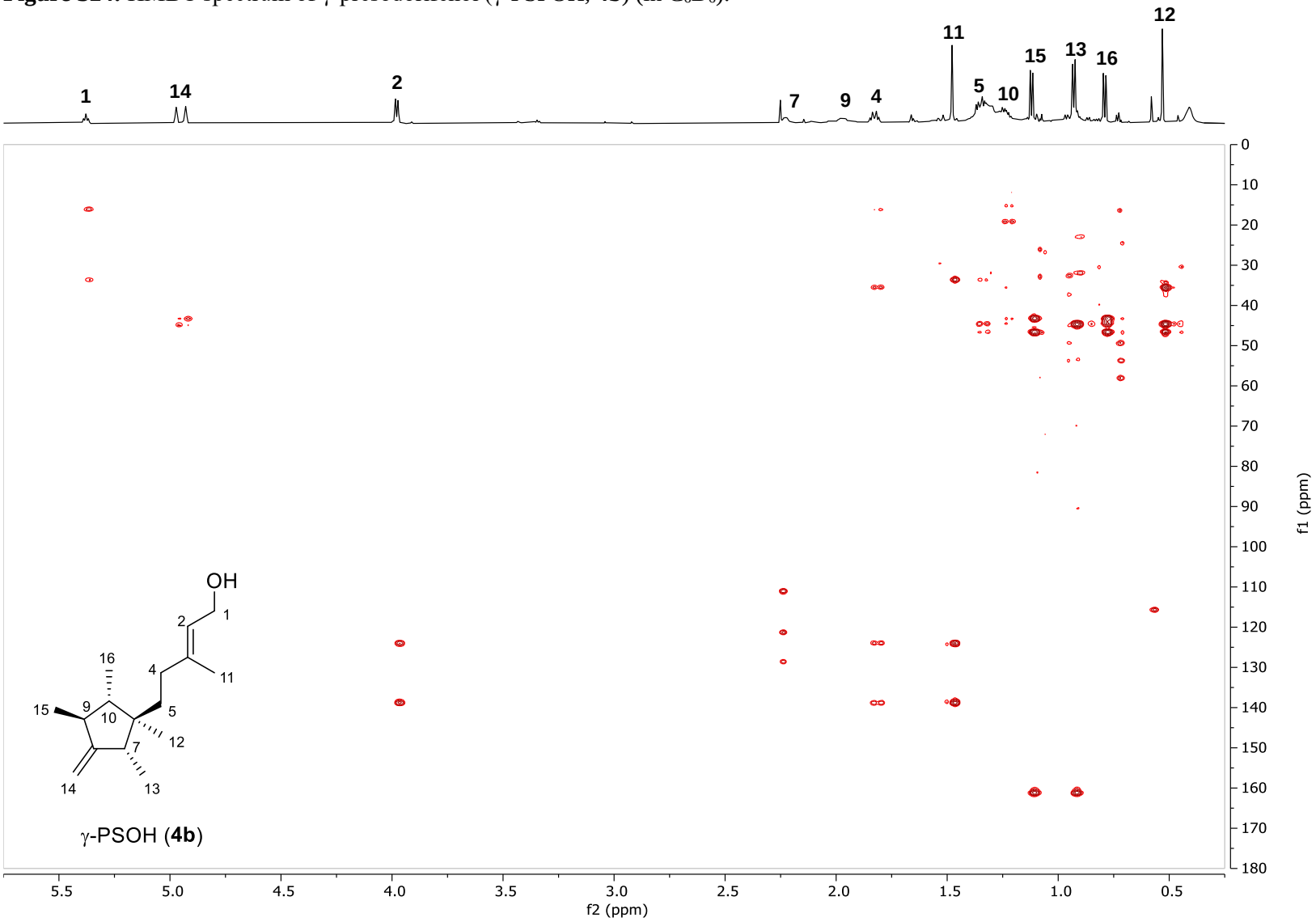


Figure S25: ^{1}H NMR spectrum of VOCs from *E. coli* expressing *Pc*-FPP-MT (*Pchl*O6_6045) and *Pc*-γ-PSPP-MT (*Pchl*O6_6042) (in C_6D_6).

