

Supporting Information for

A multidomain P450 epoxidase and a terpene cyclase from ascochlorin biosynthetic pathway in *Fusarium* sp.

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Supplementary Materials and Methods

General

Solvents and chemicals were purchased from Wako Chemicals Ltd. (Tokyo, Japan) or Kanto Chemical Co., Inc. (Tokyo, Japan), unless noted otherwise. Oligonucleotide primers were purchased from Eurofins Genetics (Tokyo, Japan), and Sigma Aldrich Japan Genosys (Table S1). PCR was performed using a TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa), with KOD FX Neo (TOYOBO) or iProof DNA polymerase (BIO-RAD). Ligations of DNA fragments were performed using an in-Fusion HD cloning kit (Clontech). Sequence analyses were performed by Eurofins Genetics (Tokyo). Total mRNA isolation of *Fusarium* sp. NBRC100844 was performed utilizing ISOGEN Reagent (NIPPON GENE CO., LTD.). Reverse transcription of mRNA was performed utilizing a SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific Inc.). Silica gel column chromatography was performed using Silica Gel 60 N (spherical, neutral, 40-50 µm) (Kanto Chemical Co., Inc.). Analytical and preparative HPLC were performed on a Shimadzu Prominence system, using a an ODS-80T_m column (4.6 i.d. x 150 mm, Tosoh Co. Ltd.) and an ODS-80T_m column (7.8 i.d. x 300 mm, Tosoh Co. Ltd.). Samples for LC-MS analysis were injected into an Shimadzu Prominence system HPLC-MicroTOF mass spectrometer (Bruker Daltonics), using electrospray ionization with a COSMOSIL 2.5C₁₈-MS-II column (2.0 i.d. x 75 mm; Nacalai Tesque, Inc.). NMR spectra were obtained at 500 MHz (¹H) and 125 MHz (¹³C) with a JEOL ECX-500 or ECZ-500 spectrometer, and chemical shifts were recorded with reference to solvent signals (¹H NMR: CDCl₃ 7.26 ppm; ¹³C NMR: CDCl₃ 77.0 ppm). Optical rotation of each metabolite was measured utilizing DIP-1000 digital polarimeter (JASCO). The ascochlorin producing strain, *Fusarium* sp. NBRC100844 was purchased from NITE. Draft genome sequencing and assembling of *Fusarium* sp. NBRC100844 was ordered from Macrogen Japan (Kyoto, Japan). Gene prediction was performed by AUGUSTUS (<http://bioinf.uni-greifswald.de/webaugustus/>). The glufosinate solution for the fungal transformation was extracted from Basta (Bayer), as previously described,¹ and used at a 50 µL/mL concentration.

Plasmids construction and fungal transformation

Aspergillus oryzae NSAR1 (*niaD*, *sC*, *ΔargB*, *adeA*) and fungal transformation vector pTAex3, pUSA, pUNA, and pAdeA are kindly provided by Prof. K. Gomi (Graduate School of Agricultural Sciences, Tohoku University) and Prof. K. Kitamoto (Graduate School of Agricultural Sciences, The University of Tokyo).² pPTRI vector was purchased from TAKARA.³ pBARI vector was constructed based on pPTRI as previously reported.⁴ Fungal transformation was performed by the protoplast–polyethylene glycol method, as reported previously.⁵

1. Plasmids construction

Primers for fungal expression plasmids construction used in this research were listed in Table S1. The

procedures of plasmids construction were summarized in Table S2, and all the ligations are performed utilizing a in-Fusion HD cloning kit.

2. Fungal transformation

A. oryzae transformants constructed for this study and their construction procedures are summarized in Table S3.

Constructions of plasmids for *in vitro* assay and mutagenesis study

Primers for *in vitro* assay and mutagenesis study were listed in Table S4. Ligations of pET28a-AscD, pColdII-AscE, and pESC-URA-AscF were summarized in Table S5. Point mutation of AscF was performed using PCR method. The reaction solution of PCR was digested with DpnI (TAKARA) and purified to linear DNA. The linear DNA was introduced to DH5 α (Clontech) to yield plasmid for mutation. The information of mutation plasmid construction was summarized in Table S5.

Preparation of the protein lysate containing AscD from *E. coli*

E. coli RosettaII (DE3) pLysS cells harboring pET28a-AscD were cultured to an OD₆₀₀ of 0.7 in LB media containing 50 μ g ml⁻¹ kanamycin and 34 μ g ml⁻¹ chloramphenicol at 37 °C. Isopropyl β -D-1-thiogalactopyranoside was then added to a final concentration of 0.4 mM to induce gene expression, and the cultures were incubated further for 16 h at 15 °C. The 200 mL *E. coli* cells were harvested by centrifugation at 5,000 g and resuspended in 4 mL buffer A [50 mM Tris-HCl buffer pH 7.5, containing 300 mM NaCl, 1 mM EDTA, and 2 mM DTT]. The cells were disrupted by sonication and the lysate was centrifuged at 12,000 g for 15 min. The soluble protein lysate was concentrated into 0.5 mL, and used for the assay immediately.

Preparation of the protein lysate containing AscE from *E. coli*

E. coli RosettaII (DE3) pLysS cells harboring pColdII-AscE were cultured to an OD₆₀₀ of 0.5 in ZYM-5052 auto-inducing medium(F. W. Studier, Structural Genomics, Methods in Molecular Biology, Vol.1091, pp 17-32, (2013)) containing 50 μ g ml⁻¹ kanamycin and 100 μ g ml⁻¹ ampicillin at 37 °C. Isopropyl β -D-1-thiogalactopyranoside, aminolevlic acid, and Fe(SO₄)₂(NH₄)₂·6H₂O was then added to a final concentration of 0.05 mM, 225 μ g/mL, and 300 μ M to induce gene expression and biosynthesis of heme, and the cultures were incubated further for 16 h at 15 °C. The 50 mL *E. coli* cells were harvested by centrifugation at 5,000 g and resuspended in 5 mL buffer B [50 mM Tris-HCl buffer pH 8.0, containing 200 mM NaCl, 20% (v/v) glycerol, and Complete Ultra Tablets Mini EDTA-free (Roche)]. The cells were disrupted by sonication and the lysate was centrifuged at 12,000 g for 15 min. The reddish soluble protein lysate was concentrated into 2.0 mL, and used for the assay immediately.

Preparation of microsome fraction containing AscF from yeast

pESC-URA-AscF was introduced into *Saccharomyces cerevisiae* INVSC1 (invitrogen) with Frozen-EZ Yeast transformation II (Zymo research). The transformant was inoculated into 10 mL Synthetic dextrose (SD)-Ura medium, and incubated at 30 °C for 2 days as Preculture1. Preculture1 was transferred into 500 mL SD-Ura medium, and incubated at 30 °C in 1 day as Preculture2. The cells in Preculture2 were centrifuged, resuspended with Synthetic Glactose (SG)-Ura medium, and transferred into 500 mL SG-Ura medium, and incubated at 30 °C for 1 day. Cells from 250 mL culture were harvested, resuspended in 10 mL of 0.1 M KPB (pH 7.4) and disrupted with glass beads. After centrifugation (10,000 g) to remove the cell debris, ultracentrifugation (100,000 g) was carried out for 1 hour to collect the microsome fraction which was suspended in 500 µL of 0.1 M KPB (pH 7.4) with 24 mM CHAPS.

Assay of AscD reaction

For assay of AscD, 200 µM FAD⁺, 20 mM NADH, 200 µM **2** or **5** were incubated with the soluble protein lysate from AscD-expressing culture with total 50 µL scale for 30 °C in 400 min, and the reaction was extracted with 500 µL of EtoAc, and the extract was evaporated in vacuo. 50 µL of methanol was added into the extract, and 20 µL was analyzed with HPLC.

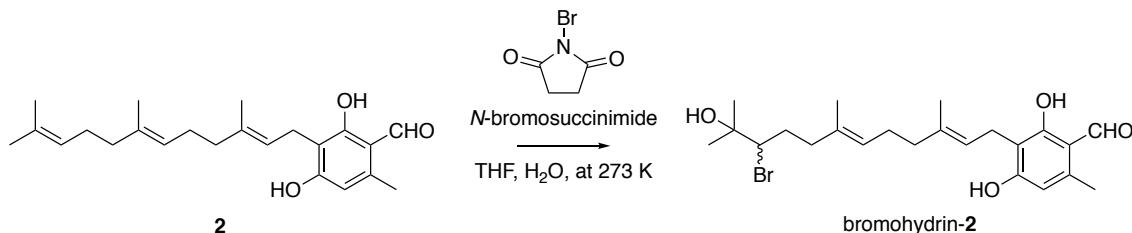
Assay of AscEF reaction

CO spectra assay for AscE was performed as the literature. For assay of AscE, 200 µM FAD⁺, 10 mM NADPH, 200 µM **2** (or 5-farnesyl-6-hydroxymellein) were incubated with the soluble protein lysate from AscE-expressing culture with total 50 µL scale for 30 °C in 12 hr, and 25 µL of the microsome fraction containing AscF was added into the reaction. After additional 12 hr incubation, enzymes in the assay were precipitated by addition of 50 µL methanol and centrifugation. 20 µL of the reaction was analyzed with HPLC.

Synthesis and preparation of racemic **4**

1. Preparation of bromohydrin-**2**

a) Synthesis of bromohydrin-**2** from **2**



Compound **2** (3.5 mg, 10 µmol) was dissolved in THF (372 µL) and H₂O (105 µL), then the solution was left on ice-water mixture while stirring until the temperature of the whole system fell to 0 °C. Recrystallized *N*-bromosuccinimide (NBS, 0.6 mg, 3 µmol) was added to solution of **2**, and stirred for 30 min at 30 °C. This operation was repeated for 6 times. Then, the reaction mixture was subjected to a

semi-preparative HPLC at once to isolate bromohydrin-**2** as clear oil.

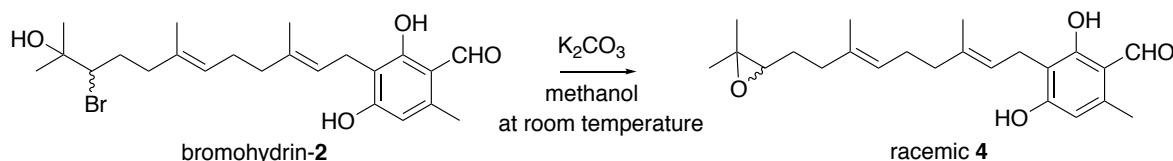
b) Isolation of bromohydrin-2

The mixture was subjected to a reverse-phase preparative HPLC, with a solvent system of milliQ (solvent A) and acetonitrile (solvent B), at a flow rate of 3.5 mL/min and a column temperature at 40 °C. Isolation was started with solvent B/solvent A (85:15), then a linear gradient from B/A (85:15) to B/A (100:0) within the following 5 min, B/A (100:0) for an additional 1min. Bromohydrin-**2** was yielded as clear oil.

6 trials (compound **2** at a total weight of 21 mg, 60 µmol) was conducted at the same time in order to accumulate enough bromohydrin-**2**, the precursor of racemic **4**.

2. Synthesis of racemic **4**

a) Dehydrochlorination of bromohydrin-2



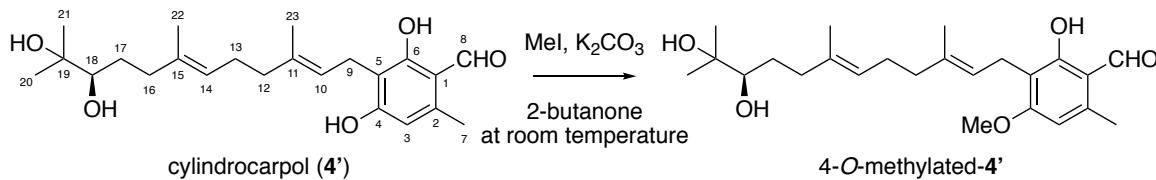
Bromohydrin-**2** prepared in last step was added into a slurry of methanol (2 mL) and K₂CO₃ (50 mg, 360 µmol), and stirred for 30 min at room temperature to yield racemic **4**.

b) Purification of racemic **4**

The reaction slurry containing racemic **4** was evaporated to remove the methanol firstly, and the residue was extracted with ethyl acetate from water. The extract was subjected to a silica-gel column chromatography and eluted using hexane:ethyl acetate (1:9) to yield racemic **4** (1.7 mg, 4.6 µmol) as a clear oil.

The mosher analysis of cylindrocarpol (4')⁷

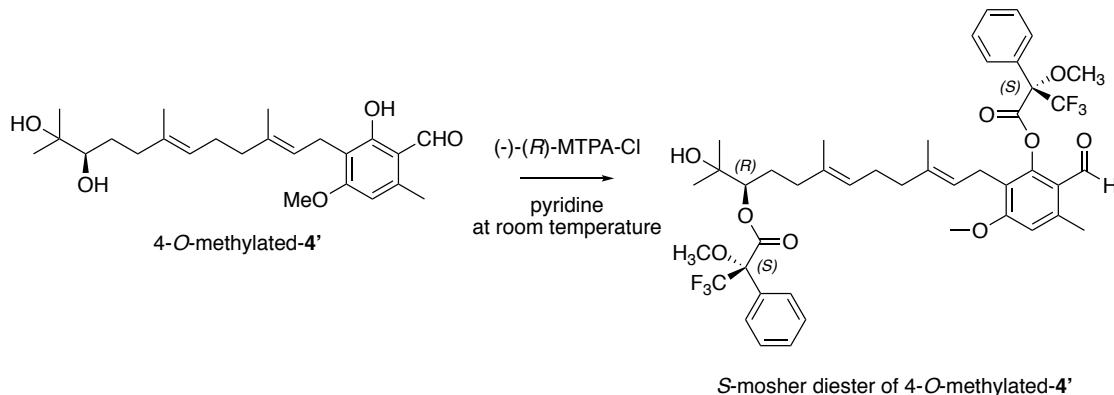
1. Synthesis of 4-*O*-methylated-4'



A mixture of **4'** (2.0 mg), MeI (20 μ l), and K₂CO₃ (20 mg) in 2-butanone (0.3 ml) was stirred at room temperature for 15 h. The reaction mixture was diluted with EtOAc (5 ml) and washed with H₂O (5 ml) twice, and the organic layer was collected and concentrated to afford the methylated derivative (2.0 mg, pale yellow oil).

¹H NMR (partial assignment, 500 MHz, CDCl₃) 12.4 (1H, s, 6-OH), 10.1 (1H, s, H-8), 6.28 (1H, s, H-3), 5.17 (1H, m, H-10), 5.16 (1H, m, H-14), 3.89 (3H, s, 4-OMe), 3.34 (1H, m, H-18), 3.30 (2H, m, H-9), 2.56 (3H, s, H-7), 2.09 (2H, m, H-16), 2.02 (2H, m, H-13), 1.96 (2H, m, H-12), 1.76 (3H, brs, H-23), 1.59 (3H, brs, H-22), 1.41, 1.58 (2H, m, H-17), 1.19 (3H, brs, H-20), 1.14 (3H, brs, H-21).

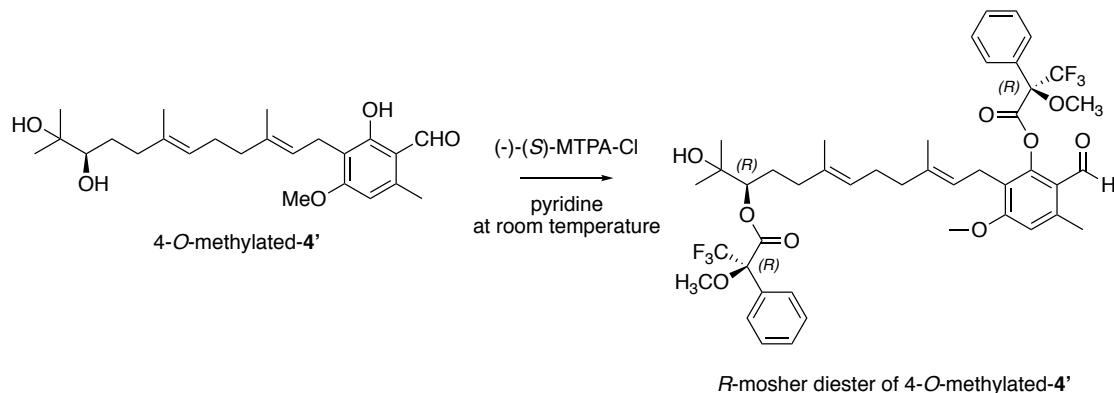
Synthesis of *S*-mosher diester of 4-*O*-methylated-4'



4-*O*-methylated-4' (1.0 mg) was dissolved in pyridine (0.3 ml, dehydrated) and treated with excess amount of (-)-(R)-MTPA-Cl (10 μ l) at room temperature for 1 h. The reaction mixture was diluted with EtOAc and washed with H₂O and saturated NaHCO₃. The organic layer was concentrated and purified by preparative HPLC (isocratic in 100% CH₃CN) to afford a *S*-mosher diester of 4-*O*-methylated-4' (ca. 0.5 mg).

