Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

Biosynthesis of lagopodins in mushroom involves complex network of oxidation reactions

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1. Supporting Methods

1.1 Reagents, strains and general techniques for DNA manipulation.

All of the chemicals were purchased from Tokyo Chemical Industry Co. Ltd., Sigma-Aldrich and Wako Pure Chemical Industries, Ltd. unless otherwise specified. Purchased chemicals were of reagent grade and used without further purification. *Coprinopsis cinerea* ku3-24[1] was used for analyzing lagopodin biosynthetic pathway. *Escherichia coli* XL1-Blue (Agilent Technologies) and *E. coli* TOP10 (Thermo Fisher Scientific Inc.) were used for plasmid propagation by standard procedures. Overproduction of recombinant proteins was carried out in *E. coli* BL21 (DE3) (Thermo Fisher Scientific Inc.). DNA restriction enzymes were used as recommended by the manufacturer (Thermo Fisher Scientific Inc.). PCR was carried out using PrimeSTAR GXL DNA polymerase (TAKARA Bio Inc.) as recommended by the manufacturer. Sequences of PCR products were confirmed through DNA sequencing (Macrogen Japan Corporation). *Saccharomyces cerevisiae* strain BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2-Δ1 trp1 pep4::HIS3 prb1 Δ1.6R can1 GAL*)[2] was used as the yeast expression host.

1.2 Biosynthetic gene cluster responsible for the production of lagopodin in *C. cinerea.*

Figure S1. The organization of the lagopodin biosynthetic gene cluster in *C. cinerea* ku3-24.[3]

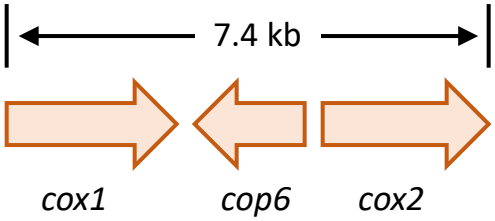


Table S1. Deduced functions of the open reading frames (ORFs) in the lagopodin biosynthetic gene cluster in *C. cinerea* ku3-24.[3]

Gene name	Protein name	Proposed protein function (most informative protein[a])
cox1 (CC1G_03562)	Cox1	Cytochrome P450
cop6 (CC1G_03563)	Cop6	sesquiterpene synthase
cox2 (CC1G_03564)	Cox2	Cytochrome P450

[a] Deduced functions of the ORFs identified within the biosynthetic gene cluster were based on the percentage sequence identity/similarity to known proteins as determined by Protein BLAST search against the NCBI non-redundant database.[4]

1.3 Construction of plasmids for manipulating the lagopodin biosynthetic genes.

1.3.1 Construction of the disruption cassette in pKW22011 for knocking out *cop6* in *C. cinerea.*

The disruption cassette included a selection marker sandwiched in between a 5' side- and a 3' side-flanking fragment (5'-*cop6* and 3'-*cop6*, respectively) as shown in **Fig. S3**. 5'-*cop6* and 3'-*cop6* are a 1,500-base pair fragment that is homologous to the site of recombination at or near the 5' and 3' end of the target gene in the *C. cinerea* ku3-24 genome. The primer sets given in **Table S2** were used to prepare the required flanking homologous regions for each of the target genes. 5'-*cop6* and 3'-*cop6* were amplified by PCR using the *C. cinerea* ku3-24 genome as a template by using the primer sets of pKW22011-LF/pKW22011-LR and pKW22011-RF/pKW22011-RR (**Table S2**) as shown in **Fig. S3**. The *hph* gene (the hygromycin B phosphotransferase gene *hph* that confers hygromycin resistance) used in the disruption cassette was amplified from pPHT[5] by PCR using the primer set of Ma13F/Ma13R (**Table S2**). Three purified fragments (5'-*cop6*, 3'-*cop6*, and the *hph* cassette), each at 50 to 150 ng in a total volume of 45 µL, were mixed with the delivery vector pRS426[6] (2 µg) pre-digested with *Eco*R I (10 units) and *Bam*H I (10 units) at 37 °C for 30 min.

The mixture was transformed into *S. cerevisiae* strain BJ5464-NpgA to generate pKW22011 (**Fig. S2**) possessing the desired deletion cassette through *in vivo* homologous recombination. The resulting plasmid pKW22011 was recovered from the yeast transformant and transferred to *E. coli*. The plasmid was amplified in *E. coli* for subsequent characterization by restriction enzyme digestion and DNA sequencing to confirm its identity. For targeted homologous recombination, a PCR product was first amplified from the plasmid carrying a desired cassette using the corresponding primer set of pRS426-F/ pRS426-R (**Table S2**) as shown in **Fig. S2**.

Figure S2. Map of plasmid pKW22011 for knockout of *cop6* in *C. cinerea.*

1.3.2 Transformation of *C. cinerea* ku3-24.

A mutant *C. cinerea* ku3-24 strain was initially cultured on an MYG agar medium (10 g/L malt extract, 4 g/L glucose and 4 g/L yeast extract medium with 15 g/L agar) at 30 °C for 5 days.[7] Homogenized mycelia collected from a single plate were used to inoculate 200 mL of MYG medium at 30 °C for 16 h with shaking at 180 r.p.m. Grown cells were collected by filtration and washed with MM buffer pH 5.5 (0.5 M mannitol and 0.05 M maleic acid). The cells were incubated with 1 mL of MM buffer pH 5.5 containing 1 mg/mL chitinase and 50 mg/mL lysing enzyme (Sigma-Aldrich) at 30 °C for 4 h. The resulting protoplasts were filtered and subsequently centrifuged at 2,000 × g for 5 min at room temperature. The collected protoplasts were washed with MM buffer pH 5.5 and centrifuged to remove the wash solution. Approximately 0.5×10^8 to 1×10^8 of protoplasts were suspended in 200 µL of MMC buffer at pH 5.5 (0.5 M mannitol, 0.05 M maleic acid and 0.05 M calcium chloride). Then 40 µL of PEG solution at pH 8.0 (400 mg/mL polyethylene glycol 8,000, 50 mM calcium chloride and 50 mM Tris-HCl) was added to the protoplast suspension. The mixture was subsequently combined with 5 µg of the DNA fragment with which the cells were to be transformed. The mixture was incubated on ice for 30 min to allow the transformation to proceed. After incubation on ice, 1 mL of the PEG solution was added to the

reaction mixture, and the mixture was incubated at room temperature for additional 15 min. The cells were plated on the RM agar (1.5% w/v) plates containing 1 mM *p*-aminobenzoic acid in order to recover the transformed *C. cinerea* ku3-24. After 24 h at 30 °C incubation, the plate was overlaid with RM top agar (0.5% w/v) containing 0.6 mg/mL hygromycin and incubated at 30 °C for 7 days to select for desired mutant strains of *C. cinerea* ku3-24. Second selection was carried out by using an RM agar plate containing 1 mM *p*-aminobenzoic acid and 0.15 mg/mL hygromycin at 30 °C for 7 days. Replacement of the target region was confirmed by diagnostic PCR (**Fig. S4**).

1.3.3 Confirmation of targeted deletion of *cop6* by PCR.

To verify that the target gene was replaced with the cassette, the gDNA isolated from the transformants was analyzed by diagnostic PCR. Three sets of PCR primers were designed for this verification (**Figs. S3 and S4**). For the first set, one primer that anneals to the selection marker and another primer that anneals at the 3' side of the 3'-*cop6* region were designed (**Fig. S3A**, "Positive PCR" set in **Table S2**). With this primer set, wild type gDNA will not produce any PCR product with this primer set. However, a PCR product around 1.7 kb in size will be formed from the gDNA of a strain containing desired gene deletion. For the second set, one primer that anneals to the selection marker and another primer that anneals at the 5' side of the 5'-*cop6* region were designed (**Fig. S3A**, "positive PCR" set in **Table S2**). With this primer set, wild-type gDNA will not produce any PCR product with this primer set. However, a PCR product around 1.7 kb in size will be formed from the gDNA of a strain containing desired gene deletion. For the third primer set, one primer that anneals near the 3' end of the target gene and another primer that anneals approximately 500 bp inside of the target gene (**Fig. S3B**, "Negative PCR" set in **Table S2**). Results of the PCR analyses are given in **Fig. S4**.

Figure S3. A schematic diagram showing the generation of the Δ*cop6* strain from *C. cinerea* ku3-24, and the primer design for the confirmation of a knockout strain by PCR using the gDNA as a template. (A) Confirmation for the generation of knockout strain. (B) Identification for the presence of wild type strain.

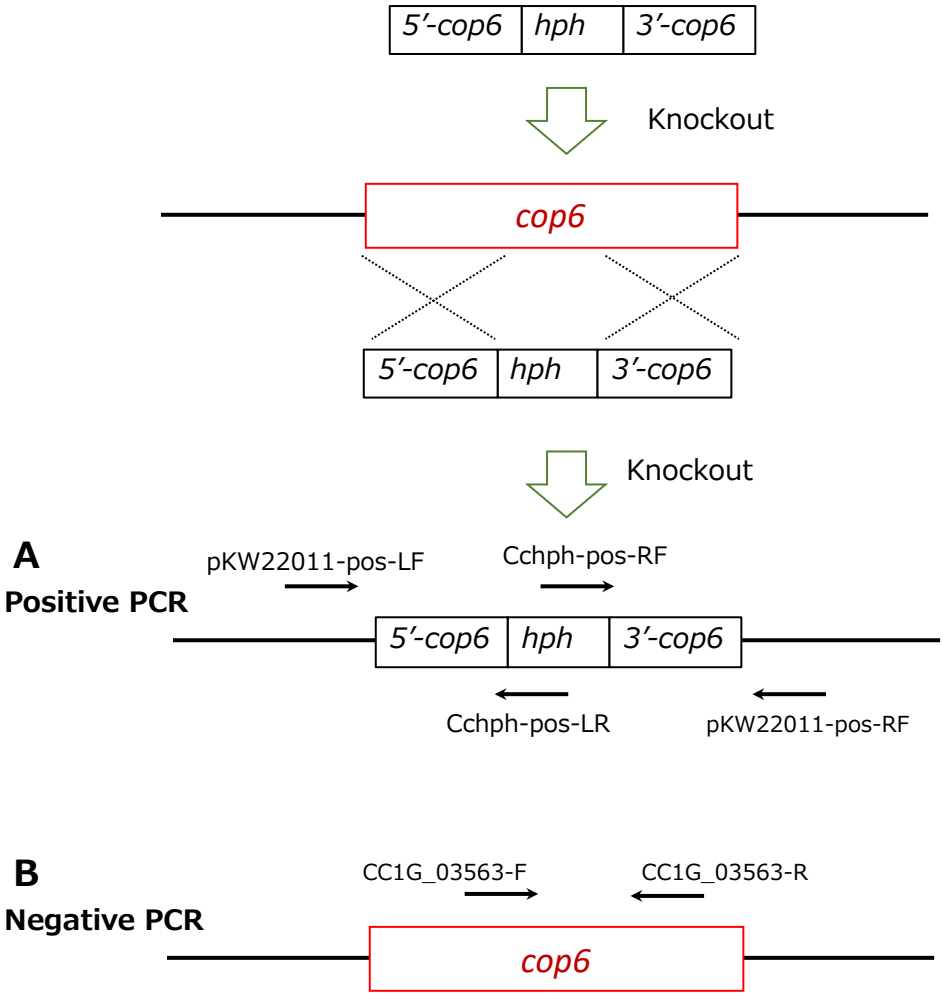
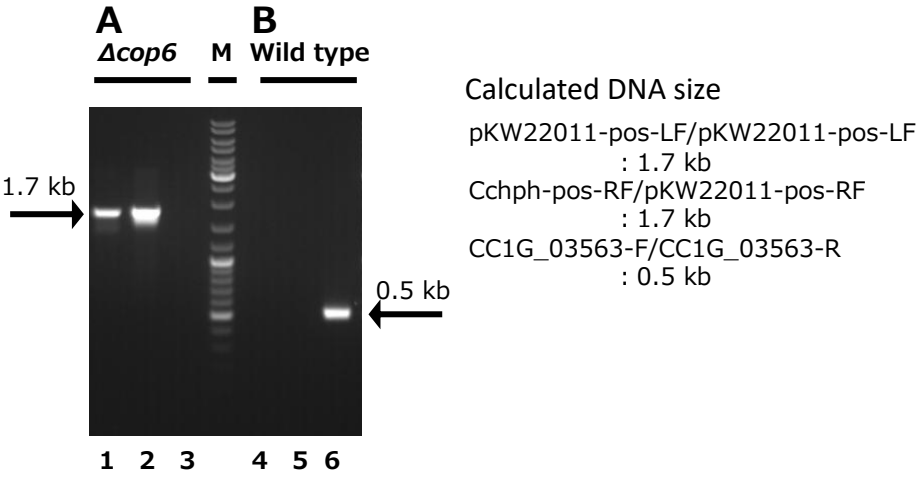


Figure S4. PCR analysis for confirming the deletion of *cop6* using the gDNA of Δ*cop6* strain and the wild-type *C. cinerea* ku3-24 as templates, respectively. Amplifications of PCR products from Δ*cop6* and the wild-type strains were performed using the primer sets described in **Fig. S3**. The sequences of the primer sets are listed in **Table S2**. Lane M, molecular weight marker. (A) With the gDNA from the Δ*cop6* strain, positive PCR as indicated in **Fig. S3A** generated correct amplicons for the left arm (lane 1: the primer set of pKW22011-pos-LF/Cchph-pos-LR) and the right arm (lane 2: the primer set of Cchph-pos-RF/pKW22011-pos-RF). No PCR product was obtained for negative PCR as indicated in **Fig. S3B** performed with the primer set CC1G_03563-F/CC1G_03563-R (lane 3). (B) With the gDNA from the wild-type strain no PCR product was obtained for positive PCRs for left arm (lane 4: primer set of pKW22011-pos-LF/Cchph-pos-LR) and right arm (lane 5: primer set of Cchph-pos-RF/pKW22011-pos-RF). However, correct amplicon was generated for negative PCR using the primer set CC1G_03563-F/CC1G_03563-R (lane 6).



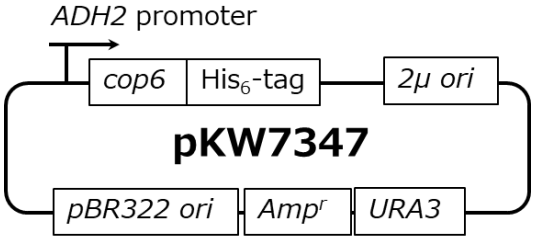
1.3.4 Cloning of the lagopodin biosynthetic genes.

The open reading frames (ORFs) of the lagopodin biosynthetic genes were determined and cloning primers were designed based on the previous study that employed predicted gene models based on the *C. cinerea* genome sequence information provided by the Broad Institute.[3] To construct vectors for expression of the cytochrome P450 genes *cox1* and *cox2* in *E. coli* or yeast, we isolated total RNA from *C. cinerea* ku3-24 using the Ambion RNAqueous kit (Thermo Fisher Scientific Inc.). The SMARTer RACE cDNA Amplification kit (Clontech Laboratories, Inc.) was used for synthesizing cDNA according to the protocol supplied by the manufacturer. The *C. cinerea* ku3-24 strain was cultured in a MYG liquid medium at 30 °C for 7 days with shaking at 180 r.p.m. Total RNA was isolated from the mycelium using the Ambion RNAqueous kit (Thermo Fisher Scientific Inc.). The mycelia weighing 100 mg were suspended in 100 μL of the elution buffer and 1 mL of the lysis/binding solution provided by the kit and subsequently flash-frozen in liquid nitrogen. The frozen mixture was ground with a refrigerated mortar and pestle for 2 min. The resulting cell powder was then allowed to thaw into a lysate solution. This solution was centrifuged with 13,000 × *g* at 4 °C for 2 min. The supernatant was mixed with 700 μL of 64% (v v^{-1}) ethanol to a final ethanol concentration of 32% and allowed to stand for 30 seconds on ice. The resulting supernatant was loaded onto a spin column, and the RNA was eluted from the column with the elution buffer provided by the kit. To examine the quantity and quality of the isolated total RNA, the RNA solution was checked by agarose gel electrophoresis. Subsequently, DNase (3.0 units) was added to the isolated RNA to digest the genomic DNA at 37 °C for 30 min. The target ORFs were determined based on the database annotation and expressed in suitable heterologous hosts to obtain recombinant enzymes in their active forms.

1.3.5 Construction of pKW7347, 7348 and 7349 for expression of *cop6*, *cox1* and *cox2* in *S. cerevisiae*.

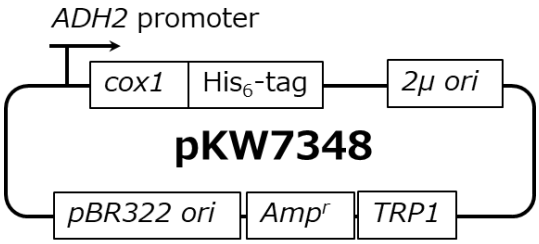
For molecular cloning of *cop6* from *C. cinerea* ku3-24, this strain was cultured in a MYG liquid medium at 30 °C for 7 days with shaking at 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *cop6* gene was amplified from the cDNA by PCR with two primers 7347_1F and 7347_1R (**Table S2**). The vector for gene expression in the *S. cerevisiae* strain BJ5464-NpgA derived from pXW55[8] was amplified by PCR using the 7347_2F/7347_2R primer set (**Table S2**). These purified fragments, each at 50 to 150 ng in a total volume of 45 μL, were mixed *in situ* by the endogenous homologous recombination activity of *S. cerevisiae*. The resulting plasmid was amplified in *E. coli* for restriction digestion analysis. The identity of the resulting vector pKW7347 (**Fig. S5**) was confirmed by DNA sequencing (Macrogen Japan Corporation).

Figure S5. Map of plasmid pKW7347 for the production of Cop6 in *S. cerevisiae* strain BJ5464-NpgA.



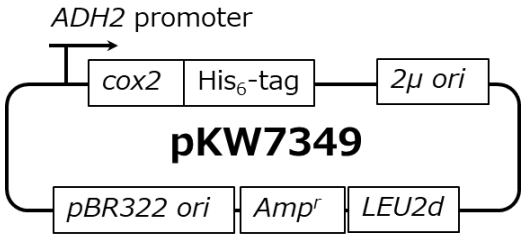
The *cox1* from the cDNA was amplified by PCR with two primers 7351_1F/7351_1R (**Table S2**) to yield 1.8 kb PCR products. The gene was cloned into the TOPO cloning vector (Thermo Fisher Scientific, Inc.) as recommended by the manufacturer to obtain the initial plasmid TOPO7348 (1st step). The *cox1* was amplified from TOPO7348 by PCR using the 7348_1F/7348_1R primer set (**Table S2**). The vector for gene expression in BJ5464-NpgA derived from pXW06[8] was amplified by PCR using the 7348_2F/7348_2R primer set (**Table S2**). These purified fragments were mixed *in situ* by the endogenous homologous recombination activity of *S. cerevisiae*. The resulting plasmid was amplified in *E. coli* for restriction digestion analysis. The identity of the resulting vector pKW7348 (**Fig. S6**) was confirmed by DNA sequencing (Macrogen Japan Corporation). This plasmid was used for expression of *cox1* for *in vivo* reaction (See Section **1.4.5**).

Figure S6. Map of plasmid pKW7348 for the production of Cox1 in *S. cerevisiae* strain BJ5464-NpgA.



The *cox2* was amplified from the cDNA by PCR with two primers 7349_1F and 7349_1R (**Table S2**). The vector for gene expression in BJ5464-NpgA derived from pXW02[8] was amplified by PCR using the 7349_2F/7349_2R primer set (**Table S2**). These purified fragments were mixed *in situ* by the endogenous homologous recombination activity of *S. cerevisiae*. The resulting plasmid was amplified in *E. coli* for restriction digestion analysis. The identity of the resulting vector pKW7349 (**Fig. S7**) was confirmed by DNA sequencing (Macrogen Japan Corporation). This plasmid was used for expression of *cox2* for *in vivo* and *in vitro* reactions (See Sections **1.4.3**, **1.4.7** and **Figs. S10**).

Figure S7. Map of plasmid pKW7349 for the production of Cox2 in *S. cerevisiae* strain BJ5464-NpgA.