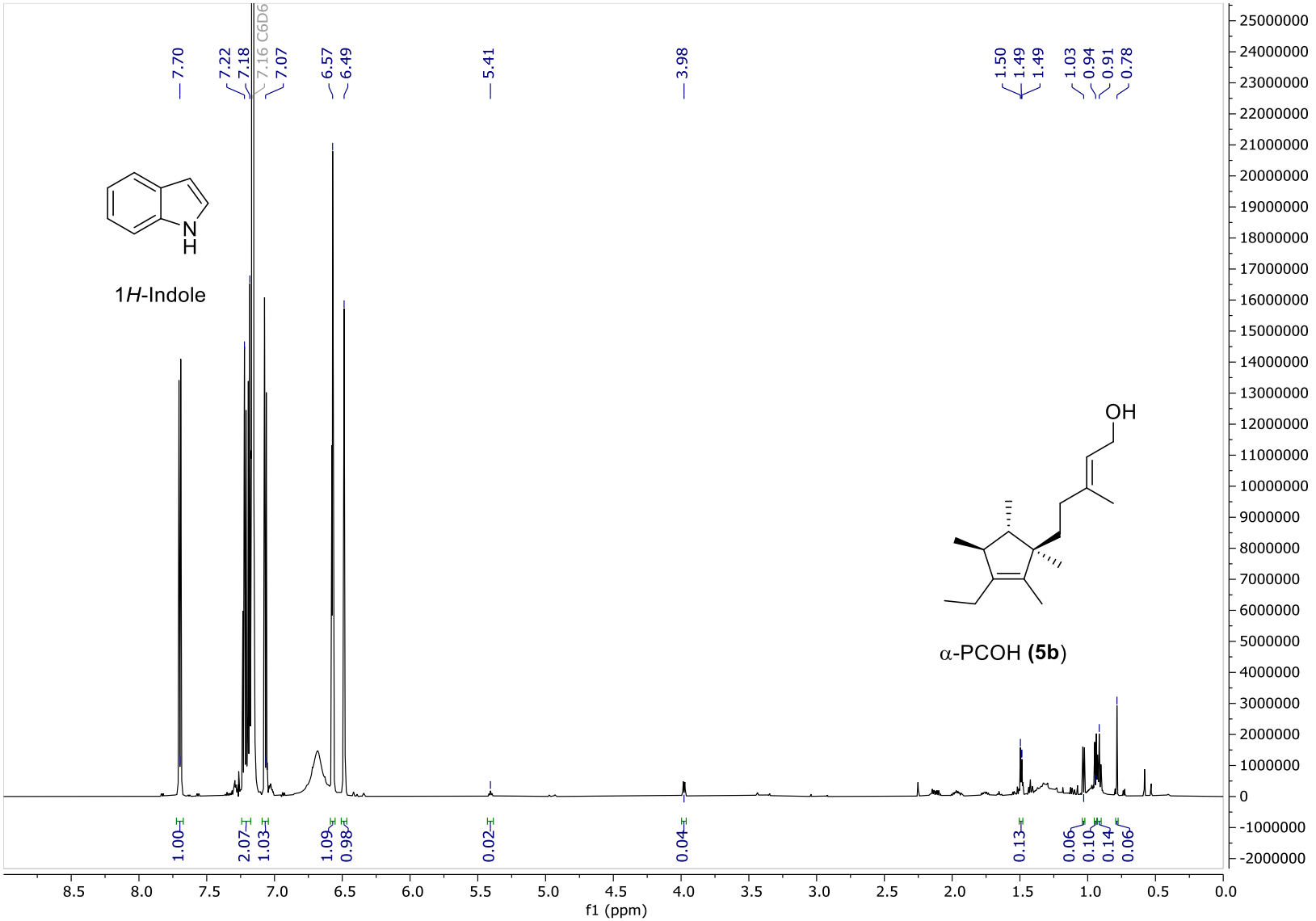


Figure S26: ^{1}H NMR spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

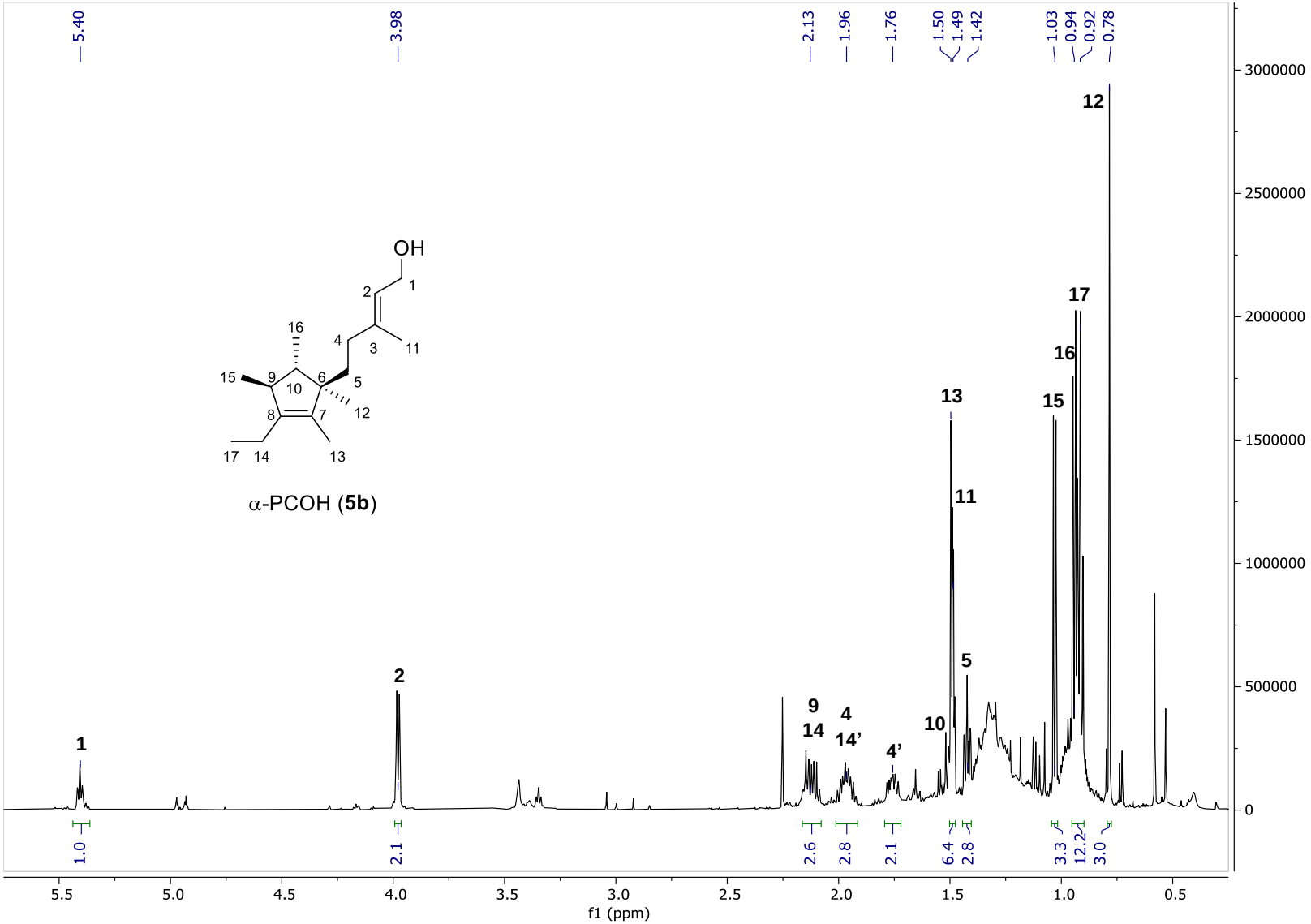


Figure S27: *dqf*-COSY spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

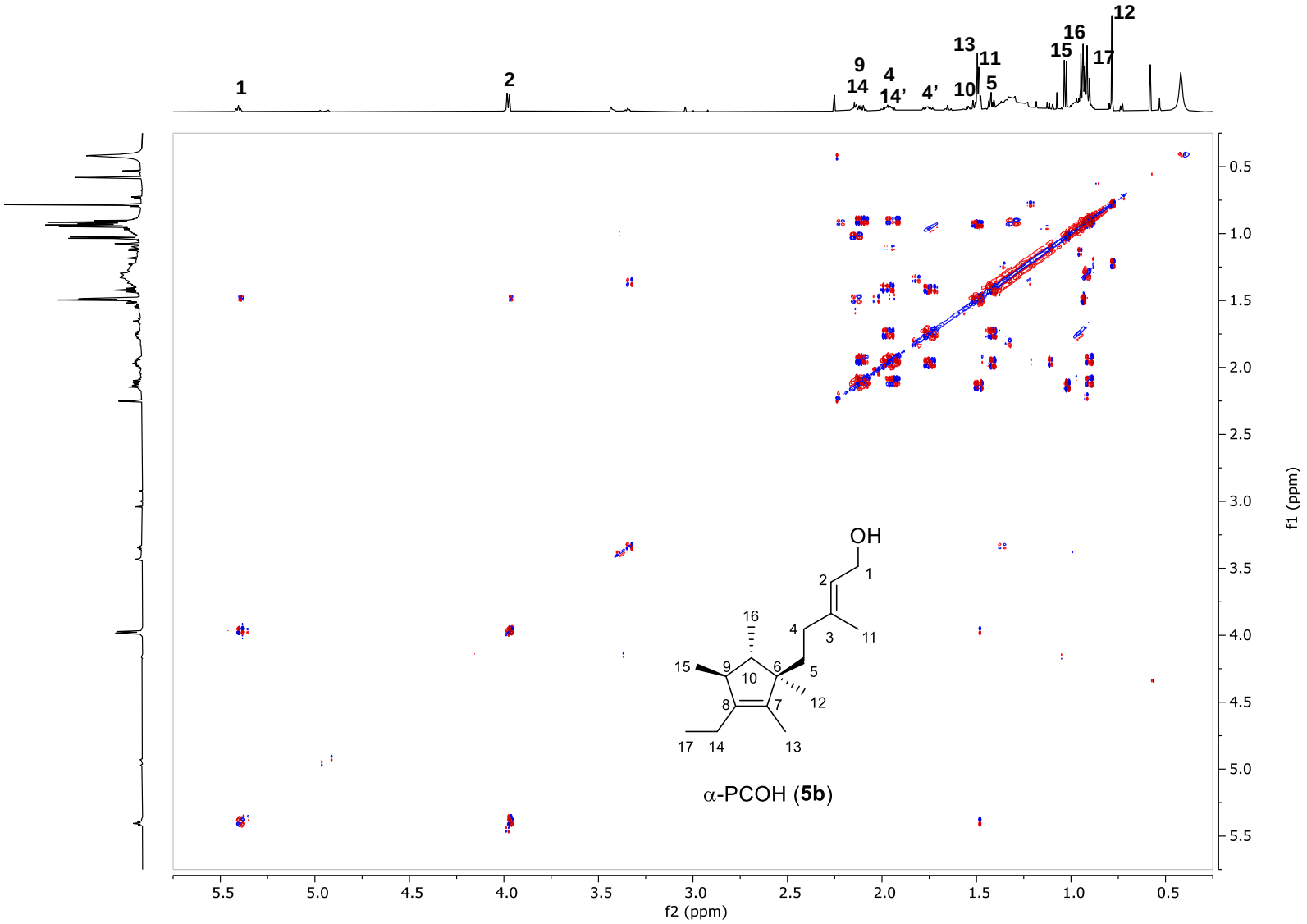


Figure S28: *dqf*-COSY spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