¹H NMR (partial assignment, 500 MHz, CDCl₃) 9.99 (1H, s, H-8), 6.64 (1H, s, H-3), 5.00 (1H, m, H-14), 4.97 (1H, m, H-18), 4.95 (1H, m, H-10), 3.89 (3H, s, 4-OMe), 3.66 (3H, s, 4-Mosher-OMe or 6-Mosher-OMe), 3.56 (3H, s, 4-Mosher-OMe or 6-Mosher-OMe), 3.10 (2H, m, H-9), 2.63 (3H, s, H-7), 1.99 (2H, m, H-13), 1.91 (2H, m, H-12), 1.83 (2H, m, H-16), 1.62 (3H, s, H-23), 1.56, 1.66 (2H, m, H-17), 1.49 (3H, s, H-22), 1.20 (3H, brs, H-20), 1.14 (3H, brs, H-21).

2. Synthesis of *R*-mosher diester of 4-*O*-methylated-4'



In the same manner, 4-*O*-methylated-4' (1.0 mg) was treated with excess amount of (+)-(S)-MTPA-Cl (10

μl) at room temperature for 1 h. The reaction mixture was diluted with EtOAc and washed with H_2O and saturated NaHCO_3 . The organic layer was concentrated and subjected to NMR analysis without purification.

^1H NMR (partial assignment, 500 MHz, CDCl_3) 9.99 (1H, s, H-8), 6.64 (1H, s, H-3), 5.04 (1H, m, H-14), 4.96 (1H, m, H-18), 4.75 (1H, m, H-10), 3.88 (3H, s, 4-OMe), 3.66 (3H, s, 4-Mosher-OMe or 6-Mosher-OMe), 3.57 (3H, s, 4-Mosher-OMe or 6-Mosher-OMe), 3.11 (2H, m, H-9), 2.63 (3H, s, H-7), 2.01 (2H, m, H-13), 1.93 (2H, m, H-16), 1.92 (2H, m, H-12), 1.63, 1.71 (2H, m, H-17), 1.62 (3H, brs, H-23), 1.53 (3H, brs, H-22), 1.15 (3H, brs, H-20), 1.11 (3H, brs, H-21).

Analytical conditions

Metabolites from the *Aspergillus oryzae* transformants and *Fusarium* sp. NBRC100844 were analyzed by HPLC, with a solvent system of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), at a flow rate of 1.0 ml/min and a column temperature of 40 °C. Separation was performed with solvent B/solvent A (50:50), a linear gradient from 50:50 to 80:20 within the following 15 min, a linear gradient from 80:20 to 100:0 within the following 5 min, 100:0 for 5 additional min.

Isolation of the metabolites from *Fusarium* sp. NBRC100844

Fusarium sp. was cultured at 30 °C for 7 days in 2 L flasks containing 1 L DPY medium. After filtering, the mycelia were lyophilized to dryness. Dried mycelia were extracted by acetone. The extraction solution was concentrated *in vacuo* to remove the solvent. The extract (146 mg) was subjected to silica-gel column chromatography and eluted stepwise using a hexane:acetone gradient (100:0 to 70:30). Fractions containing **6**, **5**, **1a**, and **7a** were further purified by reverse-phase preparative HPLC equipped with an Ultimate AQ-C18 column (Welch Inc., Ellicott, MO, USA, 5 μm , 10 mm i.d. x 250 mm) using acetonitrile-water (70:30) as the eluting solvent (flow rate 3.0 mL/min) to yield **5** (2.1 mg), **6** (3.8 mg), **1a** (24.9 mg) and **7a** (10.1 mg). For, **1b** and **7b**, *Fusarium* sp. was cultured at 30°C for 7 days in two 2 L flasks containing 1 L CD-NaBr medium (NaNO_3 0.3%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.002%, Glucose 2%, NaBr 0.35%, pH 5.5) for each flask. After filtering, the mycelia were lyophilized to dryness. Dried mycelia were extracted by acetone. The extraction solution was concentrated *in vacuo* to remove the solvent. The extract (720 mg) was subjected to silica-gel column chromatography and eluted stepwise using a hexane: acetone gradient (95:5 to 70:30). Fractions that contained **1b** and **7b** were further purified by reverse-phase preparative HPLC using acetonitrile-water (70:30) as the eluting solvent (flow rate 3.0 mL/min) to yield **1b** (7.0 mg), **7b** (35 mg).

Isolation of the metabolites accumulated in *Aspergillus oryzae* transformants

Isolation of AscABC product (**2**) from *A. oryzae* transformant

The extract from 3 L culture of *A. oryzae* NSAR1 harboring *ascABC* was subjected to silica-gel column chromatography and eluted using chloroform:methanol (100:0). Fractions that contained **2** yield 21.0 mg of a clear oil.

Isolation of AscABCEF product (**5**) from *A. oryzae* transformant

The extract from 1 L culture of *A. oryzae* NSAR1 harboring *ascABCEF* was subjected to silica-gel column chromatography and eluted stepwise using a chloroform:methanol gradient (100:0 to 90:10). Fractions that contained **5** were further purified by reverse-phase preparative HPLC (40% aqueous acetonitrile, 3.0 mL/min) to yield 5.2 mg of a clear oil.

Isolation of AscABCE product (**4'**) from *A. oryzae* transformant

The extract from 1 L culture of *A. oryzae* NSAR1 harboring *ascABCE* and *ascF* (W130A, W131A) was subjected to silica-gel column chromatography and eluted stepwise using a chloroform:methanol gradient (100:0 to 90:10). Fractions that contained **4'** were further purified by reverse-phase preparative HPLC (30% aqueous acetonitrile, 3.5 mL/min) to yield 5.0 mg of a clear oil.

Isolation of AscABCEFG product (**6**) from *A. oryzae* transformant

The extract from 1 L culture of *A. oryzae* NSAR1 harboring *ascABCEFG* was subjected to silica-gel column chromatography and eluted stepwise using a chloroform:methanol gradient (100:0 to 90:10). Fractions that contained **6** were further purified by reverse-phase preparative HPLC (40% aqueous acetonitrile, 3.0 mL/min) to yield 0.1 mg of a white solid.

Summary of analytical data

Ascochlorin (**1a**): White solid; for NMR data see Table S6 (S13) and Figures S18 and S19; HR-ESI-MS found *m/z* 403.1682 [M - H] (calcd 403.1676 for C₂₂H₂₈O₄Cl). The NMR data were in good agreement with the reported data.

3-Bromoascochlorin (**1b**): White solid; for NMR data see Table S6 (S13) and Figures S20 and S21; HR-ESI-MS found *m/z* 447.1176 [M - H] (calcd 447.1171 for C₂₃H₂₈O₄Br). The NMR data were in good agreement with the reported data.

dechloroascochlorin (**6**): White solid; for NMR data see Table S6 (S13) and Figures S22 and S23; HR-ESI-MS found *m/z* 369.2071 [M - H] (calcd 369.2066 for C₂₂H₂₉O₄). The NMR data were in good agreement with the reported data.

12,13-dihydroascochlorin (**7a**): White solid; for NMR data see Table S6 (S14) and Figures S24 and S25; HR-ESI-MS found m/z 405.1838 [M - H]⁻ (calcd 405.1833 for C₂₃H₃₀O₄Cl). The NMR data were in good agreement with the reported data.

3-bromo-12,13-dihydroascochlorin (**7b**): White solid; for NMR data see Table S6 (S14) and Figures S26 and S27; HR-ESI-MS found m/z 449.1310 [M - H]⁻ (calcd 449.1333 for C₂₃H₃₀O₄Br). The NMR data were in good agreement with the reported data.

dechloro-12,13-dihydroascochlorin (**5**): White solid; for NMR data see Table S6 (S14) and Figures S28 and S29; HR-ESI-MS found m/z 371.2225 [M - H]⁻ (calcd 371.2228 for C₂₃H₃₁O₄). The NMR data were in good agreement with the reported data.

LL-Z1272 β (**2**): Clear oil; for NMR data see Table S6 (S15) and Figures S30 and S31; HR-ESI-MS found m/z 355.2259 [M - H]⁻ (calcd 355.2279 for C₂₂H₃₁O₅). The NMR data were in good agreement with the reported data.

cylindrocopol (**4'**): Clear oil; for NMR data see Table S6 (S15) and Figures S32 and S33; HR-ESI-MS found m/z 389.2307 [M - H]⁻ (calcd 389.2333 for C₂₃H₃₃O₅). The NMR data were in good agreement with the reported data.

4-*O*-methylated-**4'**: pale yellow oil; for NMR data see “The mosher analysis of cylindrocopol” and Figures S34, S35, S36, S37 and S38; HR-ESI-MS found m/z 405.2625 [M + H]⁺ (calcd 405.2636 for C₂₄H₃₇O₅).

S-mosher diester of 4-*O*-methylated-**4'**: for partial NMR data see “The mosher analysis of cylindrocopol” and Figures S39 and S40; HR-ESI-MS found m/z 859.3248 [M - H]⁻ (calcd 859.3251 for C₄₄H₅₀O₉F₆Na).

R-mosher diester of 4-*O*-methylated-**4'**: for partial NMR data see “The mosher analysis of cylindrocopol” and Figures S41 and S42; HR-ESI-MS found m/z 859.3249 [M - H]⁻ (calcd 859.3251 for C₄₄H₅₀O₉F₆Na).

Table S1. Primers used for fungal expression plasmids construction

Primer	Sequence (5' to 3')
SmaI-ascA-Fw	TCGAGCTCGGTACCCATGGGTGCCACAACGAG
SmaI-ascA-Rv	CTACTACAGATCCCCTTATTCCCTCGGTGAGCATT
ascA-Mid-Fw	GCAAACAGGCTTCTGTTG
ascA-Mid-Rv	CAGAAGCCTGTTGCCGAC
pAdeA-SpeI-Fw	TAGAGGATCTACTAGCGATATCATGGTGTGATC
pAdeA-SpeI-Rv	AATCCATATGACTAGCTTCTATAATAGACTAGCGTG
SmaI-ascB-Fw	TCGAGCTCGGTACCCATGGGTTCTGCCATGG
SmaI-ascB-Rv	CTACTACAGATCCCCCTACTTCTCAGGTATCCAATC
SmaI-ascC-Fw	TCGAGCTCGGTACCCATGGCCCCAAACGC
SmaI-ascC-Rv	CTACTACAGATCCCCATGCGAATAGTGCAGGGAG
pTA_Prm_Fw1	GCTCGCGAGCGCGTCCACTGCATCATCAGTCTAG
pTA_Tmn_Rv1	AACCGCGCTCGCGAGCAAGTACCATACAGTACCGCG
KpnI-ascE-Fw	CTGAATTGAGCTCGGTACCATGAGTTGCGAAATTCTC
KpnI-ascE-Rv	ACTACAGATCCCCGGGTACCTTAAGAGAAAATCTCGCTGGC
KpnI-ascF-Fw	CTGAATTGAGCTCGGTACCATGGCATTCAACGAC
KpnI-ascF-Rv	ACTACAGATCCCCGGGTACCTTAAAGCTCTTCTTC
pUSA-Prm-Fw1	GCTCGCGAGCGCGTTCGATATCATGGTGTGATC
pUSA-Tmn-Rv1	AACCGCGCTCGCGAGCCTTCTATAATAGACTAGCGTG
SmaI-ascG-Fw	TCGAGCTCGGTACCCATGGATAACCTATCGTACTCG
SmaI-ascG-Rv	CTACTACAGATCCCCTTACATCTTCTACGCTTAACTCAAG
pPTRI-HIndIII-Fw	TGATTACGCCAAGCTCGATATCATGGTGTGATC
pPTRI-HindIII-Rv	GCAGGCATGCAAGCTCTTCTATAATAGACTAGCGTG
KpnI-ascD-Fw	CTGAATTGAGCTCGGTACCATGGCTGCCAAATT
KpnI-ascD-Rv	ACTACAGATCCCCGGGTACCTTAAGCCGAGACGTCAAC
ascF-M1-Fw	GACGGCTCATTATAGCGCGGCGTGTGGATGGC
ascF-M1-Rv	GCTATAATGAGCCGTCGCGC
ascF-M2-Fw	CATTATAGCTGGCGTCGGCGCGATGGCAACGGC
ascF-M2-Rv	CGACGCCAGCTATAATGAG
ascF-M3-Fw	GAACATCAGCTACGGAGCGGCTCGTATAACCTGGCCCGAG
ascF-M3-Rv	TCCGTAGCTGATGTTCAAGGC
ascF-M4-Fw	GCCGGCAATCTCCTTGCAGGAATCACCACCGTC
ascF-M4-Rv	AAGGAAGATTGCCGGCTC

Table S2. Plasmids constructed for fungal transformation and their PCR conditions

plasmid	vector	insert	primer 1	primer 2	PCR template
pUNA-ascA	pUNA digested with SmaI	ascA-F	SmaI-ascA-Fw	ascA-Mid-Rv	gDNA
		ascA-R	ascA-Mid-Fw	Smal-ascA-Rv	
pAdeA-ascA	pAdeA digested with SpeI	Prm-ascA-F	pAdeA-SpeI-Fw	ascA-Mid-Rv	pUNA-ascA
		ascA-R-Tmn	ascA-Mid-Fw	pAdeA-SpeI-Rv	
pUNA-ascB	pUNA digested with SmaI	ascB	Smal-ascB-Fw	Smal-ascB-Rv	cDNA
pTAex3-ascC	pTAex3 digested with SmaI	ascC	Smal-ascC-Fw	Smal-ascC-Rv	gDNA
PTAex3-ascB+ascC	pTAex3 digested with SmaI	ascB+Tmn	Smal-ascB-Fw	pTA_Tmn_Rv1	pUNA-ascB
		Prm+ascC	pTA_Prm_Fw1	Smal-ascC-Rv	pTAex3-ascC
pUSA-ascE	pUSA digested with KpnI	ascE	KpnI-ascE-Fw	KpnI-ascE-Rv	gDNA
pUSA-ascF	pUSA digested with KpnI	ascF	KpnI-ascF-Fw	KpnI-ascF-Rv	gDNA
pUSA-ascF+ascE	pUSA digested with KpnI	ascF+Tmn	KpnI-ascE-Fw	pUSA-Tmn-Rv1	pUSA-ascE
		Prm+ascE	pUSA-Prm-Fw1	KpnI-ascF-Rv	pUSA-ascF
pUNA-ascG	pUNA digested with SmaI	ascG	Smal-ascG-Fw	Smal-ascG-Rv	gDNA
pPTRI-ascG	pPTRI digested with HindIII	Prm-ascG-Tmn	pPTRI-HIndIII-Fw	pPTRI-HindIII-Rv	pUNA-ascG
pUSA-ascD	pUSA digested with KpnI	ascD	KpnI-ascD-Fw	KpnI-ascD-Rv	cDNA
pBARI-ascD	pBARI digested with HindIII	Prm-ascD-Tmn	pPTRI-HIndIII-Fw	pPTRI-HindIII-Rv	pUSA-ascD
pUSA-ascF(mutant1)+ascE	pUSA digested with KpnI	ascF(mutant1)-F	KpnI-ascF-Fw	ascF-M1-Fw	pUSA-ascF+ascE
		ascF(mutant1)-R+ascE	ascF-M1-Rv	KpnI-ascE-Rv	
pUSA-ascF(mutant2)+ascE	pUSA digested with KpnI	ascF(mutant2)-F	KpnI-ascF-Fw	ascF-M2-Fw	pUSA-ascF+ascE
		ascF(mutant2)-R+ascE	ascF-M2-Rv	KpnI-ascE-Rv	
pUSA-ascF(mutant3)+ascE	pUSA digested with KpnI	ascF(mutant3)-F	KpnI-ascF-Fw	ascF-M3-Fw	pUSA-ascF+ascE
		ascF(mutant3)-R+ascE	ascF-M3-Rv	KpnI-ascE-Rv	
pUSA-ascF(mutant4)+ascE	pUSA digested with KpnI	ascF(mutant4)-F	KpnI-ascF-Fw	ascF-M4-Fw	pUSA-ascF+ascE
		ascF(mutant4)-R+ascE	ascF-M4-Rv	KpnI-ascE-Rv	

