Supporting Information

The Fumagillin Biosynthetic Gene Cluster in *Aspergillus fumigatus* Encodes a Cryptic Terpene Cyclase Involved in the Formation of β-*trans*-Bergamotene

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Experimental Procedures

Strains and Culture conditions

Aspergillus fumigatus strains used in this study are listed in Table S1. Strains were maintained on Czapek-Dox agar or glucose minimal agar (GMM)[1] media and stored as 30% glycerol stocks at -70°C. For production of **1** and other metabolites, *A. fumigatus* and mutant strains were cultured in CYA medium (Czapek-Dox agar supplemented with 5 g/L yeast extract). Total RNA for RT-PCR is extracted from *A. fumigatus* grown on Czapek-Dox liquid medium with 5 g/L yeast extract (CYB) after 4 days of cultivation. For chemical complementation of ΔAf370 and ΔAf520 mutants, purified **3** or **5** is supplemented to the CYA medium at 100 or 40 mg/L respectively.

Chemicals and chemical analysis

Labeled [2-^{13}C]-malonate was purchased from Cambridge Isotope Laboratories, Inc. All solvents and other chemicals used were of analytical grade. All LC-MS analyses were performed on a Shimadzu 2010 EV LC-MS (Phenomenex® Luna, 5μ, 2.0 × 100 mm, C18 column) using positive and negative mode electrospray ionization with a linear gradient of 5–95% MeCN-H_2O in 30 minutes followed by 95% MeCN for 15 minutes with a flow rate of 0.1 mL/min. GC-FID was composed of a model 5890A gas chromatograph (Hewlett Packard) and a model 7673A automatic injector, sampler and controller (Hewlett Packard). Samples were separated through a DB-FFAP capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies). Oven temperature was programmed from 50 to 200°C at 10°C/min, with a 4 minutes hold. Injection was performed at 280°C in the split ratio 1:1; 1 μL of sample was injected. A flow of 2.0 mL/min of helium was used as carrier gas. Flame ionization detection (FID) was performed at 300°C. GC-MS for analysis was performed on an Agilent 6890-5975 GC-MS with Autosampler (Agilent Technologies) with DB-1 capillary column (50 m, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies). Oven temperature was programmed from 100 to 300°C at 20°C/min, with a 4 minutes hold. Injection was performed at 280 ^{o}C in the split ratio 10:1; 1 μL of sample was injected. A flow of 1.5 mL/min of helium was used as carrier gas. Electronic impact was recorded at 70 eV. ^{1}H, ^{13}C and 2D NMR spectra were obtained on Bruker AV500 spectrometer with a 5 mm dual cryoprobe at the UCLA Molecular Instrumentation Center.

General Molecular Biology Experiments

General molecular cloning techniques were as described elsewhere.[2] PCR was performed using Phusion® DNA Polymerase (New England Biolabs) or Takara Primer star HS polymerase (Clonetech, USA). DNA restriction enzymes were used as recommended by the manufacturer (New England Biolabs). PCR products were confirmed by DNA sequencing. *E. coli* TOP10 (Invitrogen) and XL1-Blue were used for cloning, following standard recombinant DNA techniques. RNA extraction was performed using a RiboPure Yeast Kit (Ambion) and ImProm-II™ Reverse Transcription System for RT-PCR (Invitrogen) was used to synthesize complementary DNA (cDNA) from total RNA. *Saccharomyces cerevisiae* strain BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2- Δ1 trp1 pep4::HIS3prb1 Δ1.6R can1 GAL*) was used as the yeast expression host. In vivo yeast recombination cloning was performed by transforming the *S. cerevisiae* BJ5464-NpgA with DNA fragments with > 35 bp overlaps and includes a 2μ plasmid backbone (derived from YEplac195 or YEplac112) using *S.c* EasyComp Transformation kit (Invitrogen).

In silico analysis

The *A. fumigatus* HR-PKS gene sequences are retrieved by performing BLASTP search of the *A. fumigatus* Af293 genome on the NCBI database using a β-ketoacyl synthase domain of a HR-PKS (e.g. *A. terreus* LovB) as query sequence. Genes in vicinity of each HR-PKS gene on the *a. fumigatus* chromosomes are analyzed on the NCBI Map Viewer and a gene (AFUA_8G00410) encode for type II methionine aminopeptidase was identified downstream of the HR-PKS gene AFUA_8G00370 on chromosome VIII. Functional domains in the translated protein sequences were predicted using Conserved Domain Search (NCBI) or InterproScan (EBI). Gene structure predictions (AFUA_8G00370, 380 and 520) were performed using the FGENESH program (Softberry) and manually checked by comparing with homologous gene/proteins in the GenBank database using BLASTX analysis. CLUSTALW or BIOEDIT software package is used for multiple sequence alignment. Protein structure and transmembrane helices predictions are performed with Phyre2 server,[3] PsiPred,[4] TMHMM,[5] and MEMSAT-SVM.[6]

Generation of *Aspergillus fumigatus* AFU8G_0370 andAFU8G_0520 deletion mutants

The DNA cassette used for gene replacement was generated by fusion PCR, using *A. parasiticus pyrG* gene as the selection marker, as outlined elsewhere.[7] Fusion PCRs were done using Takara Primer star HS polymerase (Clonetech, USA). The gene replacement DNA cassette for partial deletion of the Afu8g00370 open reading frame, encoding a putative polyketide synthase, was created as follows: First, about 1.2 kb of 5'UTR and 1.2 kb of 3'UTR of this gene were amplified from *A. fumigatus* genomic DNA using primers afu8g370-p1 and afu8g370-p2, and afu8g370-p3 and afu8g370-p4 (all primers used in this study are listed in Table S2). *A. parasiticus pyrG* gene was PCR amplified from genomic DNA using primers AparapyrGF-Linker and AparapyrGR-Linker. The resulting PCR product was ligated into pJET blunt cloning vector (Fermentas, USA) creating plasmid pSD38.1. Then *A. parasiticus pyrG* gene was re-amplified from plasmid pSD38.1 using primers afu8g370-p5 & afu8g370-p6. All three fragments were then fused by PCR, using primers afu8g370-p1 & afu8g370-p4. Polyethylene glycol mediated transformation of *A. fumigatus akuB*^{ku80} (*pyrG1*) strain[8] (a gift from Dr. Cramer) was performed as described previously.[7] The gene replacement resulted in a partial deletion of Afu8g00370, from the translation start site (+1) to +2010 bp. The partial deletion was confirmed by PCR (data not shown) and Southern blot (Figure S1). The resulting transformant was designated TSD49.1.

The gene Afu8g00520 (ortholog of *ubiA* encoding a putative prenyltransferase) was deleted following a similar strategy. Approximately, 1.4kb fragments corresponding to the 5'UTR and 3'UTR of Afu8g00520 were amplified from *A. fumigatus* genomic DNA using primer pairs afu8g520-p1 and afu8g520-p2, and afu8g520-p3 and afu8g520-p4, respectively. Next, the *A. parasiticus pyrG* gene was amplified from plasmid pSD38.1 using primers afu8g520-p5 and afu8g520-p6. All three fragments were fused by PCR using the nested primers afu8g520-nestF and afu8g520-nestR. *A. fumigatus akuB*^{ku80} was also used as host strain for fungal transformation using this fusion PCR product. The gene replacement resulted in a complete deletion of Afu8g00520 coding region. The deletion was also confirmed by PCR (data not shown) and Southern blot (Figure S6). Several transformants were obtained. The resulting transformant was designated TSD50.1.

An isogenic control strain was also obtained by transforming *A. fumigatus akuB*^{ku8} with a wild-type copy of the *A. fumigatus pyrG* gene. The DNA fragment containing *pyrG* used for this transformation was PCR amplified from p1439 (Stinnett et al., 2007) with primers CoLinkerF_641 and ColinkerR_642. The resulting transformant was designated TSD51.1.

Construction of plasmid for *fma*-PKS expression in *S. cerevisiae*

The *fma*-PKS expression plasmid, pHCfmaPKS, was constructed using in vivo yeast recombination cloning. The complete *fma-PKS* cDNA was constructed by three overlapping fragments (Table S4). The middle largest exon region (4479 bp) that was predicted to not contain any intron was amplified from *A. fumigatus* gDNA using primer pair FG370-P2-F with FG370-P2-R (fragment designated as FG370-P2). The 3′ and 5′ end regions were predicted to contain introns and were thus amplified from cDNA. RNA was extracted from a four-day old culture in CYB medium using the RiboPure Yeast Kit (Ambion) following the manufacturer's instructions and residual gDNA in the total RNA was digested with DNase (2 U/μL) (Invitrogen) at 37 ^{o}C for four hours. The 3′ and 5′ *fma*-PKS cDNA was synthesized using ImProm-II™ Reverse Transcription System with the reverse primers FG370-P1-R and FG370-P3-R, respectively (Table S4). The cDNA was used as template for PCR. The 3′ and 5′ intron-free cDNA fragments were amplified using primer pairs FG370-P1-F/FG370-P1-R-T_{ADH2} and P_{ADH2}-FG370-P3-F/FG370-P3-R3 (designated as P_{ADH2}-FG370-P3 and FG370-P1-T_{ADH2} respectively). For in vivo yeast recombination, the three overlapping fragments, P_{ADH2}-FG370-P3, FG370-P2 and FG370-P1-T_{ADH2}, were combined with a PmlI/NdeI-linearized YEplac195-derived 2μ expression plasmid (containing *URA3* marker) flanking with P_{ADH2} promoter and T_{ADH2} terminator (also encode a 6×His-tag and stop codon);[9] co-transfomation of *S. cerevisiae* BJ5464-NpgA using an *S. c* EasyComp™ Transformation kit (Invitrogen) yield the expression plasmid pHCfmaPKS by in vivo recombination. The resulting pHCfmaPKS plasmid was recovered by Zymoprep™ Yeast Plasmid Miniprep (Zymo Research) for propagation in *E. coli* XL1 and verified by sequencing.

Construction of plasmid for *fma*-AT expression in *S. cerevisiae*

An intron-free *fma-AT* was constructed from cDNA which was synthesized with the reverse primer pHis8-FG380-R using ImProm-II™ Reverse Transcription System. The cDNA was used

as template for PCR and amplified by using primer pair pHis8-FG380-F and pHis8-FG380-R, which contained EcoRI and NotI restriction sites, respectively. To introduce 8×His-tag to the N-terminal of *fma*-AT, the PCR product was digested with EcoRI and NotI and ligated into the EcoRI/NotI-digested pHis8 expression vector,[10] to yield plasmid pHCHis8fmaAT. This plasmid was used as template for PCR using primer pair ADH2p-380-F with ADH2t-380-R. The resulting PCR product and an NdeI/HindIII-linearized YEplac112-derived 2 μ expression plasmid (containing *TRP1* marker) were combined; co-transformation of *S. cerevisiae* BJ5464-NpgA using an *S. c* EasyComp™ Transformation kit (Invitrogen) yield the expression plasmid pHCfmaAT by in vivo recombination. The resulting pHCfmaAT plasmid was recovered by Zymoprep™ Yeast Plasmid Miniprep (Zymo Research) for propagation in *E. coli* XL1 and verified by sequencing.

Expression and purification of *fma*-PKS and *fma*-AT from *S. cerevisiae*

For 1 L culture, the cells were grown at YPD (10 g/L yeast extract, 20 g/L peptone) supplemented with 1% dextrose and incubated at 28 ^{o}C with shaking for 72 hours. The cells were harvested by centrifugation (3,750 rpm at 4°C for 10 mins) and the cell pellet was resuspended in 20 mL lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication on ice in one minute intervals until homogenous. To remove cellular debris, the homogenous mixture was centrifuged at 17,000 rpm for 1 hour at 4°C. Ni-NTA agarose resin was added to the supernatant (2 mL) and the solution was stirred at 4°C overnight. Soluble fma-PKS was purified by gravity-flow column chromatography with increasing concentrations of imidazole in Buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM–250 mM imidazole, pH 7.9). Purified protein was concentrated and buffer was exchanged into Buffer B (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, pH 8.0) using an Amicon Ultra-15 Centrifugal Filter Unit and stored in 10% glycerol. The purified *fma*-PKS and *fma*-AT were analyzed by SDS-PAGE (Figure S2A) and their concentration were calculated to be 3.5 mg/L and 18.0 mg/L, respectively, using the Bradford assay with BSA as a standard.