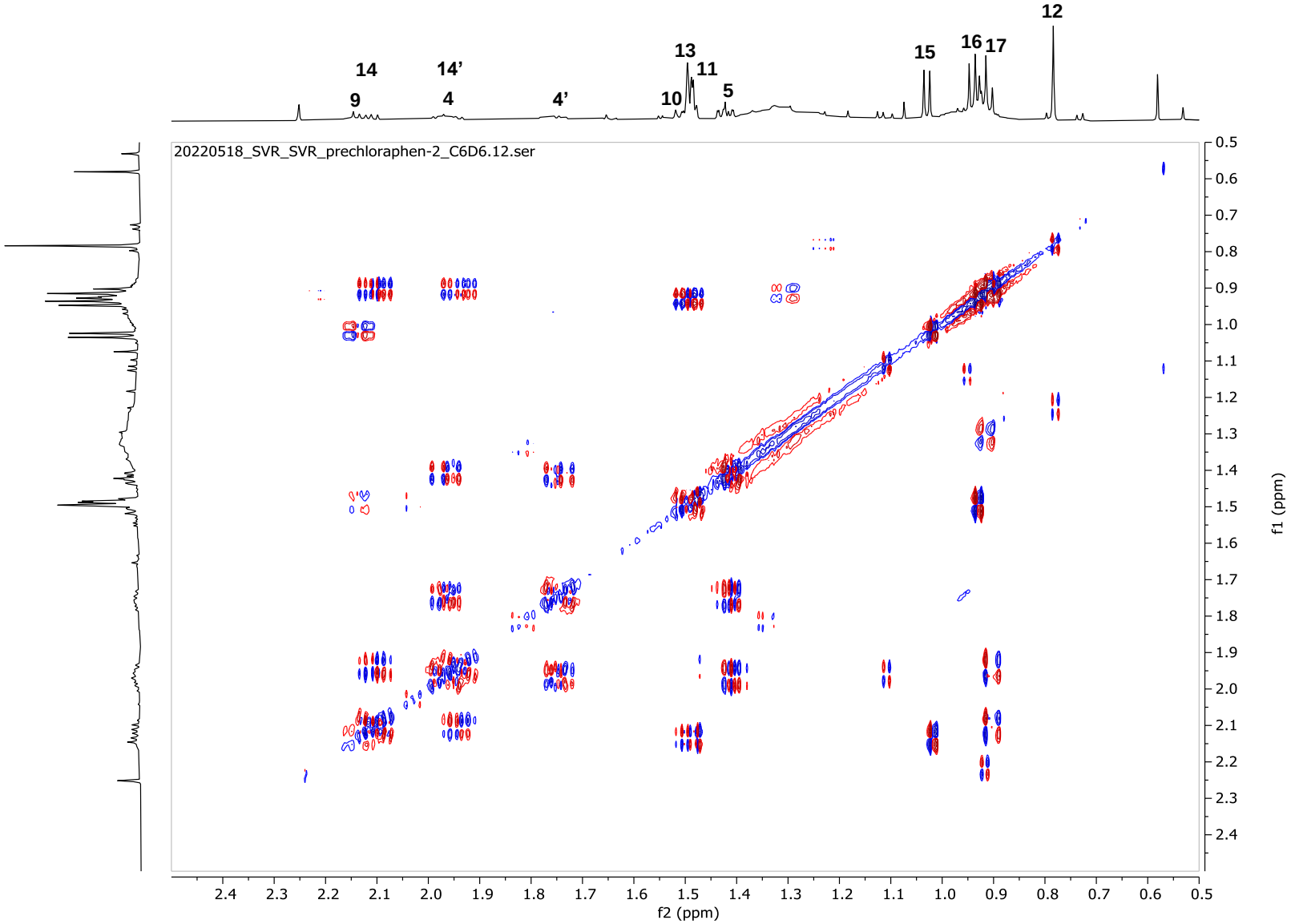


Figure S29: NOESY spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

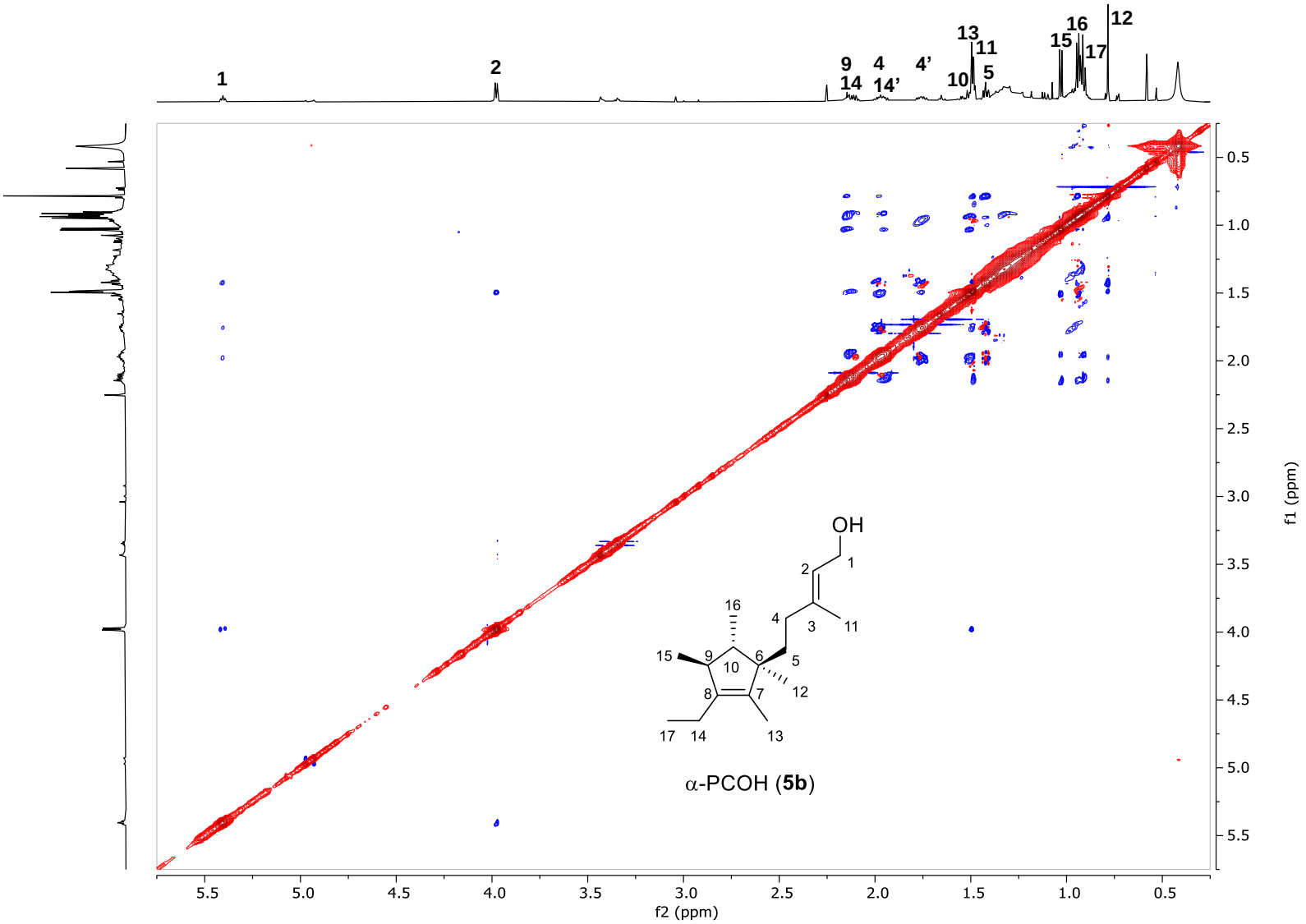


Figure S30: NOESY spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

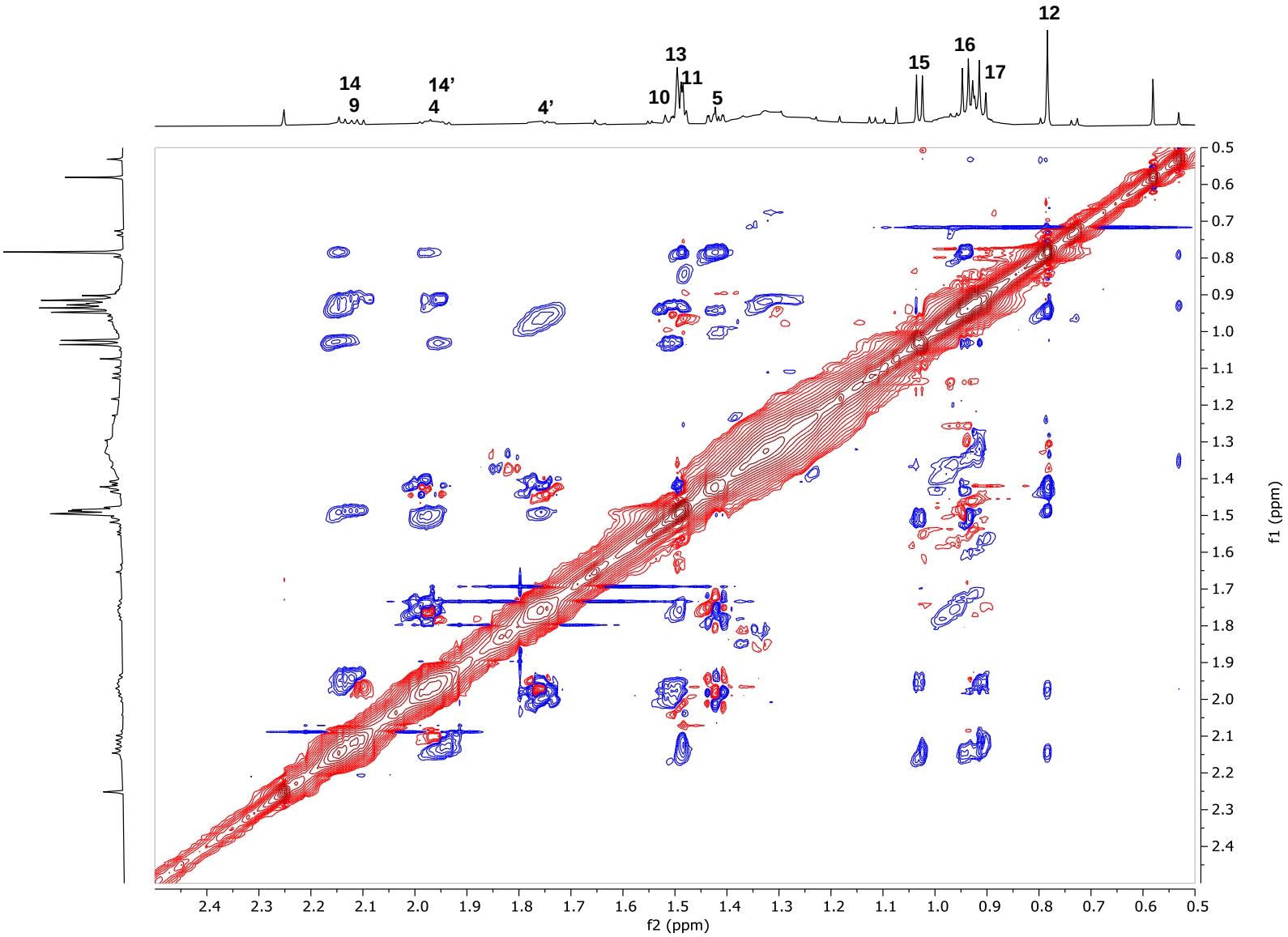


Figure S31: HSQC spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