Table S3. A. oryzae transformants constructed in this study

Strain constructed	Host strain	Plasmid used in transformation
A. oryzae/ascA	A. oryzae NSAR1	pAdeA-ascA
A. oryzae/ascABC	A. oryzae/ascA	pTAex3-ascB+ascC
A. oryzae/ascABC	A. oryzae/ascABC	pUSA-ascF+ascE
A. oryzae/ascABC	A. oryzae/ascABC	pPTRI-ascG
A. oryzae/ascABC	A. oryzae/ascABC	pBARI-ascD
A. oryzae/ascABC(W127A)	A. oryzae/ascABC	pUSA-ascF(mutant1)+ascE
A. oryzae/ascABC(W130A, W131A)	A. oryzae/ascABC	pUSA-ascF(mutant2)+ascE
A. oryzae/ascABC(W207A, W209A)	A. oryzae/ascABC	pUSA-ascF(mutant3)+ascE
A. oryzae/ascABC(W228A)	A. oryzae/ascABC	pUSA-ascF(mutant4)+ascE

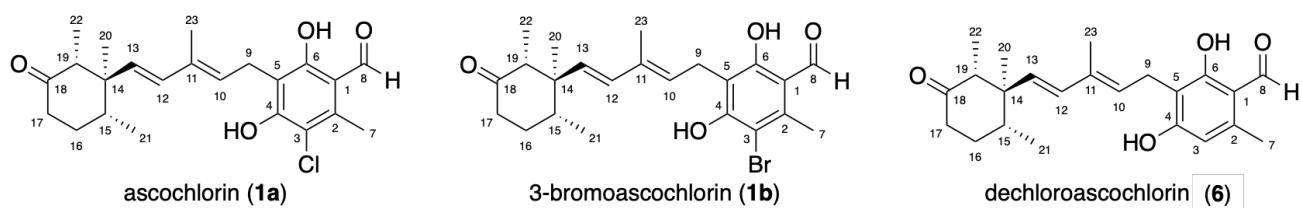
Table S4. Primers used for cloning and mutagenesis study

Primer	Sequence (5' to 3')
NdeI-AscD-Fw	CGCGCGGCAGCCATATGGCTGCCAAATTCCCAAGAAG
HindIII-AscD-Rv	GTCGCGCCGCAAGCTTAAGCCGAGACGTCAACACAG
NdeI-AscE-Fw	ATCATCATCATCATATGAGTTGCGAAATTCTCGC
HindIII-AscE-Rv	GCAGGTGACAAGCTCTACTAAGAGAAAATCTGCTGGC
EcoRI-AscF-Fw	TTGAAAATTGCAATTcATGGCATTCAACGACGTCCCTC
BglII-AscF-Rv	TTAAGAGCTCAGATCtAAAGCTCCTTCCCTCATGTCG
AscF-W130A-Fw	CATTATAGCTGGGCGTCGGCGTGGATGGGCAACGGCATTG
AscF-W130A-Rv	CAATGCCGTTGCCCATCCACGCCGACGCCAGCTATAATG
AscF-W131A-Fw	CATTATAGCTGGGCGTCGTGGCGATGGGCAACGGCATTG
AscF-W131A-Rv	CAATGCCGTTGCCCATGCCACGCCAGCTATAATG
AscF-W207W209A-Fw	GAACATCAGCTACGGAGCGGCTGCGTATACTGGCCGAG
AscF-W207W209A-Rv	CTCGGGCCAGGTATAACGCAAGCCGCTCCGTAGCTGATGTT
AscF-W228A-Fw	GCCGGCAATCTCCTTGCGGGAATCACCAACCGTC
AscF-W228A-Rv	GACGGTGGTGAATCCCGCAAGGAAGATTGCCGGC
AscF-E61A-Fw	CCAACAACTTGCCTGGcGATGACGTATGCCTTGATGTGG
AscF-E61A-Rv	CCACATACAAGGCATACGTCATCtgCCAGGCAAAGTTGTTGG
AscF-E61Q-Fw	CCAACAACTTGCCTGGcAGATGACGTATGCCTTGATGTGG
AscF-E61Q-Rv	CCACATACAAGGCATACGTCATCTgCCAGGCAAAGTTGTTGG
AscF-D235A-Fw	GAATCACCAACCGTCTGCGcCATTGTGTACGCCATTGTC
AscF-D235A-Rv	GACAATGGCGTACACAATGgCGCAGACGGTGGTATTGTC
AscF-D235N-Fw	GAATCACCAACCGTCTGCaACATTGTGTACGCCATTGTC
AscF-D235N-Rv	GACAATGGCGTACACAATGTtGCAGACGGTGGTATTGTC

Table S5. Plasmids constructed for in vitro assay

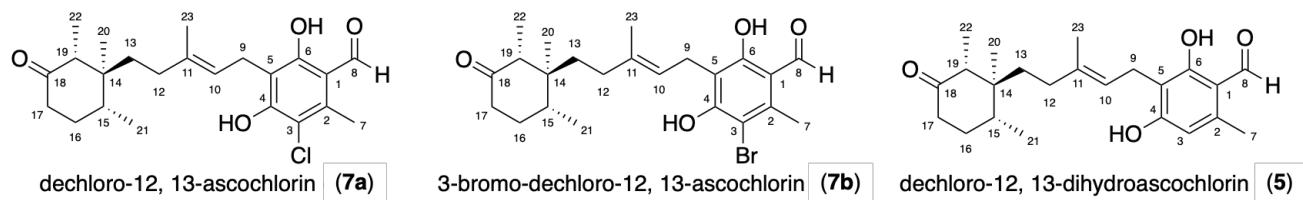
Plasmid	Vector	Insert	Primer1	Primer2	PCR template
pET28a-AscD	pET28a digested with NdeI and HindIII	AscD	NdeI-AscD-Fw	HindIII-AscD-Rv	cDNA of AscD
pColdII-AscE	pColdII digested with NdeI and HindIII	AscE	NdeI-AscE-Fw	HindIII-AscE-Rv	cDNA of AscE
pESC-URA-AscF	pESC-URA digested with EcoRI and BglII	AscF	EcoRI-AscF-Fw	BglII-AscF-Rv	cDNA of AscF
pESC-URA-AscF(W130A)	-	-	AscF-W130A-Fw	AscF-W130A-Rv	pESC-URA-AscF
pESC-URA-AscF(W131A)	-	-	AscF-W131A-Fw	AscF-W131A-Rv	pESC-URA-AscF
pESC-URA-AscF(W207W209A)	-	-	AscF-W207W209A-Fw	AscF-W207W209A-Rv	pESC-URA-AscF
pESC-URA-AscF(W228A)	-	-	AscF-W228A-Fw	AscF-W228A-Rv	pESC-URA-AscF
pESC-URA-AscF(E61A)	-	-	AscF-E61A-Fw	AscF-E61A-Rv	pESC-URA-AscF
pESC-URA-AscF(E61Q)	-	-	AscF-E61Q-Fw	AscF-E61Q-Rv	pESC-URA-AscF
pESC-URA-AscF(D235A)	-	-	AscF-D235A-Fw	AscF-D235A-Rv	pESC-URA-AscF
pESC-URA-AscF(D235N)	-	-	AscF-D235N-Fw	AscF-D235N-Rv	pESC-URA-AscF

Table S6. NMR data of the isolated compounds



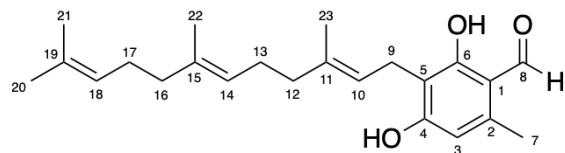
Position	ascochlorin (1a)		3-bromoascochlorin (1b)		dechloroascochlorin (6)	
	δ_{H} (mult, Hz)	δ_{C} (Hz)	δ_{H} (mult, Hz)	δ_{C} (Hz)	δ_{H} (mult, Hz)	δ_{C} (Hz)
1	-	113.9	-	114.4	-	111.9
2	-	137.9	-	139.9	-	142.2
3	-	113.3	-	106.2	6.22 (1H, s)	110.6
4	-	156.3	-	157.0	-	163.8
5	-	113.8	-	113.9	-	113.5
6	-	162.3	-	162.8	-	161.6
7	2.60 (3H, s)	14.3	2.65 (3H, s)	14.3	2.49 (3H, s)	18.1
8	10.13 (1H, s)	193.4	10.16 (1H, s)	193.6	10.1 (1H, s)	193.1
9	3.53 (2H, d, $J = 7.4$ Hz)	22.4	3.56 (2H, d, $J = 7.4$ Hz)	22.6	3.50 (2H, d, $J = 7.4$ Hz)	21.4
10	5.52 (1H, t, $J = 7.3$ Hz)	127.7	5.52 (1H, t, $J = 7.5$ Hz)	127.7	5.52 (1H, t, $J = 7.2$ Hz)	127.5
11	-	134.2	-	134.3	-	136.4
12	5.89 (1H, d, $J = 16.0$ Hz)	133.3	5.90 (1H, d, $J = 16.0$ Hz)	133.4	5.92 (1H, d, $J = 16.1$ Hz)	132.9
13	5.36 (1H, d, $J = 16.0$ Hz)	135.8	5.38 (1H, d, $J = 16.0$ Hz)	135.8	5.40 (1H, d, $J = 15.8$ Hz)	135.4
14	-	48.6	-	48.6	-	48.6
15	1.95-1.92 (1H, m)	41.0	1.98-1.92 (1H, m)	41.0	1.98-1.90 (1H, m)	40.9
16	1.95-1.92 (1H, m)	31.3	1.98-1.92 (1H, m), 1.61 (1H, m)	31.3	1.98-1.90 (1H, m) 1.62 (1H, m)	31.2
17	2.39 (2H, m)	41.7	2.39 (2H, m)	41.7	2.38 (2H, m)	41.7
18	-	212.9	-	212.9	-	212.9
19	2.39 (1H, m)	53.7	2.39 (1H, m)	53.7	2.38 (1H, m)	53.7
20	0.69 (3H, s)	10.5	0.70 (3H, s)	10.5	0.70 (3H, s)	10.4
21	0.83 (3H, d, $J = 6.8$ Hz)	16.5	0.83 (3H, d, $J = 6.7$ Hz)	16.5	0.84 (3H, d, $J = 6.7$ Hz)	16.4
22	0.80 (3H, d, $J = 6.6$ Hz)	9.0	0.81 (3H, d, $J = 6.6$ Hz)	9.1	0.81 (3H, d, $J = 6.7$ Hz)	9.0
23	1.92 (3H, s)	12.8	1.92 (3H, s)	12.8	1.94 (3H, s)	12.8

¹H NMR: 500 MHz, ¹³C NMR: 125 MHz (in CDCl₃)

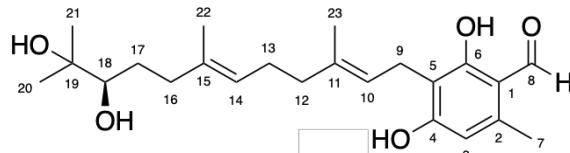


Position	12,13-dihydroascocochlorin (7a)		3-bromo-12,13-dihydroascocochlorin (7b)		dechloro-12,13-dihydroascocochlorin (5)	
	δ_H (mult, Hz)	δ_C (Hz)	δ_H (mult, Hz)	δ_C (Hz)	δ_H (mult, Hz)	δ_C (Hz)
1	-	113.3	-	114.3	-	112.2
2	-	137.8	-	139.8	-	142
3	-	113.8	-	106.2	6.24 (1H, s)	110.8
4	-	156.4	-	157.1	-	163.9
5	-	114.4	-	114.5	-	113.3
6	-	162.4	-	162.9	-	162.5
7	2.45 (1H, m)	50.6	2.45 (1H, m)	50.6	2.48 (3H, s)	18.1
8	10.1 (1H, s)	193.4	10.2 (1H, s)	193.6	10.06 (1H, s)	193.4
9	3.39 (2H, d, $J = 7.1$ Hz)	22.2	3.41 (2H, d, $J = 7.2$ Hz)	22.5	3.37 (2H, d, $J = 7.1$ Hz)	21.3
10	5.25 (1H, t, $J = 7.1$ Hz)	121	5.24 (1H, t, $J = 7.5$ Hz)	121	5.27 (1H, t, $J = 7.2$ Hz)	121.5
11	-	136.8	-	136.8	-	138.3
12	2.00 (1H, m) 1.84 (1H, m)	32.8	1.98 (1H, m) 1.84 (1H, m)	32.8	1.97 (1H, m) 1.88 (1H, m)	32.8
13	1.39 (2H, m)	35.7	1.39 (2H, m)	35.7	1.40 (2H, m)	35.7
14	-	43.6	-	43.6	-	43.7
15	2.00 (1H, m)	36.2	1.98 (1H, m)	36.2	2.00 (1H, m)	36.2
16	1.84 (1H, m) 1.63 (1H, m)	31.1	1.83 (1H, m) 1.61 (1H, m)	31.1	1.84 (1H, m), 1.61 (1H, m)	31.1
17	2.32 (2H, m)	41.7	2.31 (2H, m)	41.7	2.33 (2H, m)	41.7
18	-	214.3	-	214.3	-	215.1
19	2.45 (1H, m)	50.6	2.45 (1H, m)	50.6	2.47 (1H, m)	50.7
20	0.56 (3H, s)	15.5	0.56 (3H, s)	15.5	0.57 (3H, s)	15.5
21	0.87 (1H, d, $J = 6.7$ Hz)	15.2	0.88 (1H, d, $J = 6.8$ Hz)	15.2	0.88 (3H, d, $J = 6.6$ Hz)	15.2
22	0.90 (3H, d, $J = 6.7$ Hz)	7.7	0.91 (3H, d, $J = 6.8$ Hz)	7.7	0.92 (3H, d, $J = 6.7$ Hz)	7.7
23	1.81 (3H, s)	16.5	1.81 (3H, s)	16.5	1.83 (3H, s)	16.5

^1H NMR: 500 MHz, ^{13}C NMR: 125 MHz (in CDCl_3)



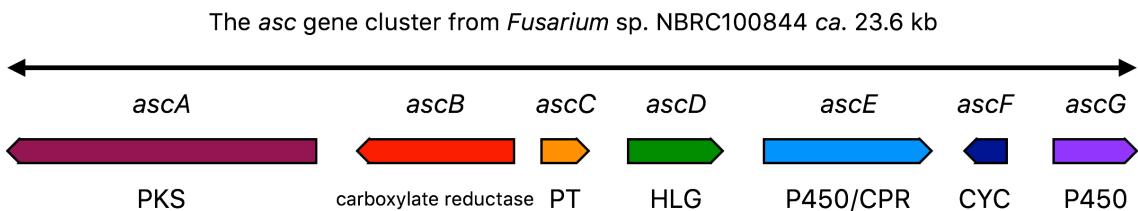
LL-Z1272 β (**2**)



cylindrocarpol (**4'**)