In vitro assays of *fma*-PKS and *fma*-AT

For in vitro synthesis of dodecapentaenoic acid **4**, 10 μM *fma*-PKS and 10 μM *fma*-AT were incubated with 2 mM malonyl-CoA, 1 mM NADPH in 100 mM PBS, pH 7.4 in a total 100 μL

reaction. The reaction was incubated at room temperature overnight and extracted with 100 μL ethyl acetate with 1% acetic acid twice. The organic phase was dried and dissolved in 20 μL MeOH for analysis on LC-MS. For in vitro synthesis of 5-dodecapentaenoyl fumagillol **5**, 1 mM fumagillol **2** was supplemented to the same reaction condition above in 100 μL volume following the same protocols. For in vitro synthesis of ^{13}C-labeled dodecapentaenoic acid, 10 μM *fma*-PKS and 10 μM *fma*-AT were incubated with 100 mM [2-^{13}C]-malonate (unlabeled malonate used for control reaction) and a malonyl-CoA regeneration system (10 mM Mg_2Cl_2, 25 mM ATP, 10 mM CoA, 25 μM MatB, 1 mM NADPH) in 100 mM PBS buffer, pH 7.4 in total 100 μL volume. The reaction was incubated at room temperature for overnight, extracted and analyzed by LCMS as above (Figure S3).

Cloning, expression, and preparation of *fma*-TC-containing microsomes for in vitro assay

The complete *fma*-PKS cDNA was constructed by two overlapping fragments using yeast in vivo recombination cloning (Table S4). The 3′ and 5′ end regions were predicted to contain introns and were thus amplified from cDNA. cDNA was synthesized using ImProm-II™ Reverse Transcription System with the anchored oligo-dT reverse primer. The cDNA was used as template for PCR. The 3’ and 5’ intron-free cDNA fragments were amplified using primer pairs P_{ADH2}-FG520-P1-F/FG520-P1-R (P_{ADH2}-FG520-P1 fragment) and FG520-P2-F/FG520-P2-R-5-T_{ADH2} (FG520-P2-T_{ADH2} fragment). For in vivo recombination, *S. cerevisiae* BJ5464-NpgA were co-transformed with three overlapping fragments, P_{ADH2}-FG520-P1, FG520-P2-T_{ADH2} and a SwaI/NdeI-linearized YEplac195-derived 2μ expression plasmid (containing *URA3* marker) flanking with P_{ADH2} promoter and T_{ADH2} terminator (no His tag) to yield the expression plasmid pHCfmaTC.

For expression of *fma*-TC, the cells were grown in YPD medium supplemented with 1% dextrose at 28°C with shaking for 48 hours. The microsomes were prepared according to the protocol described previously.[11] Briefly, the cells were harvested by centrifugation (3,750 rpm at 4°C for 10 mins) and the cell pellet was washed with 100 mL of TES buffer (50 mM Tris–HCl, pH, 7.5, 1 mM EDTA, 0.6 M sorbitol). The cells were centrifuged as above, resuspended in 100 mL of TES-M (TES supplemented with 10 mM 2-mercaptoethanol), and allowed to incubate at room temperature for 10 min. The yeast cells were centrifuged again at 3,750 rpm for 10 min, and the pellet was resuspended in 2.5 mL of extraction buffer (1% bovine serum albumin,

fraction V, 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, all dissolved in TES). Zirconia/silica beads (0.5 mm in diameter, Biospec Products) were added until skimming the surface of the cell suspension. Cell walls were disrupted manually by hand shaking in a cold room for 10 min at 30-s intervals separated by 30-s intervals on ice. Cell extracts were transferred to a 50-mL centrifuge tube, the Zirconia/silica beads were washed three times with 5 mL of extraction buffer, and the washes were pooled with the original cell extracts. Finally, microsomes were obtained by differential centrifugation at 10,000*g* for 10 min at 4°C to remove cellular debris followed by centrifugation at 100,000*g* for 70 min at 4°C. The microsomal pellets were weighed prior to resuspension in 1.5 mL of TEG-M buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 20% glycerol, and 1.5 mM 2-mercaptoethanol) and stored frozen at -80 °C.

For in vitro microsomes assay, 1 or 10 mg/mL (wet weight) microsomal fractions containing *fma*-TC and 1 mM FPP were incubated with or without 5 mM $MgCl_2$ in a 100 μL reaction. The reaction was incubated at room temperature for overnight and extracted with 100 μL hexanes twice. The organic phase was dried and re-dissolved in 100 μL hexanes for analysis by GC-FID or GC-MS. For the measurement of the conversion rate of FPP into **3**, 10 mg/mL microsome fractions containing *fma*-TC, 1 mM FPP and 5 mM $MgCl_2$ were incubated at room temperature. The reactions were stopped at 15 min, 30 min, 1 hours and 2 hours by adding 100 μL hexanes and extracted twice, respectively. The organic phase was dried and re-dissolved in 5 μL DMF for analysis by GC-FID. The concentration of β-*trans*-bergamotene produced was measured by the peak area compared to the standard curve. The conversion rate of **3** was measured to be 4.4 $\mu M min^{-1}$ in the presence 10 mg/mL microsomes. The amount of protein in 10 mg/mL microsomes was calculated to be 250 μg/mL based on a modified Bradford assay against a BSA standard curve (protein samples were pre-denatured in 0.1 M NaOH).

Purification and characterization of compound 3

S. cerevisiae strain BJ5464-NpgA harboring pHCfmaTC plasmid was inoculated to 4 mL Yeast Synthetic Drop-Out medium without uracil. The cells were grown for 72 hours with constant shaking at 28°C. A 1 mL aliquot of the seed culture was inoculated to 1 L YPD medium supplemented with 1% dextrose. The culture was shaken at 28 ^{o}C for 96 hours. The cells were harvested by centrifugation (3750 rpm, 10 minuntes, 4°C). The cell pellet was extracted by actetone (100 mL) at room temperature and concentrated *in vacuo*. The acetone extract was

suspended in MeCN (15 mL) and partitioned with hexanes (15 mL × 3). The hexanes soluble fraction (55.1 mg out of 65.0 mg) was separated on a Silica gel column (230—400 mesh, hexanes) to give **3** (39.0 mg).

Compound **3** had a molecular formula $C_{15}H_{24}$, as deduced from EI-MS, and showed $[\alpha]_D^{22}$ −5.6 (*c* 2.5, $CHCl_3$). The ^{1}H NMR data showed the characteristic signals of one set of isoprenyl protons (*δ* 1.94, *br.q*, *J* = 7.7 Hz, H-9, *δ* 5.15, *tqq*, *J* = 7.0, 1.3, 1.3 Hz, H-10; *δ* 1.68, *br.s*, H-12; *δ* 1.61, *br.s*, H-13;), two olefinic methine protons (*δ* 4.62, *br.s*, H-15a and *δ* 4.54, *br.s*, H-15b) and one methyl proton (δ 0.70, *s*, H-14). The ^{13}C NMR data exhibited signals of three methyls (*δ* 25.7, 18.6 and 17.6), five methylenes (*δ* 23.5, 23.5, 23.8, 27.1 and 38.2), two methines (*δ* 38.7 and 50.2), one *tert*-methyl (*δ* 43.8), and two olefinic group (*δ* 125.1 and 130.1; *δ* 152.1 and 106.0) (Table S5). These ^{1}H, ^{13}C and EI-MS spectroscopic features were identical to those of β-*trans*-bergamotene.[12-14] The NOESY spectrum (Figure S10) showed the correlations of *δ* 1.68 (H-12) to *δ* 2.51 (H-1), *δ* 2.05 (H-5) and *δ* 2.28 (H-6β) and the correlations of 0.70 (H-14) to δ 2.24 (H-3β) and δ 1.82 (H-4) confirming the *trans*-configuration of structure. Accordingly, compound **3** was established as (−)-β-*trans*-bergamotene.

Purification and characterization of compound 5

S. cerevisiae strain BJ5464-NpgA harboring both pHCfmaPKS and pHCfmaAT plasmids was inoculated to 4 mL Yeast Synthetic Drop-Out medium without uracil and tryptophan. The cells were grown for 72 hours with constant shaking at 28°C. A 1 mL aliquot of the seed culture was inoculated 50 mL YPD (10 g yeast extract, 20 g peptone and 950 mL Milli-Q water) supplemented with 1% dextrose. Fumagillol (**2**, 2.5 mg in 100 μL MeOH) was added to the culture after 48 hours at 28°C with shaking and the cells were cultivated for another 48 hours. The cells were harvested by centrifugation (3750 rpm, 10 minuntes, 4°C). The cell pellet was extracted by acetone (100 mL) at room temperature and concentrated *in vacuo*. The acetone extract was further separated on a semi-preparative RP-18 HPLC column (Luna®, ODS-3, 5 *μ*m, semi-preparative: 250 × 10 mm) elutaed by 85% $MeCN_{aq}$ with flow rate of 2.5 mL/min to give **5** (1.1 mg, t_R: 12.1 min).

Compound **5** had the molecular formula $C_{28}H_{38}O_5$, as deduced from ESI-MS showing the quasi-molecular ion $[M+Na]^+$ at *m/z* 477. The UV spectrum showed a typical polyene pattern with maximum absorption at 359 nm. The ^{1}H and ^{13}C NMR data of **5** were similar to those in

fumagillin[15] except the signals of the polyene moiety (Table S6). Both ^{1}H and ^{13}C spectra showed the characteristic signals of one dodecapentaenoyl moiety (^{1}H data, δ 5.85−7.30 and δ 1.79, br.d, J = 6.8 Hz; ^{13}C, Table S6). The stereochemistry of H-2' to H-11' showed all *trans* based on the coupling constant (J = 14.5−15.5 Hz). The ^{1}H and ^{13}C NMR assignments of **5** were made by 2D NMR analyses and are listed in Table S6. Thus, compound **5** was established as 5-dodecapentaenoyl fumagillol.