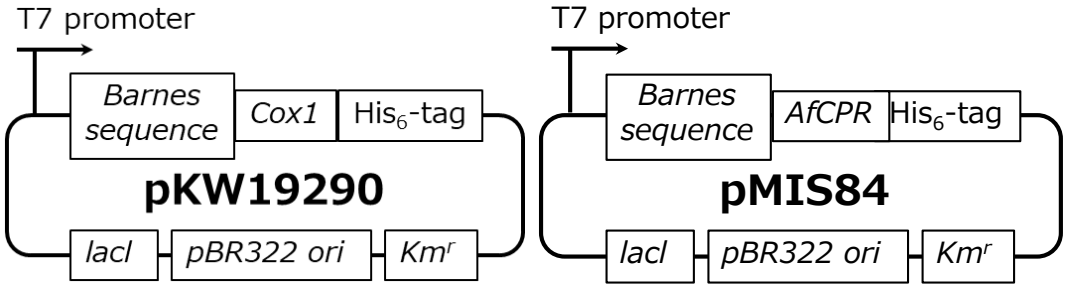


1.3.6 Construction of pKW19290 for expression of *cox1* and pMIS84 for expression of *AfCPR* in *E. coli*.

The *cox1* was amplified from pKW7348 by PCR with two primers pKW19290_Fw1 and pKW19290_Rv1 (**Table S2**). The vector for gene expression in *E. coli* derived from pET28b(+) was amplified by PCR using the P-186F/ms-188R primer set (**Table S2**). These purified fragments were ligated to generate pKW19290 (**Fig. S8**) using the GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific, Inc.). The identity of the resulting vector pKW19290 was confirmed by DNA sequencing. This plasmid was used for expression of *cox1* for purification of Cox1 for *in vitro* reactions (See Sections **1.4.1**, **1.4.8** and **Figs. S9**).

For molecular cloning of the cytochrome P450 reductase (CPR) gene from *A. fumigatus*,[9] cDNA was synthesized as described previously.[6] The *AfCPR* was amplified from cDNA by PCR with two primers pMIS84_Fw1 and pMIS84_Rv1 (**Table S2**). The vector for gene expression in *E. coli* derived from pET28b(+) was amplified by PCR using the P-311F/P-471R primer set (**Table S2**). These purified fragments were ligated to generate pMIS84 (**Fig. S8**) using the GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific, Inc.). The identity of the resulting vector pMIS84 was confirmed by DNA sequencing. This plasmid was used for expression of *AfCPR* for purification of CPR for *in vitro* reactions (See Sections **1.4.1**, **1.4.8** and **Figs. S9**).

Figure S8. Map of plasmid pKW19290 for expression of *cox1* and pMIS84 for expression of *AfCPR* in *E. coli.*

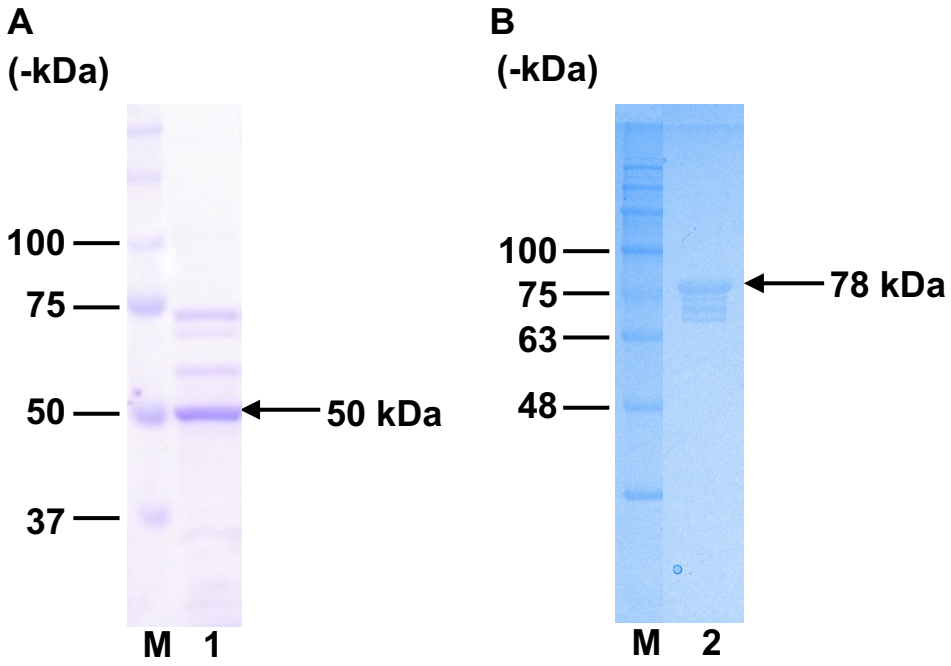


1.4 Protein production and enzymatic assays.

1.4.1 Production and purification of Cox1 and AfCPR.

Overexpression and subsequent purification of Cox1 were performed as follows: BL21 (DE3) harboring the plasmid pKW19290 was grown overnight in 10 mL of TB medium with 50 μg /mL kanamycin at 37 °C. Each liter of fresh TB medium with 100 μg/mL kanamycin was inoculated with 5 mL of the overnight culture and incubated at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.6. Then expression of each gene was induced with 100 μM isopropylthio-β-D-galactoside (IPTG) at 15 °C. Incubation was continued for another 24 h, after which cells were harvested by centrifugation at 10,000 × *g* for 10 min. All subsequent procedures were performed at 4 °C or on ice. Harvested cells were resuspended in disruption buffer [0.1 M Tris-HCl (pH 7.4), 0.1 M NaCl, and 20 mM imidazole]. Cells were disrupted by French Press, and the lysate was clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant and precipitate were recovered as the soluble and insoluble fraction, respectively. The soluble fraction containing proteins was applied onto a Ni-NTA (QIAGEN) column. After washing the column with a wash buffer containing 10 mM imidazole and 0.1 M NaCl in 0.1 M Tris-HCl (pH 7.4), the target protein was eluted with an elution buffer containing 0.1 M imidazole and 0.1 M NaCl in 0.1 M Tris-HCl (pH 7.4). The elution fractions containing the desired protein were pooled and dialyzed against the storage buffer [0.1 M Tris-HCl (pH 7.4), 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M NaCl and 25% (v/v) glycerol]. Protein concentration was estimated using the Bio-Rad protein assay kit with bovine immunoglobulin G as a standard. Purified protein samples were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide Tris-HCl gel stained with Coomassie Brilliant Blue R-250 stain solution (CBB; nacalai tesque) (**Fig. S9**). The purified protein was pooled and further concentrated with Amicon Ultra centrifugal concentrator (Millipore). Essentially the same protocol was used to overexpress and purify AfCPR from BL21 (DE3) harboring the plasmid pMIS84.

Figure S9. SDS–PAGE analyses of the nickel affinity chromatography fraction containing Cox1 and AfCPR. (A) Cox1 and (B) AfCPR analyses of the partially purified enzymes. The gels were stained with CBB. Lane M: molecular weight marker; 1: Cox1 (50 kDa); lane 2: AfCPR (50 kDa).



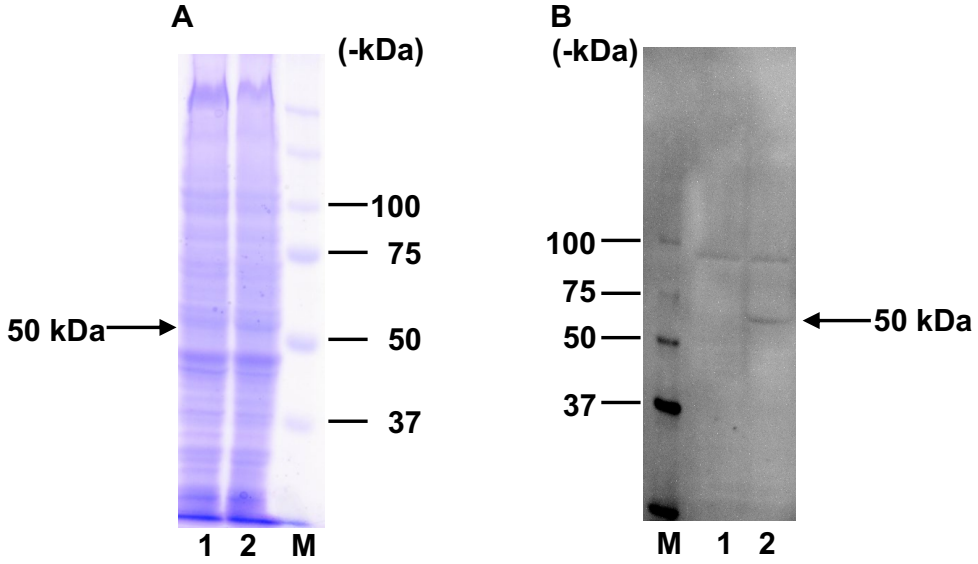
1.4.2 Preparation of *S. cerevisiae* microsomal fraction containing Cox2.

S. cerevisiae strain BJ5464-NpgA was transformed with pKW7349 (**Fig. S7**). Selected cells were grown in 2 mL of SC medium prepared using YNB with ammonium sulfate with DO supplement (Clontech Laboratories, Inc./TAKARA Bio Inc.) without L-leucine at 30 °C for 48 h with shaking at 180 r.p.m. The culture was transferred to 100 mL of fresh SC medium without L-leucine and the culture was incubated at 30 °C for 48 h. YPD medium (500 mL) was inoculated with the culture (100 mL) and incubated at 30 °C for 15 h. The cells were harvested by centrifugation at 2,500 × *g*. All subsequent procedures were performed at 4˚C or on ice. Harvested cells were resuspended in 10 mL of resuspension buffer [100 mM Tris-HCl (pH 7.4), 0.1 M NaCl], and the cells were disrupted by sonication. The lysate was clarified by centrifugation at 10,000 × g. Then, the supernatant was fractionated by ultracentrifugation at 100,000 × *g* for 1 h. The pellet was resuspended in 2 mL of TEG buffer [20 % (v/v) glycerol, 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA] to yield a microsomal fraction.[10]

1.4.3 Western blotting analysis for gene expression in yeast.

Western blotting analysis was performed to confirm the expression of *cox2* from the plasmid pKW7349 constructed as described earlier for the production of C-terminal hexahistidine-tagged Cox2. Subsequently, a standard western blotting analysis was carried out on the gel with the chemiluminescent system (Thermo Fisher Scientific Inc.) using an alkaline phosphatase-based detection method to identify the presence of our target enzymes (**Fig. S10**). Anti-poly-histidine mouse monoclonal antibody HIS-1 (Sigma-Aldrich) was used for visualization of the target protein.

Figure S10. Protein gels for isolation of Cox2 as a microsomal fraction. (A) SDS–PAGE and (B) Western blotting analyses of the microsomal fraction containing Cox2. Lane M: molecular weight marker; lane 1: culture and protein preparation of *S. cerevisiae* strain BJ5464-NpgA harboring pRS426 without *cox2*; lane 2: Cox2 (50 kDa).



1.4.4 Chemical complementation of the Δ*cop6/C. cinerea* ku3-24 mutant strain for rescued biosynthesis of 2.

For isolation of the biosynthetic product **2**, the Δ*cop6/C. cinerea* ku3-24 mutant strain was incubated in 30 mL of YMG medium for 5 days with shaking at 180 r.p.m. The culture was supplemented with 10 μM of **3** and incubated for an additional 2 h at 30 °C. 250 μL of the liquid medium was extracted with ethyl acetate (200 μL). The ethyl acetate extracts were combined and concentrated *in vacuo*, and the dried residue was dissolved in DMF (50 μL). The resulting solution

was subjected to LC–MS analysis (**Fig. 2**). For a larger-scale isolation of the product, 300 mL of the liquid medium was extracted with ethyl acetate (300 mL). The ethyl acetate extract was concentrated *in vacuo* to give an oily residue, which was fractionated by silica gel flash column chromatography with 0–100% *n*-hexane/ethyl acetate gradient elution, and then subsequently 0–100% ethyl acetate/MeOH gradient elution. The fractions containing the desired material were pooled and dried. The residue was purified by reversed-phase HPLC (Nacalai Tesque Inc., Cosmosil 5C18 MS-II 5 μm, 20 × 250 mm) using a stepwise elution with 80% MeOH in H_2O followed by 90% MeOH in H_2O over 80 min at a flow rate of 8.0 mL/min. The fractions containing **2** were collected, and this procedure afforded 0.1 mg of **2**. **3** was prepared according to the procedure given in Section **1.4.6**.

1.4.5 Bioconversion experiments with the *S. cerevisiae* strain BJ5464-NpgA harboring a plasmid carrying a gene of interest.

Bioconversion experiments using fed **3** were performed using *cox1* expressed in *S. cerevisiae* strain BJ5464-NpgA. The *cox1* expressed in *S. cerevisiae* strain BJ5464-NpgA was grown for 2 days in 50 mL of the fresh SC medium without uracil and L-tryptophan, and the culture was incubated at 30 °C for 2 days with shaking at 220 r.p.m. Fresh YPD medium (2 mL) was inoculated with each of the culture (20 μL) and incubated at 28 °C for 4 days with shaking at 250 r.p.m. The culture was supplemented with 10 μM of **3**, and incubation was continued for another 9 h. The resultant culture was filtered to separate the liquid medium and the cells. 250 μL of the liquid medium was extracted with ethyl acetate (200 μL). The ethyl acetate extracts were combined and concentrated *in vacuo*, and the dried residue was dissolved in DMF (50 μL). The resulting solution was subjected to LC–MS analysis (**Fig. 3ii**). LC–MS analysis was performed with a Thermo Scientific Exactive liquid chromatography mass spectrometer using both positive and negative electrospray ionization. Samples were analyzed using an ACQUITY UPLC 1.8 μm, 2.1 × 50 mm C18 reversed-phase column (Waters) and separated on a linear gradient of 10–80% (v/v) CH_3CN in H_2O supplemented with 0.05% (v/v) formic acid at a flow rate of 500 μL/min to yield **4**. For bioconversion experiments by using other substrates or enzyme Cox2, essentially the same procedure described here was followed.

1.4.6 Engineered biosynthesis and isolation of 3, 4, 5, 6, 7 and 8 from the *S. cerevisiae* strain BJ5464-NpgA expressing *cox*1, *cox2* and *cop6*.

Engineered biosynthesis of **3**, **7** and **8** was performed using *S. cerevisiae* strain BJ5464-NpgA expressing *cox2* and *cop6* from the plasmids pKW7347 and 7349. The engineered *S. cerevisiae* strain BJ5464-NpgA was grown at 28 °C for 2 days in 2 × 20 mL of the fresh SC medium without uracil and L-leucine with shaking at 250 r.p.m. Fresh YPD medium (3 × 1 L) was inoculated with each of the culture (10 mL) and incubated at 28 °C for 7 days with shaking at 250 r.p.m. The culture was centrifuged to separate the supernatant from the cells. Subsequently, 3 liters of the supernatant was extracted with ethyl acetate（3 × 3 L）. Mycelia were extracted with 2 × 100 mL of acetone for isolation of **3**, **7** and **8**. The ethyl acetate and acetone extracts were separately concentrated *in vacuo* to give an oily residue, which were combined and then were dissolved with 15 mL of 90% CH_3OH. The solution was fractionated by silica gel (KANTO CHEMICAL CO., INC., Silica gel 60N 100-210μm) flash column chromatography with 0–100% hexane/ethyl acetate gradient elution. The sample was further purified by a reversed-phase HPLC (Nacalai Tesque Inc., COSMOSIL 5C18 MS-II, 20 × 250 mm) using an isocratic elution system of 70 % CH_3OH (v/v) in H_2O with 0.05% (v/v) formic acid at a flow rate of 8.0 mL/min to afford **8** (3.0 mg/L) and 80 % CH_3OH (v/v) in H_2O with 0.05% (v/v) formic acid to afford **7** (3.2 mg). The fractionated sample by silica gel flash column chromatography was further purified by a preparative TLC (Merck & Co., Inc., PLC Silica gel 60 F254, 0.5 mm) by using 100% hexane to afford **3** (4.7 mg/L). The chemical structures were confirmed by high-resolution electrospray ionization mass spectrometry (HRESIMS) and NMR.

Engineered biosynthesis of **4**, **5** and **6** was performed using *S. cerevisiae* strain BJ5464-NpgA expressing *cox1*, *cox2* and *cop6* from the plasmids pKW7347, 7348 and 7349. The engineered *S. cerevisiae* strain BJ5464-NpgA was grown at 28 °C for 2 days in 20 mL of the fresh SC medium without uracil, L-tryptophan and L-leucine with shaking at 250 r.p.m. Fresh YPD medium (3 × 0.5 L) was inoculated with each of the culture (5 mL) and incubated at 28 °C for 4 days with shaking at 250 r.p.m. The culture was centrifuged to separate the supernatant from the cells. Subsequently, 1.5 liters of the supernatant was extracted with ethyl acetate（3 × 1.5 L）. Mycelia were extracted with 70 mL of acetone for isolation of **4**, **5** and **6**. The ethyl acetate and acetone extracts were separately concentrated *in vacuo* to give an oily residue, which were

combined and then were dissolved with 15 mL of 90% CH_3OH. The solution of CH_3OH was extracted with hexane (3 × 15 mL) and then extracted with hexane (10 × 7 mL). The hexane layer was fractionated by silica gel flash column chromatography with 0–100% hexane/ethyl acetate gradient elution. The sample was further purified by a reversed-phase HPLC (Nacalai Tesque Inc., COSMOSIL 5C18 MS-II, 20 × 250 mm) using an isocratic elution system of 77 % CH_3OH (v/v) in H_2O with 0.05% (v/v) formic acid at a flow rate of 8.0 mL/min to afford **4** (12.3 mg/L) and **5** (5.1 mg). The water layer was concentrated and then was dissolved with 10 mL of 60% CH_3OH. The solution was extracted with CH_2Cl_2 (4 × 7 mL). The extract was concentrated and then fractionated by silica gel flash column chromatography with 0–100% hexane/ethyl acetate gradient elution. The sample was further purified by a reversed-phase HPLC (Nacalai Tesque Inc., COSMOSIL 5C18 MS-II, 20 × 250 mm) using an isocratic elution system of 60 % CH_3OH (v/v) in H_2O with 0.05% (v/v) formic acid at a flow rate of 8.0 mL/min to afford **6** (12.0 mg/L). Other biosynthetic products isolated in this study were also purified using essentially the same experimental procedure, and their chemical structures were confirmed by high-resolution electrospray ionization mass spectrometry (HRESIMS) and NMR.

1.4.7 Preparation of 10 by bioconversion of 6 using the *S. cerevisiae* strain BJ5464-NpgA expressing *cox2*.

The yeast strain BJ5464-NpgA transformed with the plasmid pKW7349 carrying *cox2* was shaken at 200 r.p.m. for 2 days at 30 °C in 3 mL of SC medium without leucine. 200 µL of the culture was used to inoculate five of the 100 mL baffled flask containing 20 mL of YPD medium for a total volume of 100 mL. The flasks were shaken at 250 r.p.m. for 3 days at 28 °C. Subsequently, **6** was added such that its final concentration came to 66 µg/mL, and the culture was continued to be incubated under the same condition for additional 4 days. After the incubation, the culture was centrifuged at 8,000 g for 5 min. to remove the mycelium, and the media was extracted with 100 mL of ethyl acetate for three times. The extract was pooled, dried and subjected to HPLC fractionation by isocratic elution with 45% CH_3OH (v/v) in H_2O with 0.05% (v/v) formic acid using the Cosmosil 5C18 MS-II 5 µm, 20 × 250 mm column (Nacalai Tesque Inc.). A crude sample of **10** (0.4 mg) was obtained, which was used as an authentic reference to obtain the LC traces (**Fig.**

S12) used for analyzing the enzymatic assays. The chemical structure was confirmed by HRESIMS and NMR.

1.4.8 *In vitro* assay for examining the activity of Cox1 against 3, 7 and 8.