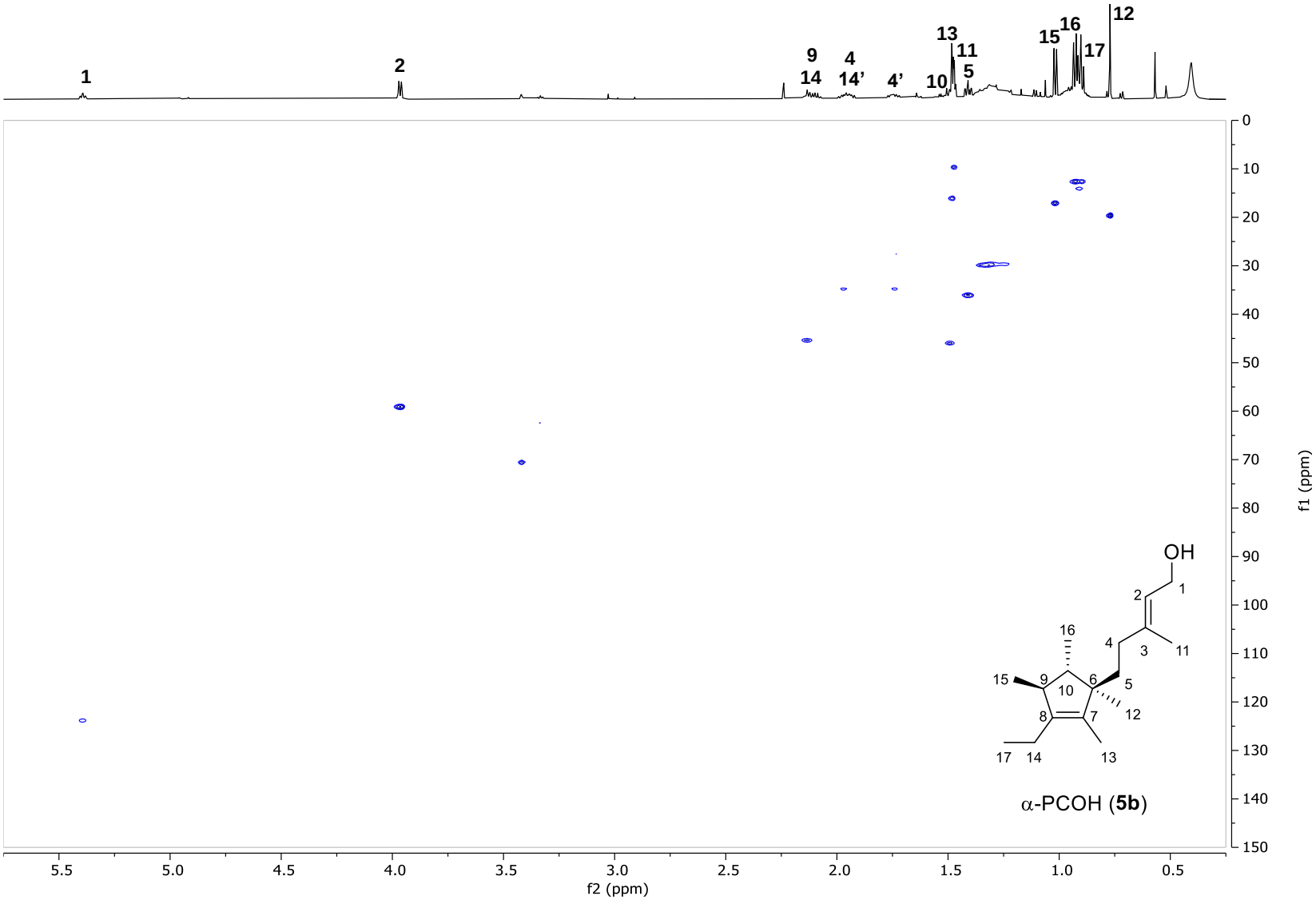


Figure S32: HMBC spectrum of α-prechlororaphenol (α-PCOH, **5b**) (in C_6D_6).

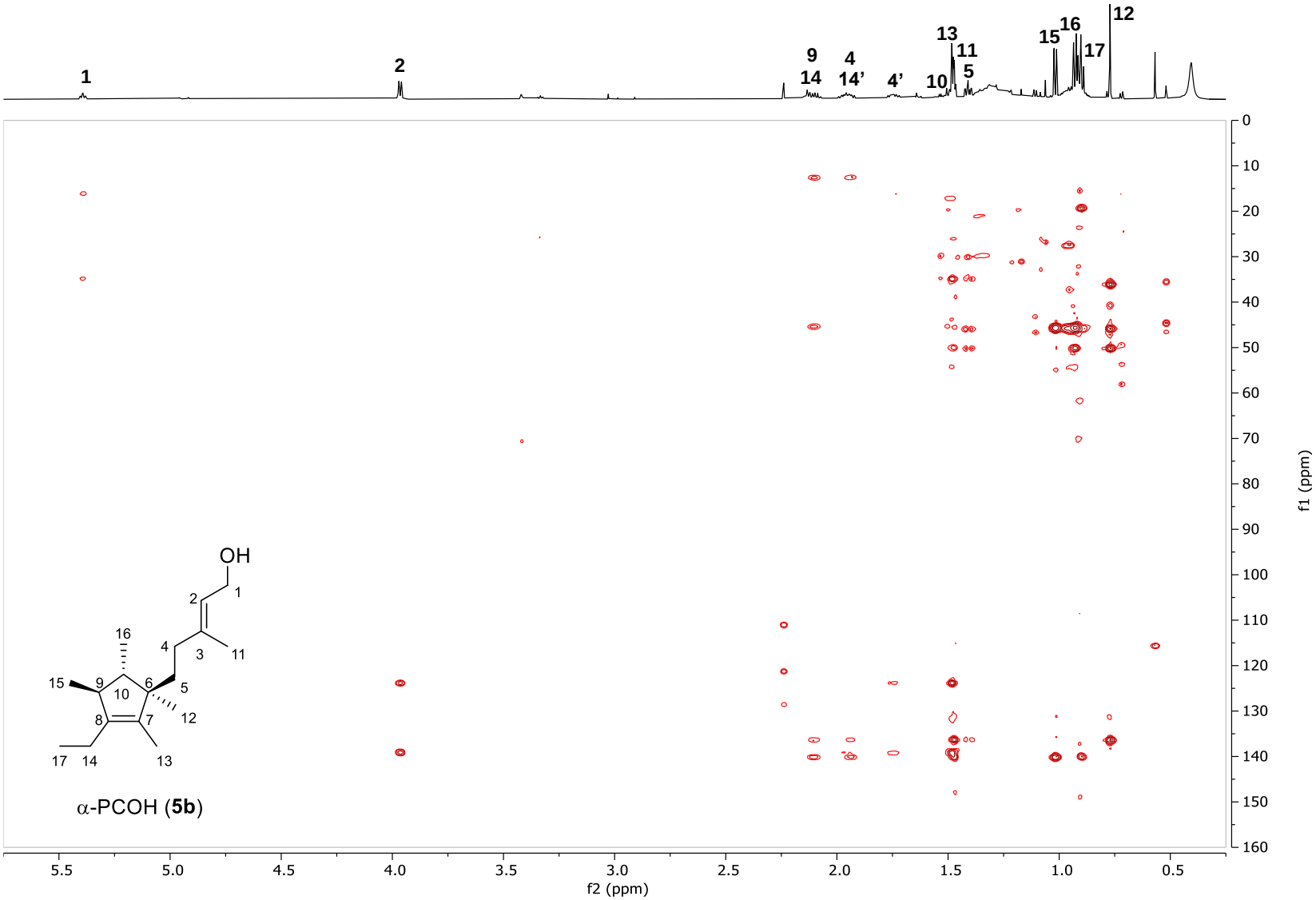


Figure S33: ^{1}H NMR spectrum of VOCs from *E. coli* expressing the chlororaphen (**6**) biosynthetic gene cluster *Pchl*O6_6042 till *Pchl*O6_6045 (in C_6D_6).