Supplementary Tables

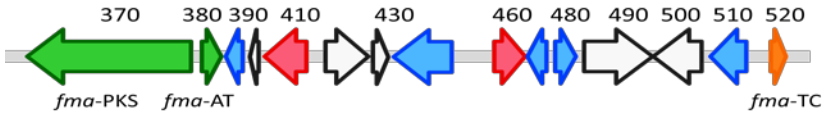
Table S1. Strains used in this study

A. fumigatus strain	Genotype	Source
ku80	*akuB*^{ku80}; *pyrG1*	Gift from Dr. Robert Cramer
TSD49.1	*akuB*^{ku80}; ΔAfu8g00370::*pyrG* $^{A.parasiticus}$	This study
TSD50.1	*akuB*^{ku80}; ΔAfu8g00520::*pyrG* $^{A.parasiticus}$	This study
TSD51.1	*akuB*^{ku80}; *pyrG1, pyrG* $^{A.fumigatus}$	This study

Table S2. Primers used in this study

Primer name	Sequence (5'→ 3')
afu8g520-p1	AGATGACGAGCGTTGTCCCCATGACATTGATC
afu8g520-p2	GATAGAGGAGGGTAGCTTGTCCAGGTGTGATCCG
afu8g520-p3	CATCTTCCCTCGTTTGAGAGGCGAGCTTCCTTAGG
afu8g520-p4	ACATTGCCGTGACGGGTGCCTCGATGG
afu8g520-p5	CGGATCACACCTGGACAAGCTACCCTCCTCTATCACCGGTCGCCTCAAACAATGCTCT
afu8g520-p6	CCTAAGGAAGCTCGCCTCTCAAACGAGGGAAGATGGTCTGAGAGGAGGCACTGATGCG
afu8g520-nestF	AAGGCCACAAAGGCCACGCAGCTAAGCT
afu8g520-nestR	CGACGGATGCGGATGTCATGCGTAGCT
afu8g370-p1	GCATGCCGTCCTGCATGACTGGTCGGATGAG
afu8g370-p2	CTTCTGTTTCTAGGGTGGAGGGGAACGGGG
afu8g370-p3	AGCTGCCAGCAGACCTCAAGT
afu8g370-p4	AAGACTGTCTCGACCATCCAGCAT
afu8g370-p5	CCCCGTTCCCCTCCACCCTAGAAACAGAAGACCGGTCGCCTCAAACAATGCTCT
afu8g370-p6	ACTTGAGGTCTGCTGGCAGCTGTCTGAGAGGAGGCACTGATGCG
AparapyrGF-Linker	ACCGGTCGCCTCAAACAATGCTCTGGATCCTATGGATCTCAGAACAATATACC
AparapyrGR-Linker	GTCTGAGAGGAGGCACTGATGCGGTCGACATCACCCTTACCCAAACTA
CoLinkerF_641	ACCGGTCGCCTCAAACAATGCTCT
ColinkerR_642	GTCTGAGAGGAGGCACTGATGCG
FG370-P1-F	CTGACTCACGAGCTTGGACTACCG
FG370-P1-R	CATGCCAAATCCCATTTTCCCA
FG370-P1-R-TADH2	TGTCATTTAAATTAGTGATGGTGATGGTGATGCACCATGCCAAATCCCATTTTCCCA
FG370-P2-F	GTACTAGACAATGCGGAGCAC
FG370-P2-R	GCGATTTGCAGGCTGACTGATA
PADH2-FG370-P3-F	CTAGCGATTATAAGGATGATGATGATAAGACTAGTATGACCCTCACATATGGCCAT
FG370-P3-R	CAAGGACAGCATGTCGGACAG
pHis8-FG380-F	GACAGCGAGAATTCATGAACCGCGAGGATGTCGAG
pHis8-FG380-R	GACGGACGGCGGCCGCCTAAAAGTCGAGGTGTTTCCTCATA
ADH2p-380-F	ATCAACTATCAACTATTAACTATATCGTAATACCATATGAACCGCGAGGATGTCGAGT
ADH2t-380-R	TGGTGGTGGTGACTCGCGACCTCATACACAAGCTTAAAGTCGAGGTGTTTCCTCATAAAG
PADH2-FG520-P1-F	CTAGCGATTATAAGGATGATGATGATAAGACTAGTATGGACCGTGTGCTATCGCT
FG520-P1-R	GACCAACGTGCAGGAGATCGT
FG520-P2-F	GGATCACACCTGGACAAGCTAC
FG520-P2-R-5-TADH2	TCGTGAAGGCATCGGTCCGCACAAATTTGTCATTTCTAAGGAAGCTCGCCTCTCAAACGA
FG520-P2-R-2	TATATTTCCCGCTGCTCTTGAAG
anchored oligo-dT reverse primer	TTTTTTTTTTTTTTTTTTTTTVN

Table S3. Deduced functions of genes within the *fma* cluster

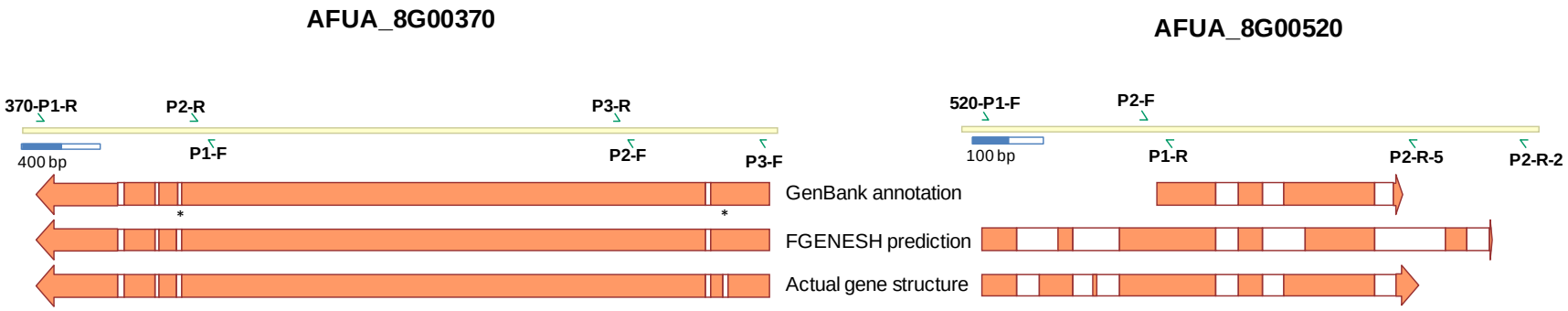


A. fumigatus Af293, Chomosome VIII (NC_007201.1, 79,000 – 115,000), 36 kbp[a]

Gene locus[b] (AFUA_8G00)	Size (gene/protein)	BLASTP homologs[c]	Identity/ similarity (%)	Conserved domain[d]	Putative function[e]
370*	7603/2435	*Peltigera membranacea* PKS1 *Chaetomium chiversii* RADS1	45/60 30/48	KS-MAT-DH-ER-KR-ACP	HR-PKS (dodecapentanoate synthase) †
380	926/292	NFIA_093920	96/97	Alpha/beta hydrolase family, pfam12697	α,β-hydrolase †
390	805/248	NFIA_093930 MAA_08362	96/98 72/89	Methyltransf_2 superfamily, pfam00891	*O*-methyltransferase
400	488/144			No conserved domain detected	misannotation, likely to encode part of 390
410	1937/561	NFIA_093940	94/96	Methionine aminopeptidase 2, cd01088	methionine aminopeptidase, type II fumagillin resistance
420	1989/622	MAA_08349	37/51	GAL4-like domain, cd00067	pathway-specific $Zn(II)_2Cys_6$ transcription factor
430	531/157	MAA_08350 ATEG_0032	72/85 45/68	EthD domain, pfam07110	dehydrogenase
440	2718/905	MAA_08358 *Thermobifida fusca* 2YLZ_A	63/77 42/57	Flavin-binding MO, pfam00743 AdoMet_MTases, cd02440	misannotation or bifunctional Baeyer-Villiger monooxygenases/ methyltransferase
460	1425/397	Pc12g00290	89/94	MetAP1, cd01086	methionine aminopeptidase, type I
470	895/275	NFIA_093950	89/94	ABM superfamily, cl10022	monooxygenase
480	930/309	NFIA_093990 *Streptomyces lavendulae* MmcH	94/97 30/48	Phytanoyl-CoA dioxygenase-like, pfam05721	non-heme iron-dependent dioxygenase
490	3155/1017	MAA_08352 *A. fumigatus* PsoA	61/78 41/59	DH-KR	Pseudogene, encode partial PKS
500	2227/721	MAA_02990 MAA_08357	69/81 69/82	Propionyl-CoA synthetase-like, cd05967	acyl-CoA ligase
510	1665/536	MAA_08345/MAA_09043	78/88	Cytochrome P450, pfam00067	P450 oxidoreductase
520*	1233/289	MAA_08344 ATEG_10144	76/84 44/57	UbiA prenyltransferase superfamily, pfam01040	β-*trans*-bergamotene synthase †

[a] The *fma* gene cluster is located adjacent to the pseurotin A gene cluster (the locus tag of PKS-NRPS gene *psoA* is AFUA_8G00540).
[b] The CDS annotation for the two genes in asterisks (*) on the GenBank database is incorrect (see Table S4 for the correct gene structure and Supplementary methods).
[c] If available, the closest characterized homologs is shown or the locus tag of the closest BLASTP homologs if there is no characterized ones.
[d] Conserved domain is based on analysis by NCBI Conserved Domain Search. For AFUA_8G00370 HR-PKS, the abbreviations for the conserved domains are KS, beta-ketoacyl synthase; AT, malonyl-CoA:transacylase; DH, dehydratase; ER, enoyl reductase; KR; ketoreductase; ACP, acyl carrier protein.
[e] Dagger (†) indicates the function of the encoded protein has been verified by gene deletion and/or in vitro experiments.

Table S4. Revised annotation of *fma*-PKS and *fma*-TC gene.



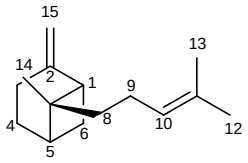
Locus tag/Gene	AFUA_8G00370 (*fma*-PKS gene)[a]	AFUA_8G00520 (*fma*-TC gene)[b]
GenBank ID (mRNA)	XM_742074.1	XM_742060.1
CDS annotation in NC_007201.1	complement (<79285..80133,80197..80511,80560..80749, 80794..86223,86283..>86887)	join(<113744..113909,113972..114042,114101..114358, 114412..>114438)
Revised (actual) CDS annotation	complement (join(79285..80133,80197..80511,80560..80733, 80794..86223,86283..86400,86464 >86887))	join(113249..>113347,113411..113503, 113559..113570, 113636..113909, 113972.. 114042,114101..114358,114419..>114481)
New GenBank ID	KC407775	KC407776

[a] The complete coding sequence of *fma*-PKS gene is constructed by yeast recombination from two cDNA fragments amplified from total RNA by RT-PCR using 370-P1-F/P1-R and 370-P3-F/P3-R primers, and an intron-less fragment amplified from genomic DNA of *A. fumigatus* using 370-P2-F/P2-R. Sequencing of the cloned 370-P1-F/P1-R and 370-P3-F/P3-R cDNA fragments revealed the correct exon regions (*misannotated introns).

[b] The complete coding sequence of *fma*-TC gene is constructed by yeast recombination from two cDNA fragments using FG520-P1-F/P1-R and FG520-P2-F/P2-R-5 primers amplified from total RNA by RT-PCR. The exon regions of 5′ fragment is determined by sequencing of the cloned FG520-P1-F/P1-R cDNA fragment, while sequencing of the 3′ cDNA fragment amplified with FG520-P2-F and FG520-P2-R-2 (located at 3′ untranslated region) revealed the correct exon regions and the stop codon.

NOTE For more details for cloning of the two genes for expression in *S. cerevisiae*, see Supplementary Methods.

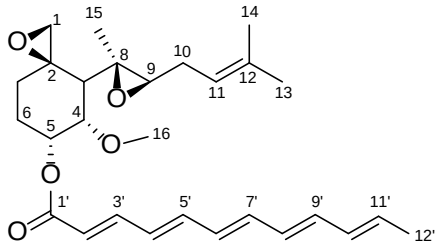
Table S5. ^{1}H and ^{13}C NMR spectroscopic data of compound **3** ($CDCl_3$, 500MHz)



3

no.	δ_H (ppm) (mult, J_{HH} (Hz))	δ_C (ppm) (mult)
1	2.51 (t, 5.0)	50.2 d
2	-	152.1 s
3α	2.56 (ddd, 18.0, 8.3, 3.0)	23.8 t
3β	2.24 (ddd, 18.0, 7.7, 2.1)	
4	1.82 m	23.5 t
5	2.05 m	38.7 d
6α	1.41 (d, 10.0)	27.1 t
6β	2.28 (br.dt, 10.0, 5.9)	
7	-	43.8 s
8	1.61 m	38.2 t
9	1.94 (br.q, 7.7)	23.5 t
10	5.15 (tqq, 7.0, 1.3, 1.3)	125.1 d
11	-	130.1 s
12	1.68 br.s	25.7 q
13	1.61 br.s	17.6 q
14	0.70 s	18.6 q
15a	4.62 br.s	106.0 t
15b	4.54 br.s	

Table S6. ^{1}H and ^{13}C NMR spectroscopic data of **5** ($CDCl_3$, 500 MHz).