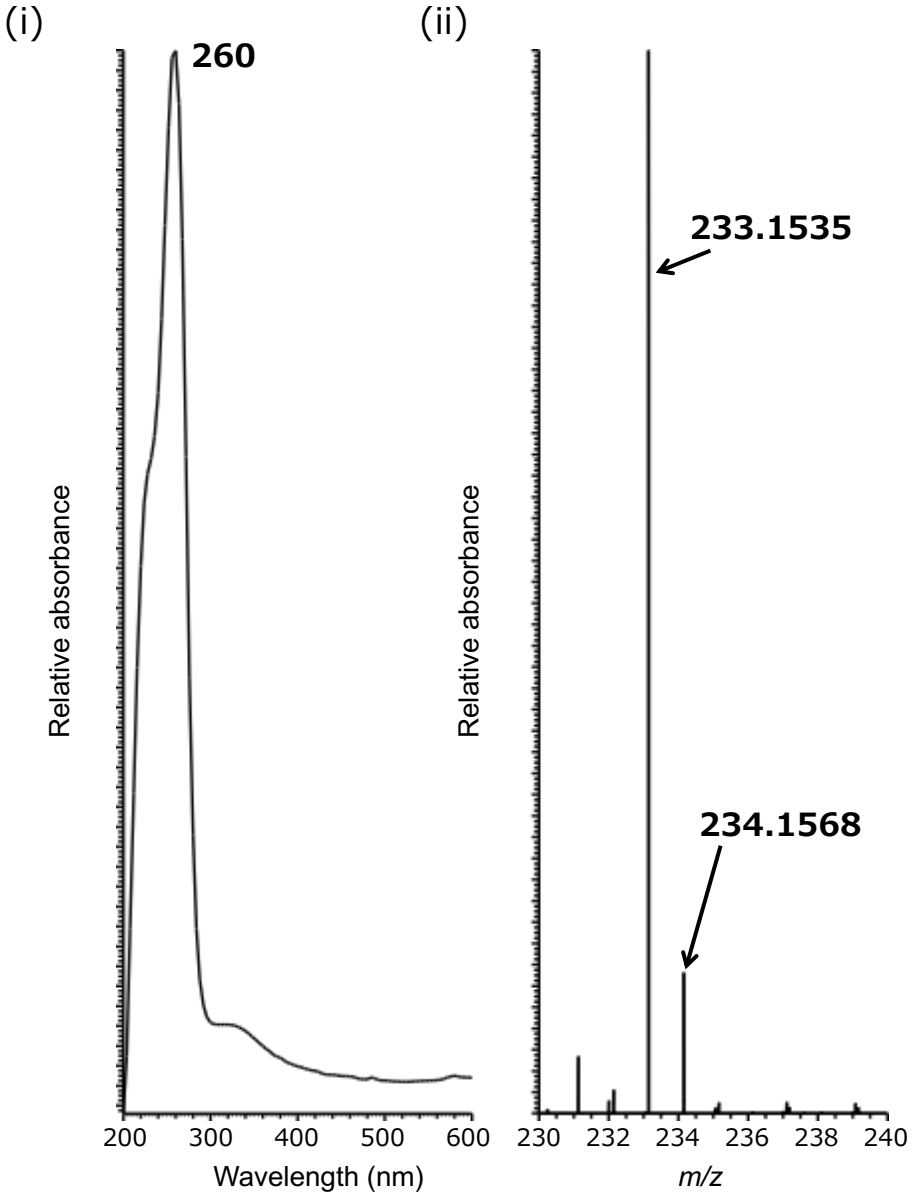
The assay mixture (100 µL) containing 0.2 mg/mL of Cox1 (**Fig. S9**), 0.2 mM of **3** or **7** or **8**, 2 mM NADPH, 0.1 M Tris-HCl (pH 7.4) and 0.98 mg/mL of NADPH-CPR[9] was incubated at 30 °C for 7 h. The reaction (100 µL) was terminated by extraction with MeOH (100 µL). The extract was subjected to LC–MS analysis. Heat-inactivated (100 °C for 5 min) Cox1 was used in reaction as a negative control. The results of the assay are given in **Fig. 4** in the main text.

1.4.9 *In vitro* assay for examining the activity of Cox2 against 3, 7 and 8.

The assay mixture (400 µL) containing 2 mg/mL of microsomal fraction containing Cox2 (**Fig. S10**), 0.2 mM of **3** or **7** or **8**, 1 mM NADPH, 0.1 M Tris-HCl (pH 7.4) and 48.8 µg/mL of NADPH-cytochrome P450 reductase was incubated at 30 °C for 7 h. The reaction (350 µL) was terminated by extraction with ethyl acetate (400 µL). The ethyl acetate extracts were combined and concentrated *in vacuo*, and the dried residue was dissolved in DMF (50 µL). Heat-inactivated (100 °C for 5 min) Cox2 was used in reaction as a negative control. The results of the assay are given in **Fig. 5** in the main text.

1.4.10 LC–HRMS analysis for the quinolone form of 8 extracted from the cultures of engineered *S. cerevisiae* strain BJ5464-NpgA expressing *cox2* and *cop6*.

Figure S11. LC traces corresponding to the *m*/*z* for the quinone form of **8**. (i) UV/VIS spectrum and (ii) HRMS spectrum. The *m*/*z* of 233 for $[C_{15}H_{20}O_2 + H]^+$ was determined to be 233.1535 for the quinone form of **8** isolated from the cultures of engineered *S. cerevisiae* strain BJ5464-NpgA expressing *cox2* and *cop6*.



2. Supporting Results

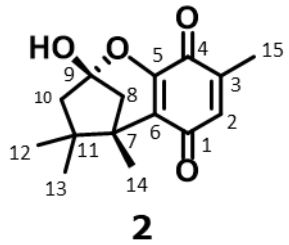
Table S2. Oligonucleotide primer sequences. DNA primers were designed on the basis of sequence data obtained from the *C. cinerea* sequence database.

Primer name	Sequence, 5'-3'
7347_1F	ATTAACTATATCGTAATACCATATGCCTGCAGCTTTGCCATACAACG
7347_1R	TAAATTAGTGATGGTGATGGTGATGAATCTCCGTGCAGCTCGCACAC
7347_2F	CATCACCATCACCATCACTAATTTAAATGACAAATTTG
7347_2R	CATATGGTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTGTAGC
7348_1F	ATTAACTATATCGTAATACCATCATATGACTTCTACAACGCAGGTTCTCATTGC
7348_1R	AAACCTAGTGATGGTGATGGTGATGTTTCTGGAGCAGGGCCGCATATTTAG
7348_2F	CATCACCATCACCATCACTAGGTTTAAACATGCCTTCACGATTTATAGTTTCCATTATCAAG
7348_2R	ATGATGGTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTG
7349_1F	ATTAACTATATCGTAATACCATATGAACATCGTCAACTCGCTCGACC
7349_1R	AACCTCAGTGATGGTGATGGTGATGAAGAGCTGAAATGGATTCTGCAGTGTCG
7349_2F	CATCACCATCACCATCACTGAGGTTTAAACATGCCTTCACGATTTATAGTTTTCATTATCAAG
7349_2R	CATATGGTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTGTAGC
7347_1F	ATTAACTATATCGTAATACCATATGCCTGCAGCTTTGCCATACAACG
7347_1R	TAAATTAGTGATGGTGATGGTGATGAATCTCCGTGCAGCTCGCACAC
7347_2F	CATCACCATCACCATCACTAATTTAAATGACAAATTTG
7347_2R	CATATGGTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTGTAGC
7348_1F	ATTAACTATATCGTAATACCATCATATGACTTCTACAACGCAGGTTCTCATTGC
7348_1R	AAACCTAGTGATGGTGATGGTGATGTTTCTGGAGCAGGGCCGCATATTTAG
7348_2F	CATCACCATCACCATCACTAGGTTTAAACATGCCTTCACGATTTATAGTTTCCATTATCAAG
7348_2R	ATGATGGTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTG
7349_1F	ATTAACTATATCGTAATACCATATGAACATCGTCAACTCGCTCGACC
7349_1R	AACCTCAGTGATGGTGATGGTGATGAAGAGCTGAAATGGATTCTGCAGTGTCG
7349_2F	CATCACCATCACCATCACTGAGGTTTAAACATGCCTTCACGATTTATAGTTTTCATTATCAAG
7349_2R	CATATGGTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTGTAGC
7351_1F	TTAACGTCAAGGAGAAAAAACTATAATGACTTCTACAACGCAGGTTCTCATTGC
7351_1R	AGCATCAGTGATGGTGATGGTGATGTTTCTGGAGCAGGGCCGCATATTTAG
ms-188R	GGAAAACAGCAAGTAGAAGAGCCATGGTATATCTCCTTCTTAAAGTTAAAC
P-186F	GCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCT
pKW19290_Fw1	CTACTTGCTGTTTTCCTTGCGCTCTCGAGTATCGTTGTGGCG
pKW19290_Rv1	GTGGTGGTGGTGGTGCGCTTTCTGGAGCAGGGCCGCATATTTAG
Ma13F	GGTACCGAGCTCGGATCCACTAGTAACG
Ma13R	GTAATACGACTCACTATAGGGCGAATTGGGC
pKW22011-LF	GACGGTATCGATAAGCTTGATATCGGATTGCTCGCCCCAAGGACTTC
pKW22011-LR	TACTAGTGGATCCGAGCTCGGTACCGGCAGTCTATTTCTGGAGCAGGGC
pKW22011-RF	TTCGCCCTATAGTGAGTCGTATTACAGGTTGCTTCGCCGGAAGCAT
pKW22011-RR	GGTGGCGGCCGCTCTAGAACTAGTGATCTAACTTTTGTGAGTTCTCCTTTCTTGGAATTAGGC
pKW22011-pos-LF	CCCATCCAAGAGCAAGAGACGC
pKW22011-pos-RR	AATTGTGCCATGGCATAGAAGAACGC
pRS426-F	AGGTCGACGGTATCGATAAGCTTGATATCG

pRS426-R	GTGGCGGCCGCTCTAGAACTAGTG
Cchph-pos-LR	AAGTCAGCTTCATTTTCCGTGTGGC
Cchph-pos-RF	CTCTCCACCTACCTCCACCACCTACG
CC1G_03563_F	CATTCACCGCCTTCGTCACCTACG
CC1G_03563_R	TAGTCGATTTGCTCGGGGAAGGC
pKW22012-pos-LF	CATGGGCGAGGTGGAGTGC
pKW22013-pos-RR	CGACTATACCCTAGGCGCAAAGACCG
pMIS84_Fw1	CTACTTGCTGTTTTCATTGTTGTCCTGGTAGTGCTCTTG
pMIS84_Rv1	GTGGTGGTGGTGCTCCGACCAGACATCCTCCTGGTAGC
P-311F	GAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCC
P-471R	GAAAACAGCAAGTAGAAGAGCCATGGTATATCTCCTTCTTAAAGTTAAAC

2.1 Chemical characterization of **2**.

Table S3. NMR data of compound **2**[11] in $CDCl_3$. The molecular formula of **2** was established by mass data [HRESIMS: *m/z* 261.1125 $(M–H)^-$, calcd. for $C_{15}H_{17}O_4^-$, 261.1132, Δ = 0.7 mmu].



2

Position	Isolated δ_H		mult. (*J* in Hz)	Reported[11] δ_H		mult. (*J* in Hz)	Isolated δ_C	Reported[11] δ_C
1							186.7	187.3
2	6.39	1H	s	6.39		d (1.0 Hz)	135.1	135.3
3							141.6	142.2
4							182.6	182.6
5							152.5	153.9
6							126.3	126.1
7							48.0	48.2
8	2.33	1H	d (12.0 Hz)	2.29	1H	d (13.0 Hz)	48.4	48.7
	1.83	1H	d (12.5 Hz)	1.82	1H	brd (13 Hz)		
9							109.1	109.4
10	2.08	1H	d (15.0 Hz)	2.01	1H	dd (13.0, 1.5 Hz)	54.6	55.6
	2.02	1H	m	1.92	1H	d (13.0 Hz)		
11							45.1	45.9
12	1.06	3H	s	1.04	3H	s	25.5	24.7
13	0.95	3H	s	0.93	3H	s	26.7	25.8
14	1.34	3H	s	1.32	3H	s	17.4	17.7
15	2.01	3H	s	1.94	3H	s	15.0	14.7

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S12. ^{1}H NMR spectrum of **2** in $CDCl_3$ (500 MHz).

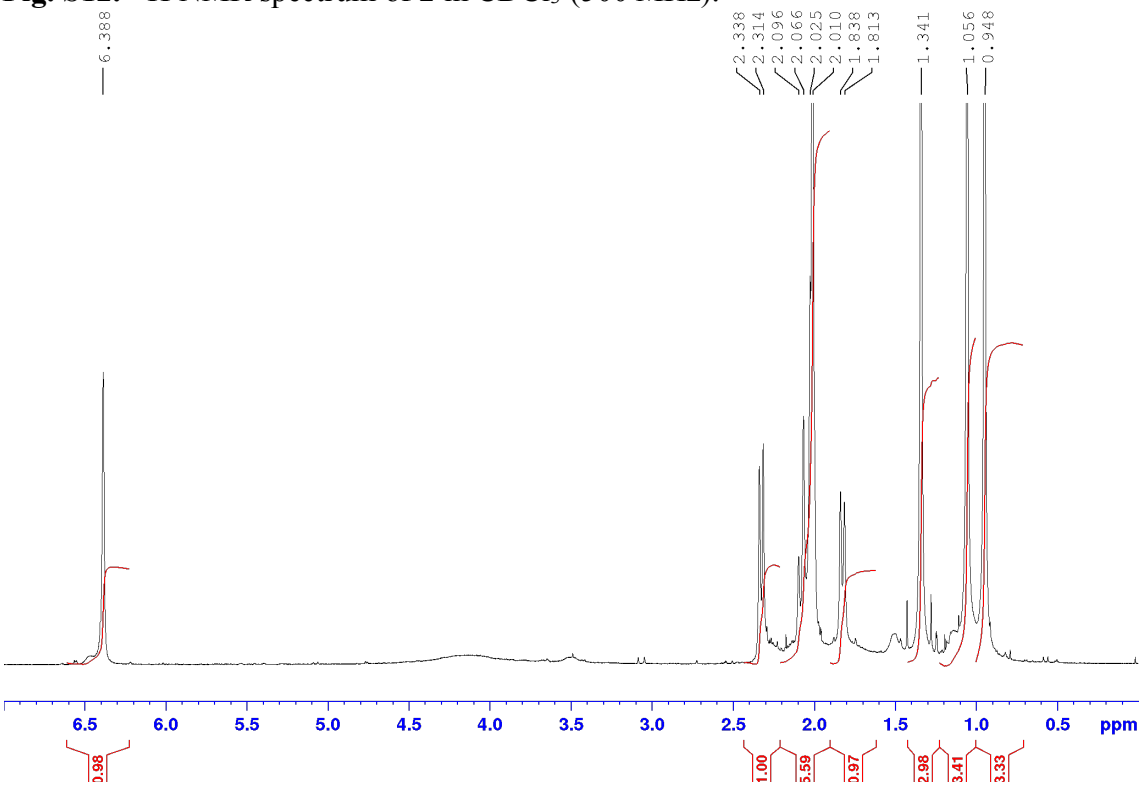
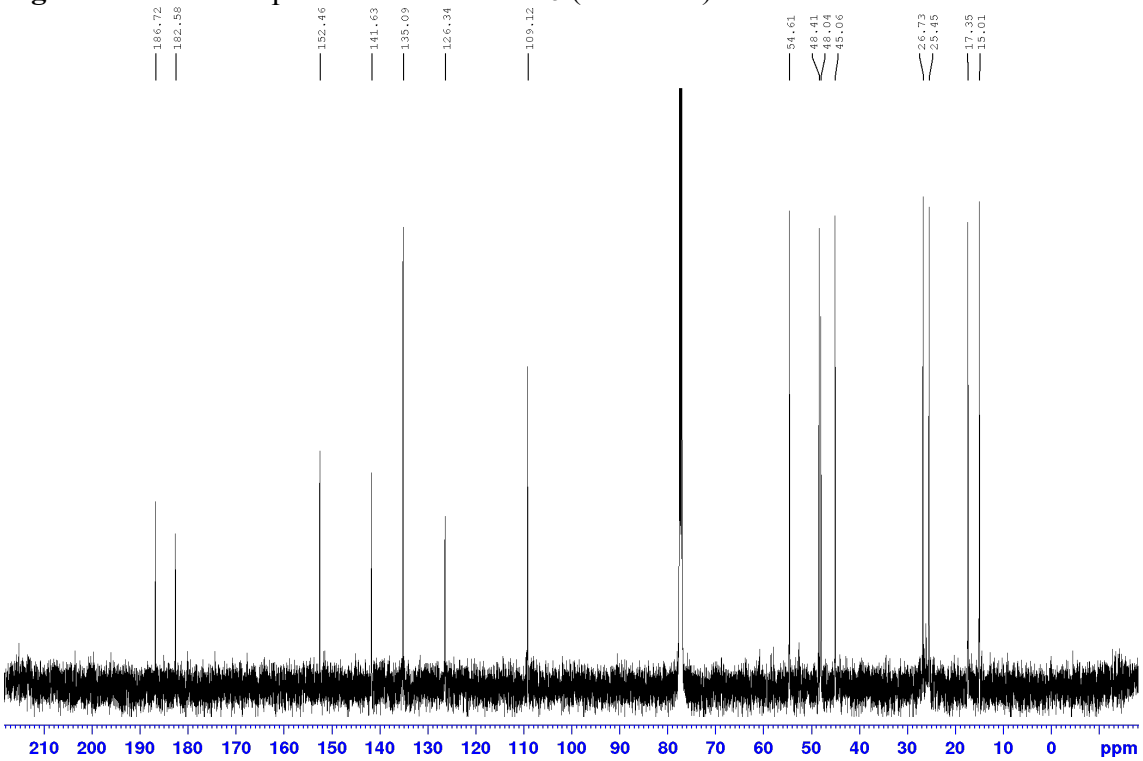
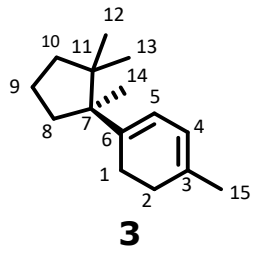


Fig. S13. ^{13}C NMR spectrum of **2** in $CDCl_3$ (125 MHz).



2.2 Chemical characterization of **3**.

Table S4. NMR data of compound **3**[12] in $CDCl_3$. The molecular formula of **3** was established by mass data [APCIMS: *m/z* 204.1874 (M^+, calcd. for $C_{15}H_{24}^+$, 2204.1874, Δ = 0 mmu]; $[\alpha]^{20}_D$: – 83.8 (*c* 0.43, CH_3OH).



3

	Isolated			Reported[12]		Isolated	Reported[12]
Position	δ_H		mult. (*J* in Hz)	δ_H	mult. (*J* in Hz)	δ_C	δ_C
1	2.18	2H	t (9.5 Hz)	2.18		25.7	25.6
2	2.04	2H	t (7.5 Hz)			29.2	29.1
3						133.8	133.5
4	5.66	1H	br s	5.66		120.0	119.9
5	5.66	1H	br s	5.68		118.5	118.4
6						142.2	142.0
7						51.4	51.2
8	2.17	1H	m	n.d.		36.5	36.3
	1.41	1H	m	n.d.			
9	1.62	2H	m	n.d.		19.5	19.4
10	1.62	1H	m	n.d.		40.4	40.2
	1.50	1H	m	n.d.			
11						43.6	43.4
12	1.01	3H	s	1.01		25.2	24.9
13	0.82	3H	s	0.82		26.9	26.7
14	1.02	3H	s	1.02		22.3	22.1
15	1.79	3H	s	1.79		23.0	22.7

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S14. ^{1}H NMR spectrum of **3** in $CDCl_3$ (500 MHz).