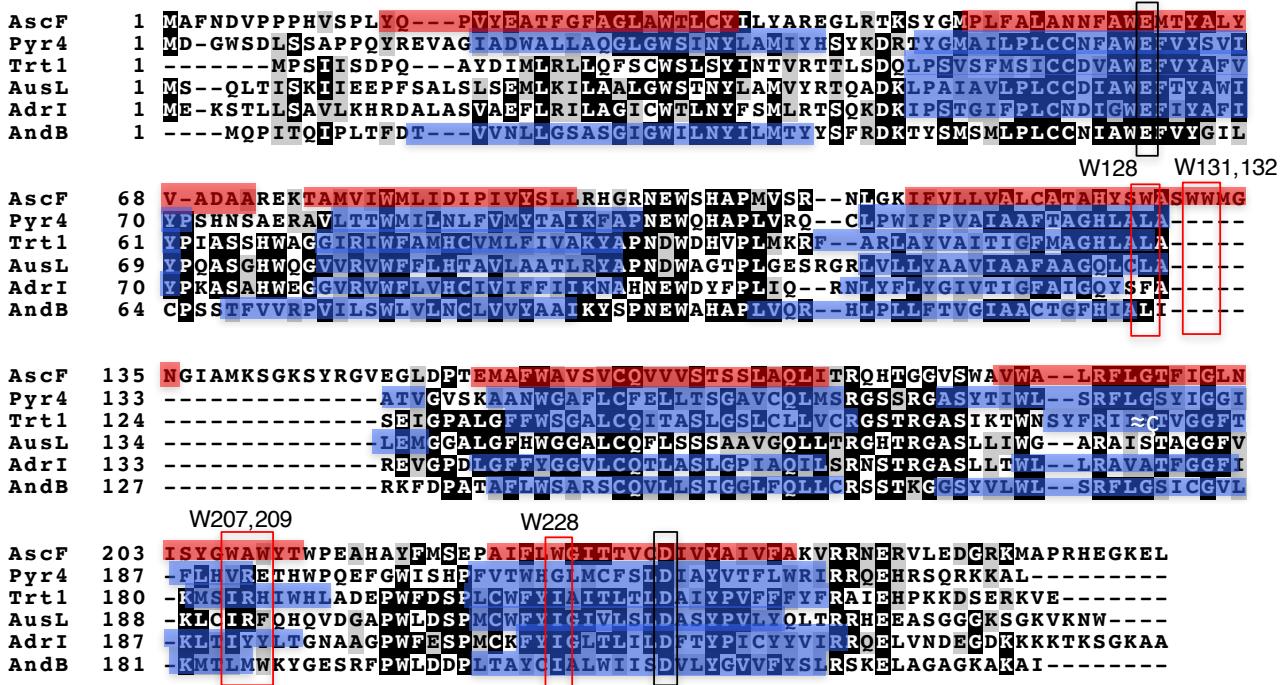
Position	LL-Z1272 β (2)		cylindrocarpol (4')	
	δ_H (mult, Hz)	δ_C (Hz)	δ_H (mult, Hz)	δ_C (Hz)
1		113.2	-	113.2
2		142.1	-	141.9
3	6.22 (1H, s)	111.0	6.19 (1H, s)	110.8
4		162.9	-	162.7
5		112.2	-	112.4
6		163.9	-	163.7
7	2.47 (3H, s)	18.0	2.47 (3H, s)	18.1
8	10.0 (1H, s)	193.0	10.0 (1H, s)	192.3
9	3.40 (2H, d, $J = 7.2$ Hz)	21.2	3.35 (2H, d, $J = 6.6$ Hz)	21.1
10	5.27 (2H, t, $J = 7.2$ Hz)	121.2	5.17 (1H, t, $J = 6.9$ Hz)	122.3
11		138.7	-	137.6
12	2.04 (2H, m)	39.7	2.05 (2H, m)	39.3
13	2.07 (2H, m)	26.7	2.08 (2H, m)	25.5
14	5.08 (1H, m)	124.5	5.12 (1H, t, $J = 7.8$ Hz)	124.8
15		135.5	-	135.2
16	2.11 (2H, m)	39.8	2.13, 2.17 (2H, m)	36.6
17	1.97 (2H, m)	26.4	1.42, 1.62 (2H, m)	29.8
18	5.08 (1H, m)	123.7	3.39 (1H, dd, $J = 12.6, 1.8$)	78.3
19		131.5	-	73.5
20	1.67 (3H, brs)	25.7	1.18 (3H, brs)	26.5
21	1.59 (3H, brs)	17.7	1.12 (3H, brs)	23.4
22	1.59 (3H, brs)	16.3	1.58 (3H, brs)	14.3
23	1.82 (3H, brs)	16.1	1.76 (3H, brs)	16.1
6-OH	12.7 (1H, s)		12.7 (1H, s)	

1H NMR: 500 MHz, ^{13}C NMR: 125 MHz (in $CDCl_3$)



Genes	Amino acids (base pairs)	Protein homologue, origin organism	Identities / Similarities	Predicted Function
<i>ascA</i>	2118 (6527)	StbA, <i>Stachybotrys bisbyi</i>	60% / 76%	Polyketide synthase (PKS)
<i>ascB</i>	1064 (3316)	StbB, <i>Stachybotrys bisbyi</i>	61% / 76%	NRPS-like carboxylate reductase
<i>ascC</i>	333 (1002)	StbC, <i>Stachybotrys bisbyi</i>	57% / 69%	UbiA prenyltransferase (PT)
<i>ascD</i>	560 (1995)	AclH, <i>Aspergillus oryzae</i> RIB40	57% / 72%	Flavin-dependent halogenase (HLG)
<i>ascE</i>	1069 (3535)	AoCYP505A3, <i>Aspergillus oryzae</i> RIB40	47% / 64%	Cytochrome P450 / Cytochrome P450 reductase (P450/CPR)
<i>ascF</i>	267 (894)	AndB, <i>Emericella variecolor</i>	30% / 49%	Terpene cyclase (CYC)
<i>ascG</i>	533 (1755)	AtaF, <i>Aspergillus terreus</i> NIH2624	33% / 51%	Cytochrome P450 (P450)

Figure S1. Schematic representation and annotation of *ascA-G*



A)

AsCE	1	-MSCEIPRPRGLPVIGNILDIDPSDAVASLCKLAEIHCGSIYKLKLAGSEKVFIS	SSRLMD
P450BM3	1	MTIKEMPQPKTFGEKLKNPLLNNDKPVQALMKADELGEIFKFEEAPGRVTRYLSSQRLIK	
AsCE	60	EVSDETRPTKLVSGPLAQRLRNAVCDS	
P450BM3	61	EACDESRFDKNLSOALKFVRDFAGDGLFTSWTHEKNWKAHNILLESFSQOAMKGYHAMM	
AsCE	120	HDLASQLVTKWAFGPHDTINVTSDFTRLTLDTIALCSMGTRFNSFYHEEMHPFIGSMIG	
P450BM3	121	VDIAVQLVOKWERLNADEHIEPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVR	
AsCE	180	LLEESGRRAPRPEWANYLLPGQAQAOYDTDIQLTRRVAAADVLA	DRRAHPNKKDILNALIV
P450BM3	181	ALDEAMNKLQRANPDDPAYDENKRQFQEDIKVMNDLVDKIIADRKA	SAGEQSDDLTHMLN
AsCE	240	CSDSKTGERMSDESILNNMIVF	
P450BM3	241	GKDPETEPLDDENI	RYQIITFLIAGHETTSGLLSFALYFLVKNPVLQKAAEAAARV
AsCE	300	RGAITIEHMSKL	LPYLEACLRET
P450BM3	301	DPVPSYKQVKOLKYVGMVLNEA	RLWPTA
AsCE	360	VALLIQIORDPAVYCPDANE	FRAERMLDENFNKL
P450BM3	355	MVLIPOLHRDKTIW	GDDVEEFRPERFE
AsCE	420	IYTAMLLQT	FNFRMHDP
P450BM3	413	YLGMMLKHFDFEDH	TNYELD
AsCE	480	ANGVNGHATPRKEQVHG	--KAVTNGHATKPMAILFGGES
P450BM3	462	-----PSTEQSAK	KVRKAENAHNTPLLVLY
AsCE	538	TVESLDSATGDMPKDRP	VVLISSSYNGOPPCNAAAQFVLNLEG
P450BM3	513	QVATLDSHAGNLPREGA	VLCKGCNVLN
AsCE	598	NRDYGPTFHRIPKL	LNSELEKNGAARLAEICLGDVIAGDIFSD
P450BM3	572	DKNWATTYQKVPAFIDET	LAQKPEGFVVKAKS
AsCE	658	TADNGD	---IGFEVQIDR
P450BM3	630	NLDIENSEDNK	-SELREDVEEA
AsCE	710	EGEGMEYRSGDHISV	PLNDWGI
P450BM3	690	DKEASYQE	GGHLGVPI
AsCE	768	LLSGYVELSQPATRK	NVLRMAACTEDEEAR
P450BM3	750	-LLQYVELQDPV	TRTQLRAMAAKTVCPHKV
AsCE	826	ESVSLSVSEYLAMLPPM	RARKYSAASSPLADASIVTLI
P450BM3	809	PACEMKFSEFIALLPSIR	PRYYSISSSPRVDEQASITV
AsCE	886	YLARLQE	GESIHVAVKPALRLFRPPSDVENT
P450BM3	868	YLAELQECDT	ITCFISTPQSEFTLPKDP
AsCE	946	DLAPAYLFIGCRDP	AKDALVVDELKQWE
P450BM3	927	SLGEAHLVFGCRSP	HEDYLYQEELENAQSEGII
AsCE	1006	EVVEKCIE	REGNFYVCGGAGV
P450BM3	984	GKKLIELDQ	GAHFWVCCGDSQMAPAVEATLMKS
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P450BM3	1043	AKDVWAG	

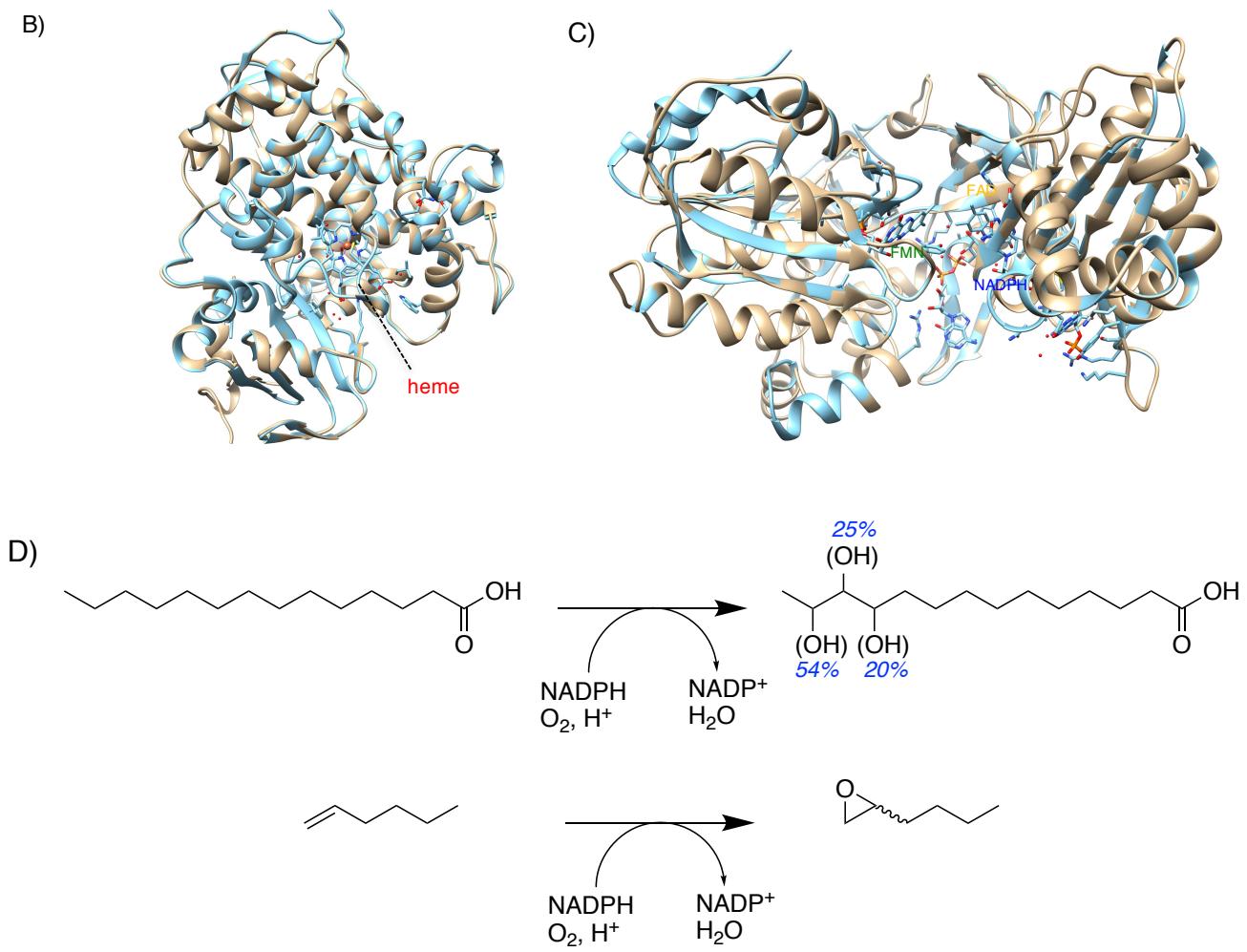


Figure S3. Comparison between AscE and P450 BM-3

Alignment between AscE and P450 BM-3 (A), homology model of P450 domain (B) and flavin- $NADP^+$ binding domain (C), and the reaction of P450 BM-3 (D). Heme, FAD, $NADP^+$, and FMN binding sites of P450BM-3^{8,9} were shown in the alignment. Homology model was built by Phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2/>). The model of AscE P450 domain (1-462) (brown) was built by using the PDB model 4H23 (cyan) as a template, and that of flavin- $NADP^+$ binding domain (504-1069) (brown) was built by using 1J9Z (cyan) as a template.