5

no.	δ_H (ppm) (mult, J_{HH} (Hz))	δ_C (ppm) (mult)	HMBC
1a	2.98 (d, 4.3)	50.9 t	59.5
1b	2.53 (d, 4.3)		
2	-	29.5 s	-
3	1.98 (d, 11.1)	48.3 d	79.2, 66.2, 61.0, 58.5, 13.9
4	3.65 (dd, 11.1, 2.7)	79.2 d	66.2, 56.6, 48.3
5	5.67 (br.s)	66.2 d	-
6a	1.96 m	25.7 t	79.2, 66.2, 59.5
6b	1.84 (ddd, 14.0, 4.4, 2.4)		
7a	2.07 (dt, 13.5, 4.4)	29.4 t	25.7
7b	1.07 (dt, 13.5, 4.4)		
8	-	58.5 s	-
9	2.58 (t, 6.2)	61.0 d	58.5, 48.3, 27.4
10a	2.35 (br.dt, 15.2, 6.0)	27.4 t	134.8, 118.7, 61.0, 58.5
10b	2.15 (dt, 15.2, 7.4)		
11	5.20 (br.t, 7.4)	118.7 d	25.7, 18.0
12	-	134.8 s	-
13	1.73 s	25.7 q	134.8, 118.7,18.0
1'	-	166.7 q	-
2'	5.86 (d, 15.5)	120.4 d	166.7, 129.4
3'	7.28 (dd, 15.5, 11.4)	144.8 d	166.7, 141.0, 129.4, 120.4
4'	6.27 (m)	129.4 d	120.4
5'	6.57 (dd, 14.6, 11.1)	141.0 d	144.8, 137.6
6'	6.26 (m)	131.1 d	131.1
7'	6.39 (dd, 14.7, 10.5)	137.6 d	141.0, 135.9, 131.1
8'	6.16 (dd, 14.5, 10.5)	130.0 d	137.6, 135.9, 131.8
9'	6.29 (m)	135.9 d	137.6, 131.8
10	6.11 (br.dd, 15.0, 10.5)	131.8 d	135.9, 132.1, 18.5
11'	5.79 (dq, 15.0, 6.8)	132.1 d	135.9, 18.5
12'	1.79 (br.d, 6.8)	18.5 q	131.8

Supplementary Figures

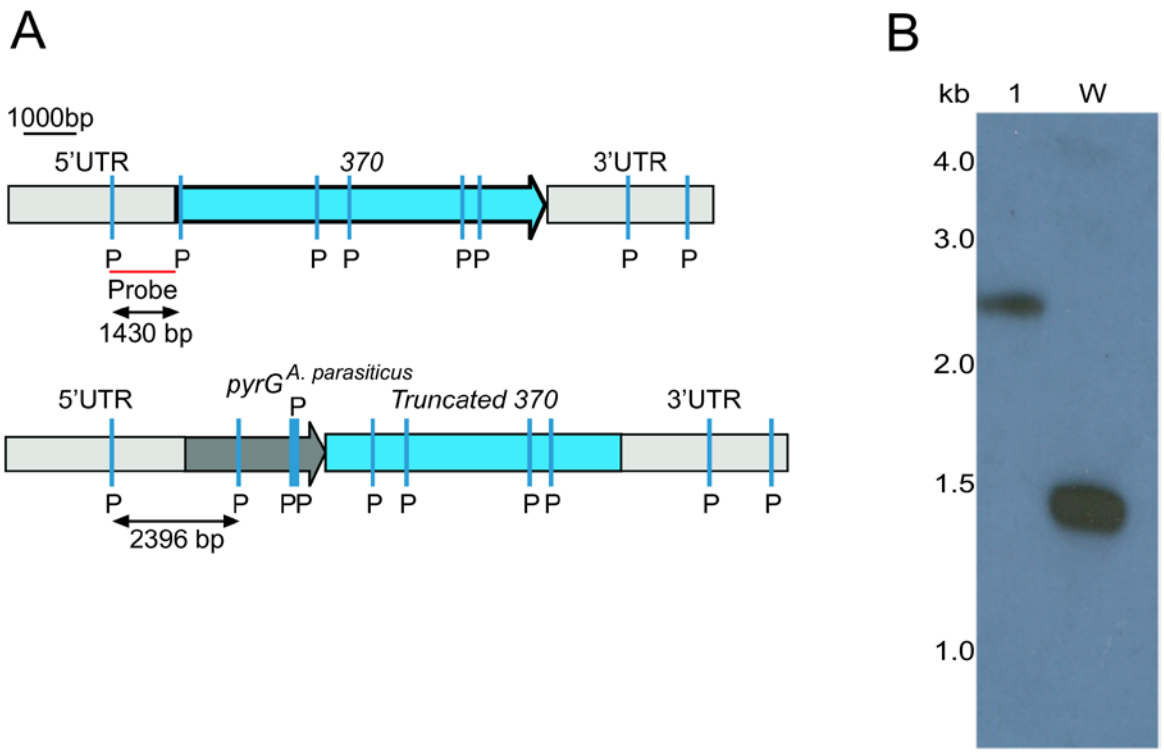


Figure S1. Targeted deletion of AFUA_8G00370 (Af370) and Southern analysis.

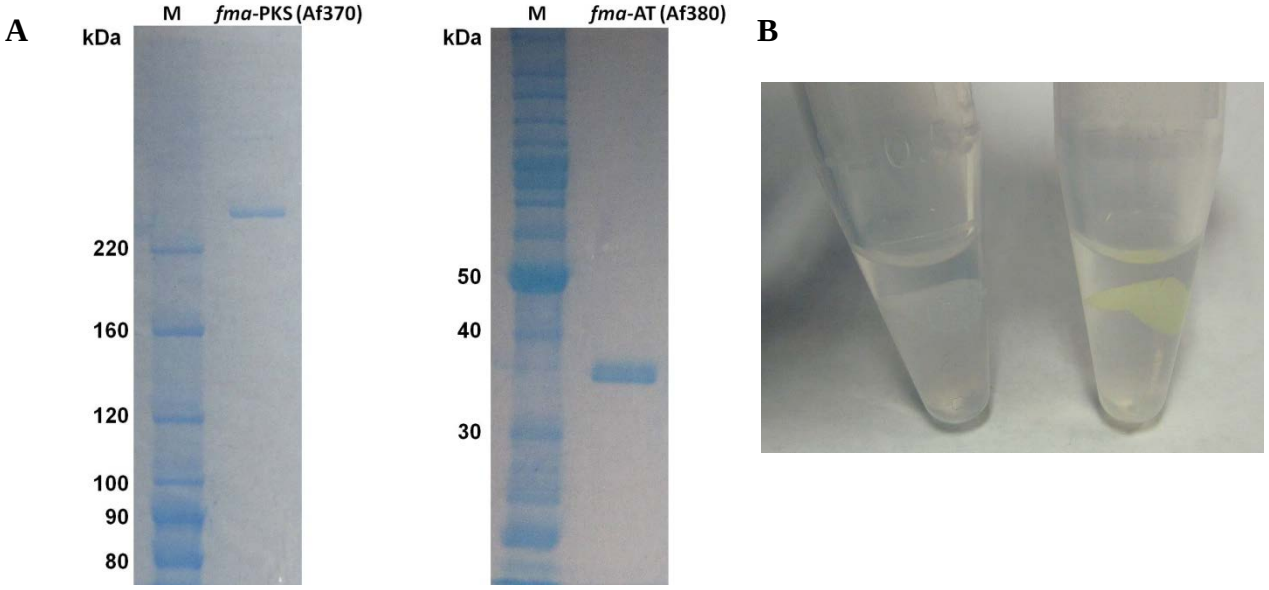


Figure S2. Recombinant *fma*-PKS (Af370) and *fma*-AT (Af380) purified from *S. cerevisiae.* (**A**) SDS-PAGE analysis of purified *fma*-PKS (264 kDa) and *fma*-AT (33 kDa). (**B**) in vitro reaction of *fma*-PKS with (left) or without (right) addition of *fma*-AT (see Supplementary Methods).

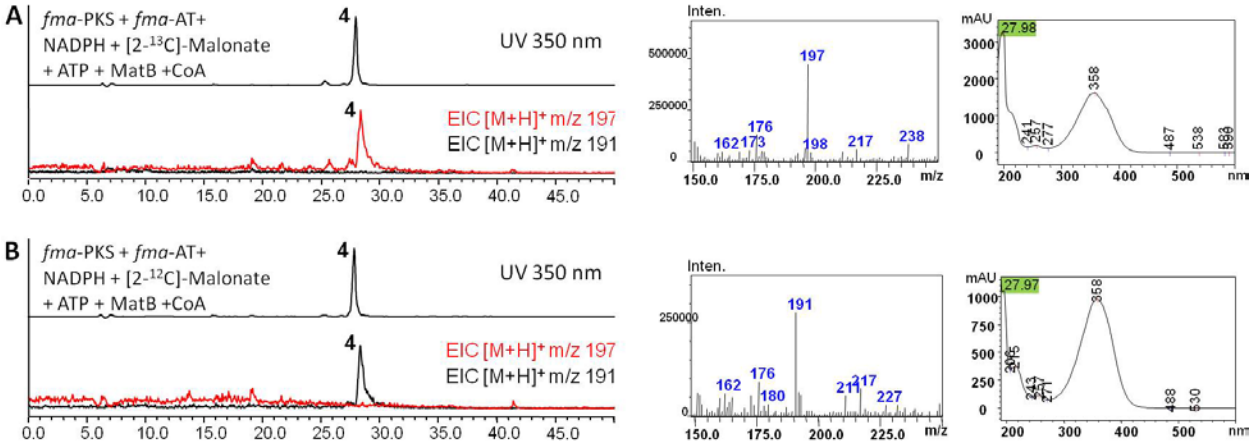


Figure S3. In vitro synthesis of ^{13}C-labeled dodecapentaenoic acid with [2-^{13}C]-malonate. (**A**) LC-MS analysis of *fma*-PKS and-AT with [2-^{13}C]-malonate. (**B**) LC-MS analysis of *fma*-PKS and-AT with unlabeled malonate as control for comparison.