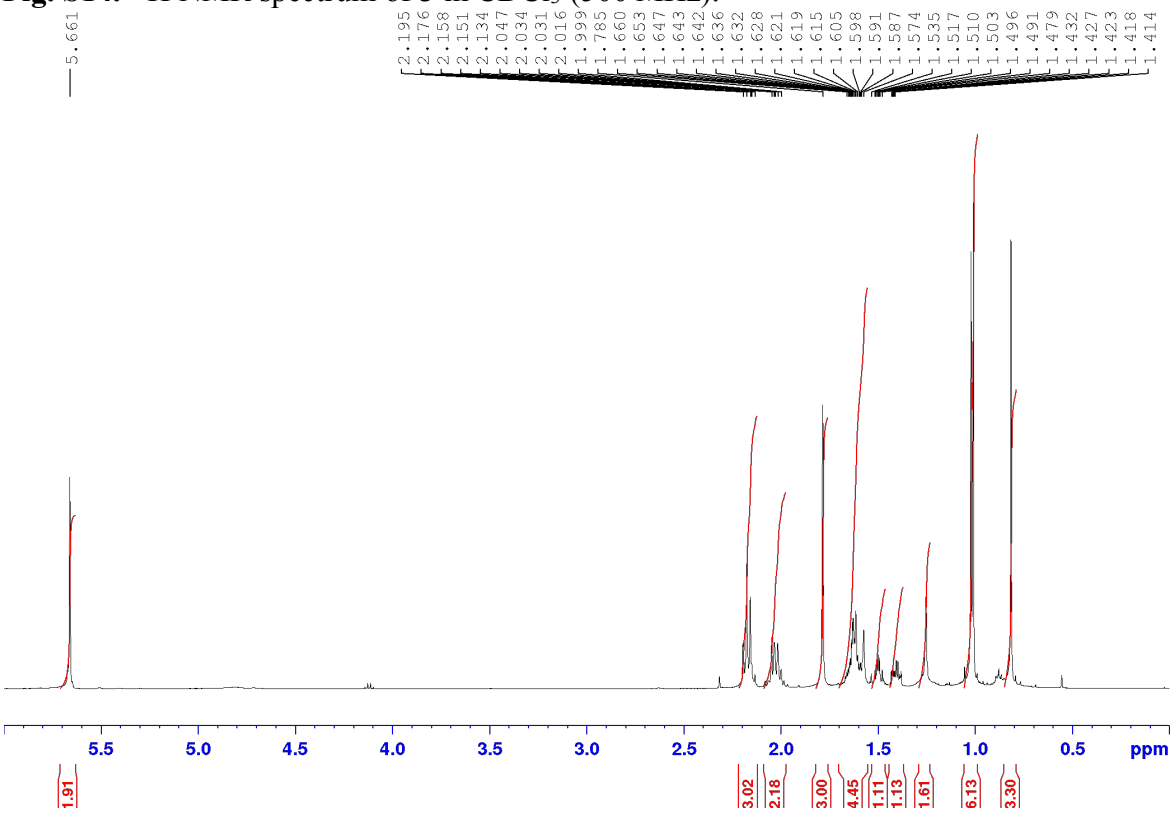
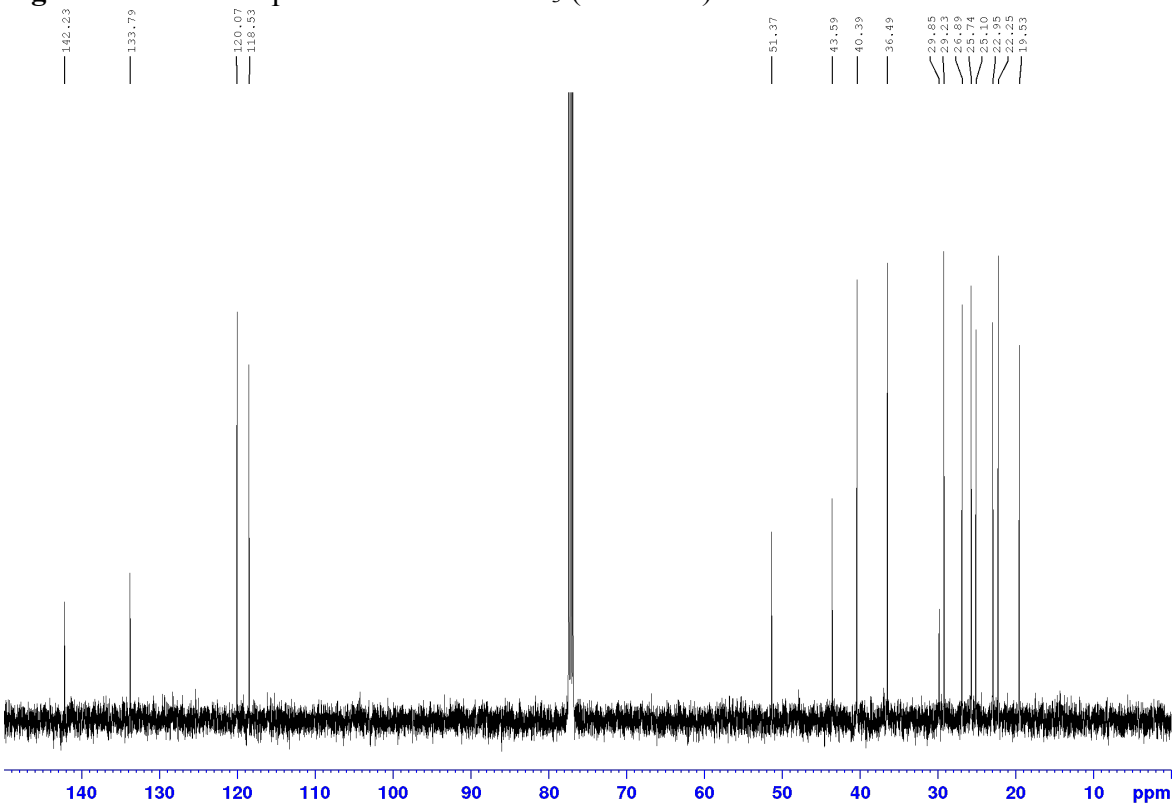
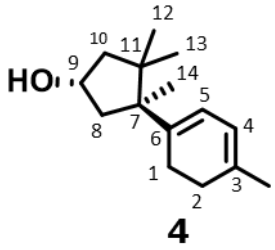


Fig. S15. ^{13}C NMR spectrum of **3** in $CDCl_3$ (125 MHz).



2.3 Chemical characterization of **4**

Table S5. NMR data of compound **4** in $CDCl_3$. The molecular formula of **4** was established by mass data [HRESIMS: *m/z* 221.1900 $(M+H)^+$, calcd. for $C_{15}H_{25}O_1^+$, 221.1900, Δ = 0 mmu].



4

Position	δ_H [ppm]		mult. (*J* in Hz)	HMBC	δ_C [ppm]
1	2.15	2H	m	2, 3, 5, 6, 7,	25.7
2	2.03	2H	m	1, 3, 4, 6	29.3
3					134.3
4	5.65	1H	d (4.5 Hz)	2, 6,	120.0
5	5.60	1H	d (5.5 Hz)	1, 3, 4, 7,	118.8
6					141.5
7					51.5
8	2.61	1H	dd (8.5, 14.5 Hz)	6, 9, 7, 14	47.3
	1.51	1H	dd (2.0, 14.5 Hz)		
9	4.40	1H	ddt (2.5, 4.5, 8.0 Hz)		70.5
9-OH	1.66	1H	br s		
10	2.00	1H	d (7.5 Hz)	9, 11, 12, 13	51.5
	1.67	1H	dd (4.5, 13.5 Hz)		
11					44.5
12	1.10	3H	s	7, 10, 11,13	25.4
13	0.81	3H	s	7, 10, 11, 12	26.9
14	1.21	3H	s	6, 7, 8, 11	23.5
15	1.78	3H	s	2, 3, 4	23.0

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S16. ^{1}H NMR spectrum of **4** in $CDCl_3$ (500 MHz).

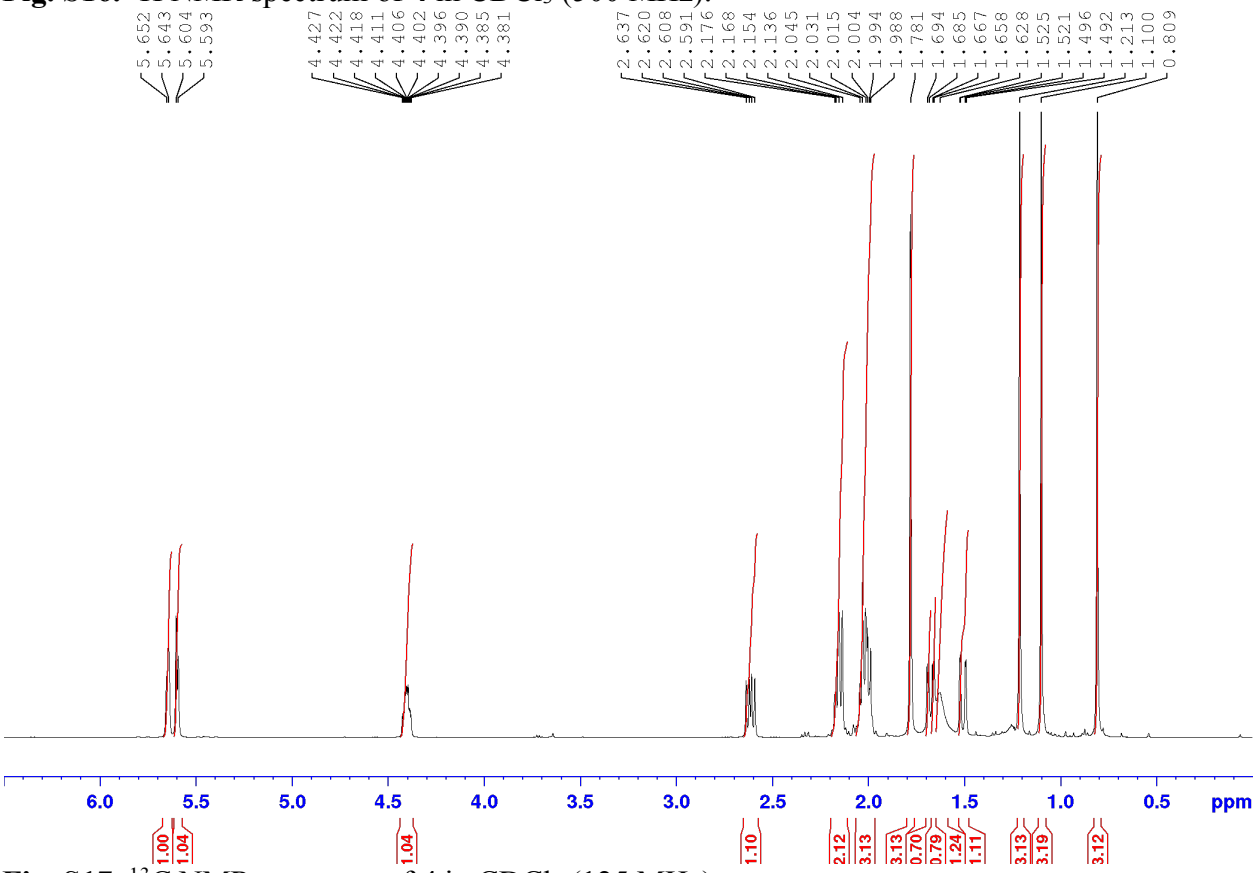


Fig. S17. ^{13}C NMR spectrum of **4** in $CDCl_3$ (125 MHz).

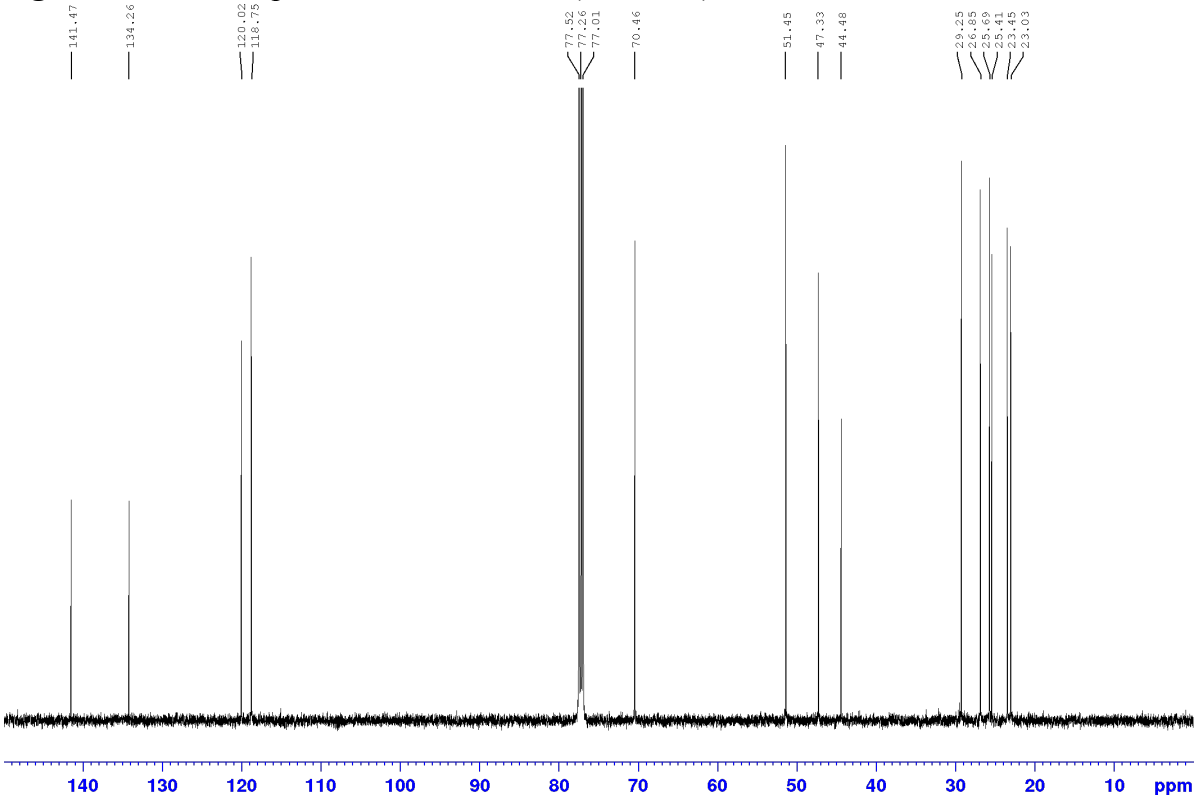


Fig. S18. COSY spectrum of **4** in $CDCl_3$ (500 MHz).

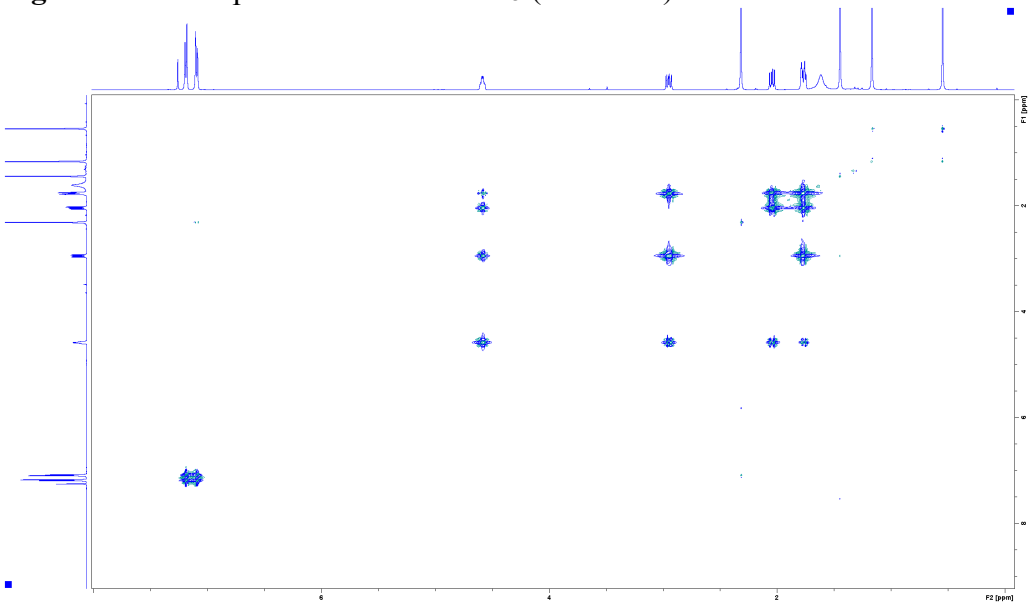


Fig. S19. HSQC spectrum of **4** in $CDCl_3$ (500 MHz).

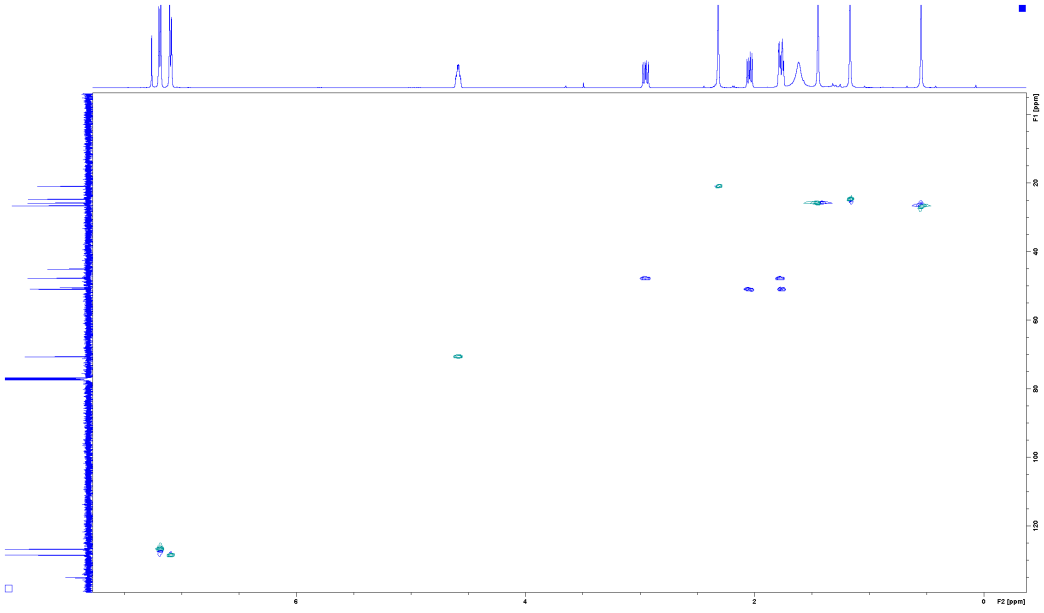
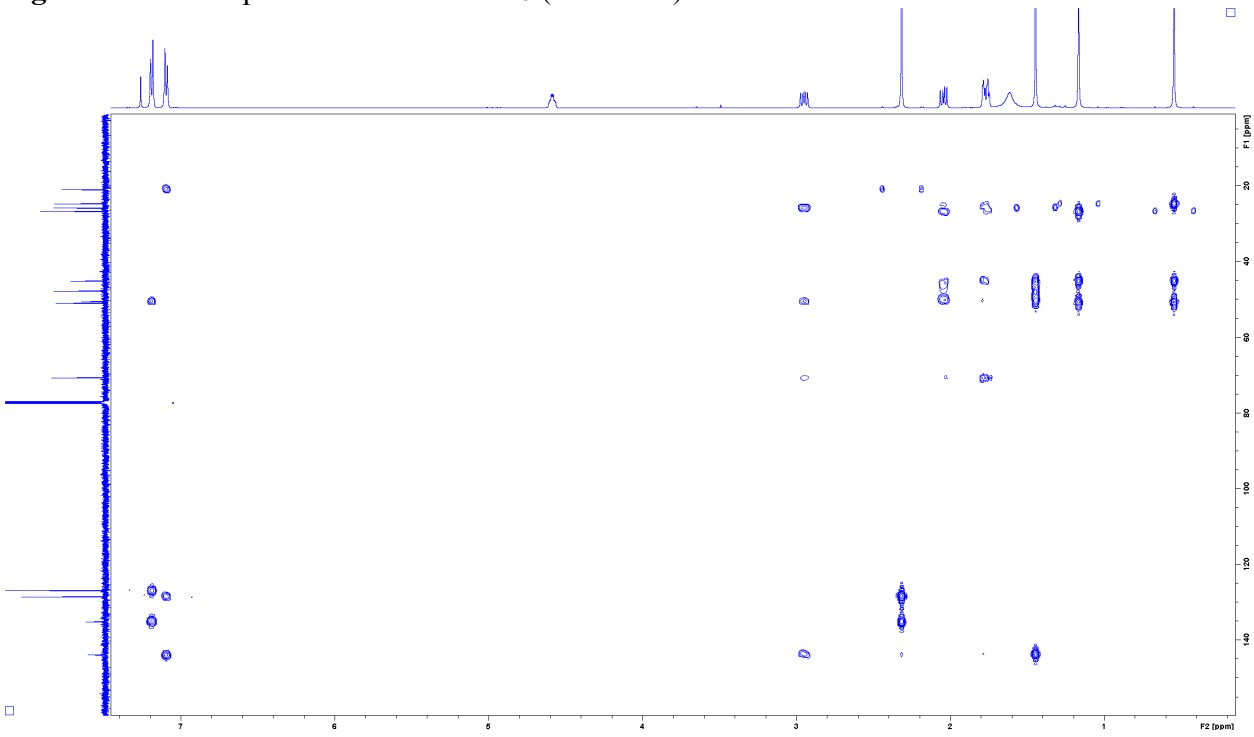


Fig. S20. HMBC spectrum of **4** in $CDCl_3$ (500 MHz).



2.4 Preparation of (*R*)- and (*S*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) esters of 4.

To a solution of **4** (1.6 mg) in CH_2Cl_2 (1.0 mL), (*R*)-MTPA chloride (3.4 μL), triethylamine (3.0 μL) and 4-dimethylaminopyridine (1 μg) were added and stirred at room temperature for 6 h. The reaction mixture was subjected to preparative TLC (20% ethyl acetate/hexane) to give the (*S*)-MTPA ester of **4**. The (R)-MTPA ester of **4** was prepared according to the same procedure. The values of Δδ[δ((*S*)-MTPA ester) – δ((*R*)-MTPA ester)] of **4** are shown in **Fig. S22**.

Figure S21. MTPA ester of 4, showing the Δδ values [Δδ (ppm) = $\delta_S - \delta_R$].

(*S*)-MTPA ester of **4**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.52 (2H, m), 7.40 (3H, m), 5.64 (1H, m), 5.58 (1H, m), 5.41 (1H, m), 3.55 (3H, s), 2.70 (1H, dd, *J* = 6.5, 15 Hz), 2.14 (3H, m), 2.02 (2H, m), 1.83 (1H, m), 1.80 (3H, s), 1.61 (1H, m), 1.05 (3H, s), 1.02 (3H, s), 0.85 (3H, s).
(*R*)-MTPA ester of **4**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.51 (2H, m), 7.40 (3H, m), 5.64 (1H, m), 5.59 (1H, m), 5.41 (1H, m), 3.54 (3H, s), 2.71 (1H, dd, *J* = 8.5, 15 Hz), 2.13 (3H, m), 2.02 (2H, m), 1.78 (3H, s), 1.73 (1H, m), 1.69 (1H, m), 1.25 (3H, s), 1.08 (3H, s), 0.84 (3H, s).

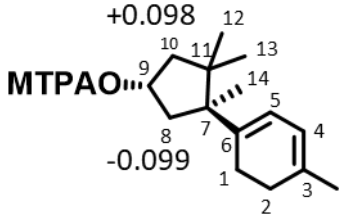


Fig. S22. ^{1}H NMR spectrum of (*S*)-MTPA ester of **4** in $CDCl_3$ (500 MHz).