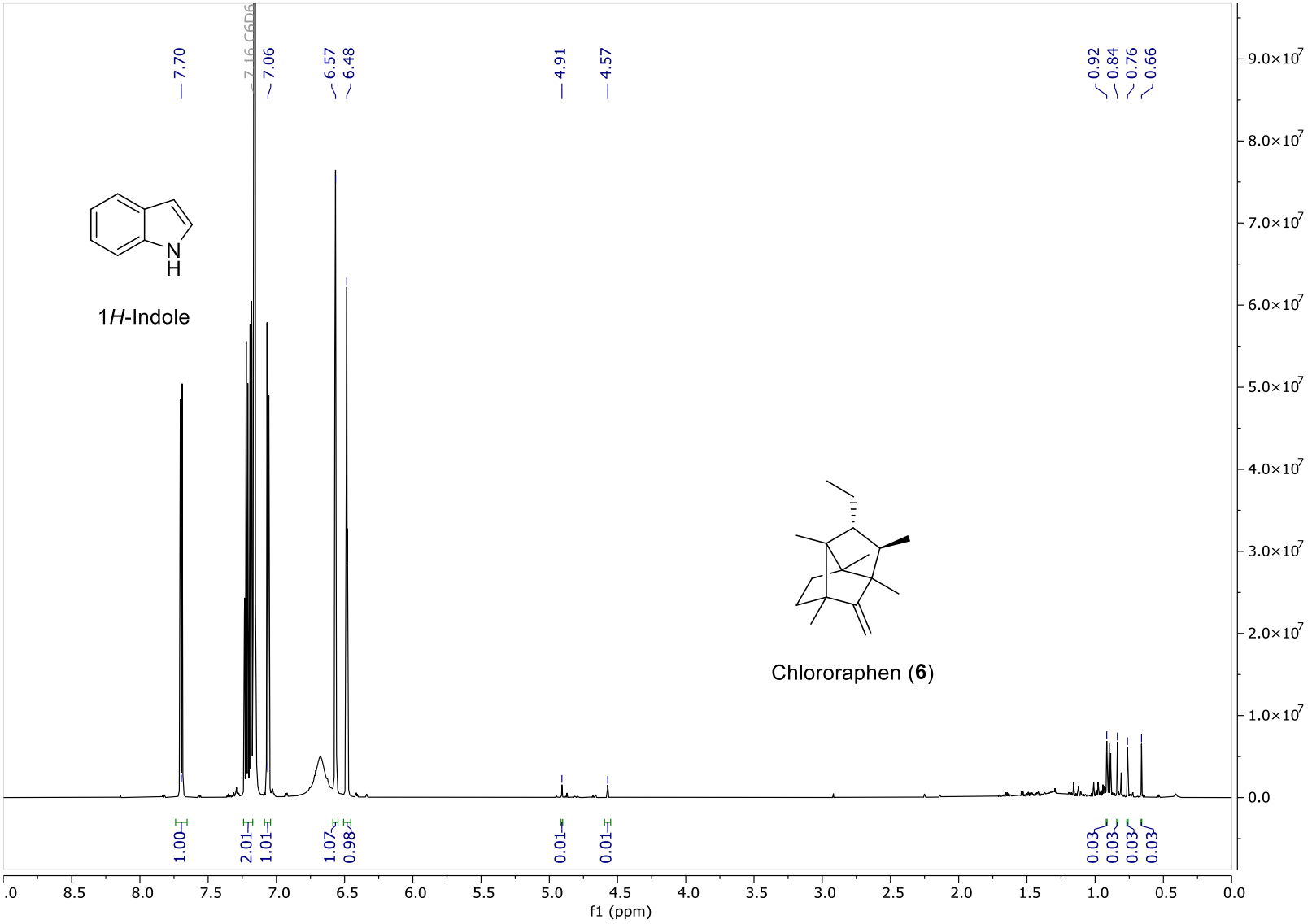


Figure S34: ^{1}H NMR spectrum of chlororaphen (**6**) (in C_6D_6).

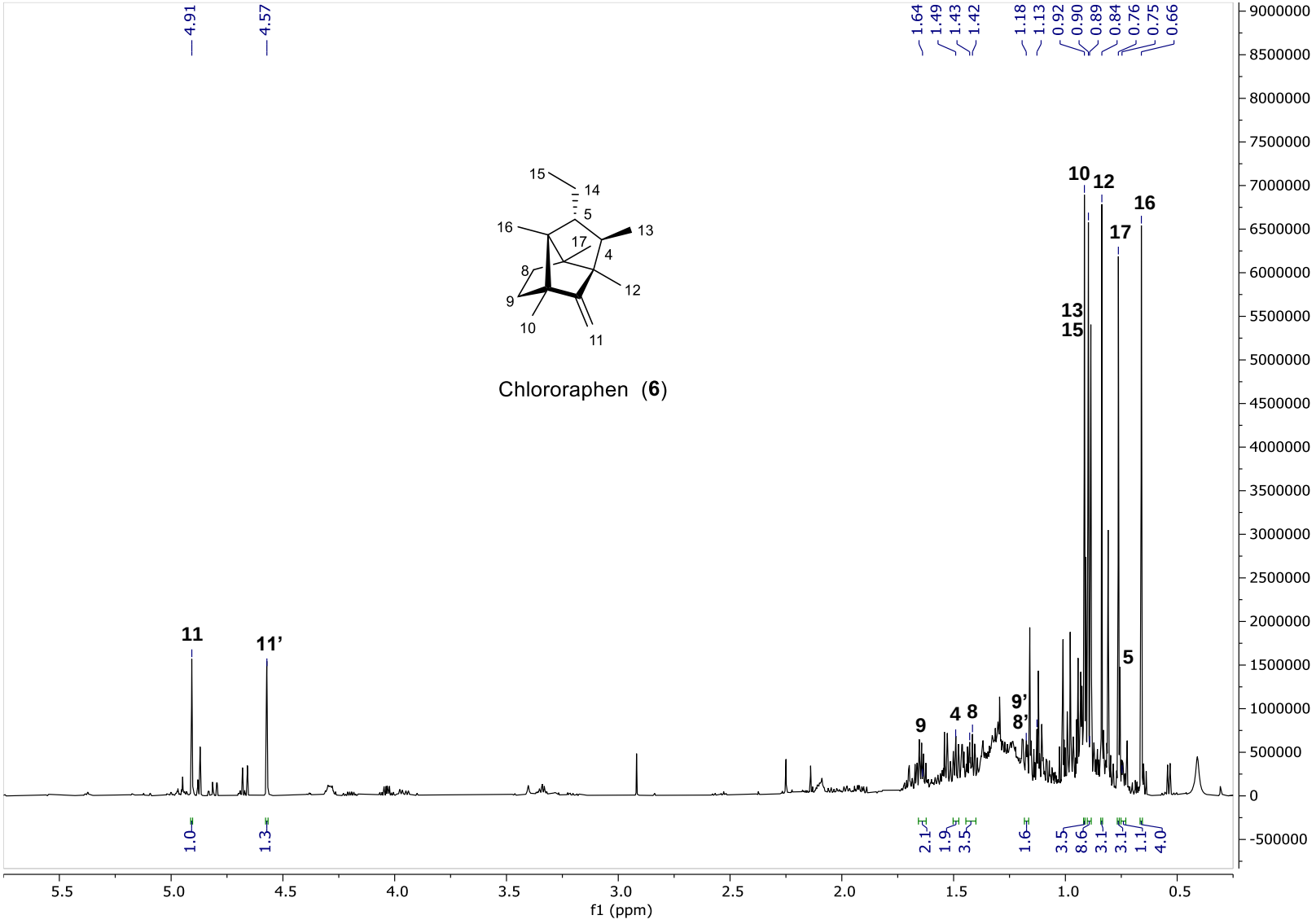


Figure S35: *dqf*-COSY spectrum of chlororaphen (**6**) (in C_6D_6).

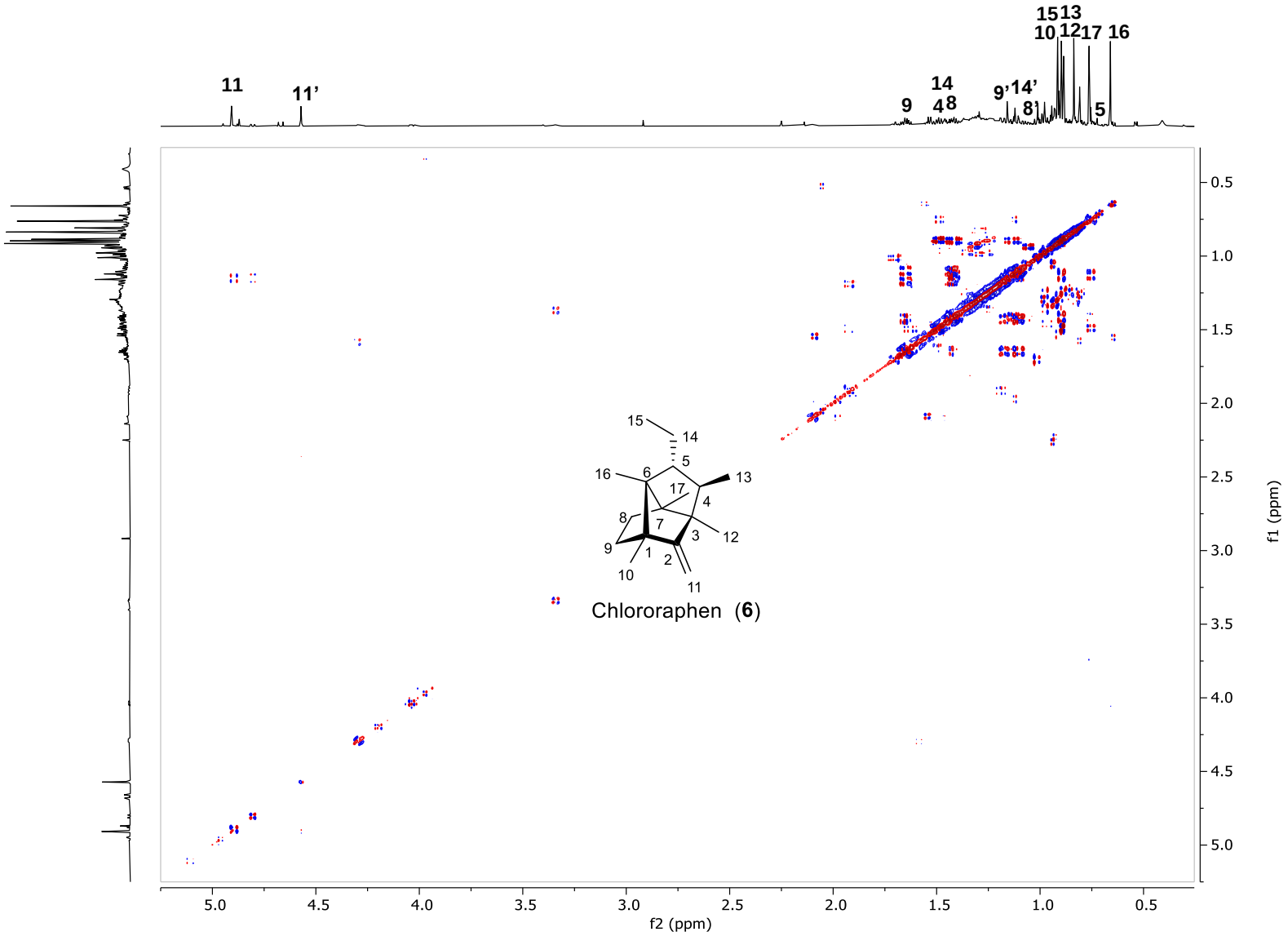


Figure S36: *dqf*-COSY spectrum of chlororaphen (**6**) (in C_6D_6)