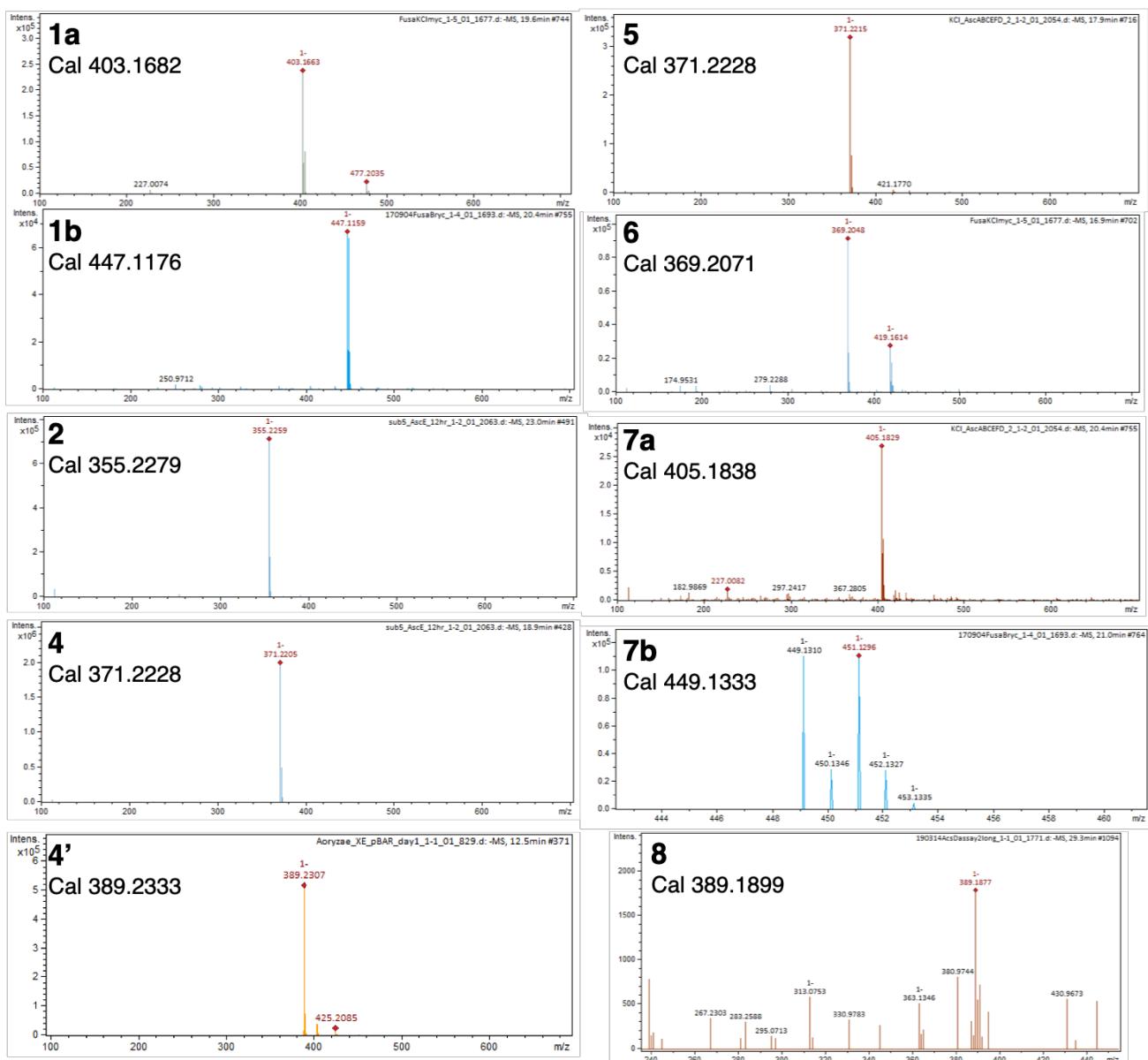


Figure S4. MS spectra of the compounds in this study

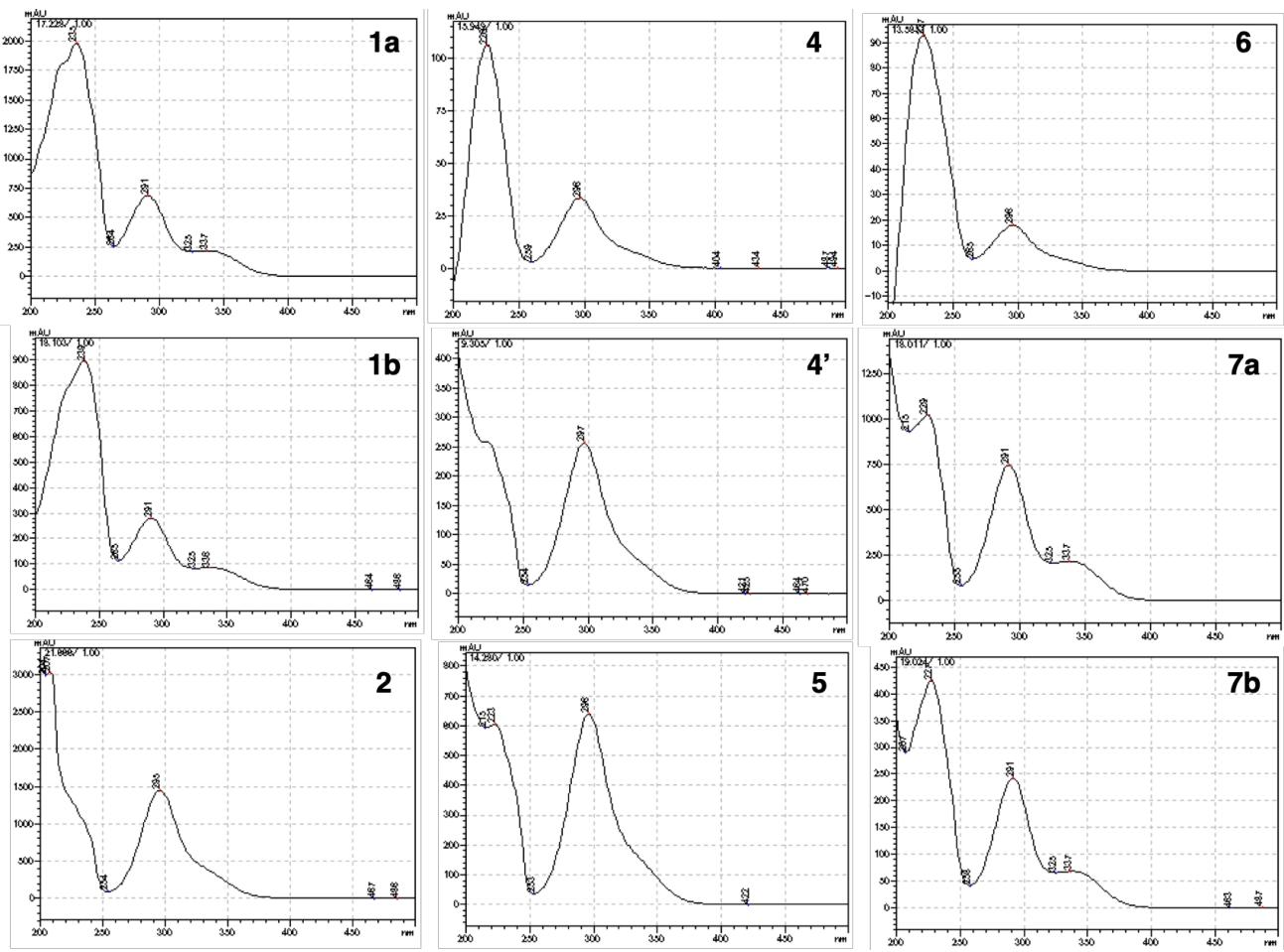


Figure S5. UV spectra of the compounds isolated in this study

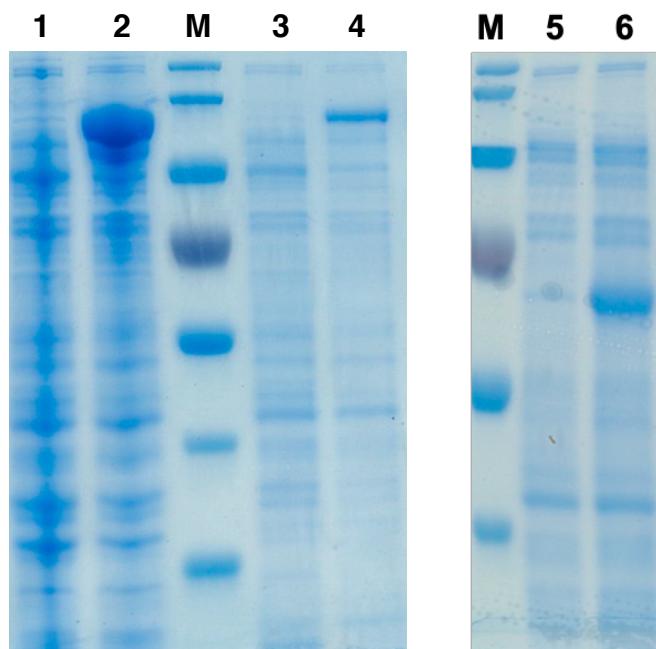


Figure S6. SDS-PAGE analysis of the recombinant AscE and AscD

From Rosetta2/pColdII-AscE (Lane2 & 4; insoluble & soluble fraction), negative control Rosetta2/pColdII (Lane1 & 3; insoluble & soluble fraction), Rosetta2/pET28a-AscD (Lane6; soluble fraction), and negative control Rosetta2/pET28a (Lane5; soluble fraction). Molecular weight of AscE is 120 kDa, and that of AscD is 63 kDa. M; Thermo Scientific PageRuler Prestained Protein Ladder (170, 130, 70 (purple), 55, 40, 35 kDa)

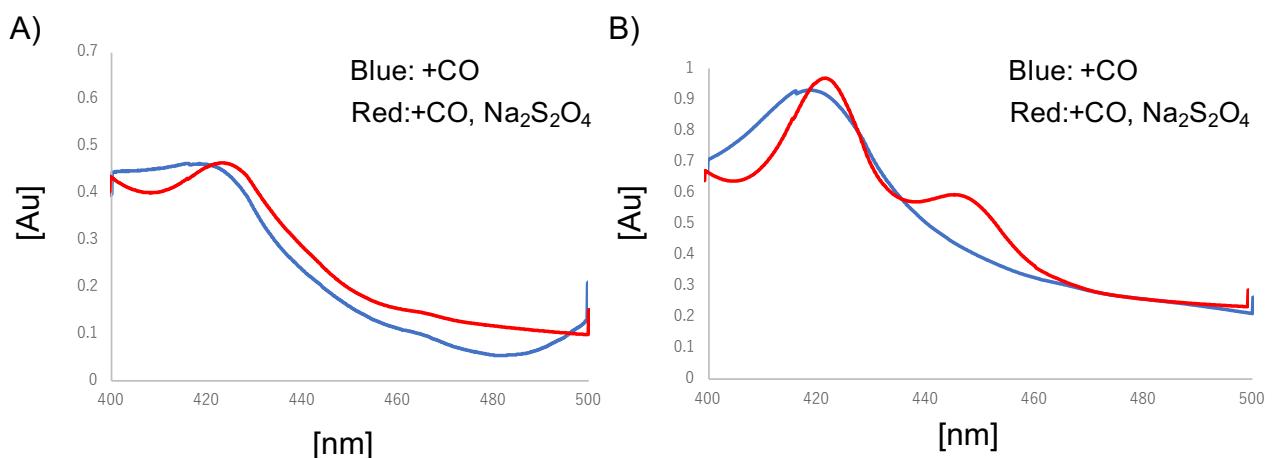


Figure S7. Absorption spectra of the recombinant AscE

Soluble fraction of Rosetta2/pColdII-AscE (B), and negative control Rosetta2/pColdII (A).

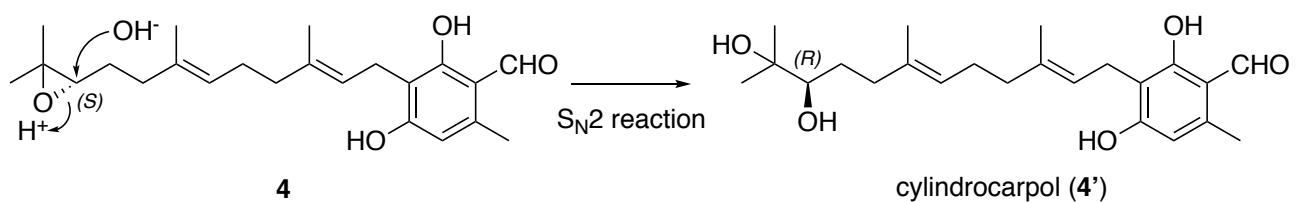


Figure S8. **4** was hydrolyzed to **4'** in S_N2 manner.

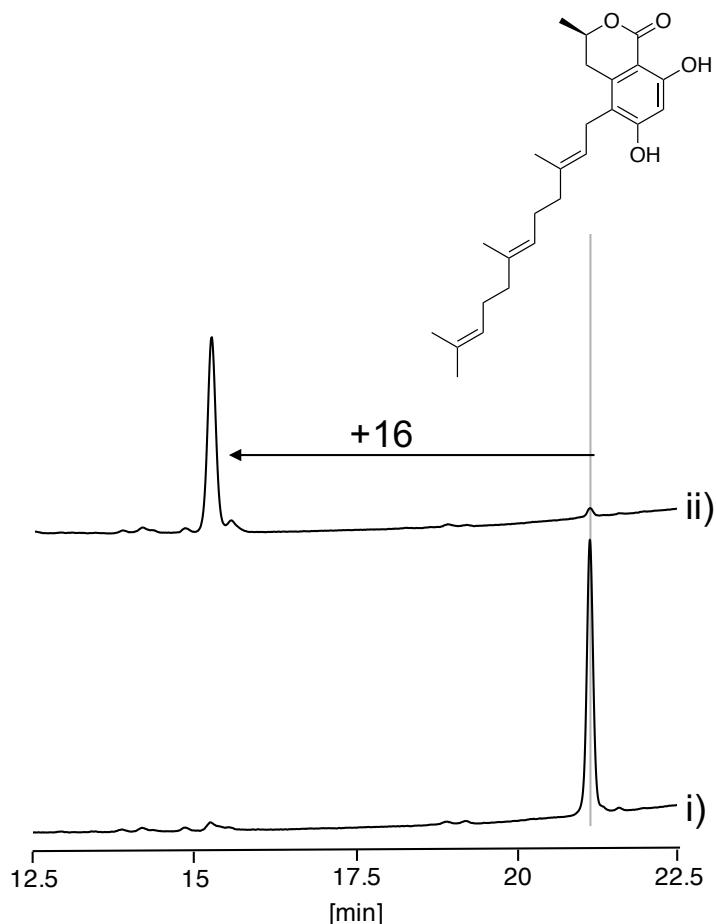


Figure S9. HPLC profile of in vitro assay of AscE by using 5-farnesyl-6-hydroxymellein as a substrate. Lane i shows a negative control, and lane ii shows complete reaction. The substrate ($m/z = 397$ [$M-H$]) was converted into the product ($m/z = 413$ [$M-H$]). The chromatograms were monitored at 295 nm.

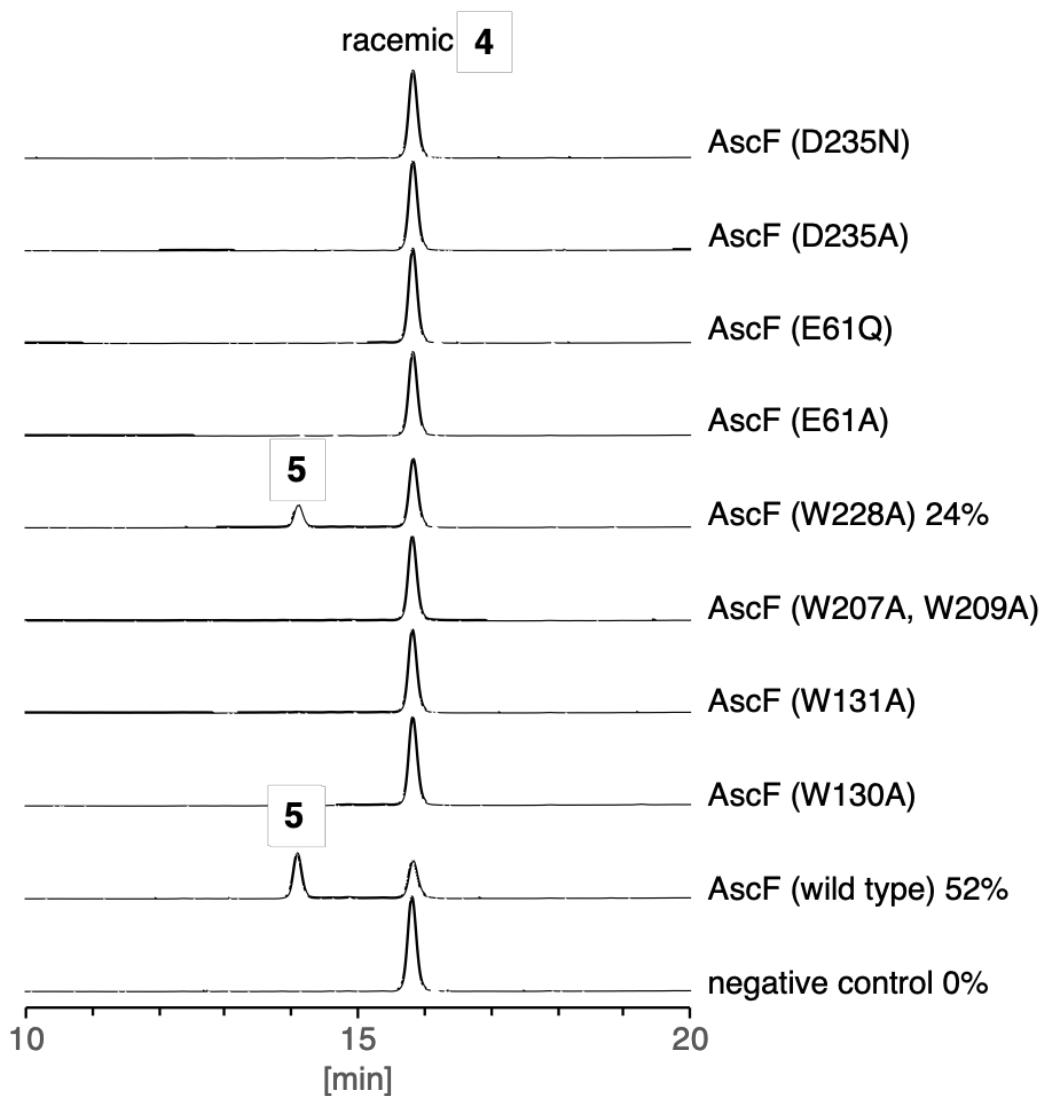


Figure S10. HPLC analysis of the AscF mutants *in vitro* assay. Percentage showed the consumption of substrate. UV absorbance was 295 nm.