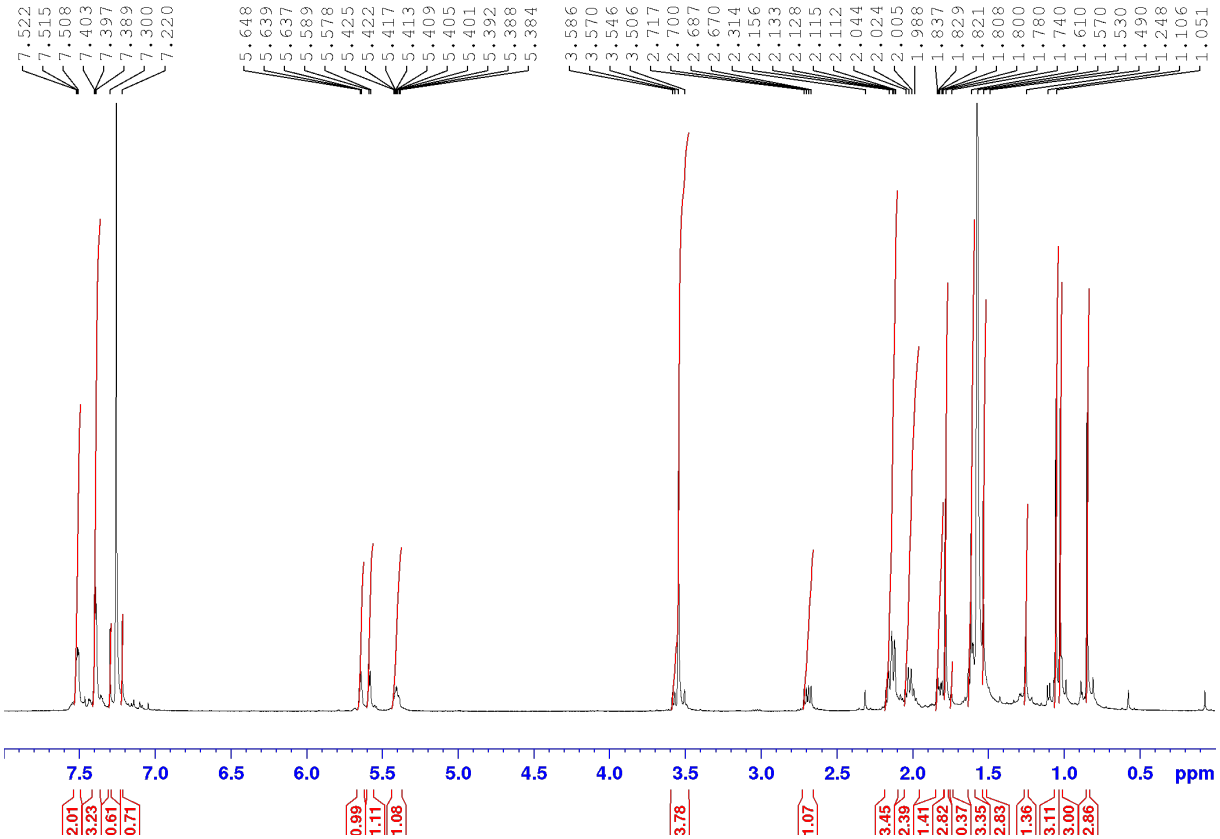


Fig. S23. COSY spectrum of (*S*)-MTPA ester of **4** in $CDCl_3$ (500 MHz).

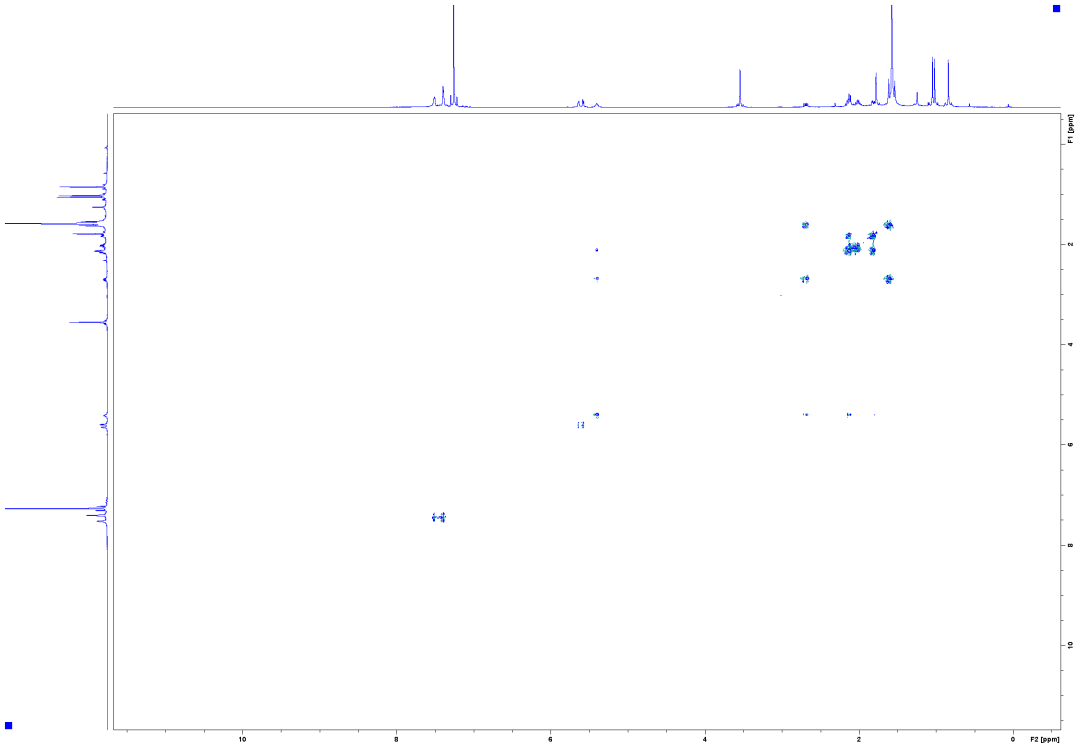


Fig. S24. HSQC spectrum of **4** in $CDCl_3$ (500 MHz).

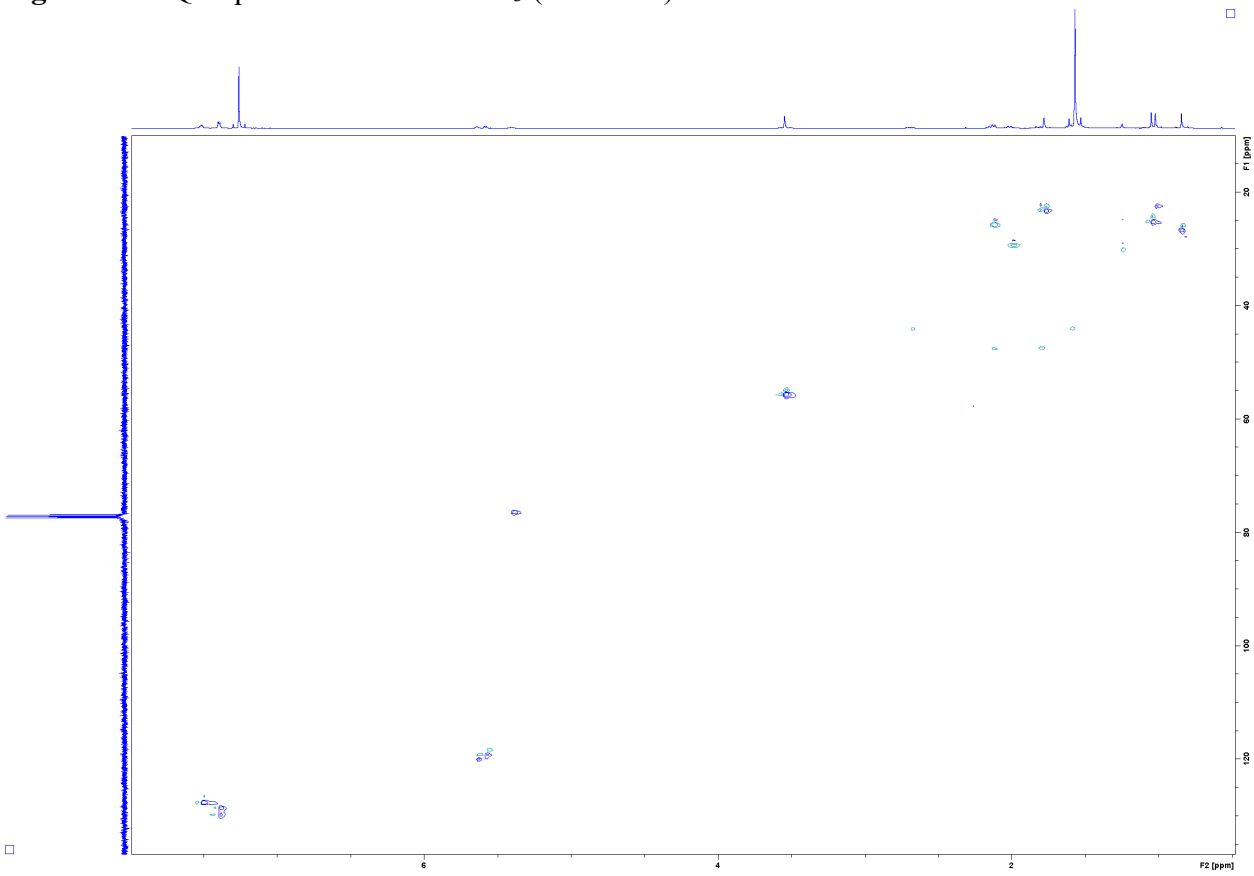


Fig. S25. ^{1}H NMR spectrum of (*R*)-MTPA ester of **4** in $CDCl_3$ (500 MHz).

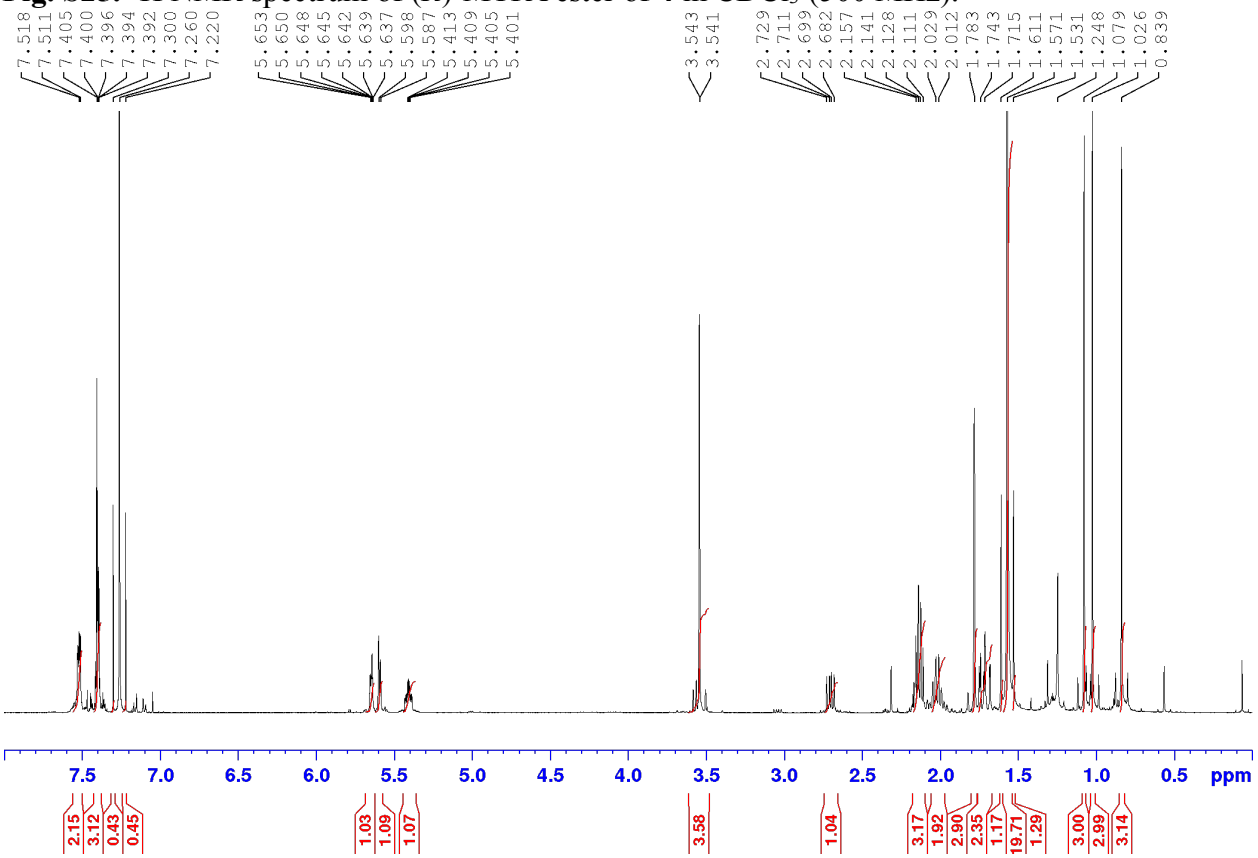


Fig. S26. COSY spectrum of (*R*)-MTPA ester of **4** in $CDCl_3$ (500 MHz).

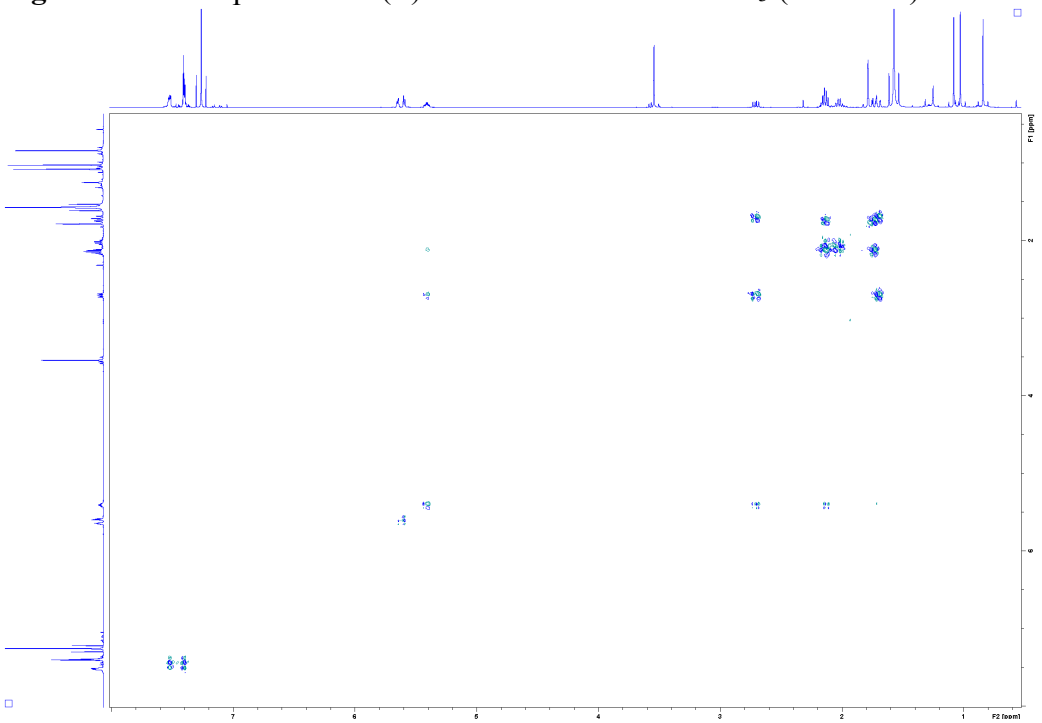
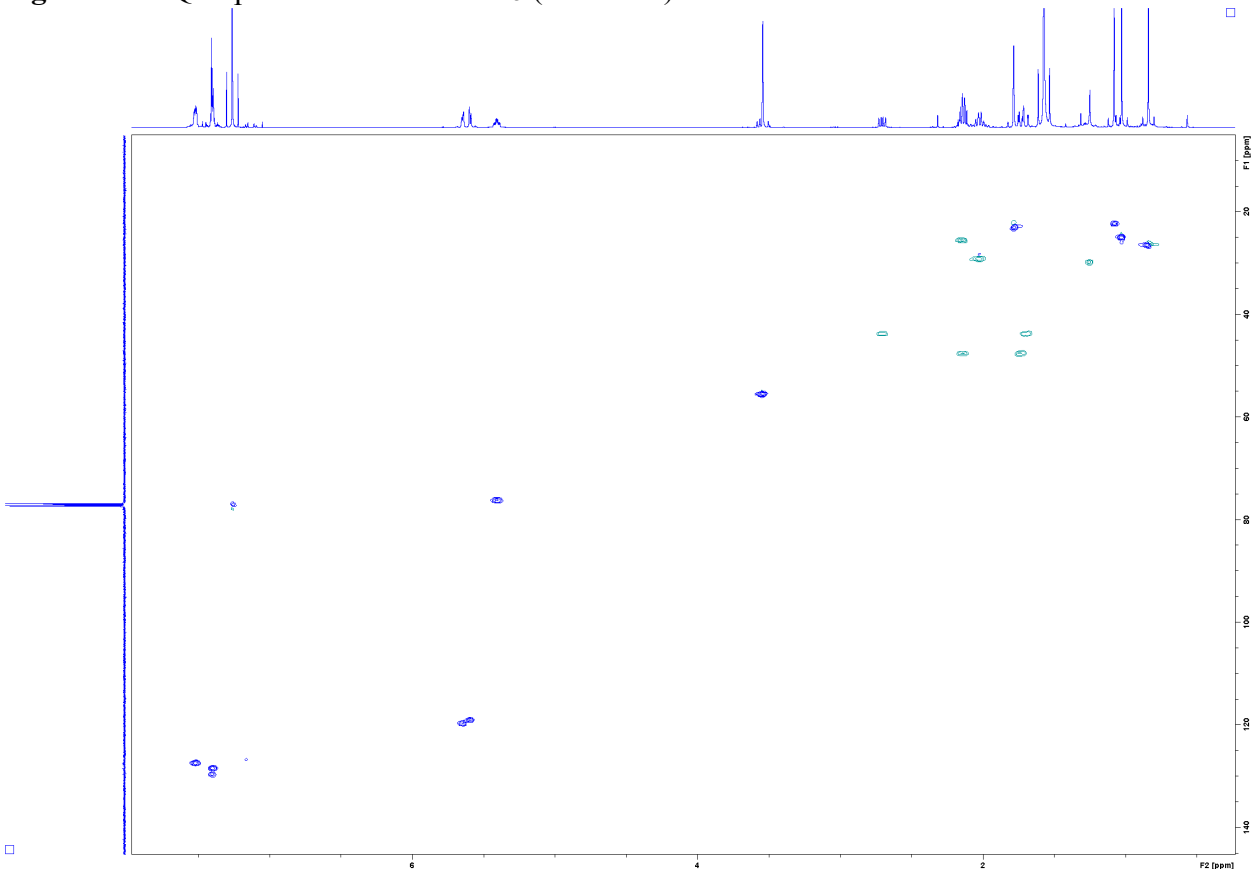
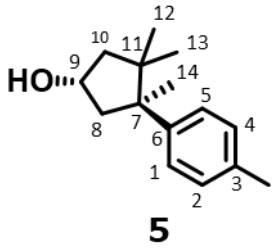


Fig. S27. HSQC spectrum of **4** in $CDCl_3$ (500 MHz).



2.5 Chemical characterization of **5**.

Table S6. NMR data of compound **5** in $CDCl_3$. The molecular formula of **5** was established by mass data [HRESIMS: *m/z* 201.1637 $(M–H_2O+H)^+$, calcd. for $C_{15}H_{21}^+$, 201.1637, Δ = 0.1 mmu].



5

Position	δ_H [ppm]		mult. (*J* in Hz)	HMBC	δ_C [ppm]
1 & 5	7.19	1H	d (8.5 Hz)	1 or 5, 3, 7	126.7
2 & 4	7.09	1H	d (8.0 Hz)	2 or 4, 6, 15	128.4
3					135.0
6					143.8
7					50.4
8	2.95	1H	dd (8.0, 14.0 Hz)	6, 7, 9, 11, 14	47.7
	1.77	1H	dd (2.5, 14.5 Hz)		
9	4.58	1H	ddt (2.5, 4.5, 8.0 Hz)	6, 9, 7, 14	70.6
9-OH	1.61	1H	br s		
10	2.00	1H	d (8.0, 14 Hz)	7, 8, 9, 12, 13	50.9
	1.76	1H	dd (5.5, 13.5 Hz)		
11					45.0
12	1.17	3H	s	7, 10, 11,13	24.6
13	0.55	3H	s	7, 10, 11, 12	26.6
14	1.45	3H	s	6, 7, 8, 11	25.7
15	2.32	3H	s	1 & 4, 3	20.8

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S28. ^{1}H NMR spectrum of **5** in $CDCl_3$ (500 MHz).