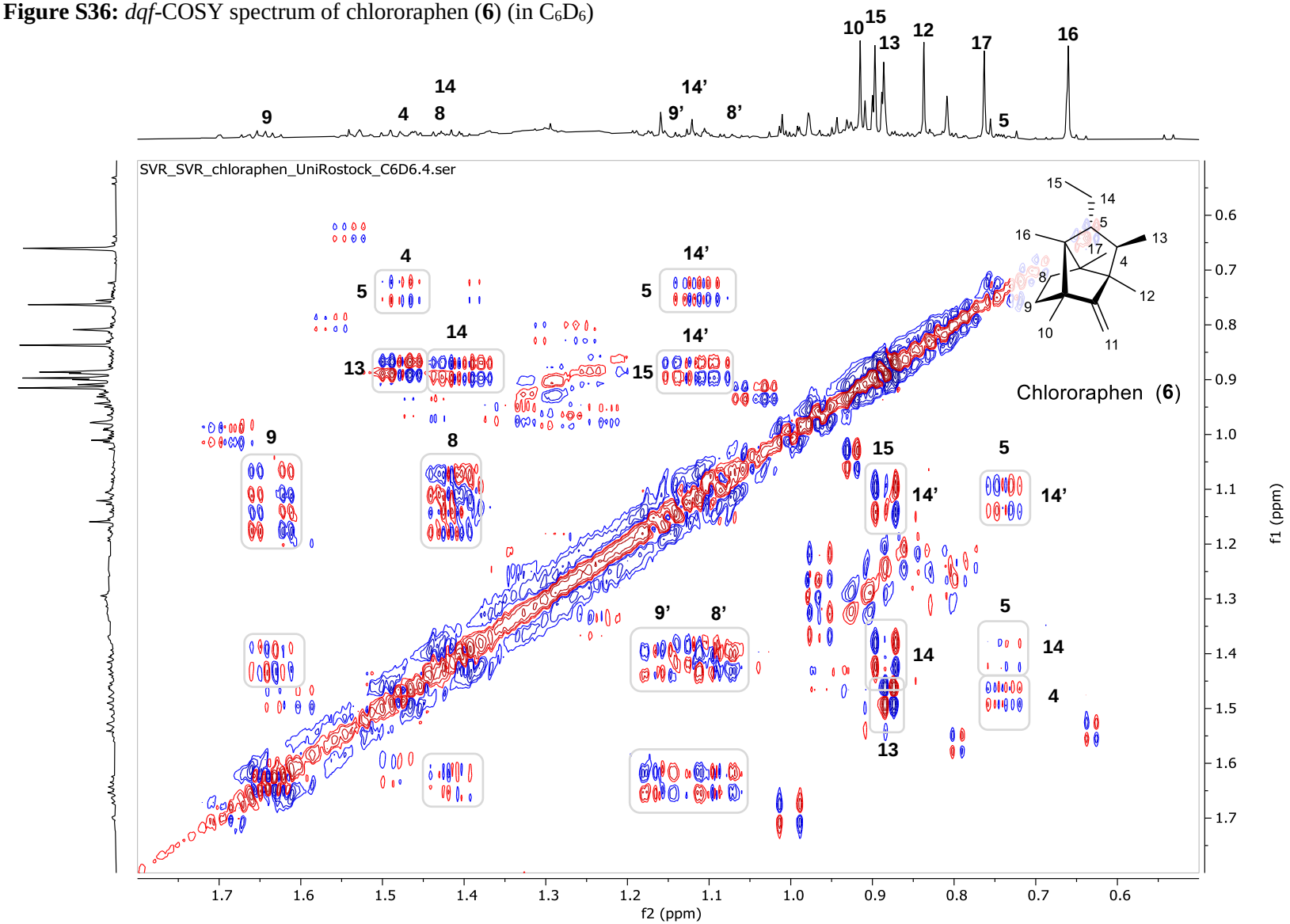


Figure S37: NOESY spectrum of chlororaphen (**6**) (in C_6D_6, d8 = 0.7 s).

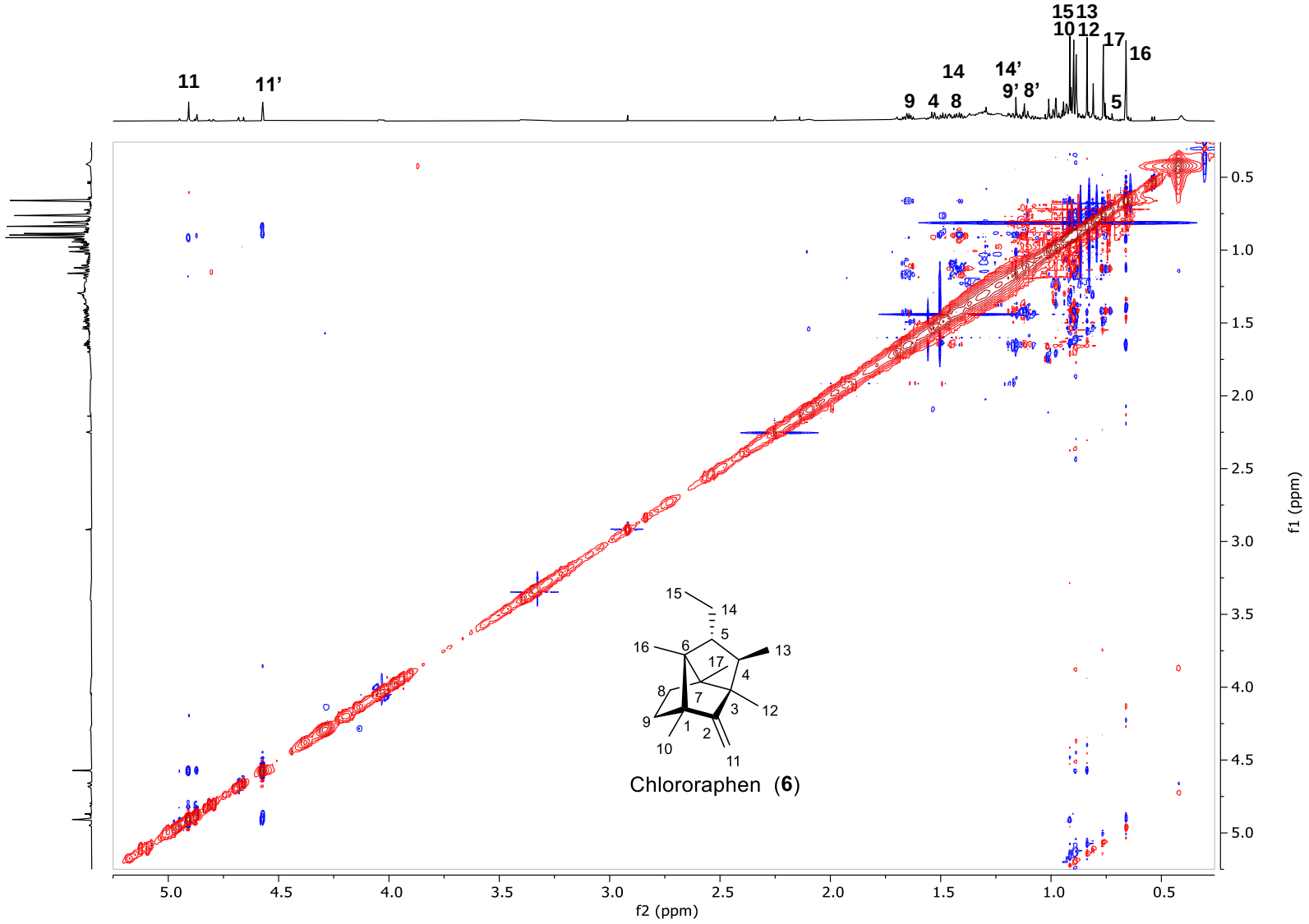


Figure S38: NOESY spectrum of chlororaphen (**6**) (in C_6D_6).