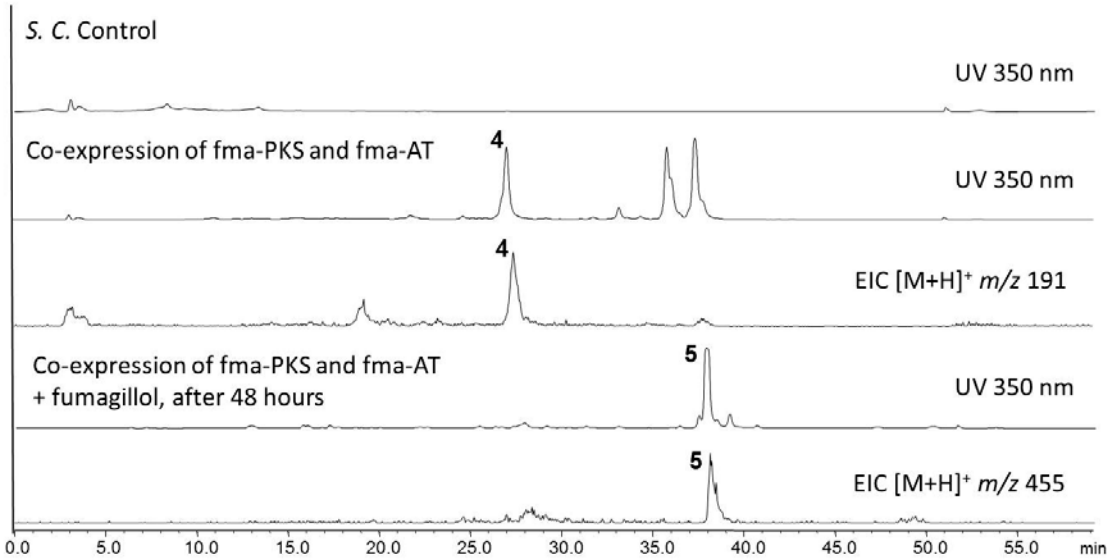
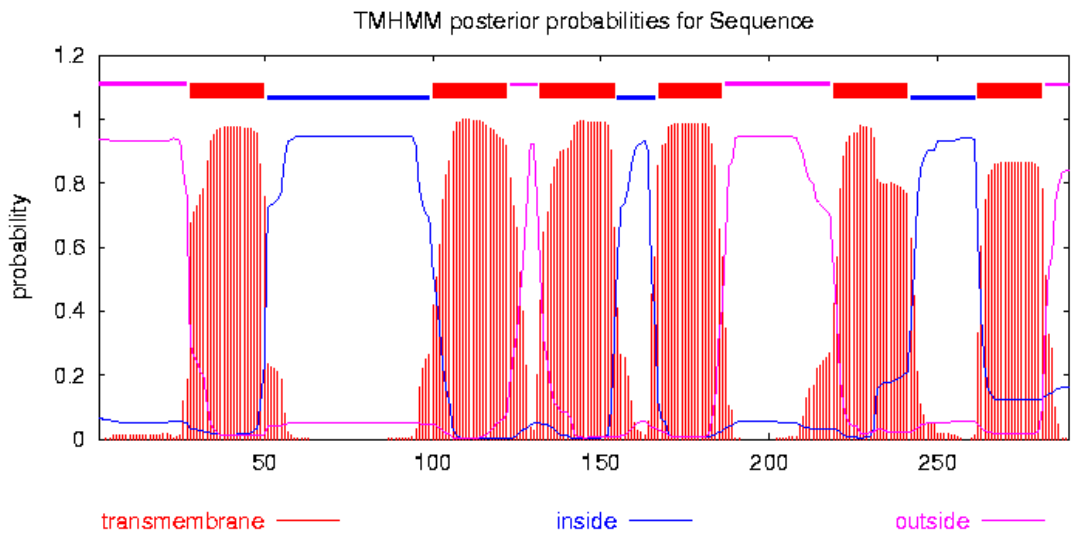
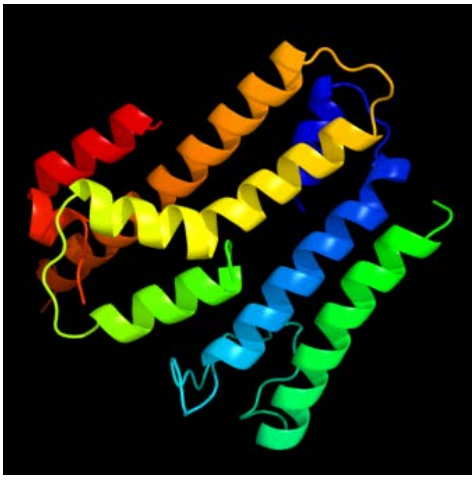


Figure S4. Production of **4** and conversion of **2** to **5** by *S. cerevisiae* expressing *fma*-KS and –AT.

A



B



Homology model based on template c3ts7B: Chain B, farnesyl diphosphate synthase from *Methylococcus capsulatus*.

188 residues (65% of Af520 (*fma*-TC) 289 residues protein sequence, aligned region shown above) have been modeled with 92.4% confidence by the single highest scoring template.

Figure S5. Protein structure prediction and analysis of *fma*-TC. (**A**) Transmembrane helices prediction of *fma*-TC with the TMHMM server (www.cbs.dtu.dk/services/TMHMM/), showing six transmembrane helices. (**B**) Homology model of *fma*-TC generated with Phyre2 protein fold recognition server (www.sbg.bio.ic.ac.uk/phyre2/) based on the top hit, *Methylococcus capsulatus* FPP synthase (PDB_ID c3ts7B), as template.

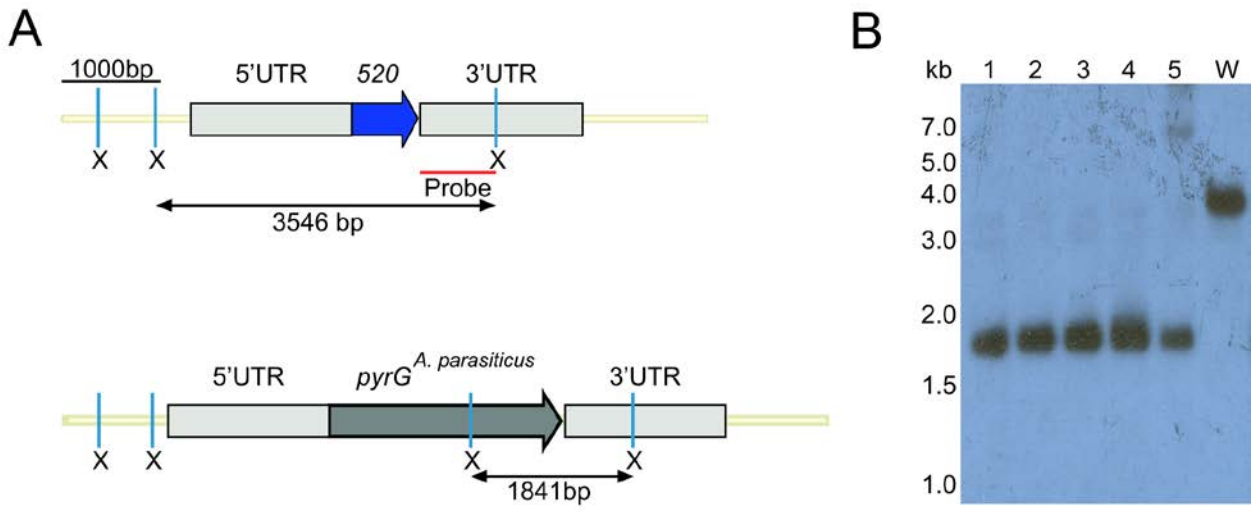


Figure S6. Targeted deletion of AFUA_8G00520 and Southern analysis.

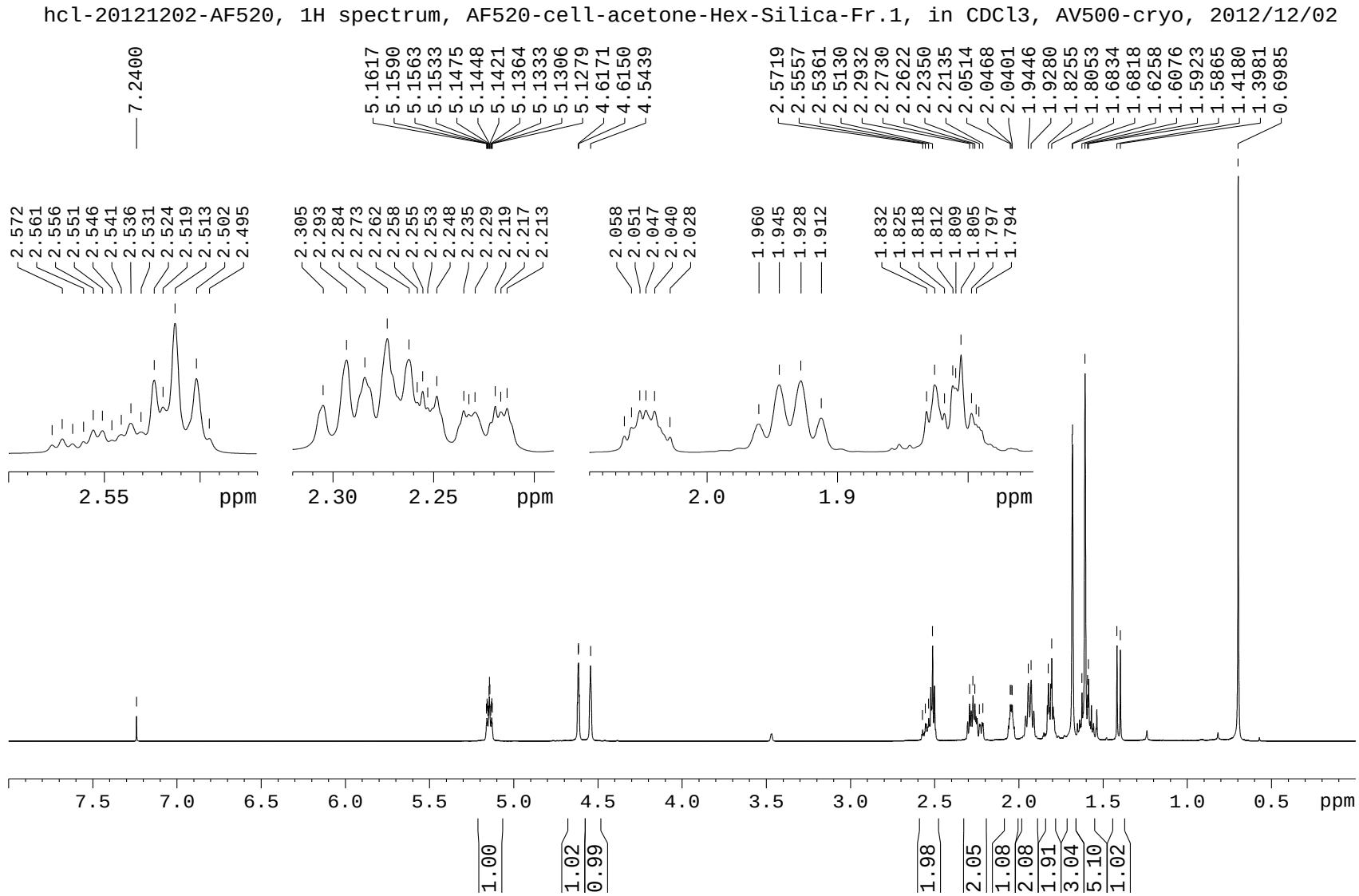


Figure S7. ^{1}H NMR spectrum of **3** ($CDCl_3$, 500 MHz).

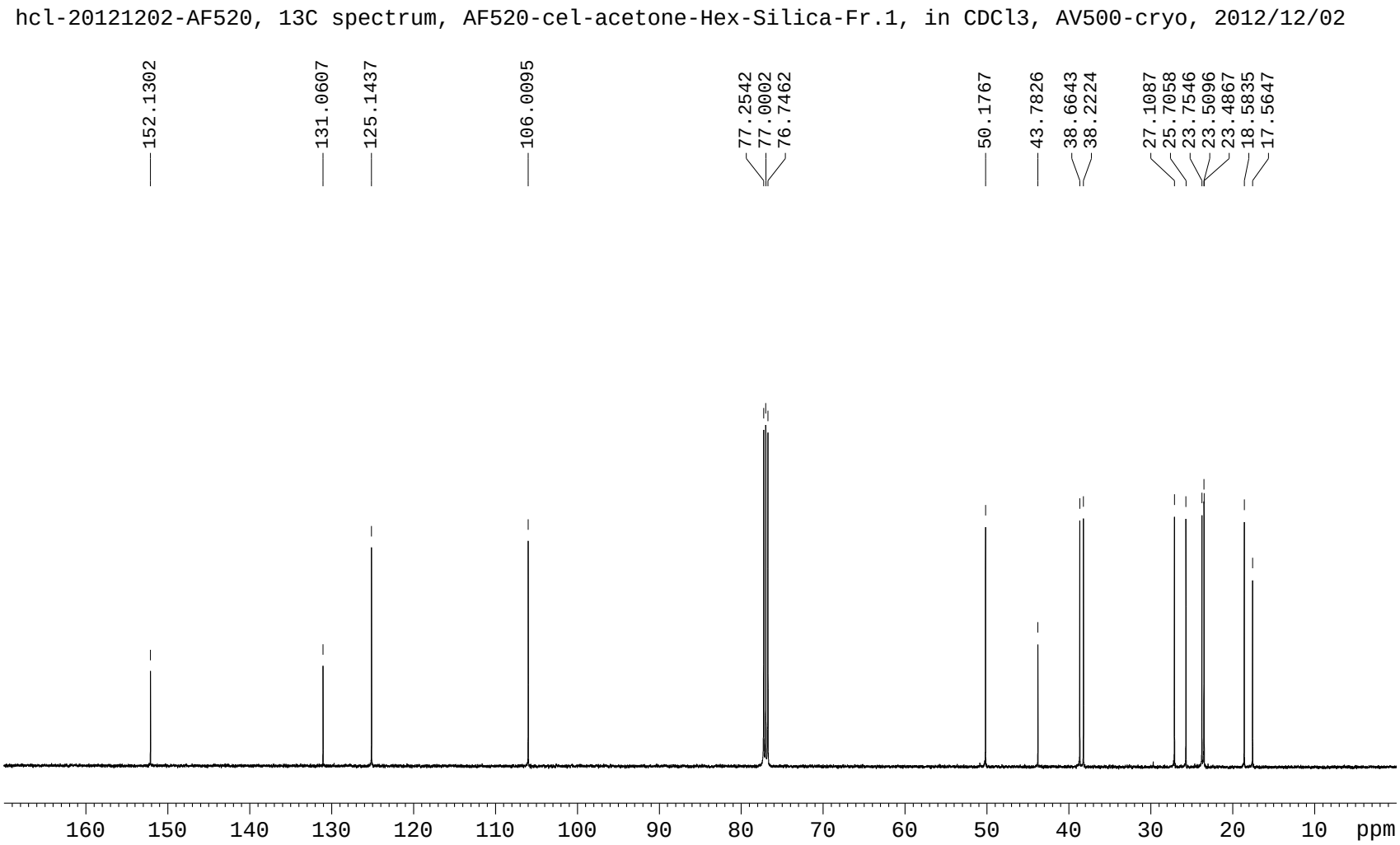


Figure S8. ^{13}C NMR spectrum of **3** ($CDCl_3$, 125 MHz).