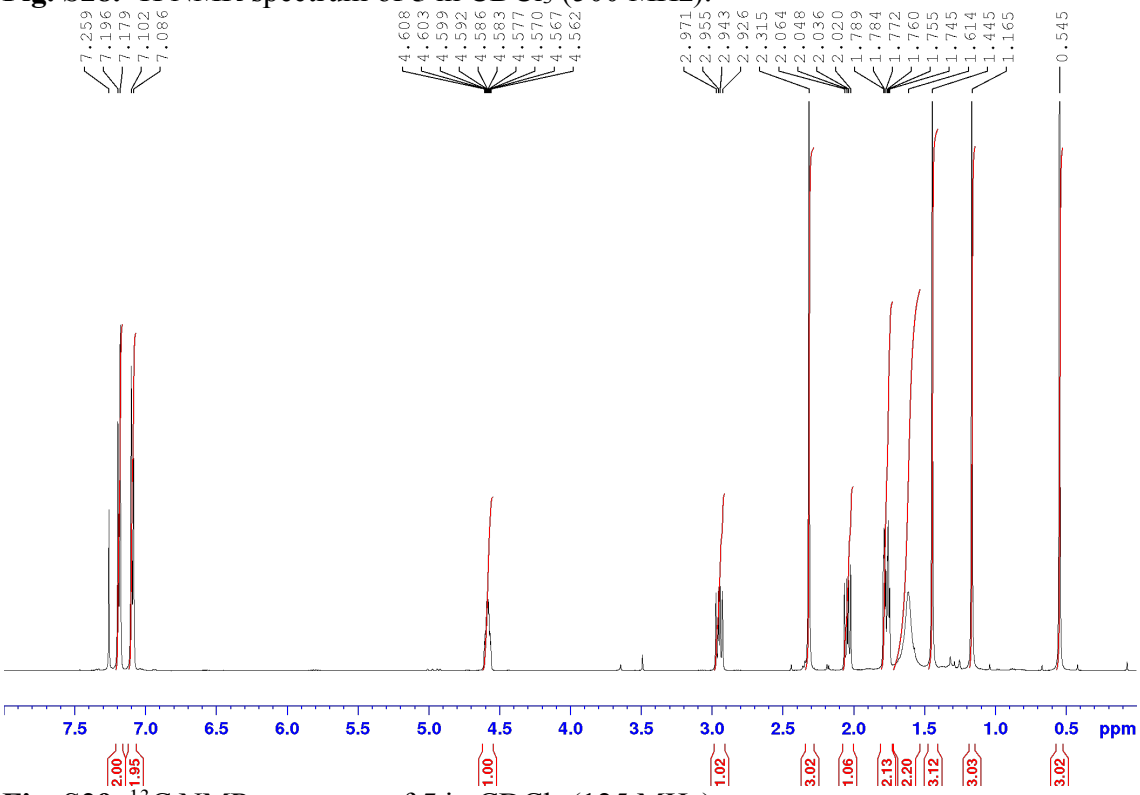


Fig. S29. ^{13}C NMR spectrum of **5** in $CDCl_3$ (125 MHz).

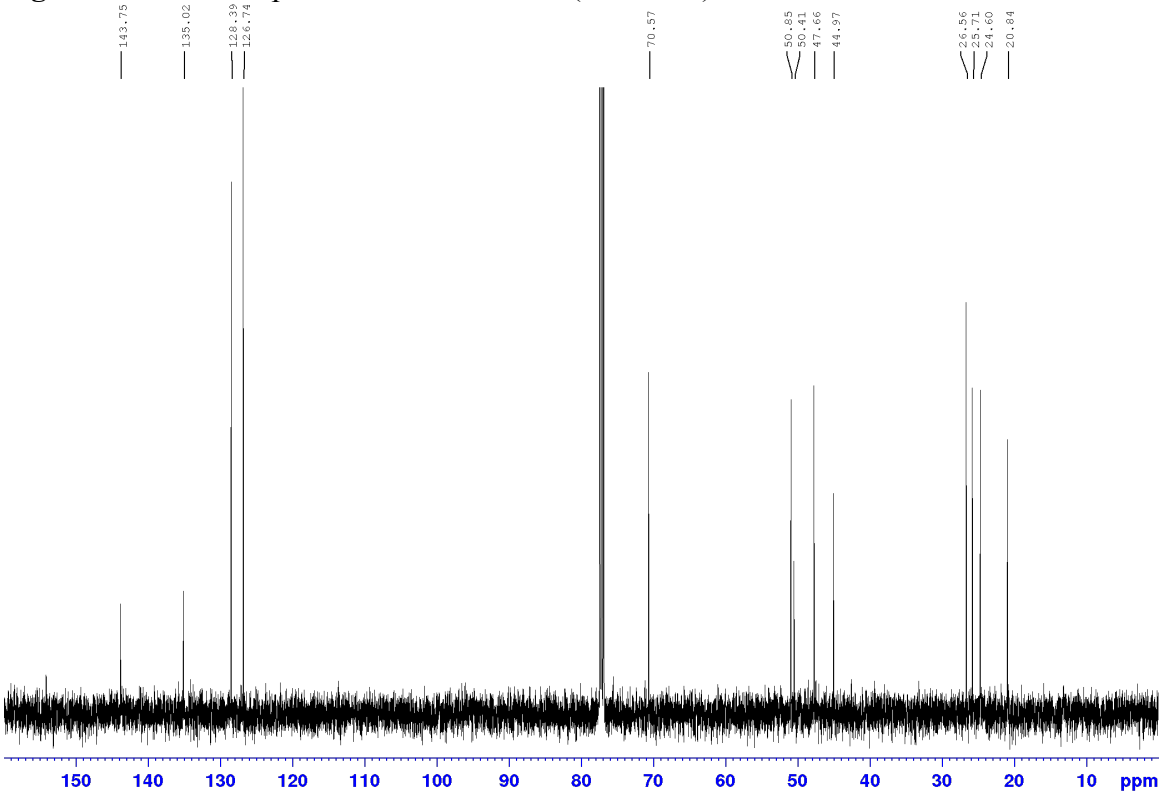


Fig. S30. COSY spectrum of **5** in $CDCl_3$ (500 MHz).

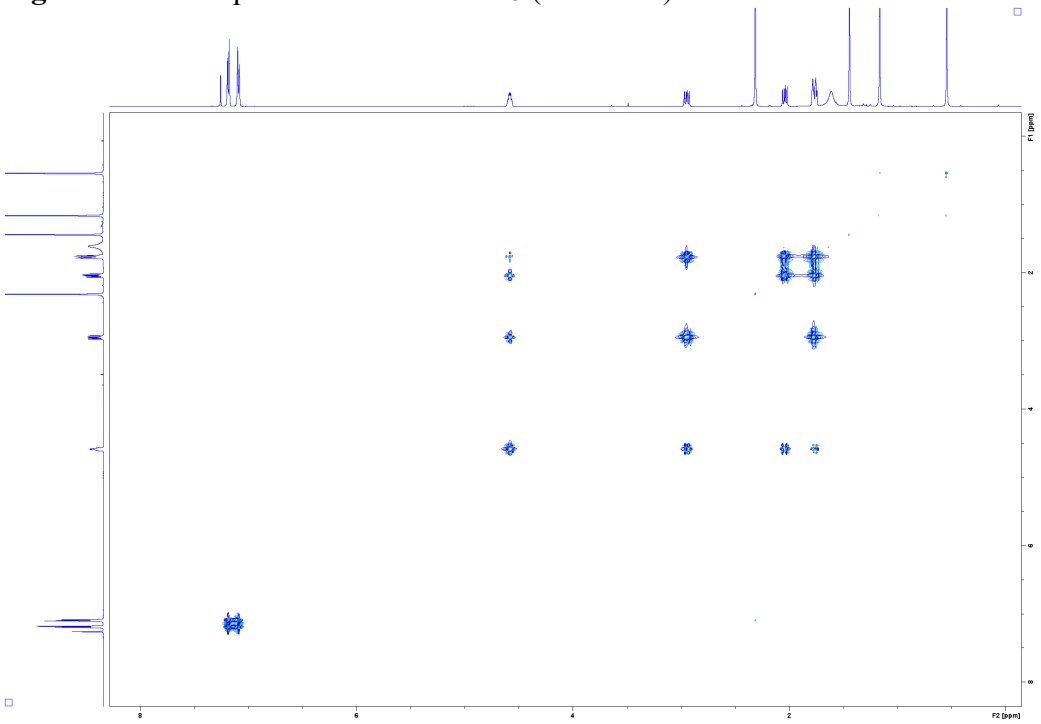


Fig. S31. HSQC spectrum of **5** in $CDCl_3$ (500 MHz).

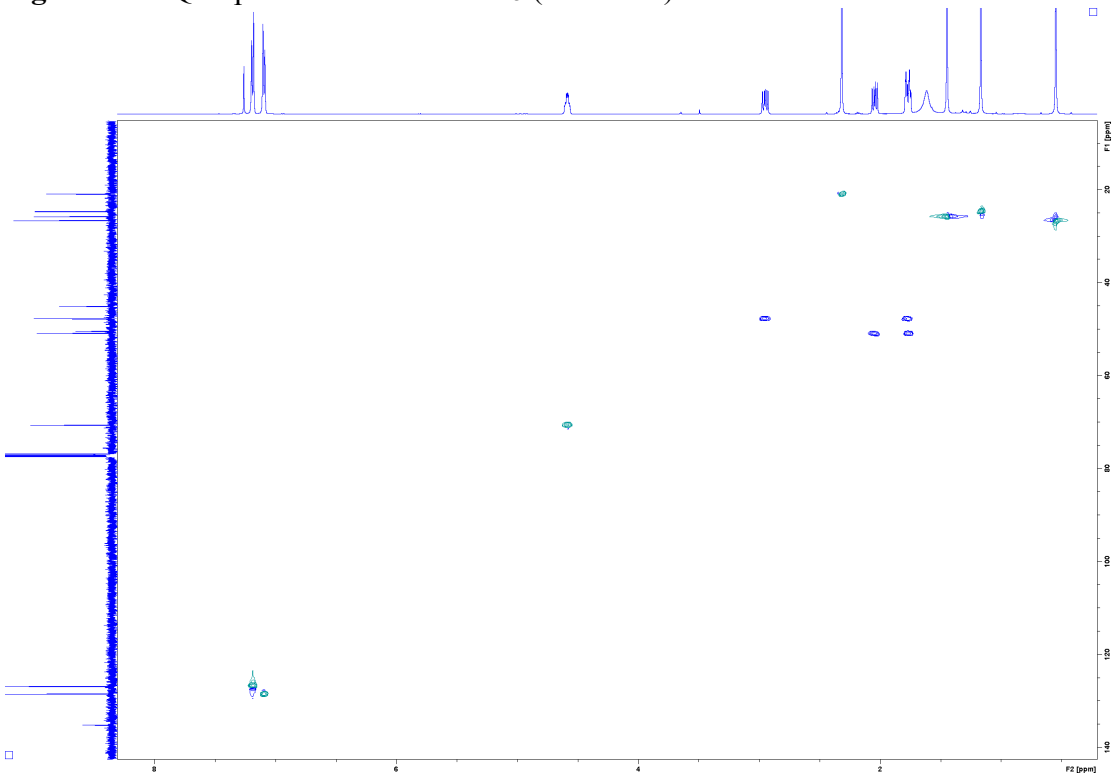
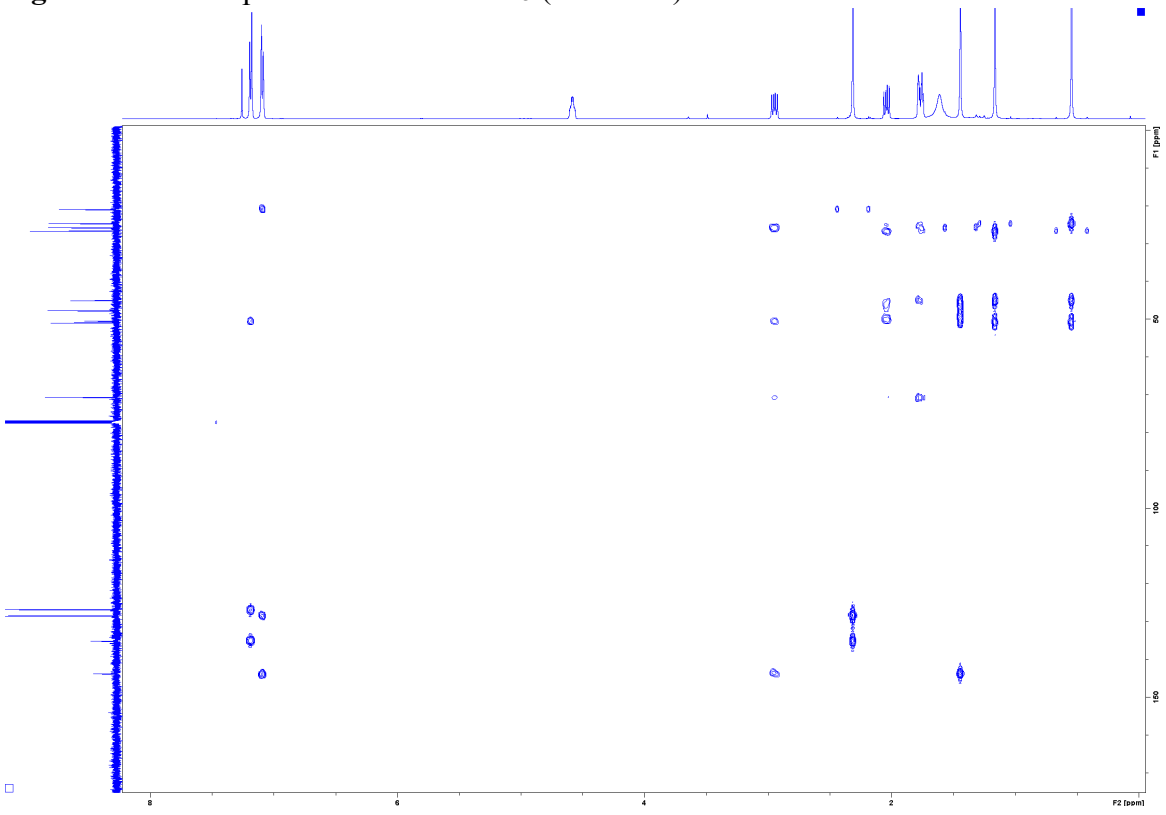
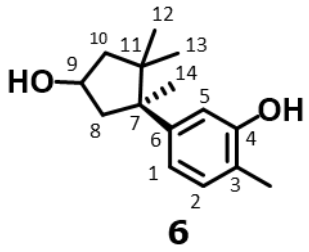


Fig. S32. HMBC spectrum of **5** in $CDCl_3$ (500 MHz).



2.6 Chemical characterization of **6**.

Table S7. NMR data of compound **6** in $CDCl_3$. The molecular formula of **6** was established by mass data [HRESIMS: *m/z* 235.1692 $(M+H)^+$, calcd. for $C_{15}H_{23}O_2^+$, 235.1693 Δ = 0.1 mmu].



6

Position	δ_H [ppm]		mult. (*J* in Hz)	HMBC	δ_C [ppm]
1	6.77	1H	d (7.5 Hz)	3, 5, 7,	119.3
2	7.02	1H	d (8.0 Hz)	4, 6, 15	130.3
3					120.9
4					153.4
4-OH	5.23	1H	br s	1, 3, 4, 7,	
5	6.74	1H	s		113.9
6					146.4
7					50.5
8	2.91	1H	dd (8.5, 14.0 Hz)	6, 9, 7, 14	47.7
	1.77	1H	dd (4.0, 14.0 Hz)		
9	4.59	1H	ddt (2.5, 4.0, 8.0 Hz)		70.7
9-OH	1.81	1H	br s		
10	2.00	1H	dd (8.0, 14.0 Hz)	7, 8, 9, 13	50.9
	1.77	1H	dd (4.0, 14.0 Hz)		
11					45.1
12	1.16	3H	s	10, 11,13	24.7
13	0.56	3H	s	10, 11, 12	26.7
14	1.42	3H	s	6, 7, 8, 11	25.9
15	2.21	3H	s	2, 3, 4	15.4

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S33. ^{1}H NMR spectrum of **6** in $CDCl_3$ (500 MHz).

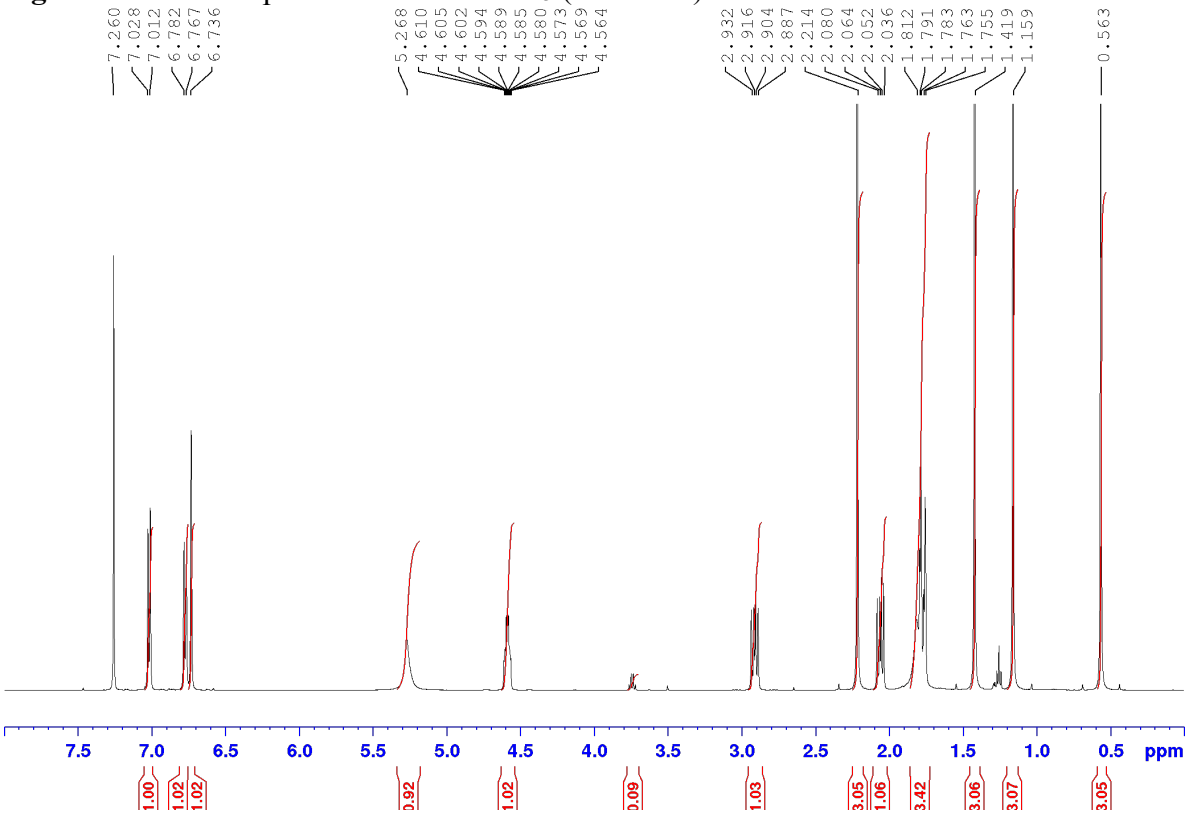


Fig. S34. ^{13}C NMR spectrum of **6** in $CDCl_3$ (125 MHz).

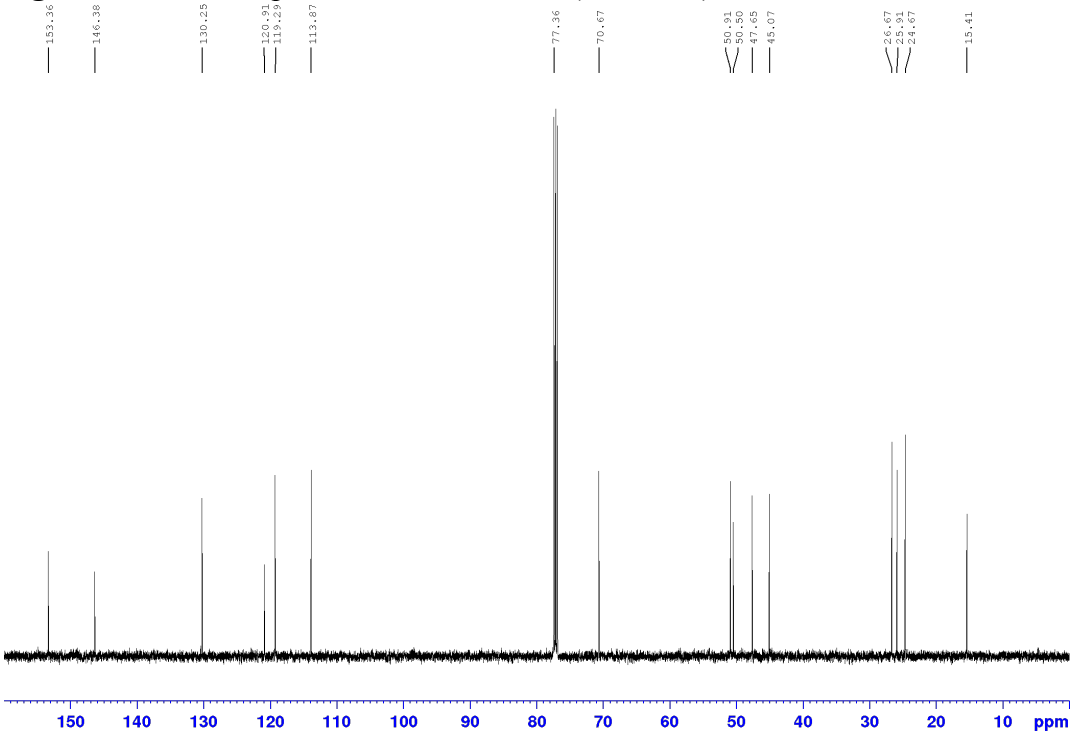


Fig. S35. COSY spectrum of **6** in $CDCl_3$ (500 MHz).