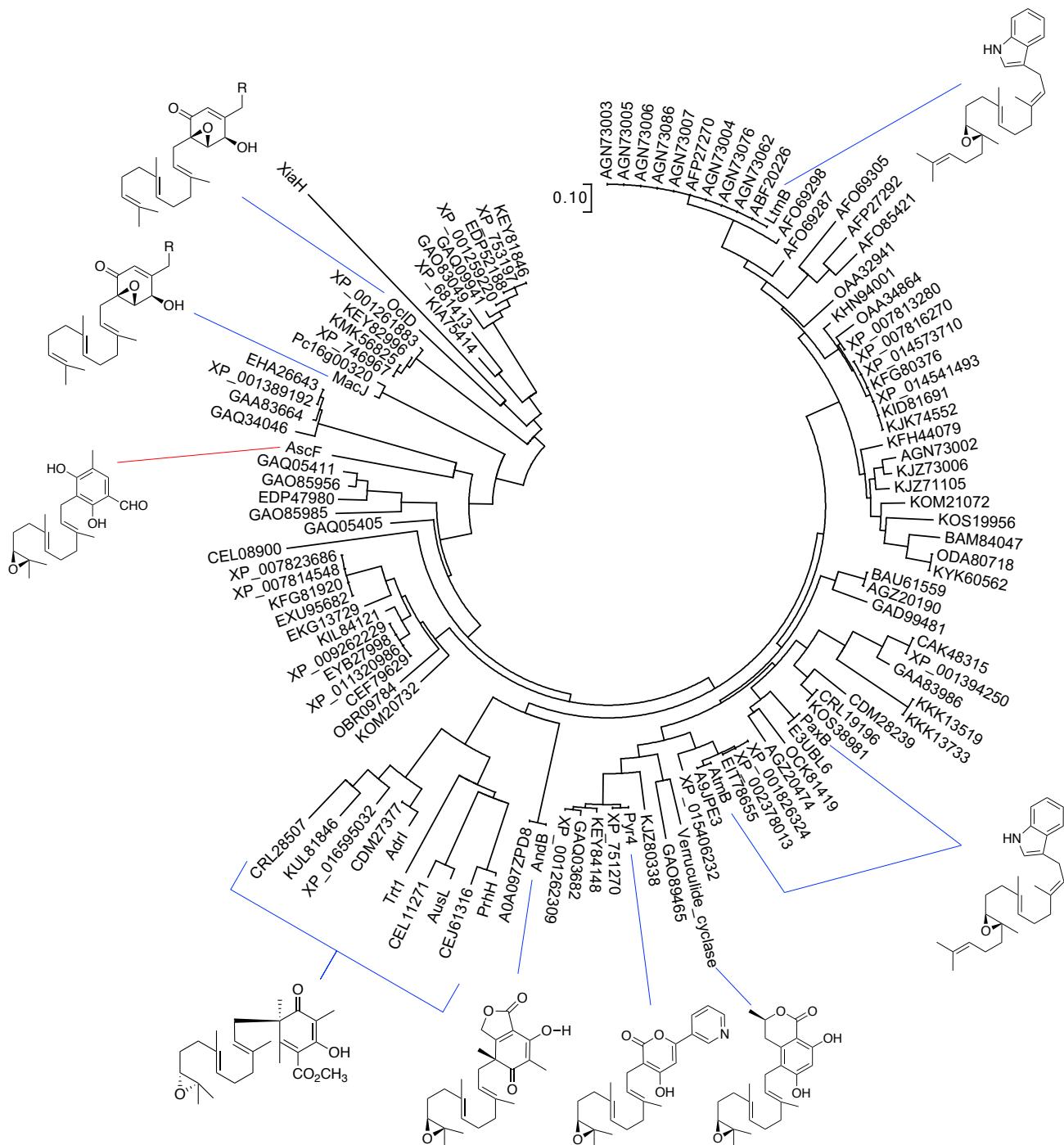


Figure S11. Phylogenetic tree analysis of meroterpenoid terpene cyclases

Multiple sequence alignment, performed with ClustalW. The scale represents 0.10 amino acid substitutions per site. XiaH from *Streptomyces* sp. SCSIO 02999 was employed as out group.

Accession numbers: AndB from *Emericella variecolor*, AtmB *Aspergillus flavus*, AusL *Aspergillus nidulans* FGSC A4, LtmB *Epichloe festucae* var. *lolii*, MacJ *Penicillium terrestris*, Adri *Penicillium chrysogenum*, PaxM *Penicillium paxilli*, LtmB *Epichloe festucae* var. *lolii*, MacJ *Penicillium terrestris*, OclD *Penicillium oxalicum* 114-2, PaxB *Penicillium paxilli*, PrhH *Penicillium brasiliense* NBRC6234, Pyr4 *Aspergillus fumigatus* F37, Trt1 *Aspergillus terreus*, AscF *Fusarium* sp., Verruculide_cyclase *Penicillium verruculosum*, AFO69287 *Periglandula ipomoeae*, AFO69298 *Epichloe*

gansuensis, AFO69305 *Aciculosporium takei*, AFO85421 *Claviceps paspali*, AFP27270 *Epichloe festucae*, AFP27292
Claviceps purpurea, AGN73002 *Epichloe aotearoae*, AGN73003 *Epichloe occultans*, AGN73004 *Epichloe siegelii*,
AGN73005 *Neotyphodium* sp. FaTG-3, AGN73006 *Neotyphodium* sp. FaTG-2, AGN73007 *Neotyphodium* sp. FaTG-2,
AGN73062 *Epichloe funkii*, AGN73076 *Epichloe coenophiala*, AGN73086 *Neotyphodium* sp. FaTG-4, AGZ20190
Penicillium crustosum, AGZ20474 *Penicillium janthinellum*, ABF20226 *Epichloe festucae* var. *lolii*, BAM84047
Tolypocladium album, BAU61559 *Penicillium simplicissimum*, CAK48315 *Aspergillus niger*, CDM27377 *Penicillium roqueforti* FM164, CDM28239 *Penicillium roqueforti* FM164, CEF79629 *Fusarium graminearum*, CEJ61316
Penicillium brasiliense, CEL08900 *Aspergillus calidoustus*, CEL11271 *Aspergillus calidoustus*, CRL19196 *Penicillium camemberti*, CRL28507 *Penicillium camemberti*, EDP47980 *Aspergillus fumigatus* A1163, EDP52188 *Aspergillus fumigatus* A1163, EHA26643 *Aspergillus niger* ATCC 1015, EIT78655 *Aspergillus oryzae* 3.042, EKG13729
Macrophomina phaseolina MS6, EXU95682 *Metarhizium robertsii*, EYB27998 *Fusarium graminearum*, GAA83664
Aspergillus kawachii IFO 4308, GAA83986 *Aspergillus kawachii* IFO 4308, GAD99481 *Byssochlamys spectabilis* No. 5, GAO83049 *Aspergillus udagawae*, GAO85956 *Aspergillus udagawae*, GAO85985 *Aspergillus udagawae*, GAO89465 *Aspergillus udagawae*, GAQ03682 *Aspergillus lentulus*, GAQ05405 *Aspergillus lentulus*, GAQ05411 *Aspergillus lentulus*, GAQ09941 *Aspergillus lentulus*, GAQ34046 *Aspergillus niger*, KEY81846 *Aspergillus fumigatus* var. RP-2014, KEY82996 *Aspergillus fumigatus* var. RP-2014, KEY84148 *Aspergillus fumigatus* var. RP-2014, KFG80376
Metarhizium anisopliae, KFG81920 *Metarhizium anisopliae*, KFH44079 *Acremonium chrysogenum* ATCC 11550, KHN94001 *Metarhizium album* ARSEF 1941, KIA75414 *Aspergillus ustus*, KID81691 *Metarhizium guizhouense* ARSEF 977, KIL84121 *Fusarium avenaceum*, KJK74552 *Metarhizium anisopliae* BRIP 53293, KJZ71105 *Hirsutella minnesotensis* 3608, KJZ73006 *Hirsutella minnesotensis* 3608, KJZ80338 *Hirsutella minnesotensis* 3608, KKK13519 *Aspergillus rambellii*, KKK13733 *Aspergillus ochraceoroseus*, KMK56825 *Aspergillus fumigatus* Z5, KOM20732 *Ophiocordyceps unilateralis*, KOM21072 *Ophiocordyceps unilateralis*, KOS19956 *Escovopsis weberi*, KOS38981 *Penicillium nordicum*, KUL81846 *Talaromyces verruculosus*, KYK60562 *Drechmeria coniospora*, B6H6U3 *Penicillium chrysogenum* ATCC 28089, OAA32941 *Aschersonia aleyrodis* RCEF 2490, OAA34864 *Metarhizium rileyi* RCEF 4871, OBR09784 *Colletotrichum higginsianum* IMI 349063, OCK81419 *Lepidopterella palustris* CBS 459.81, ODA80718 *Drechmeria coniospora*, XP_681413 *Aspergillus nidulans* FGSC A4, XP_746967 *Aspergillus fumigatus* Af293, XP_751270 *Aspergillus fumigatus* Af293, XP_753197 *Aspergillus fumigatus* Af293, XP_001259220 *Aspergillus fischeri* NRRL 181, XP_001261883 *Aspergillus fischeri* NRRL 181, XP_001262309 *Aspergillus fischeri* NRRL 181, XP_001389192 *Aspergillus niger* CBS 513.88, XP_001394250 *Aspergillus niger* CBS 513.88, XP_001826324 *Aspergillus oryzae* RIB40, XP_002378013 *Aspergillus flavus* NRRL3357, XP_007813280 *Metarhizium acridum* CQMa 102, XP_007814548 *Metarhizium acridum* CQMa 102, XP_007816270 *Metarhizium robertsii* ARSEF 23, XP_007823686 *Metarhizium robertsii* ARSEF 23, XP_009262229 *Fusarium pseudograminearum* CS3096, XP_011320986 *Fusarium graminearum* PH-1, XP_014541493 *Metarhizium brunneum* ARSEF 3297, XP_014573710 *Metarhizium majus* ARSEF 297, XP_015406232 *Aspergillus nomius* NRRL 13137, XP_016595032 *Penicillium expansum*

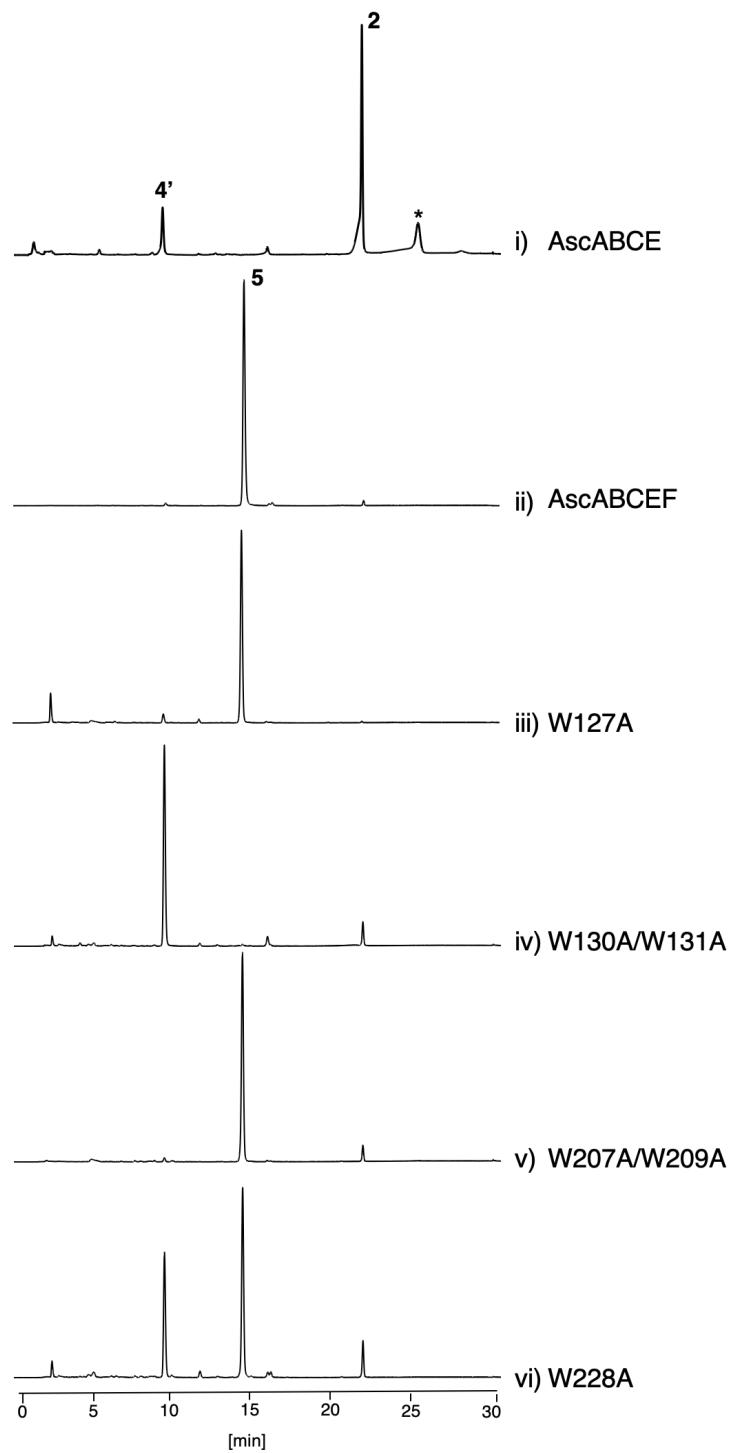


Figure S12. HPLC analysis of the product accumulated in *A. oryzae*/AscABCE i), *A. oryzae*/AscABCEF ii), W127A iii), W130A/W131A iv), W207A/W209A v), W228A vi). UV absorbance was 295 nm. The compound labeled as * is an unidentified compound which has UV_{max} at 272 and 282 nm.

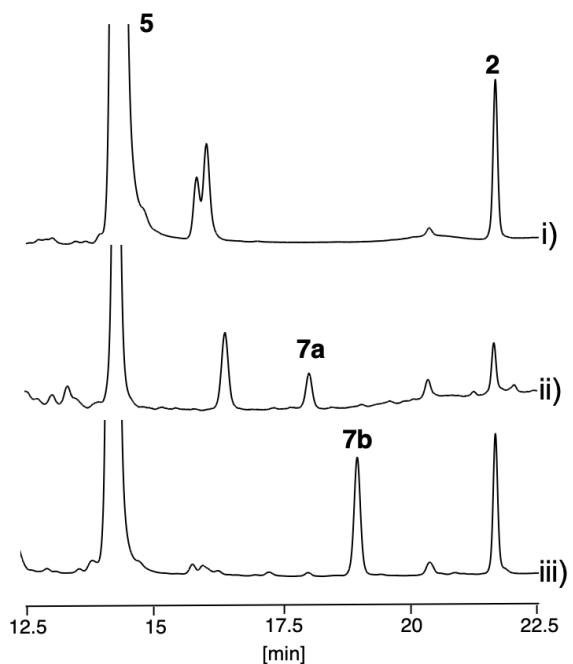


Figure S13. HPLC analysis of the products accumulated in *A. oryzae*/AscABCEF i), AscABCDEF incubated with KCl ii), incubated with KBr iii). UV absorbance was 295 nm.

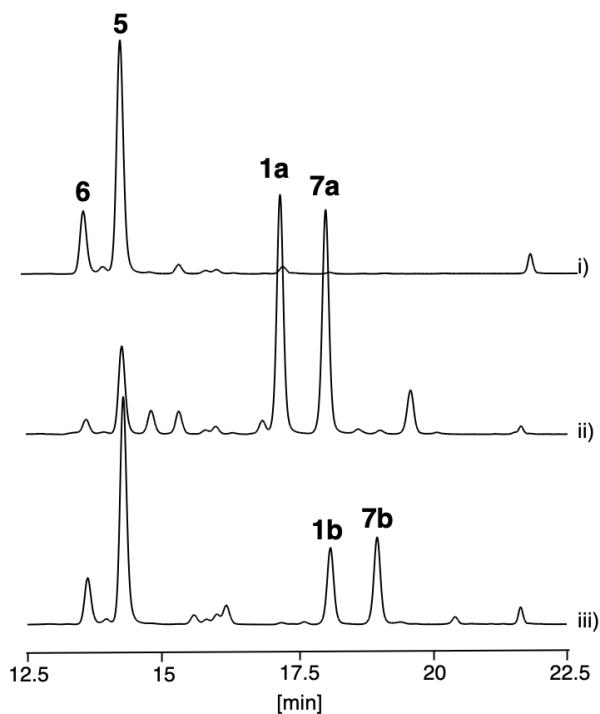


Figure S14. HPLC analysis of the products accumulated in *Fusarium* sp. incubated without any halogen source i) with KCl ii), with KBr iii). UV absorbance was 295 nm.

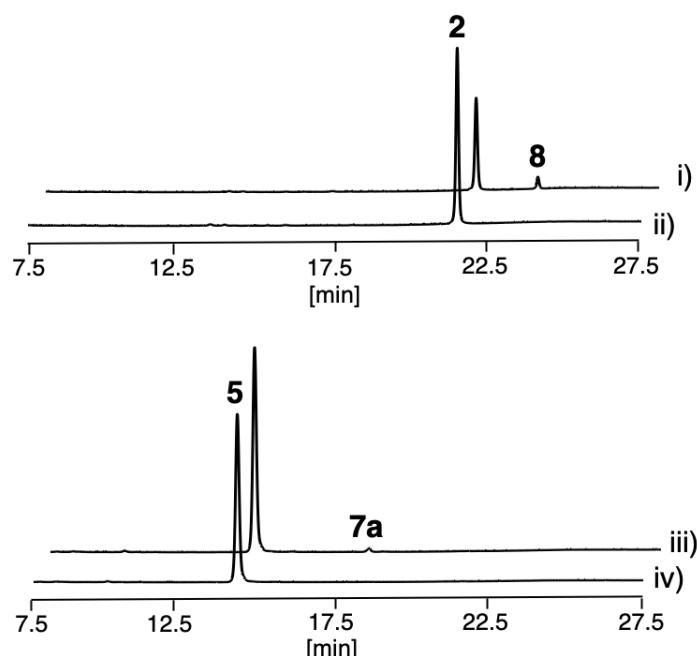


Figure S15. HPLC profile of AscD in vitro assay. **2** was incubated with soluble protein extract from Rosetta2/pET28a-AscD i), and the extract from Rosetta2/pET28 ii), and **5** was incubated with soluble protein extract from Rosetta2/pET28a-AscD iii), and the extract from Rosetta2/pET28 iv). The chromatograms were monitored at 295 nm.