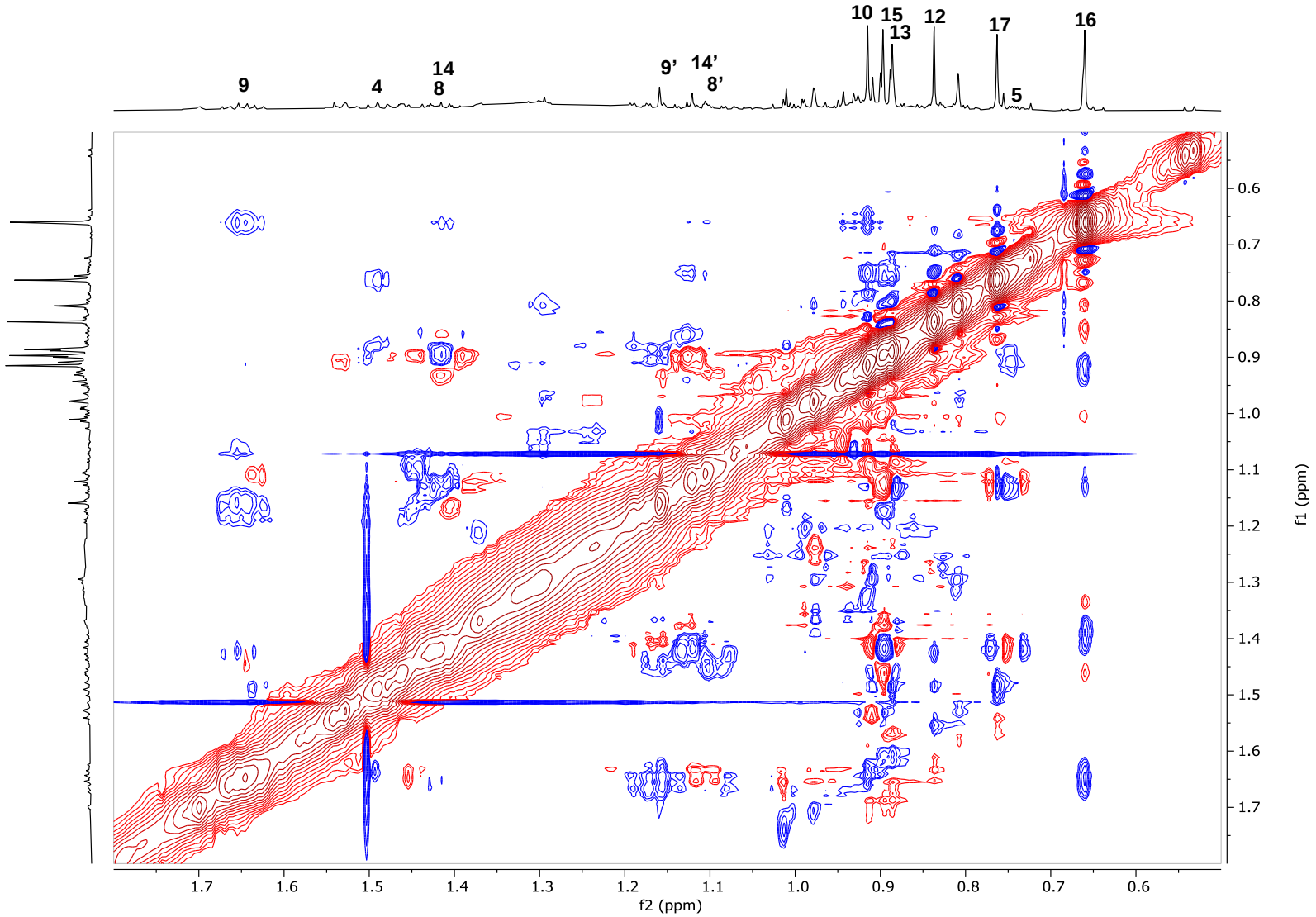


Figure S39: HSQC spectrum of chlororaphen (**6**) (in C_6D_6).

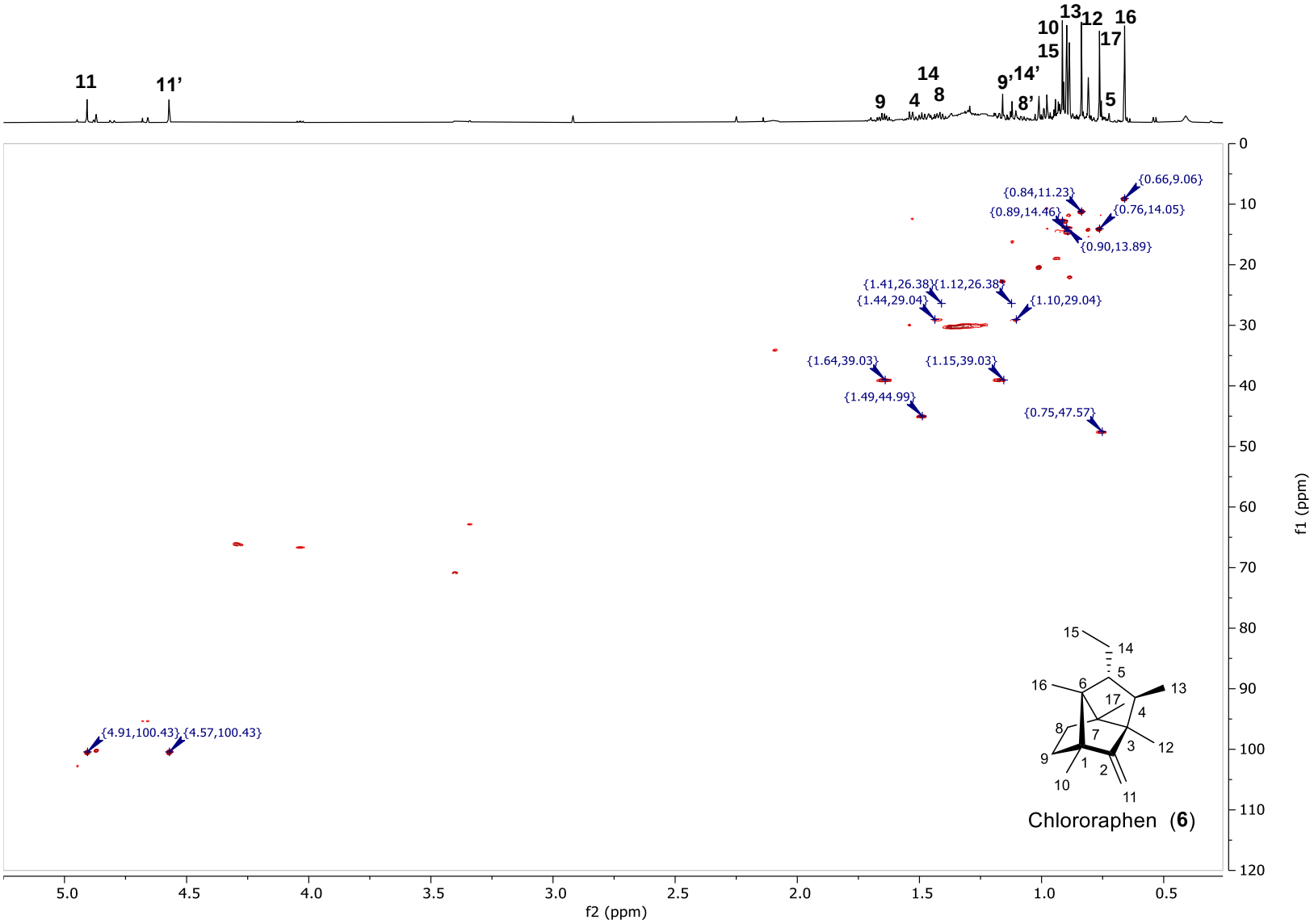


Figure S40: HMBC spectrum of chlororaphen (**6**) (in C_6D_6).