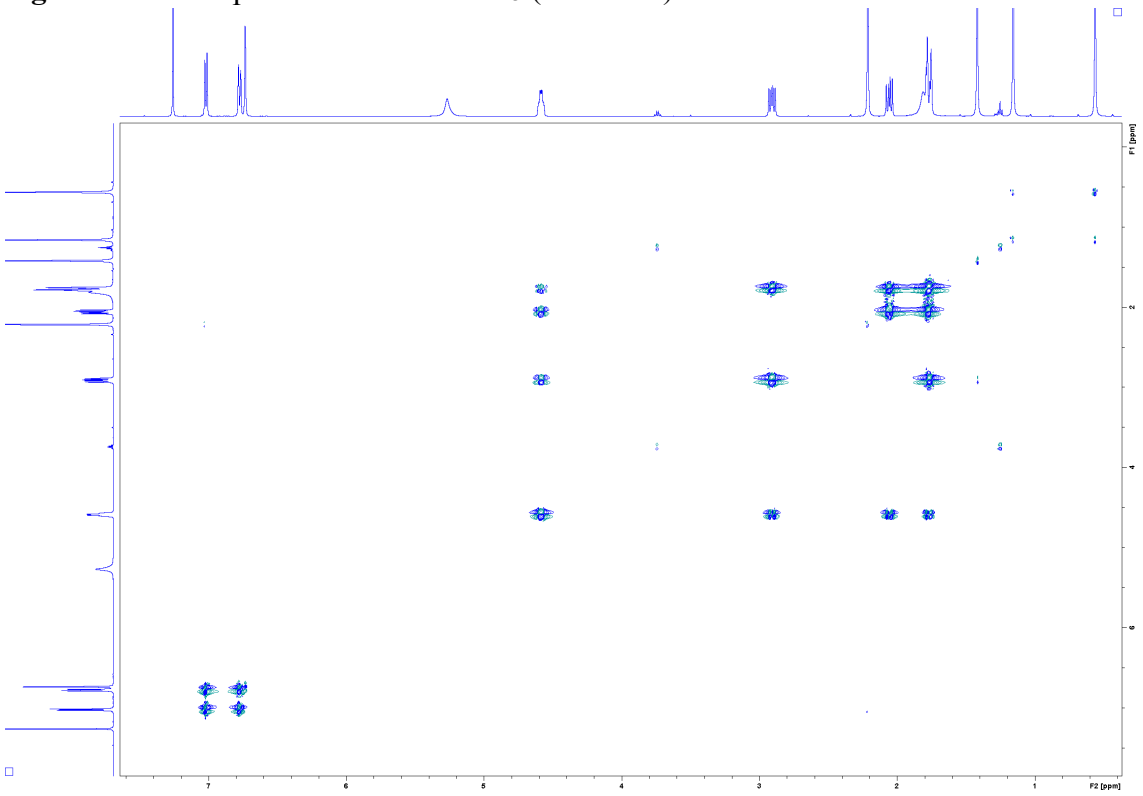


Fig. S36. HSQC spectrum of **6** in $CDCl_3$ (500 MHz).

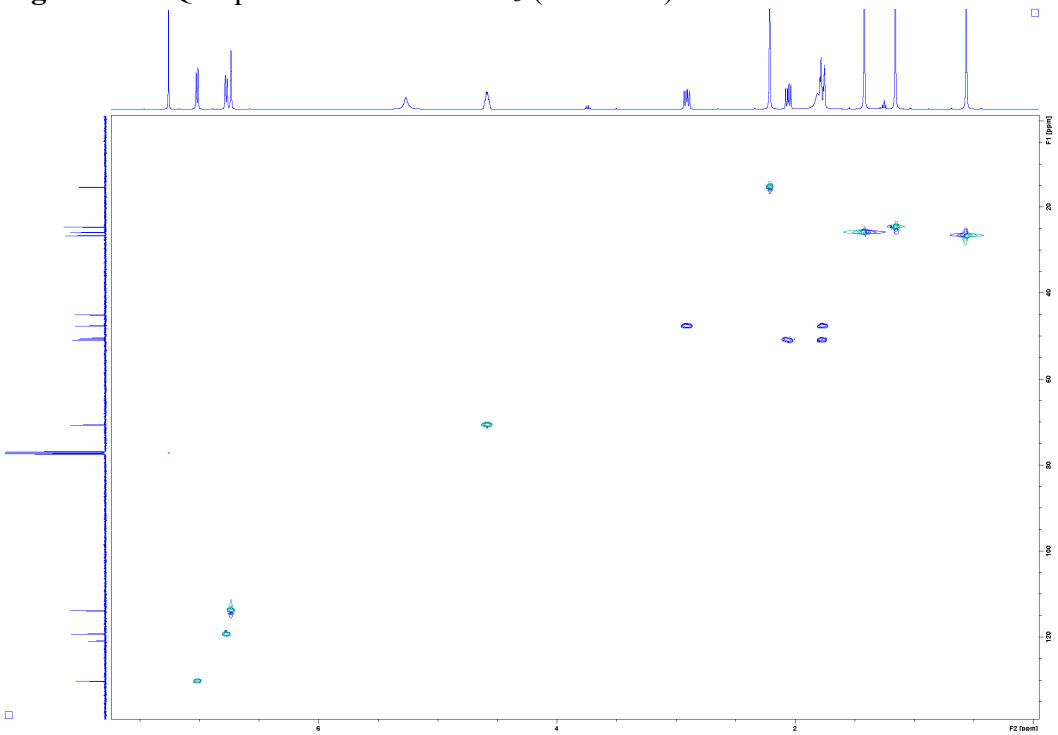
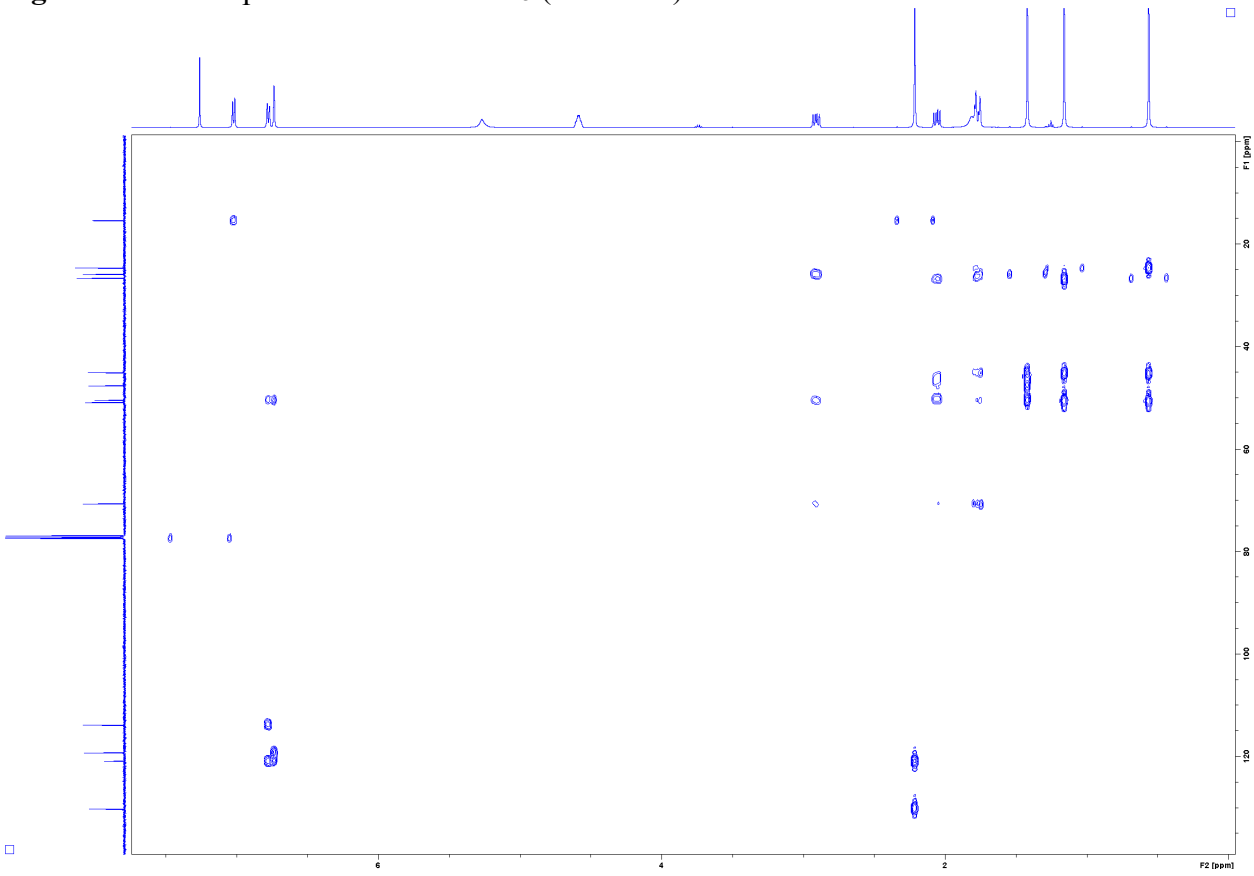
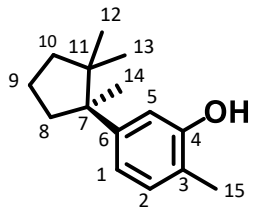


Fig. S37. HMBC spectrum of **6** in $CDCl_3$ (500 MHz).



2.7 Chemical characterization of **7**.

Table S8. NMR data of compound **7**[13] in $CDCl_3$. The molecular formula of **7** was established by mass data [$(M+H)^+$; HRESIMS: *m/z* 219.1743 $(M+H)^+$, calcd. for $C_{15}H_{23}O_1^+$, 219.1743, $\Delta = 0$ mmu].



7

	Isolated			Reported[13]		Isolated	Reported [13]
Position	δ_H		mult. (*J* in Hz)	δ_H	mult. (*J* in Hz)	δ_C	δ_C
1						148.3	146.8
1-OH	4.42						
2	6.46	1H	s	6.46	s	119.3	119.1
3						122.0	121.9
4						147.0	146.1
4-OH	4.29	1H	br s				
5	6.74	1H	s	6.74	s	116.3	116.2
6						132.1	132
7						50.9	50.8
8	2.52	1H	m	2.52	m	39.5	39.3
	1.73	1H	m	1.74	m		
9	1.73	2H	m	1.74	m	20.4	20.2
10	1.63	1H	m	1.68	m	41.2	41.1
	1.56	1H	m	1.57	m		
11						44.8	44.6
12	1.16	3H	s	1.16	s	25.6	25.4
13	0.76	3H	s	0.76	s	27.1	26.9
14	1.38	3H	s	1.38	s	23.0	22.8
15	2.16	3H	s	2.16	s	15.3	15.1

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S38. ^{1}H NMR spectrum of **7** in $CDCl_3$ (500 MHz).

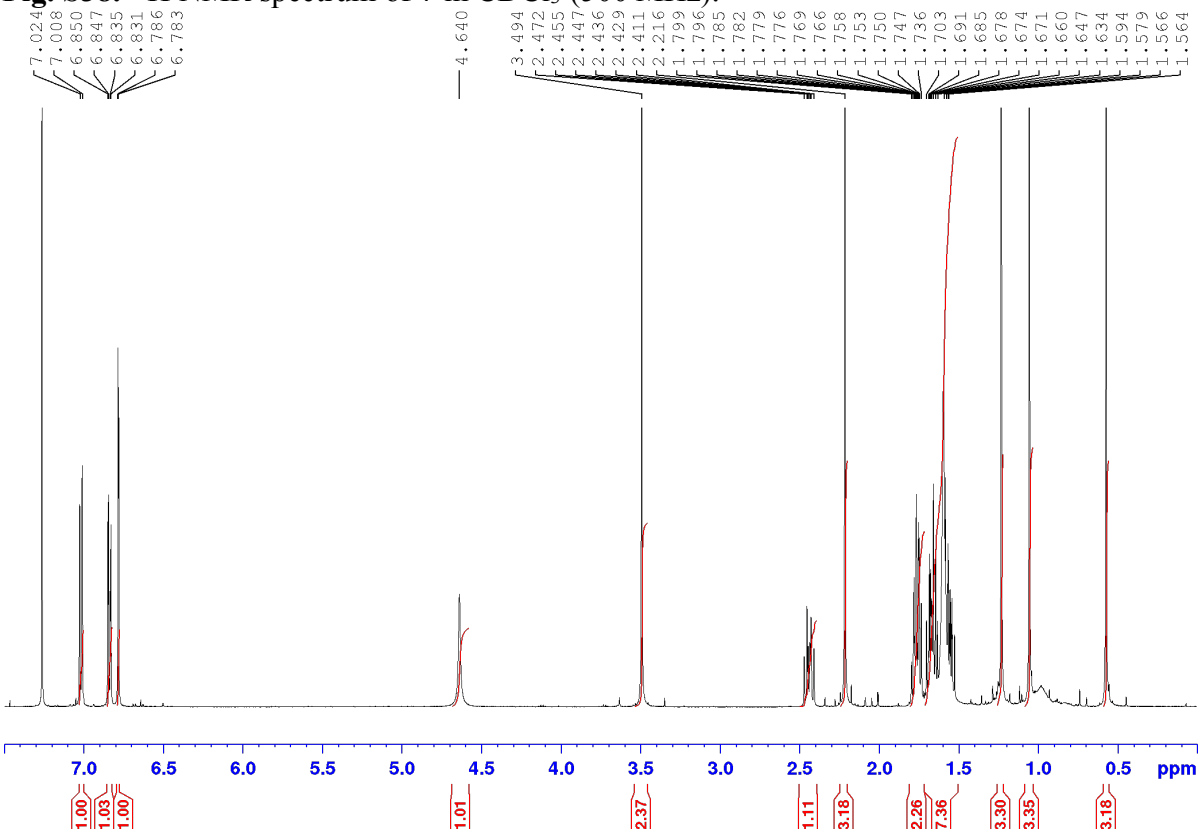
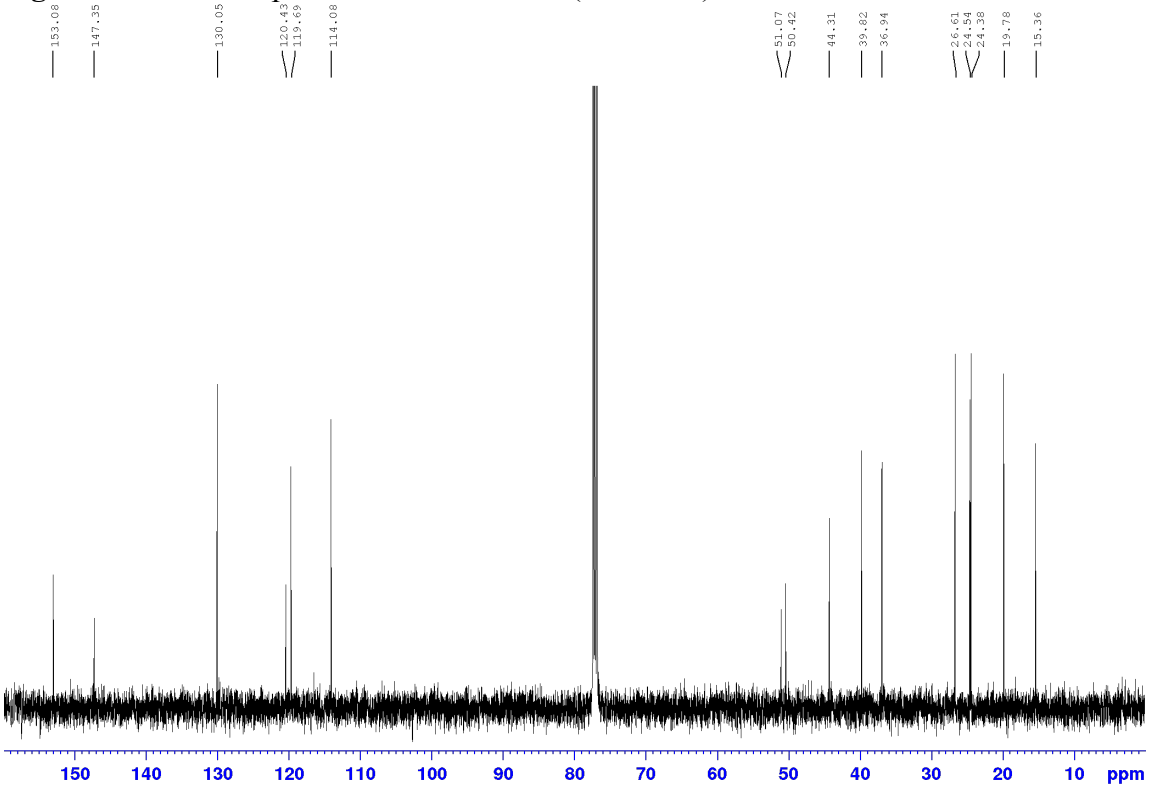
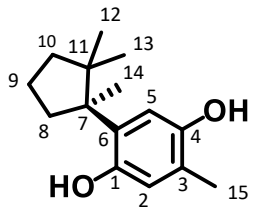


Fig. S39. ^{13}C NMR spectrum of **7** in $CDCl_3$ (125 MHz).



2.8 Chemical characterization of **8**.

Table S9. NMR data of compound **8**[14] in $CDCl_3$. The molecular formula of **8** was established by mass data [HRESIMS: *m/z* 235.1692 $(M+H)^+$, calcd. for $C_{15}H_{23}O_2^+$, 235.1693, Δ = 0.1 mmu].



8

	Isolated			Reported[14]			Isolated	Reported[14]
Position	δ_H		mult. (*J* in Hz)	δ_H		mult. (*J* in Hz)	δ_C	δ_C
1							148.3	146.8
1-OH	4.42							
2	6.46	1H	s	6.46	1H	s	119.3	119.1
3							122.0	121.9
4							147.0	146.1
4-OH	4.29	1H	br s		1H			
5	6.74	1H	s	6.74	1H	s	116.3	116.2
6							132.1	132
7							50.9	50.8
8	2.52	1H	m	2.52	1H	m	39.5	39.3
	1.73	1H	m	1.74	1H	m		
9	1.73	2H	m	1.74	2H	m	20.4	20.2
10	1.63	1H	m	1.68	1H	m	41.2	41.1
	1.56	1H	m	1.57	1H	m		
11							44.8	44.6
12	1.16	3H	s	1.16	3H	s	25.6	25.4
13	0.76	3H	s	0.76	3H	s	27.1	26.9
14	1.38	3H	s	1.38	3H	s	23.0	22.8
15	2.16	3H	s	2.16	3H	s	15.3	15.1

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S40. ^{1}H NMR spectrum of **8** in $CDCl_3$ (500 MHz).