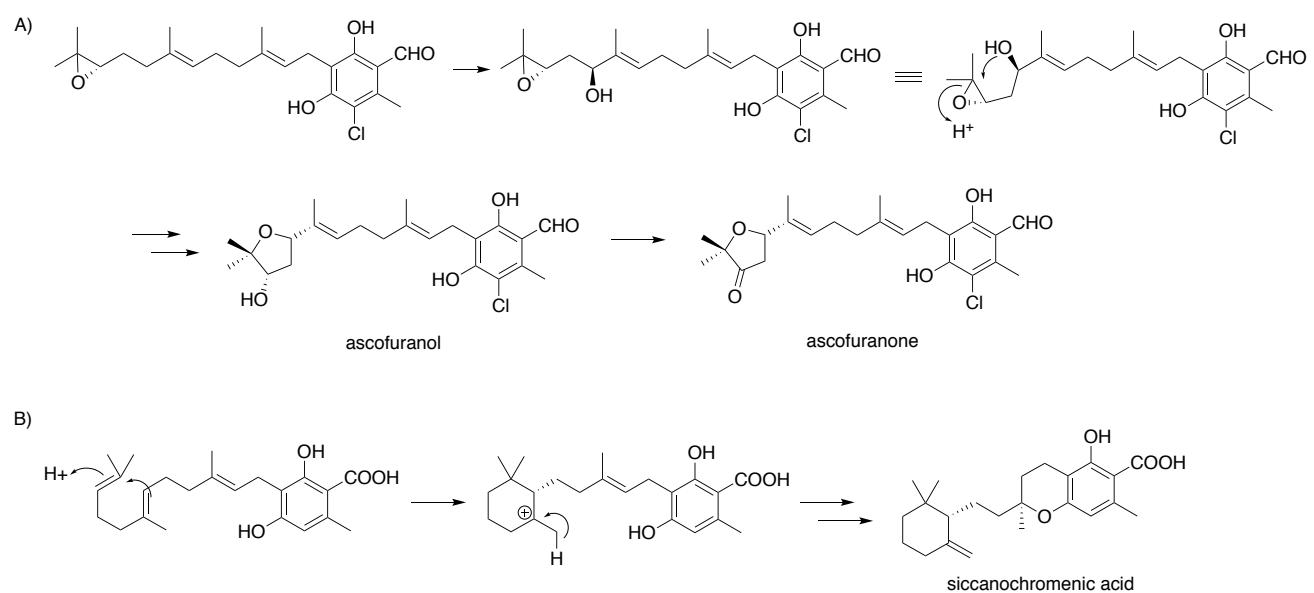


Figure S16. The biosynthetic pathway of ascofuranone (A) and putative pathway of siccanochromenic acid (B)

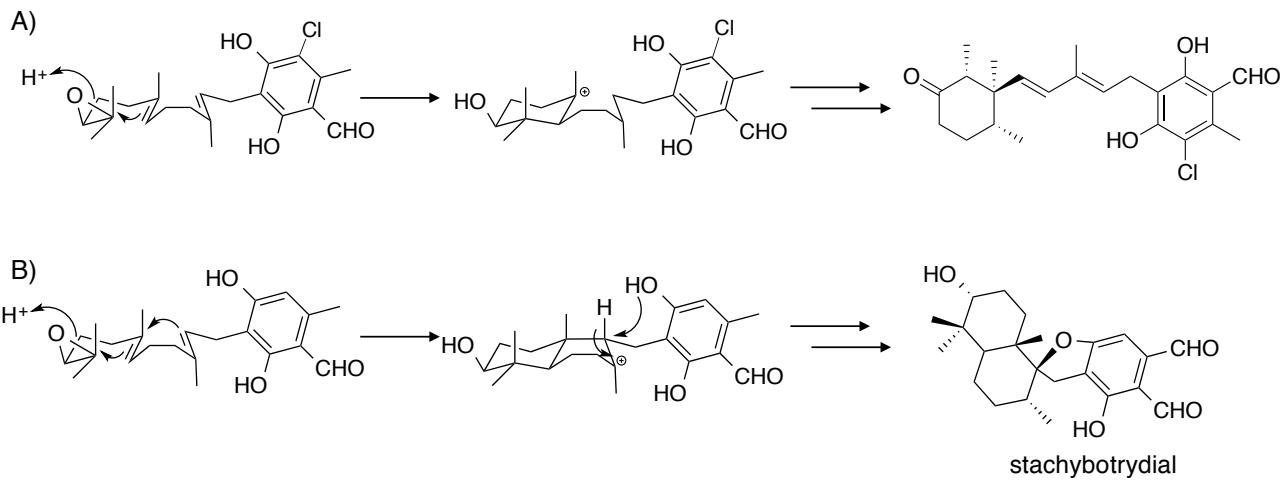


Figure S17. Comparison of terpene cyclization between ascochlorin and stachybotrydial

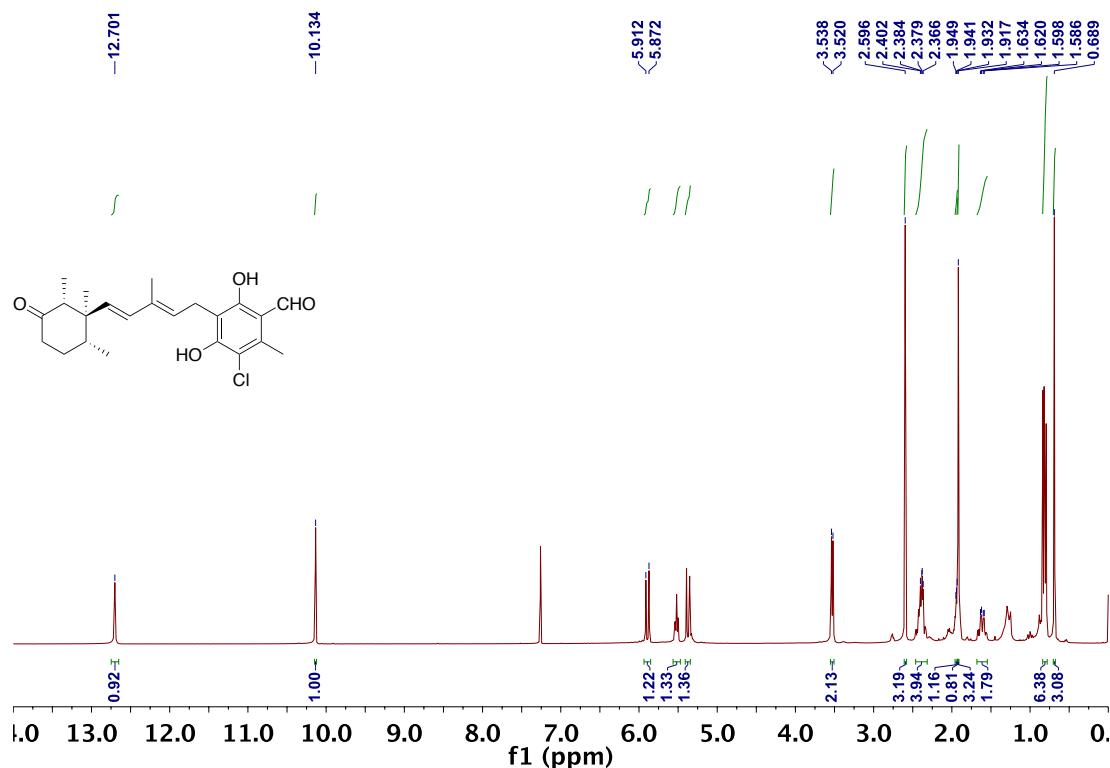


Figure S18. ^1H NMR spectrum of ascochlorin (**1a**)

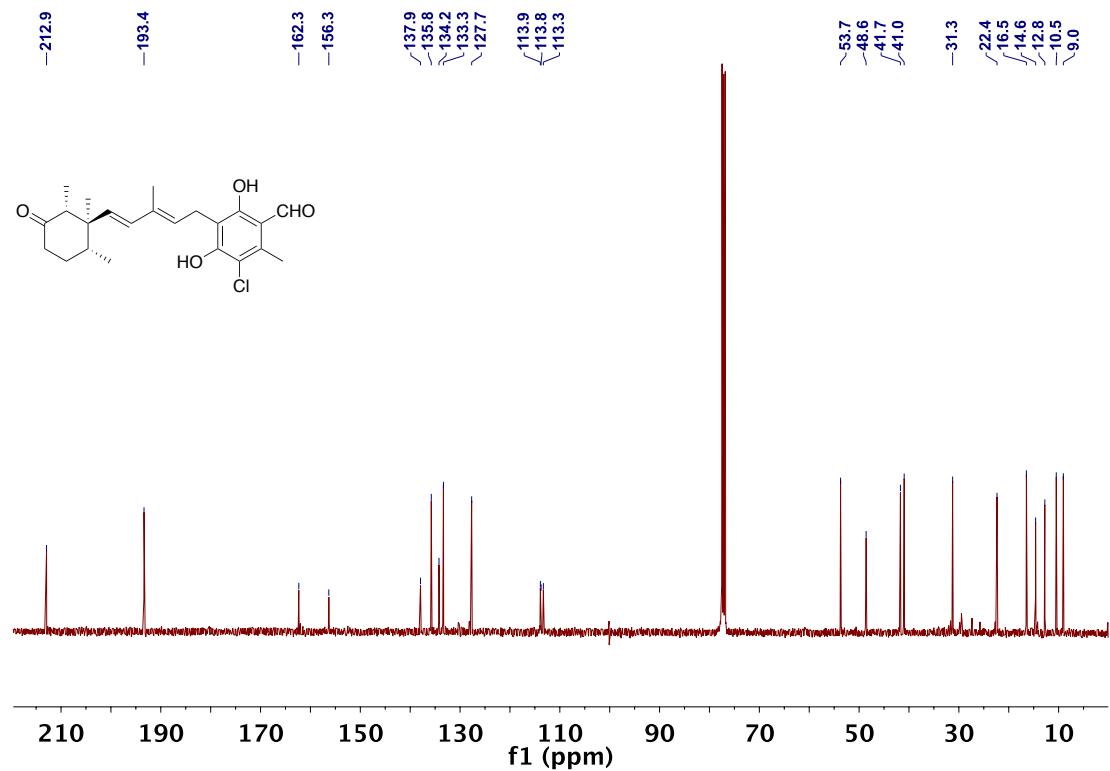


Figure S19. ^{13}C NMR spectrum of ascochlorin (**1a**)

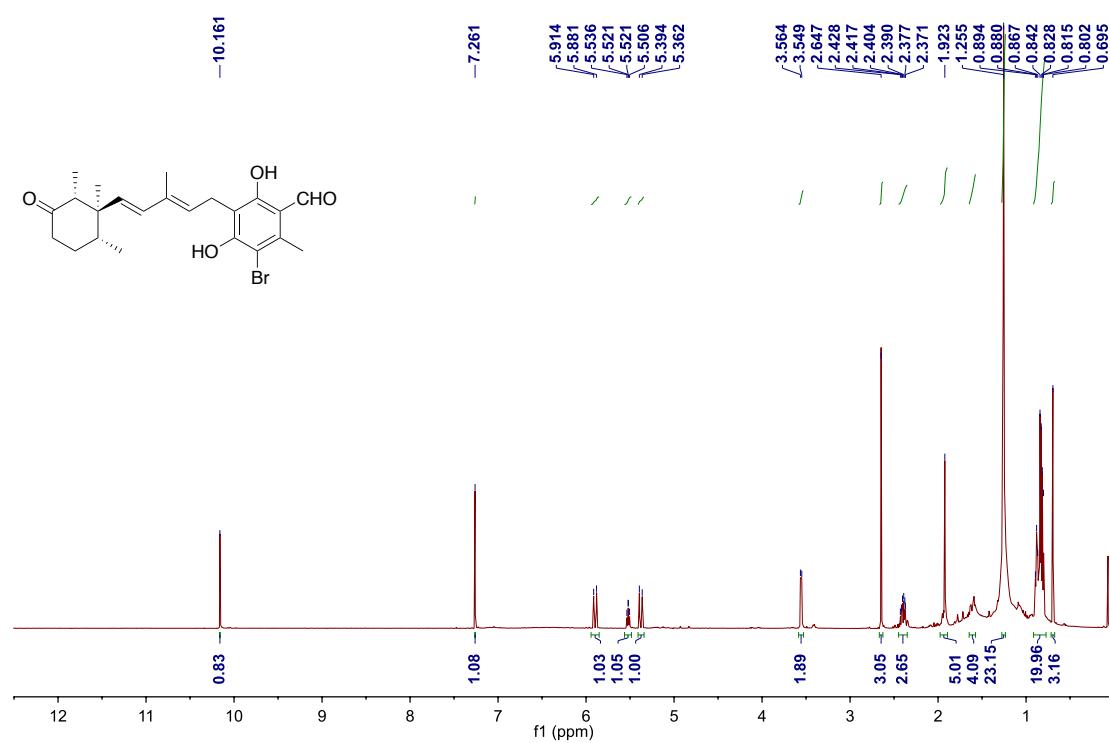


Figure S20. ¹H NMR spectrum of 3-bromoascochlorin (**1b**)

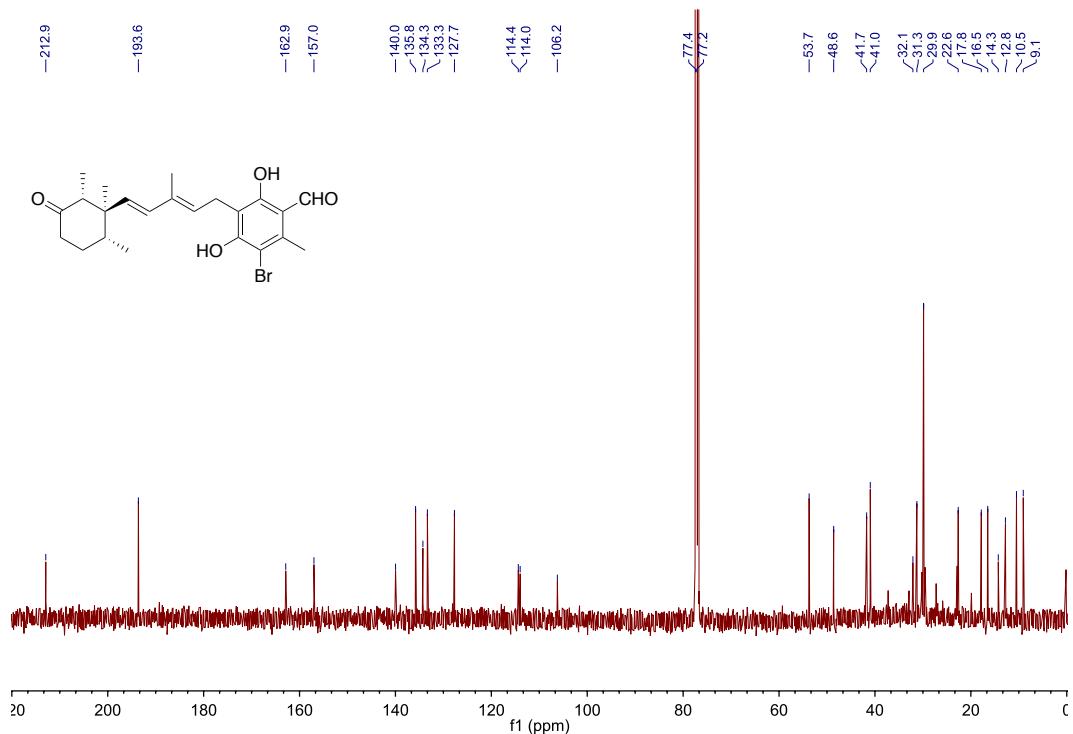


Figure S21. ¹³C NMR spectrum of 3-bromoascochlorin (**1b**)