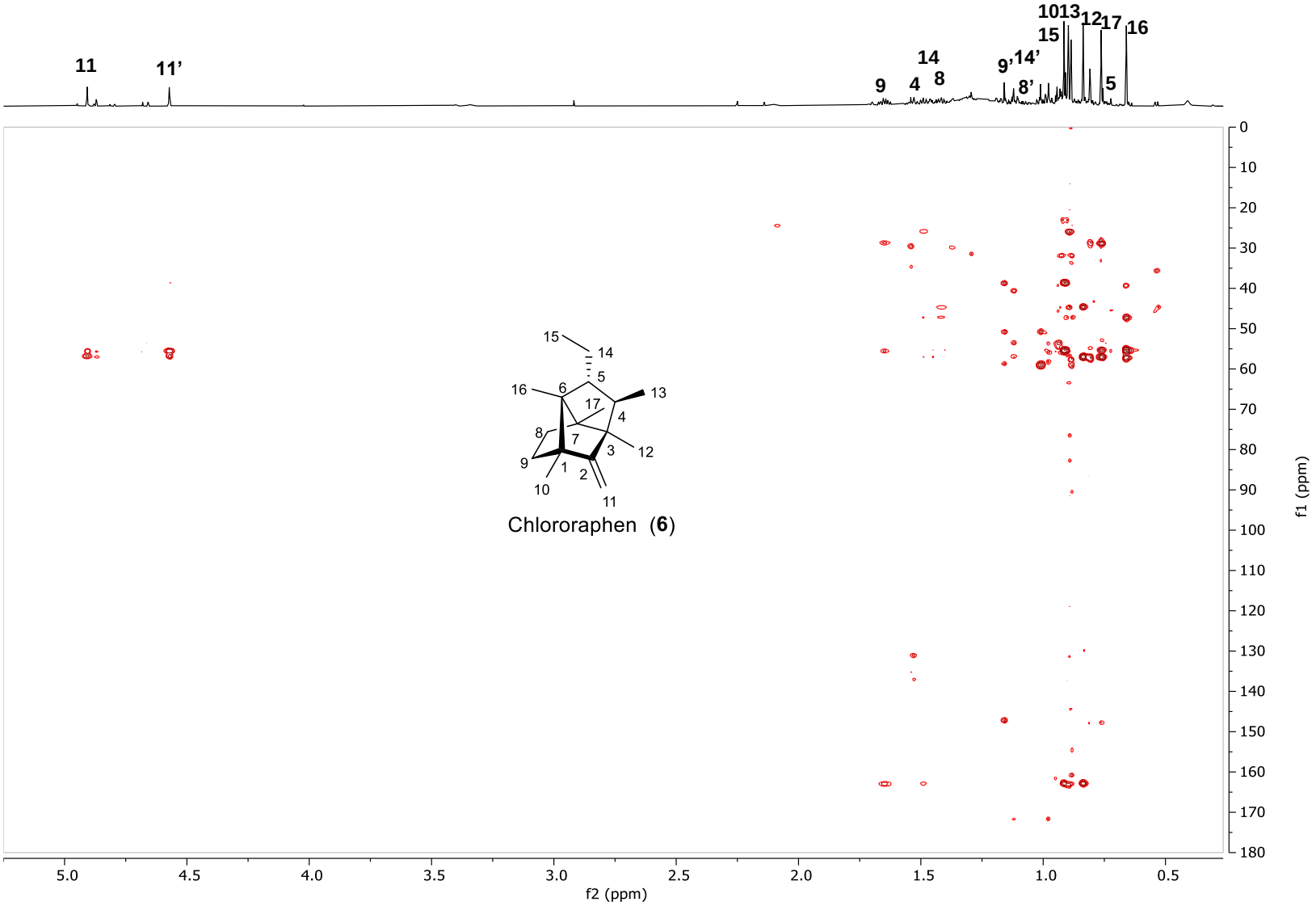


Figure S41: ^{13}C {^{1}H} NMR spectrum of chlororaphen (**6**) (in C_6D_6); NS 18'000.

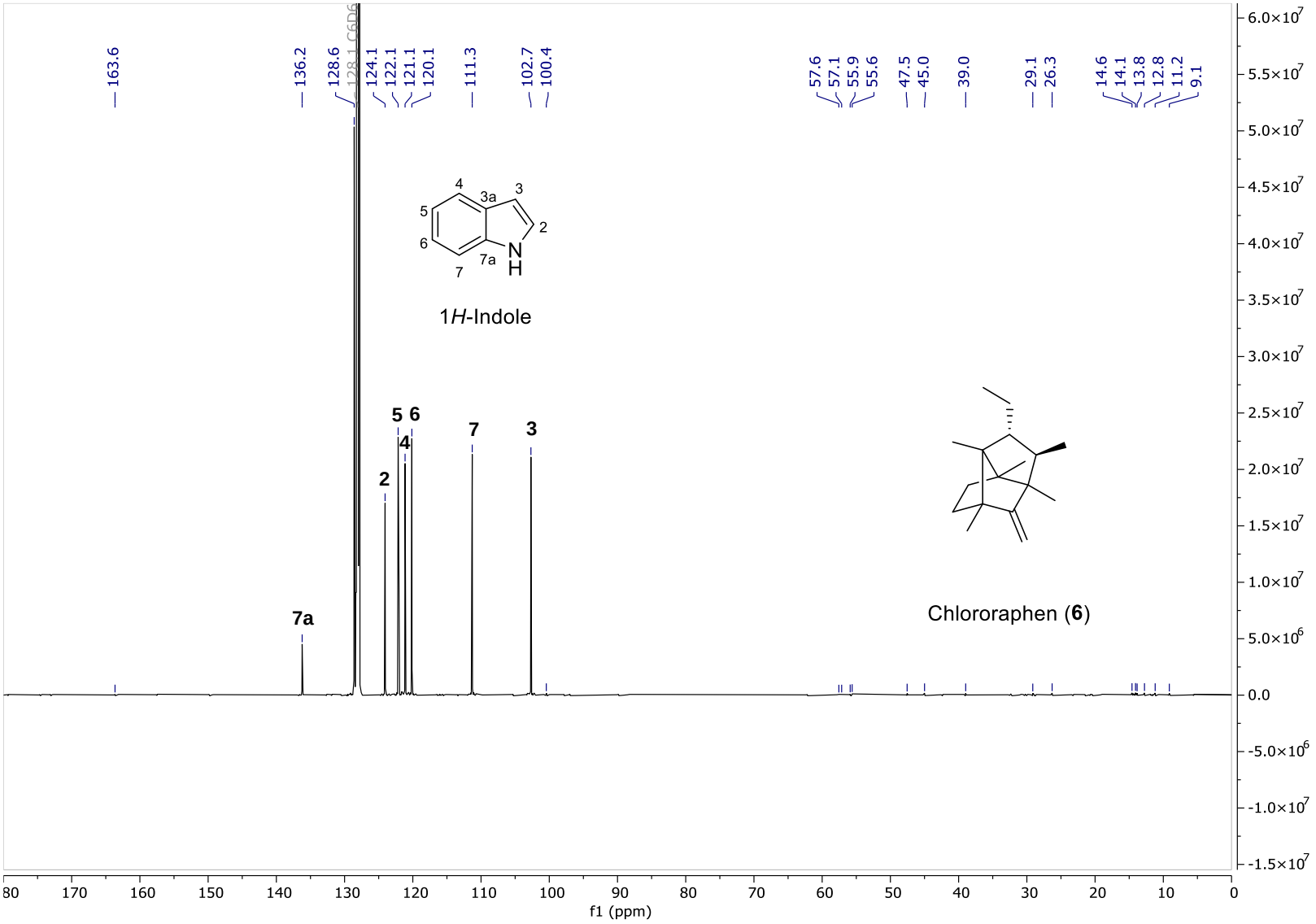
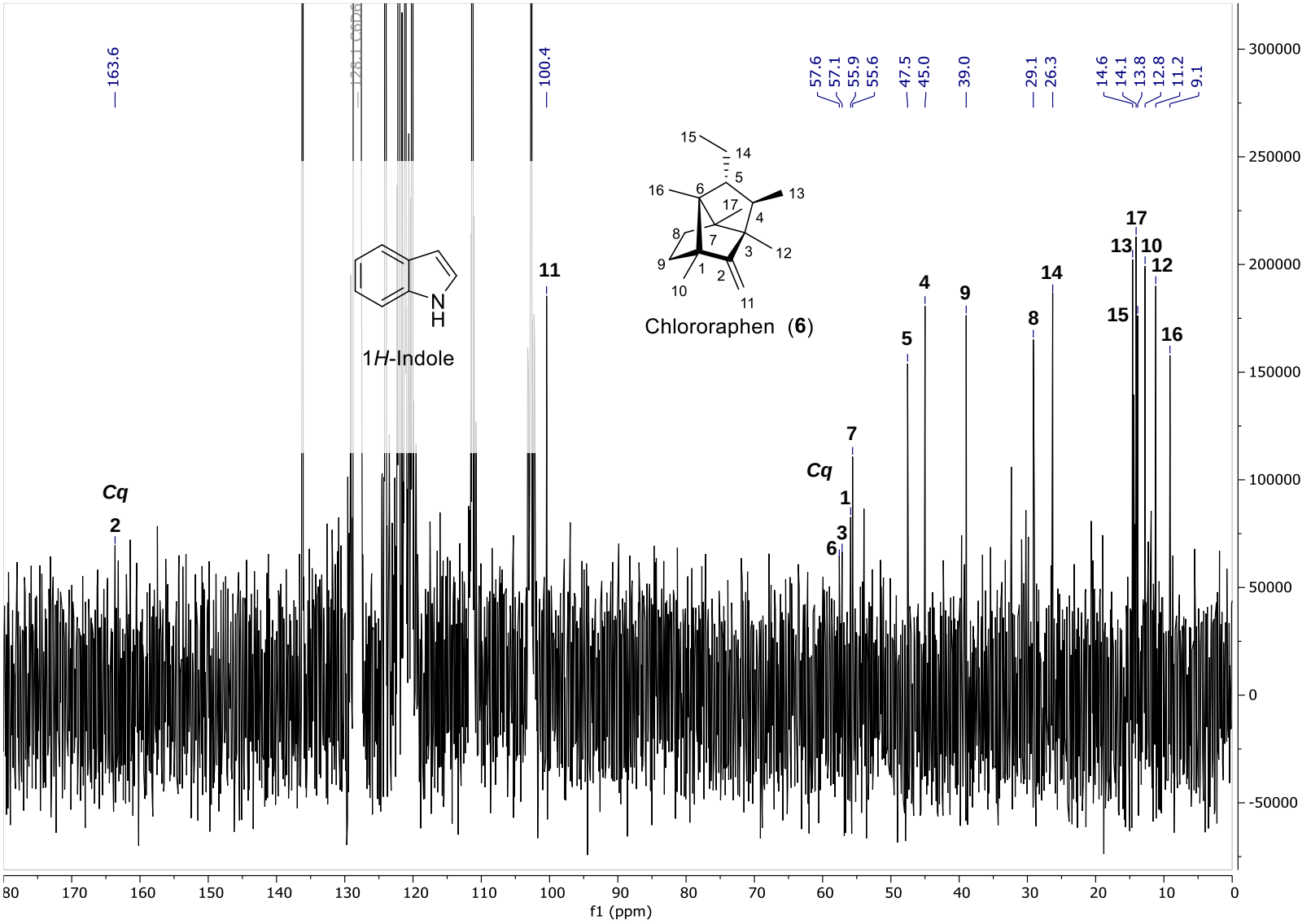


Figure S42: ^{13}C {^{1}H} NMR spectrum of chlororaphen (**6**) (in C_6D_6); NS 18’000.



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