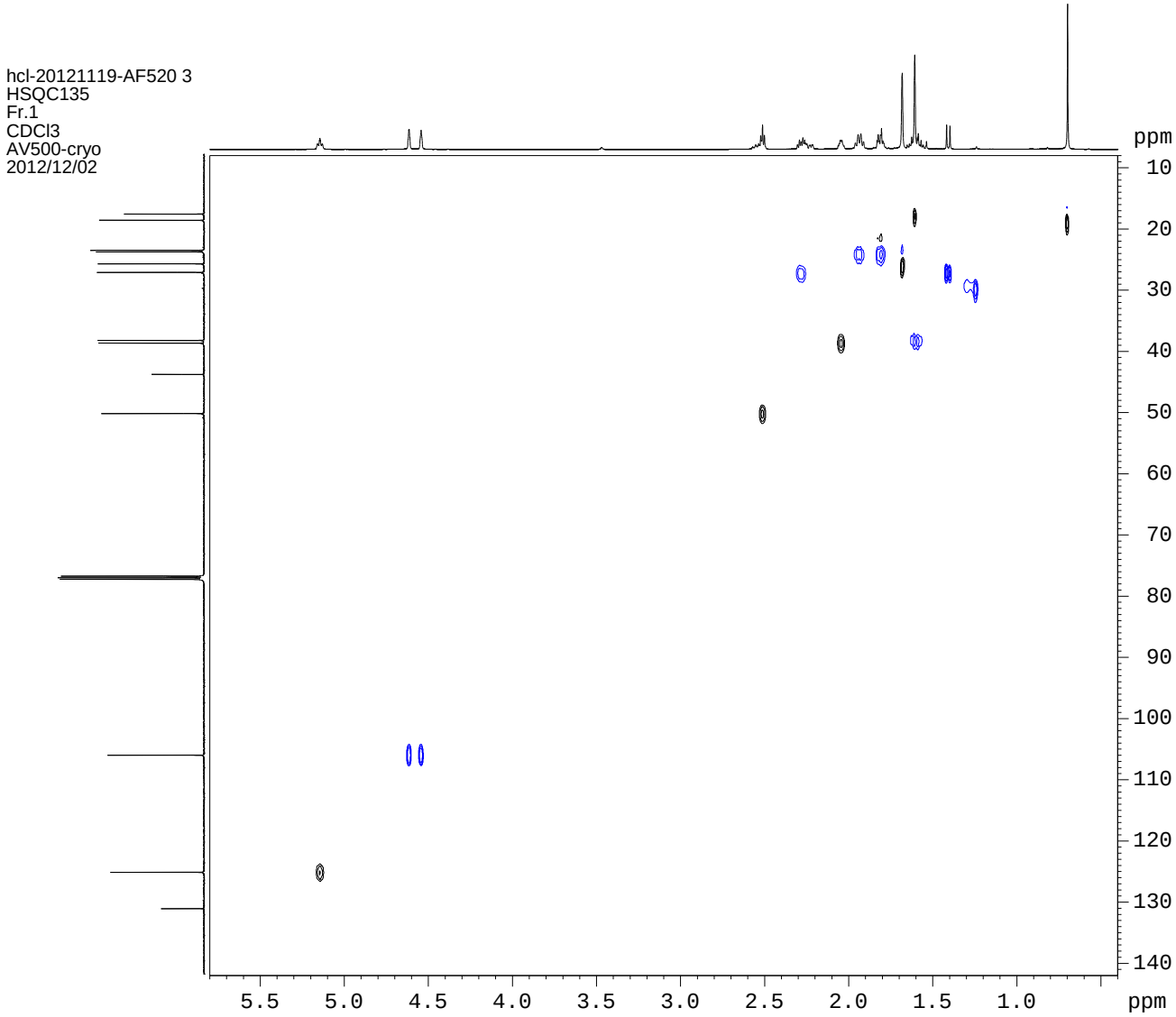


Figure S9. HSQC135 spectrum of **3** ($CDCl_3$, 500 MHz).

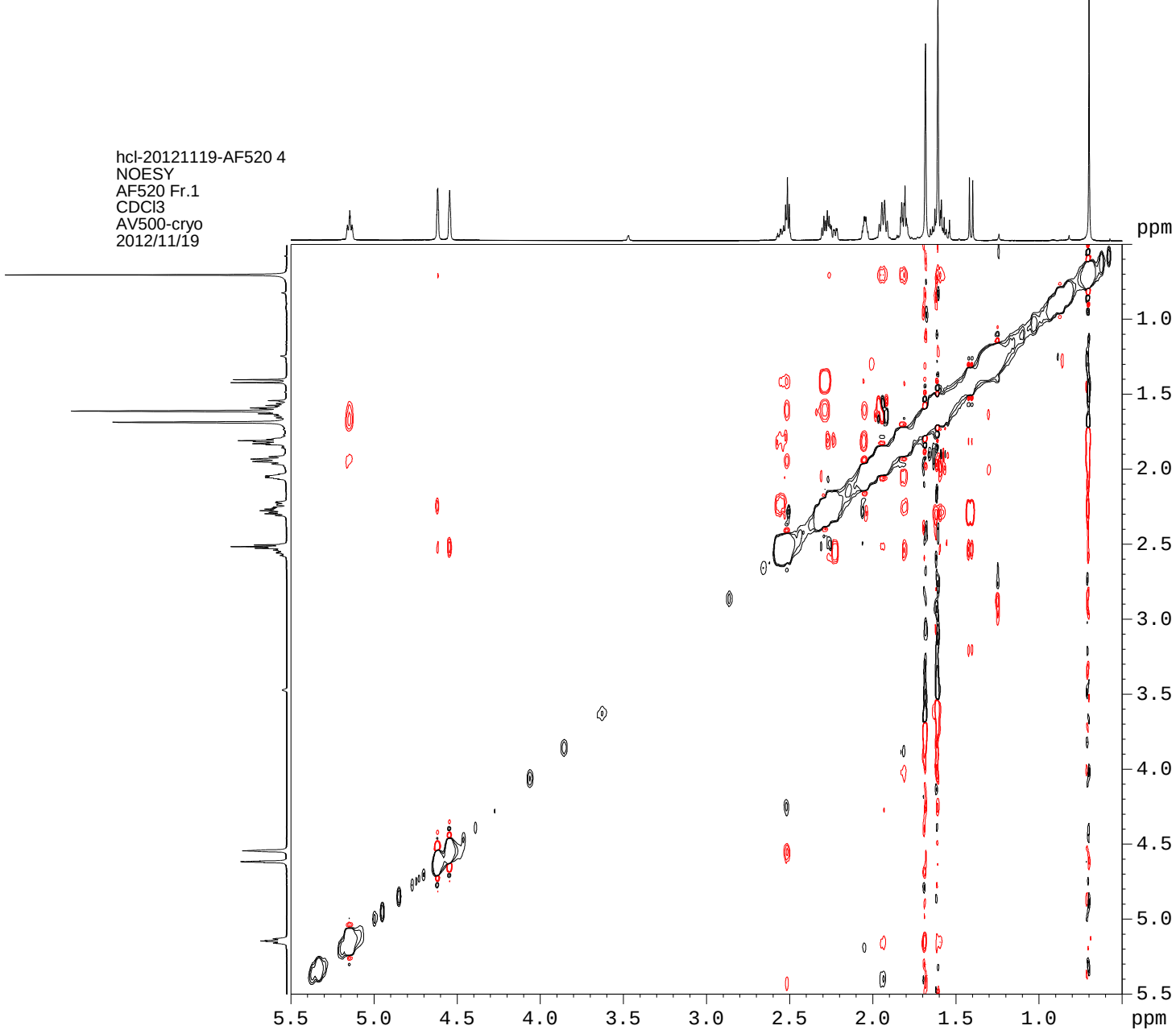


Figure S10. NOESY spectrum of **3** ($CDCl_3$, 500 MHz).

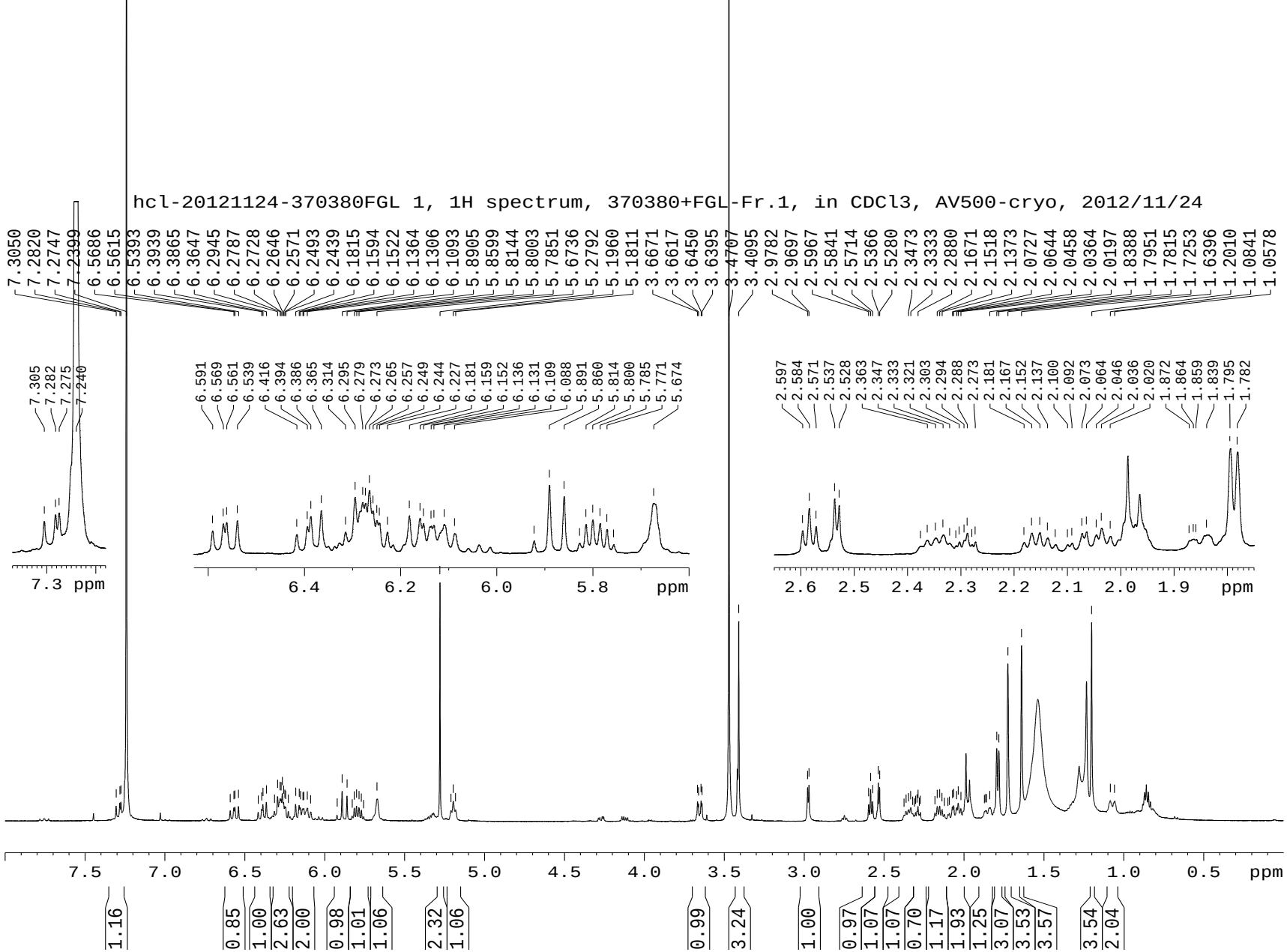


Figure S11. ^{1}H NMR spectrum of **5** ($CDCl_3$, 500 MHz).