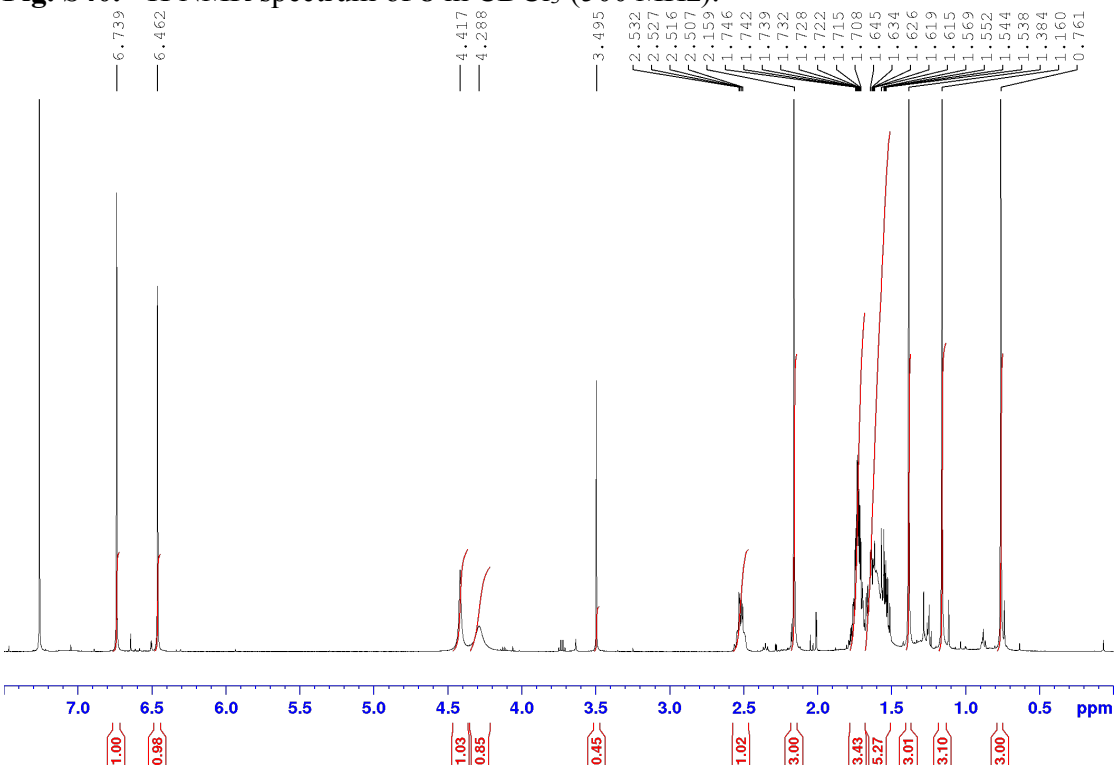
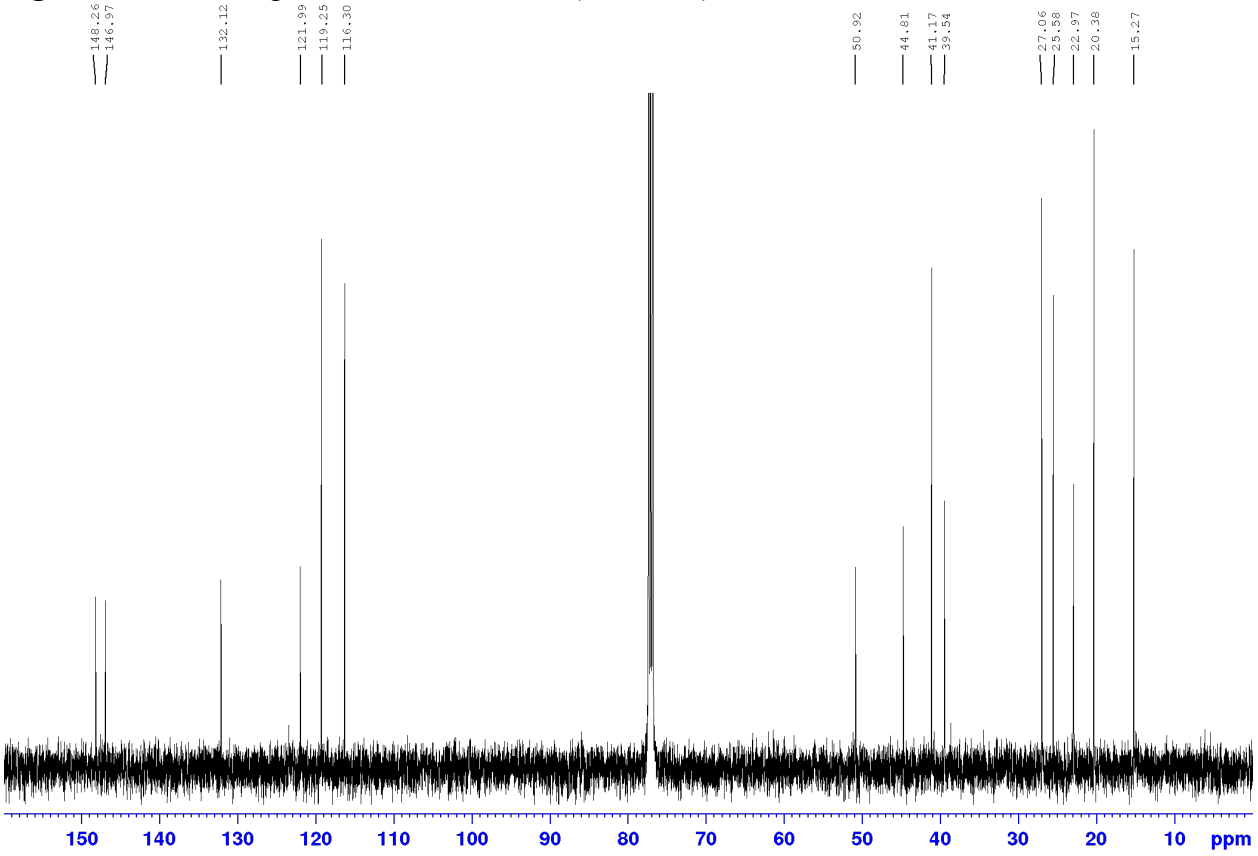
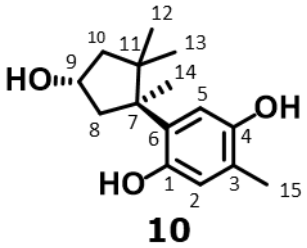


Fig. S41. ^{13}C NMR spectrum of **8** in $CDCl_3$ (125 MHz).



2.9. Chemical characterization of **10**.

Table S10. NMR data of compound **10** in $CDCl_3$. The molecular formula of **10** was established by mass data [HRESIMS: *m/z* 295.1549 $(M+HCOOH–H)^-$, calcd. for $C_{16}H_{23}O_5^-$, 295.1551, Δ = 0.2 mmu]).



10

Position	δ_H [ppm]		mult. (*J* in Hz)	HMBC	δ_C [ppm]
1					148.0
2	6.44	1H	s	4, 6	119.4
3					122.3
4					147.1
5	6.66	1H	s	1, 3	116.2
6					131.6
7					51.0
8	3.05	1H	dd (8.3, 14.3 Hz)		49.9
	1.85	1H	dd (3.3, 14.3 Hz)		
9	4.53	1H	m		69.9
10	2.01	1H	dd (8.8, 12.3 Hz)		51.6
	1.72	1H	dd (4.8, 13.8 Hz)		
11					46.5
12	1.25	3H	s	10, 11, 13	25.7
13	0.78	3H	s	10, 11, 12	26.8
14	1.55	3H	s	6, 7, 11	24.7
15	2.15	3H	s	2, 3, 4	15.3

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Fig. S42. ^{1}H NMR spectrum of **10** in $CDCl_3$ (500 MHz).

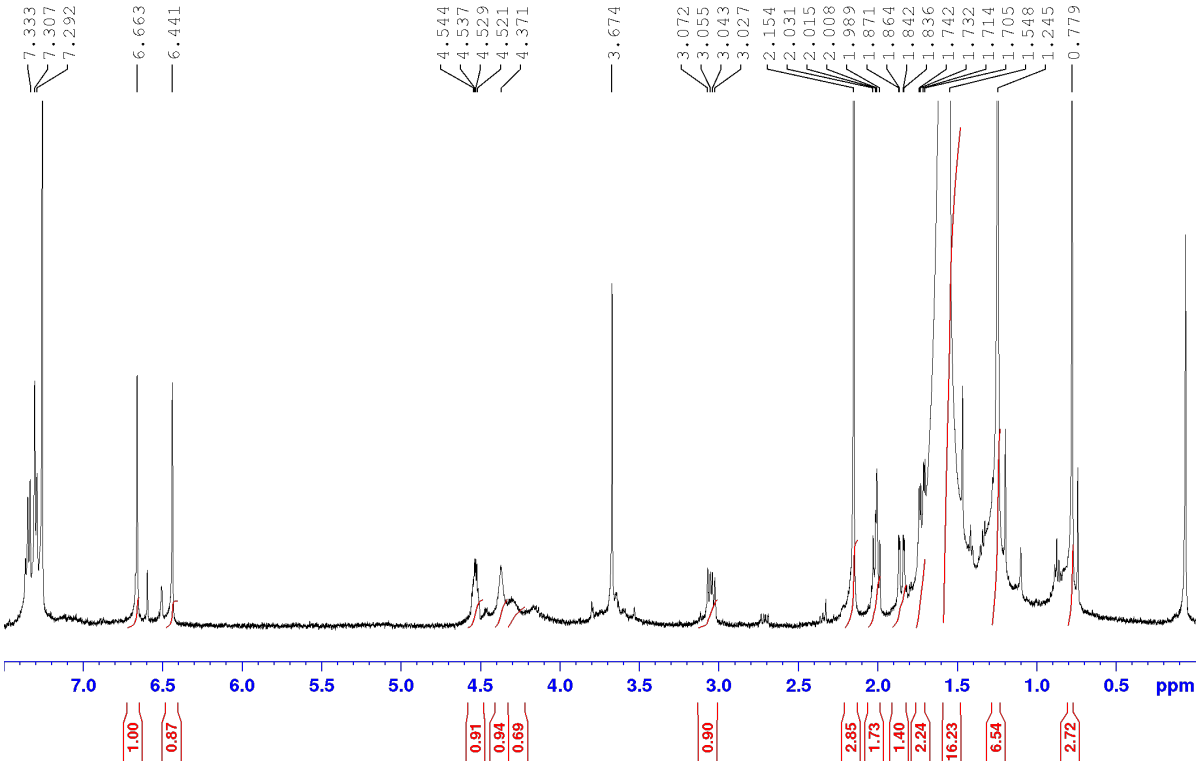


Fig. S43. ^{13}C NMR spectrum of **10** in $CDCl_3$ (125 MHz).

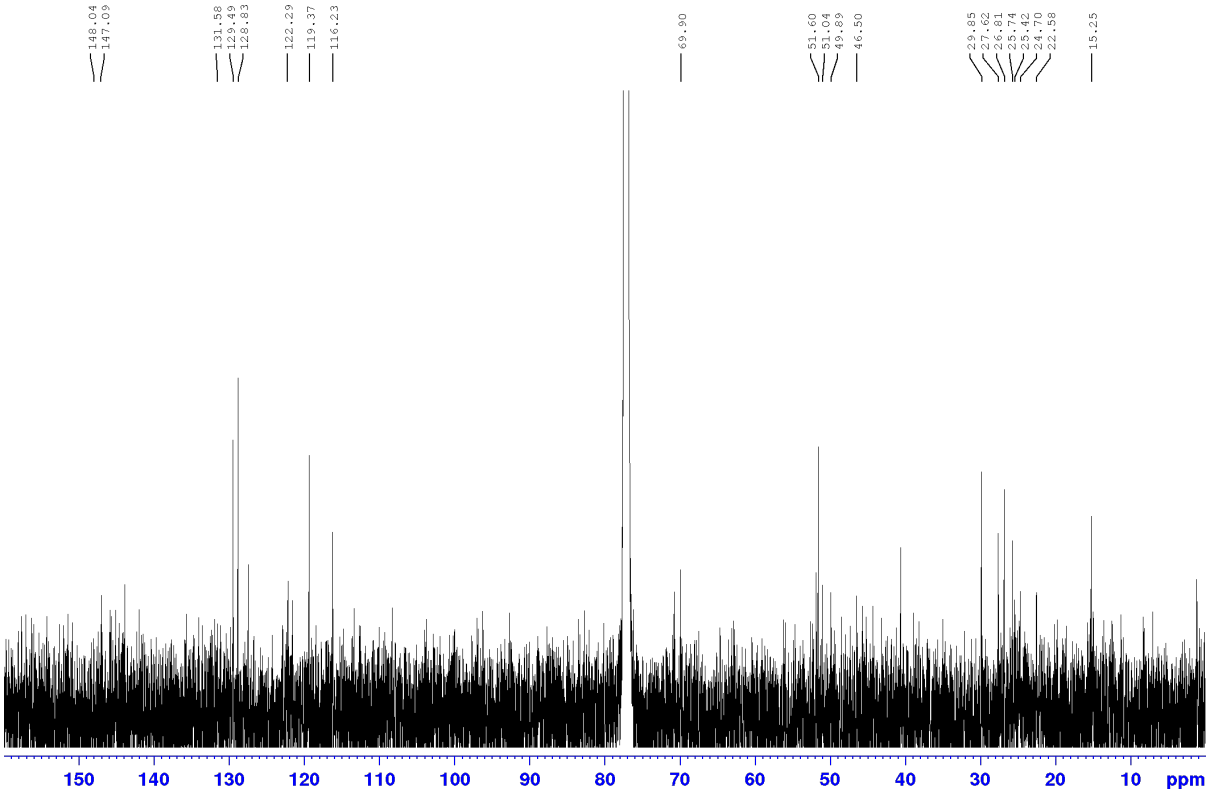


Fig. S44. COSY spectrum of **10** in $CDCl_3$ (500 MHz).

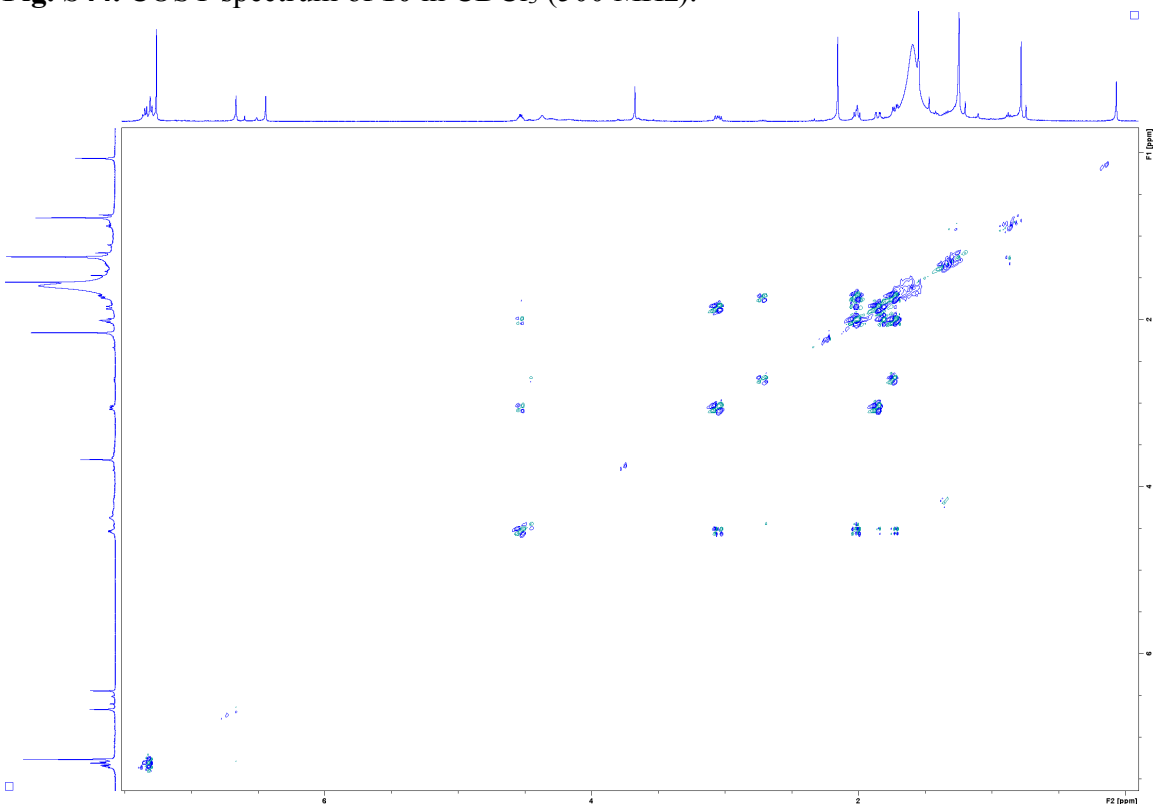


Fig. S45. HSQC spectrum of **10** in $CDCl_3$ (500 MHz).

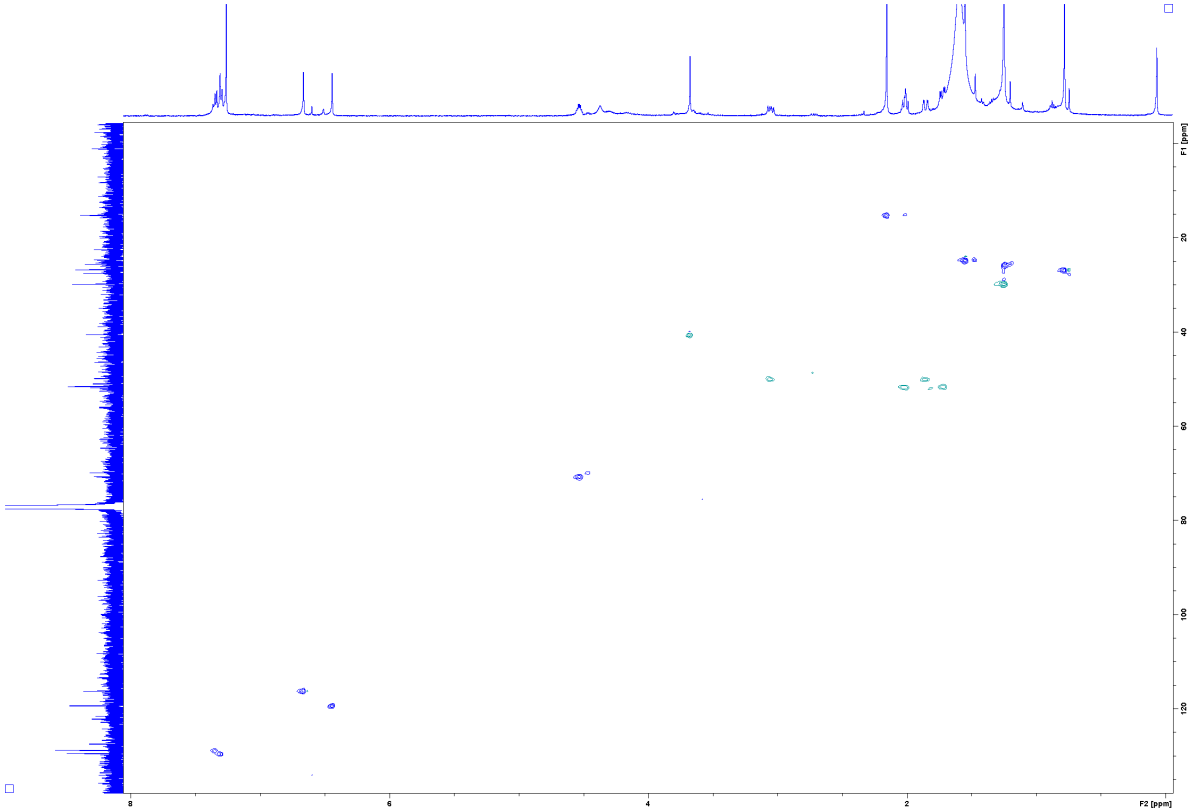
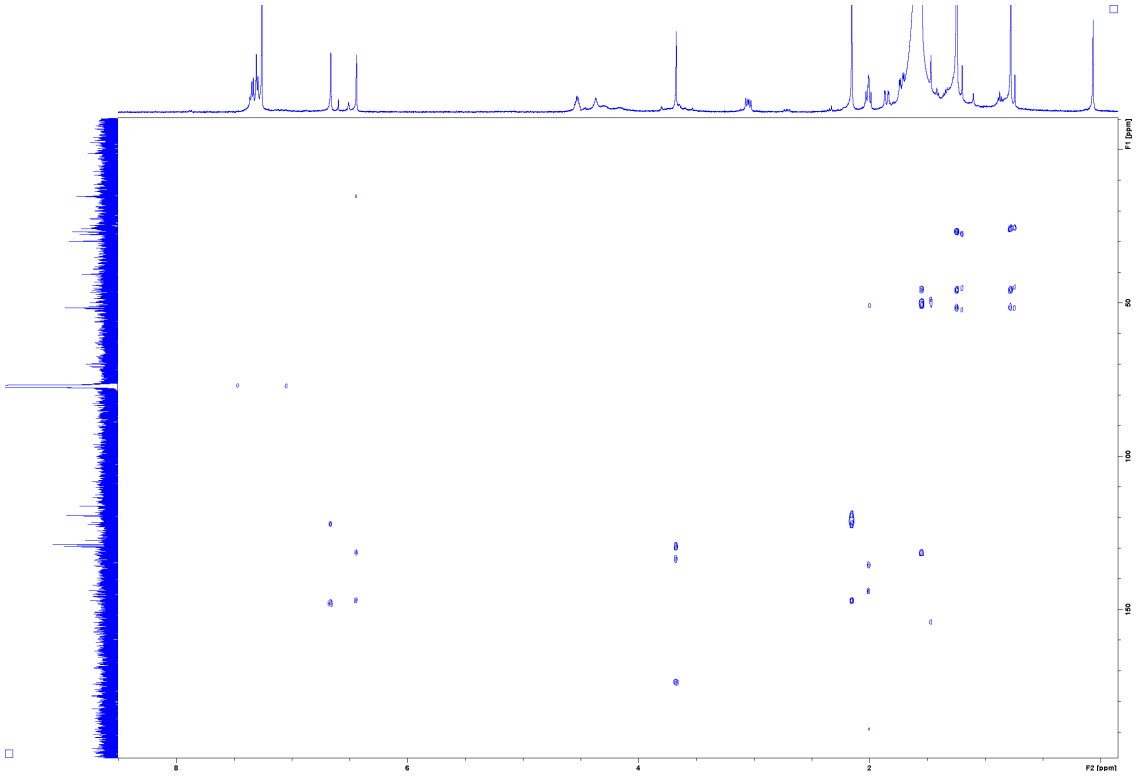


Fig. S46. HMBC spectrum of **10** in $CDCl_3$ (500 MHz).



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