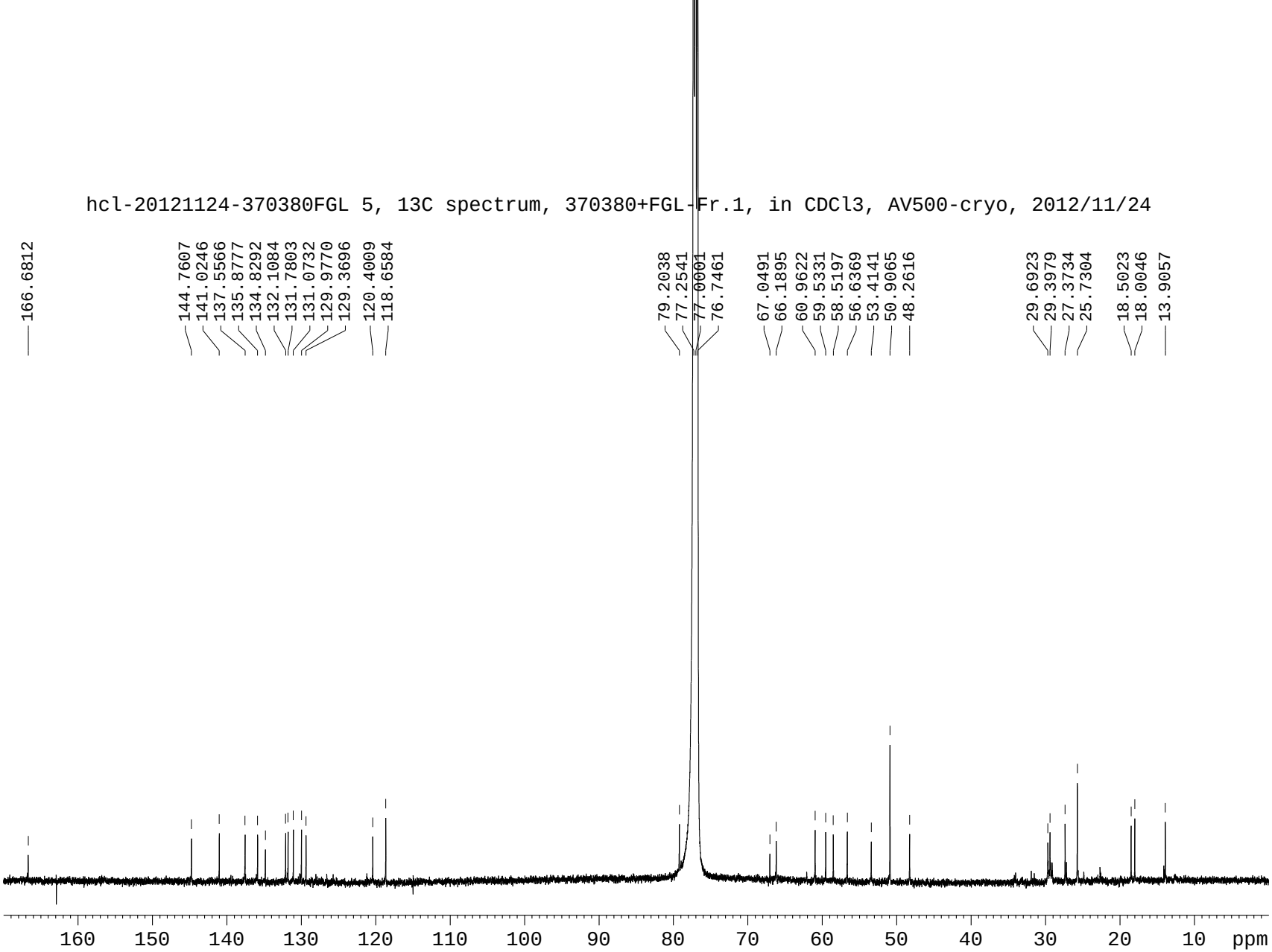


Figure S12. ^{13}C NMR spectrum of **5** ($CDCl_3$, 125 MHz).

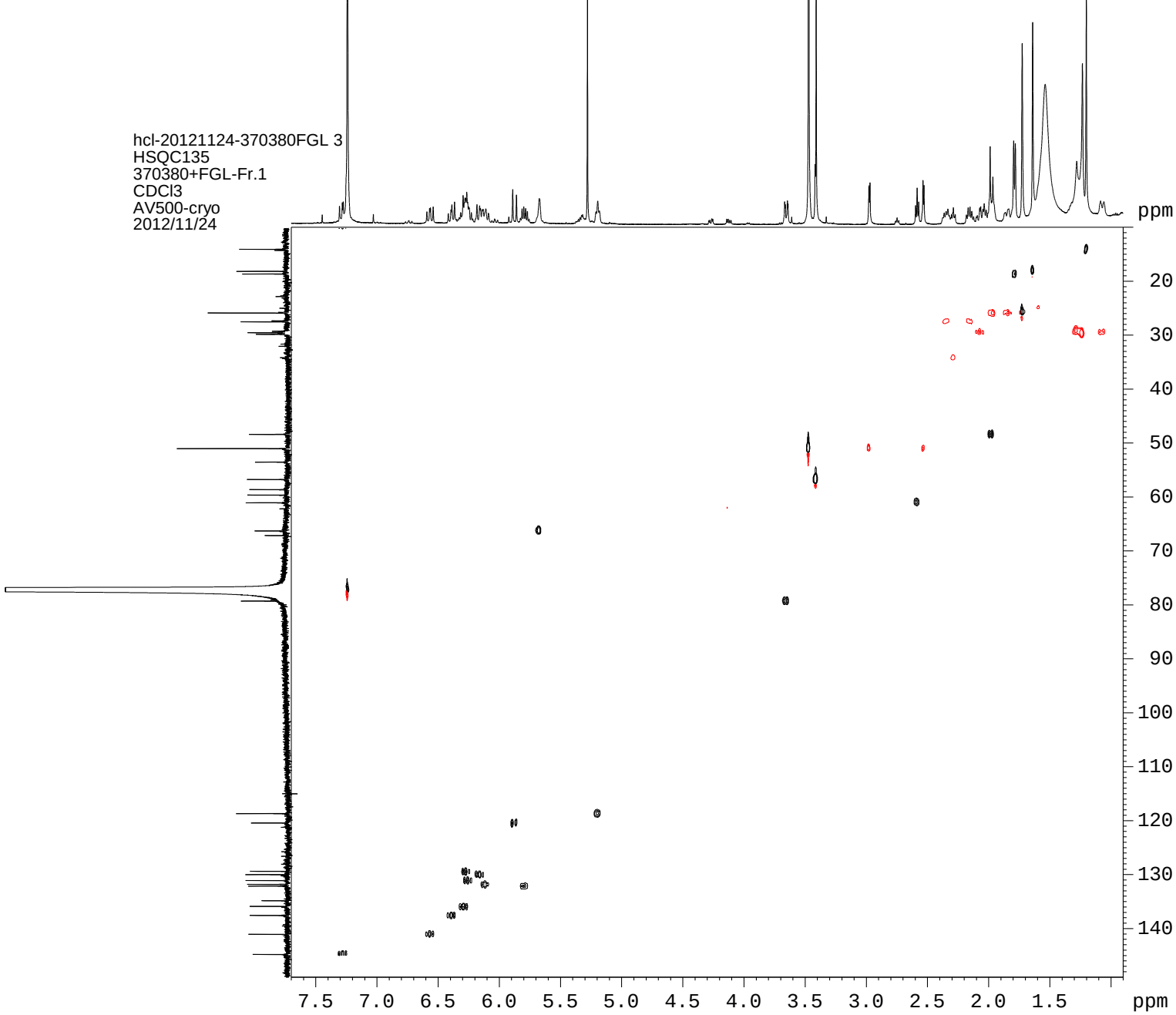


Figure S13. HSQC-135 spectrum of **5** ($CDCl_3$, 500 MHz).

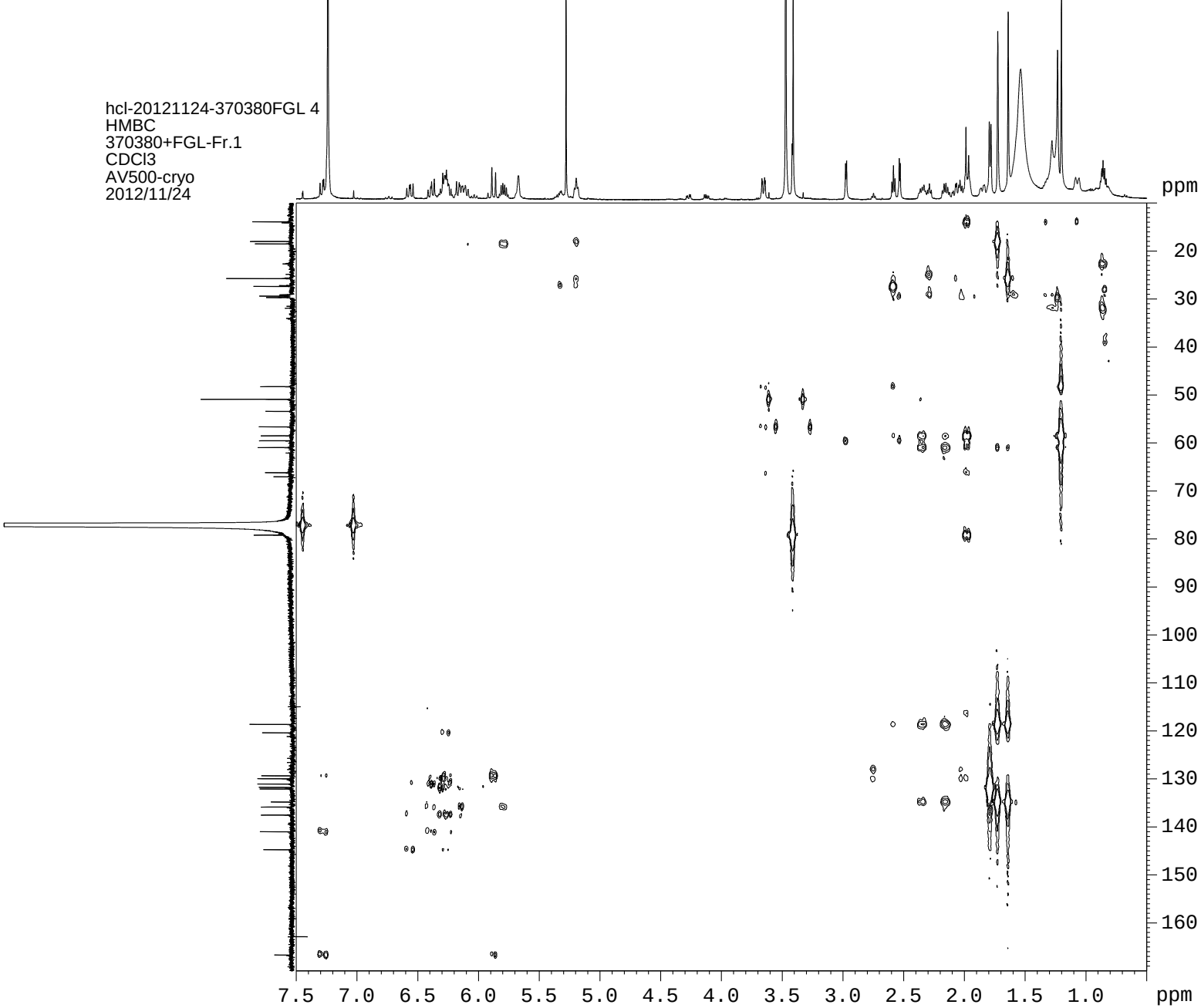


Figure S14. HMBC spectrum of **5** ($CDCl_3$, 500 MHz).

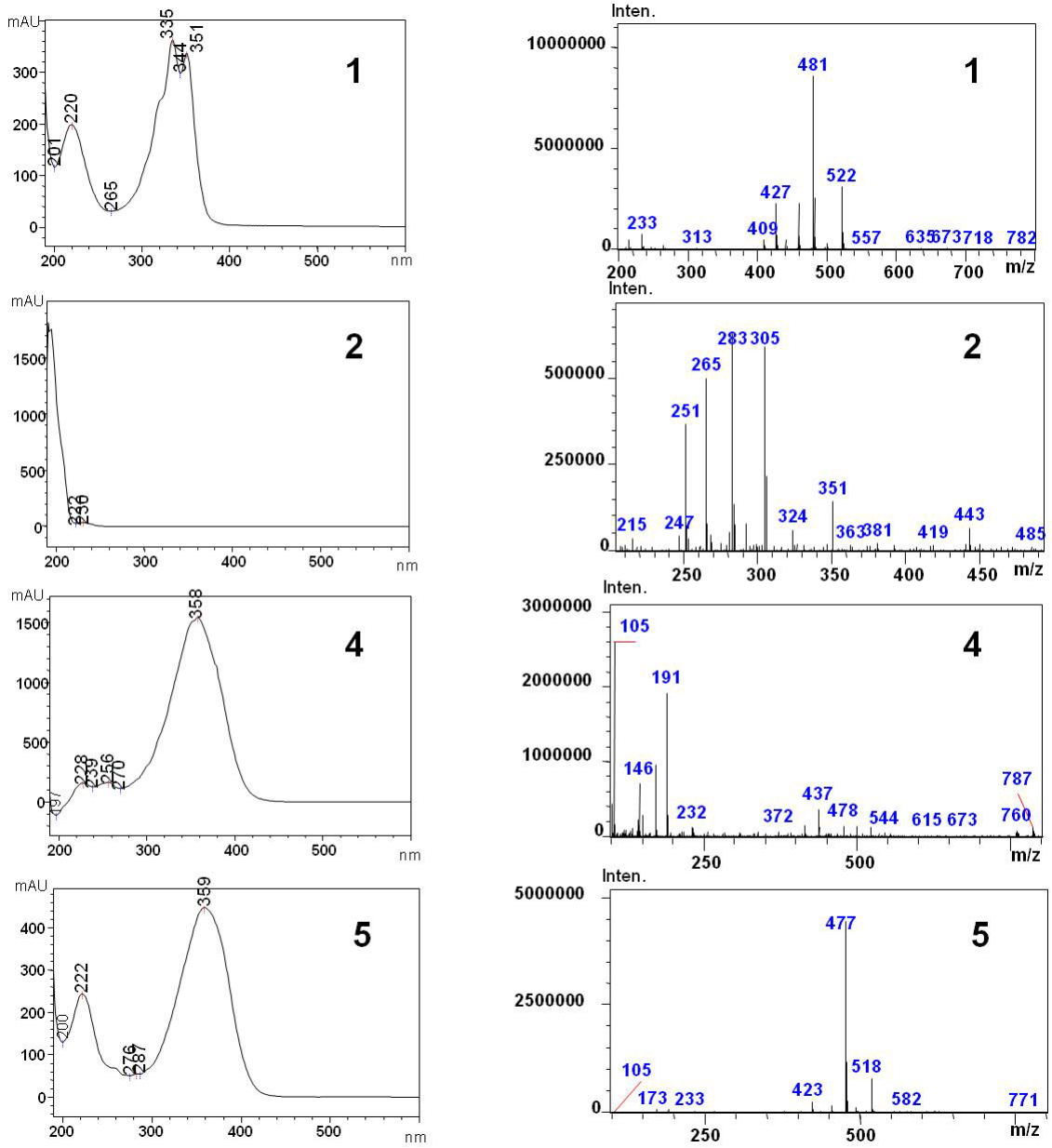


Figure S15. UV and MS spectra of **1**, **2**, **4** and **5**

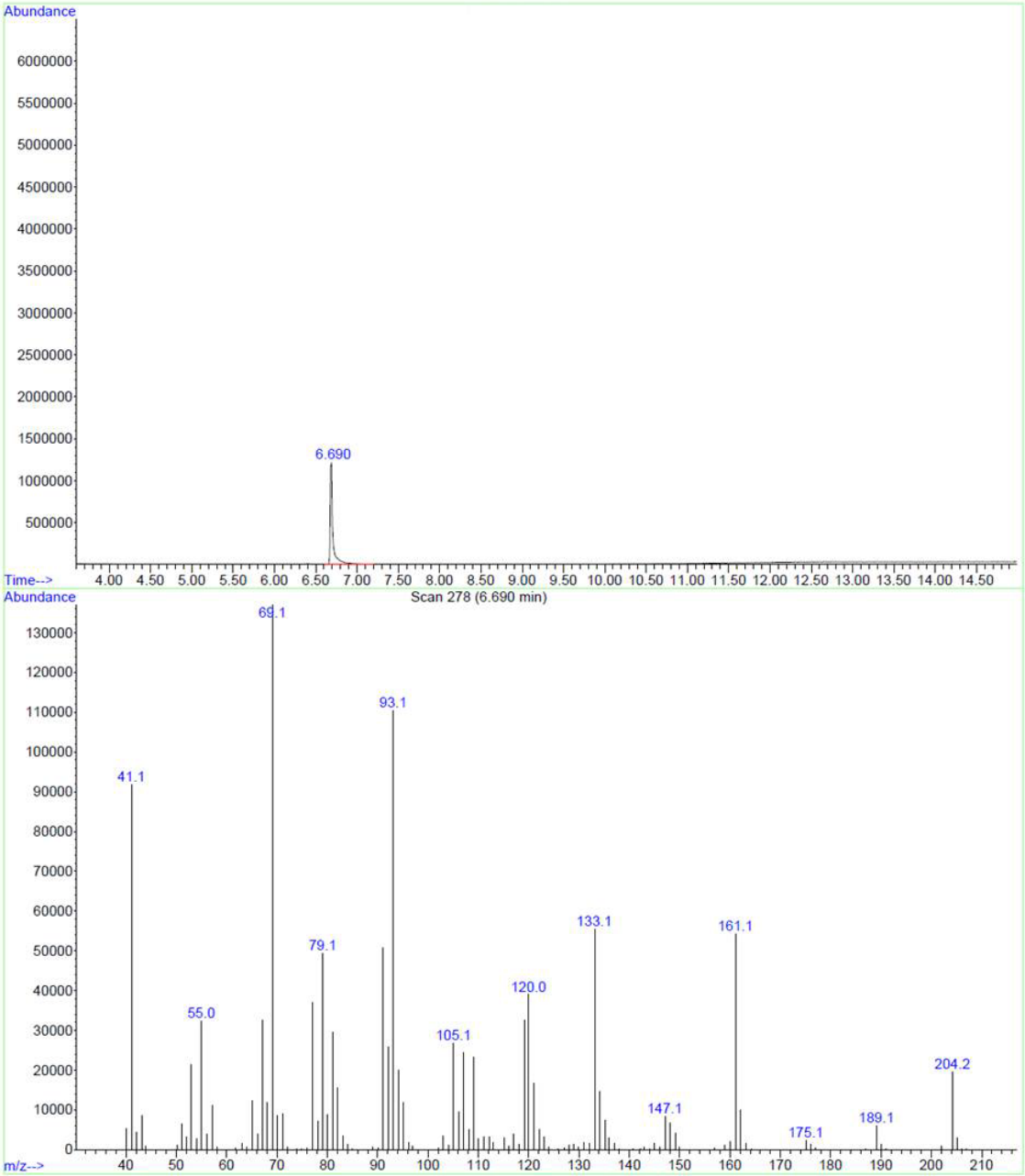


Figure S16. GC-MS data of **3** purified from *S. cerevisiae* expressing *fma*-TC.

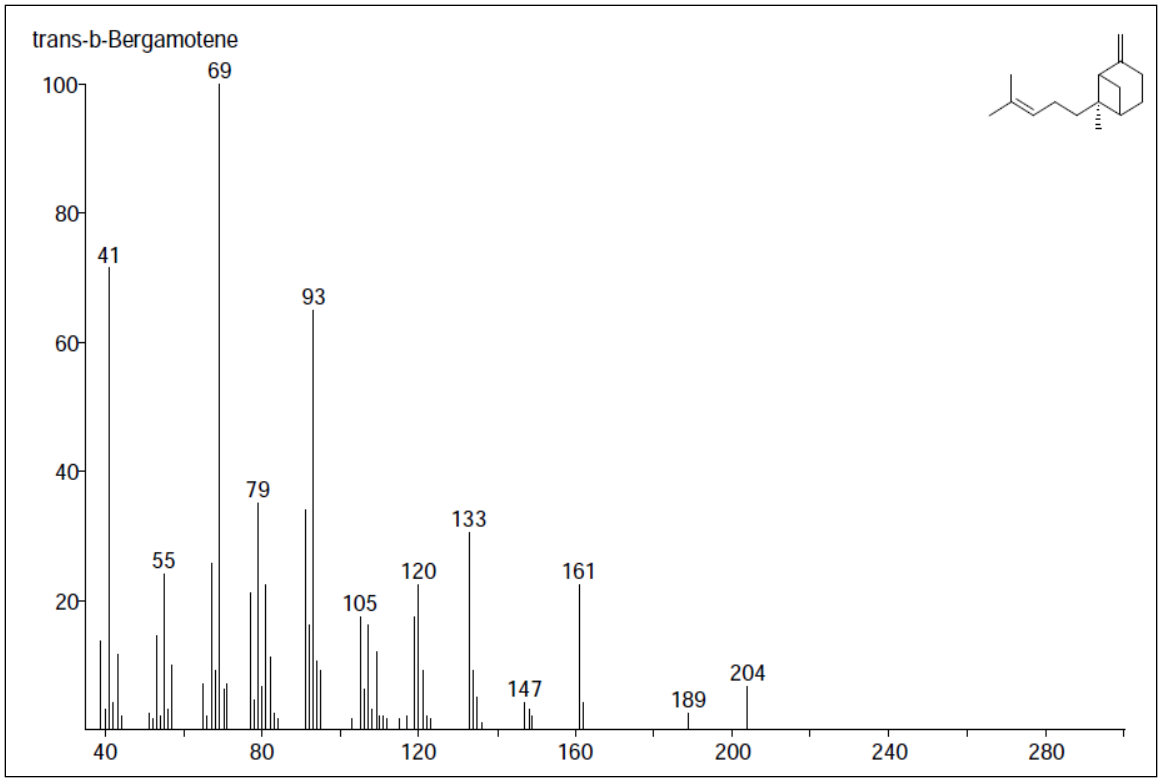


Figure S17. EI-MS of β-*trans*-bergamotene from the MassFinder 4 database (kindly provided by Prof. David Cane)

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