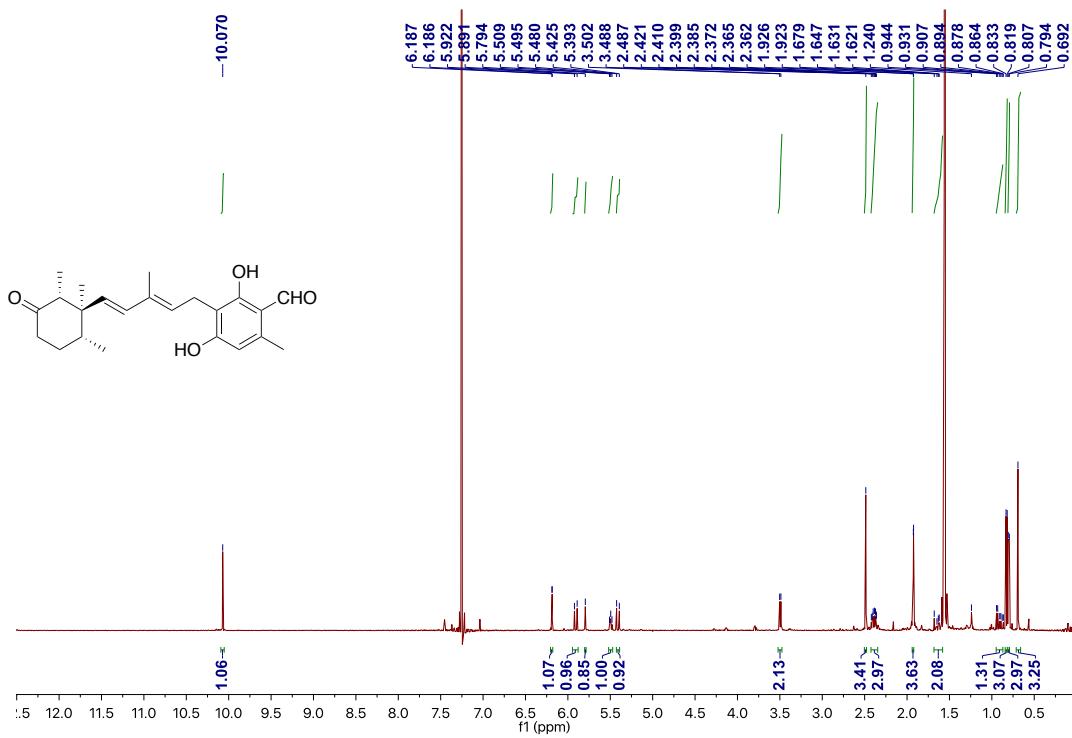


Figure S22. ^1H NMR spectrum of dechloroascochlorin (**6**)

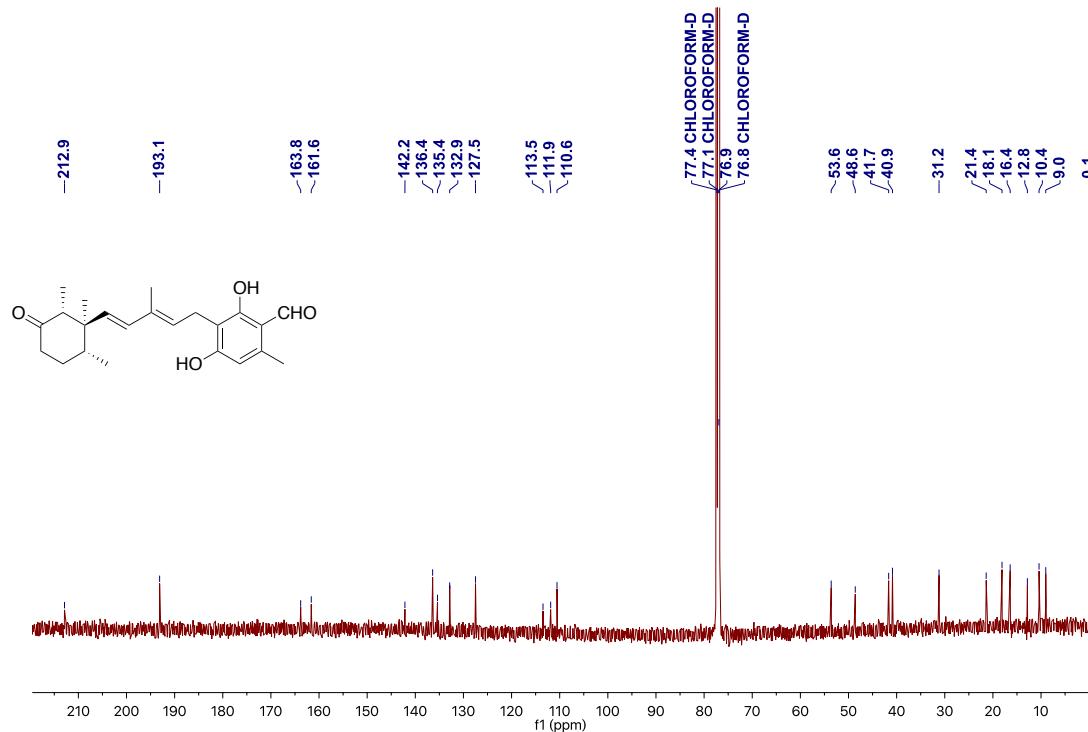


Figure S23. ^{13}C NMR spectrum of dechloroascochlorin (**6**)

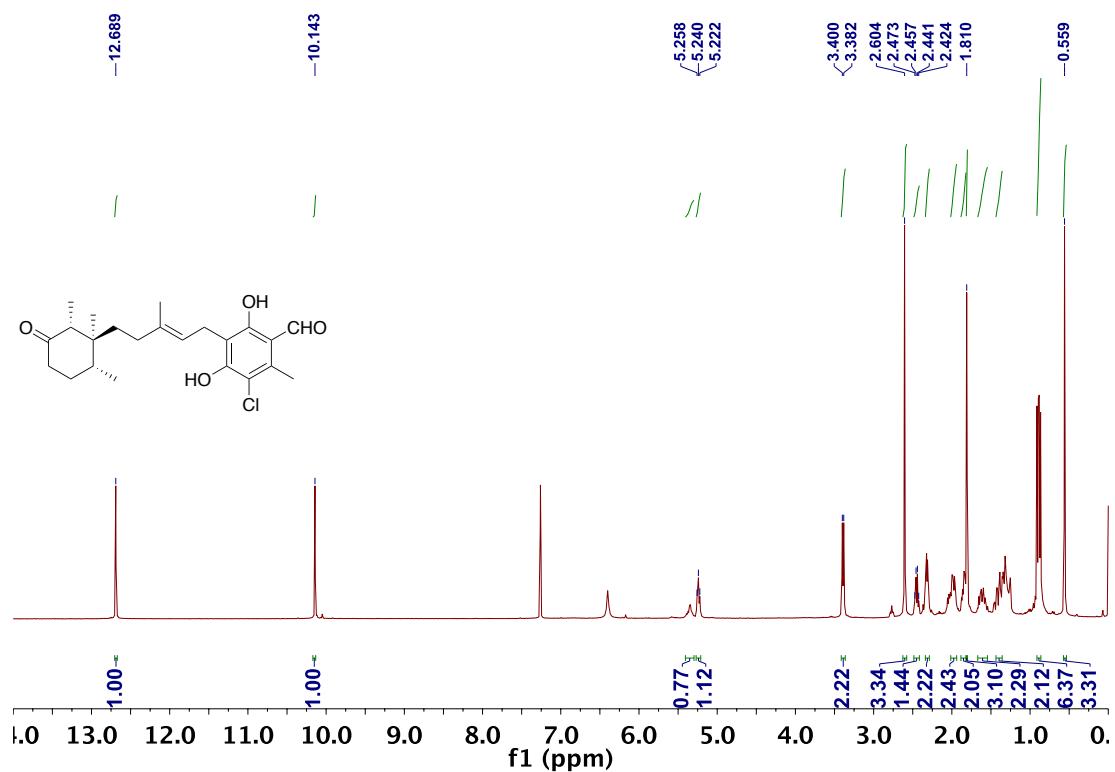


Figure S24. ¹H NMR spectrum of 12,13-dihydroascochlorin (7a)

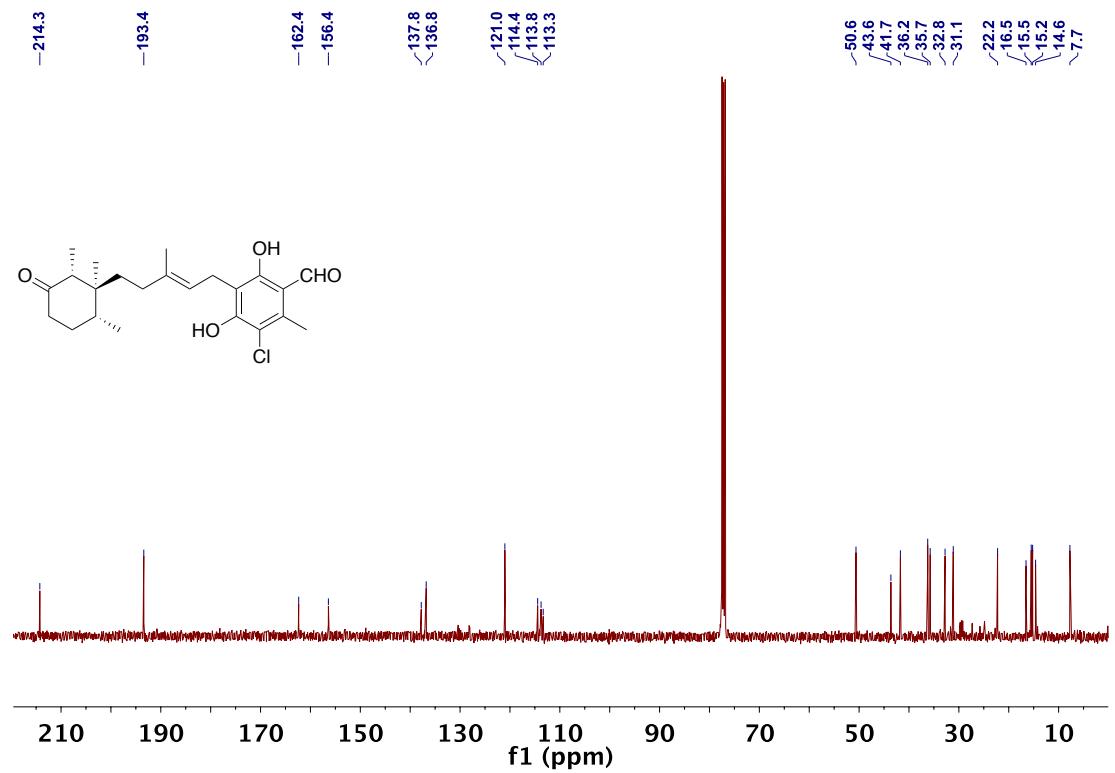


Figure S25. ¹³C NMR spectrum of 12,13-dihydroascochlorin (7a)

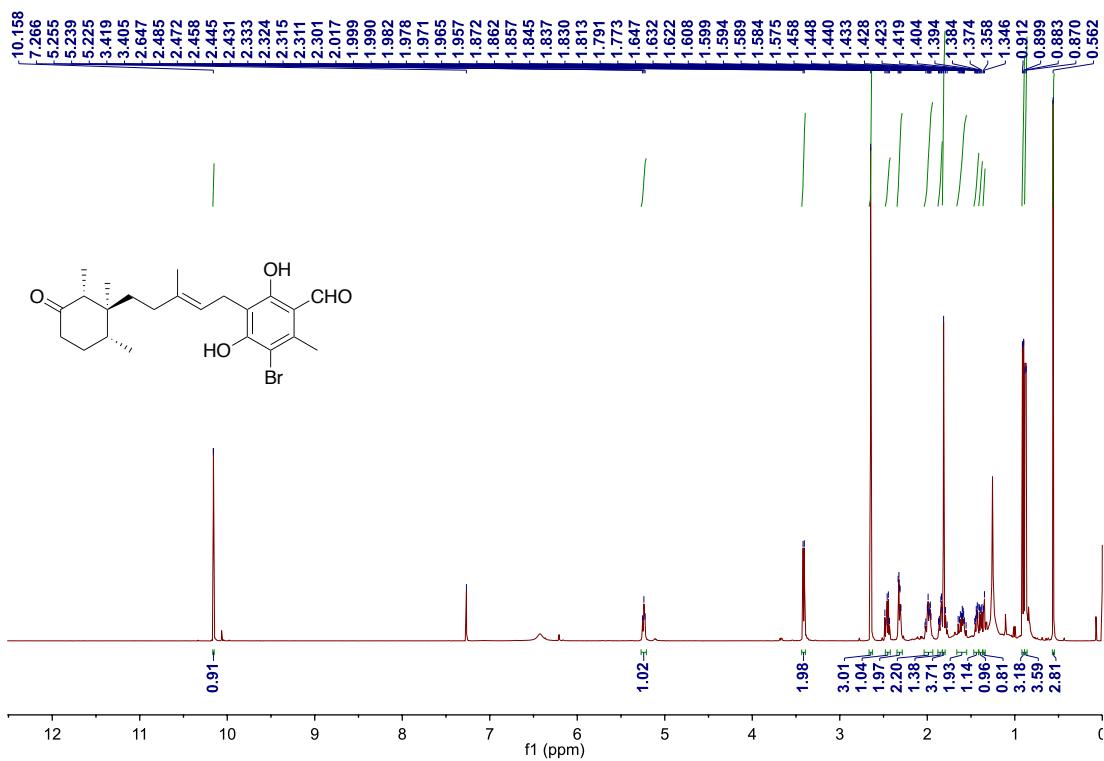


Figure S26. ¹H NMR spectrum of 3-bromo-12,13-dihydroascochlorin (7b)

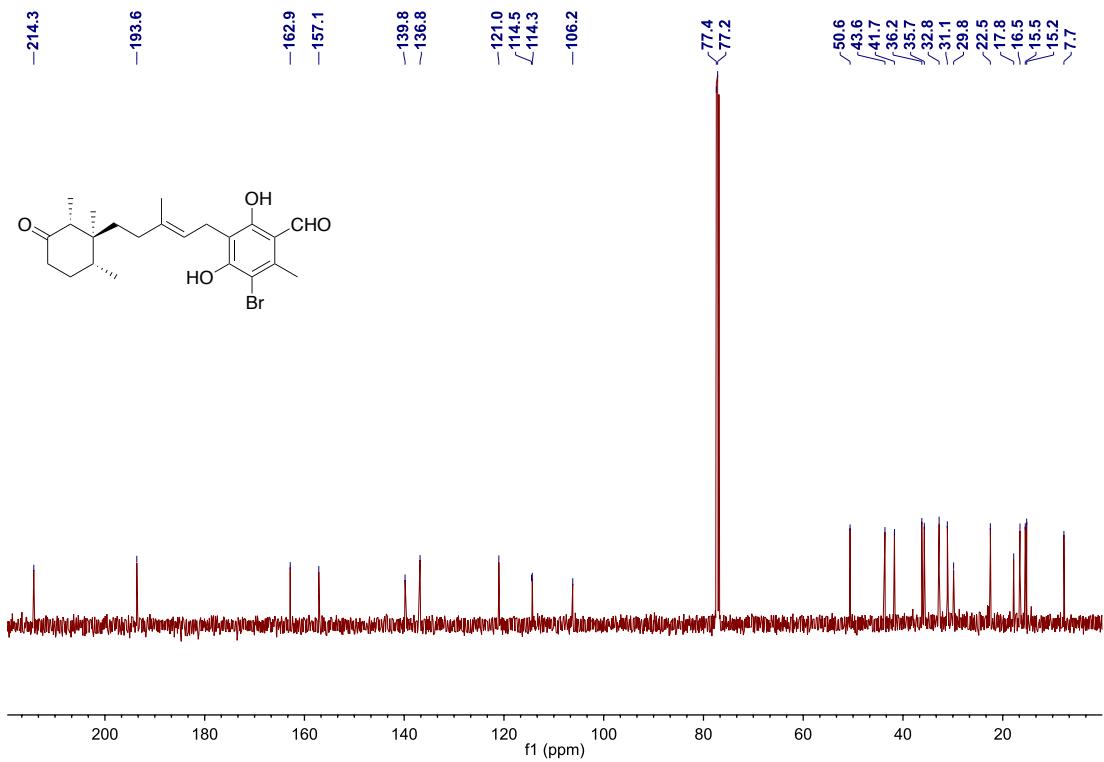


Figure S27. ¹³C NMR spectrum of 3-bromo-12,13-dihydroascochlorin (7b)

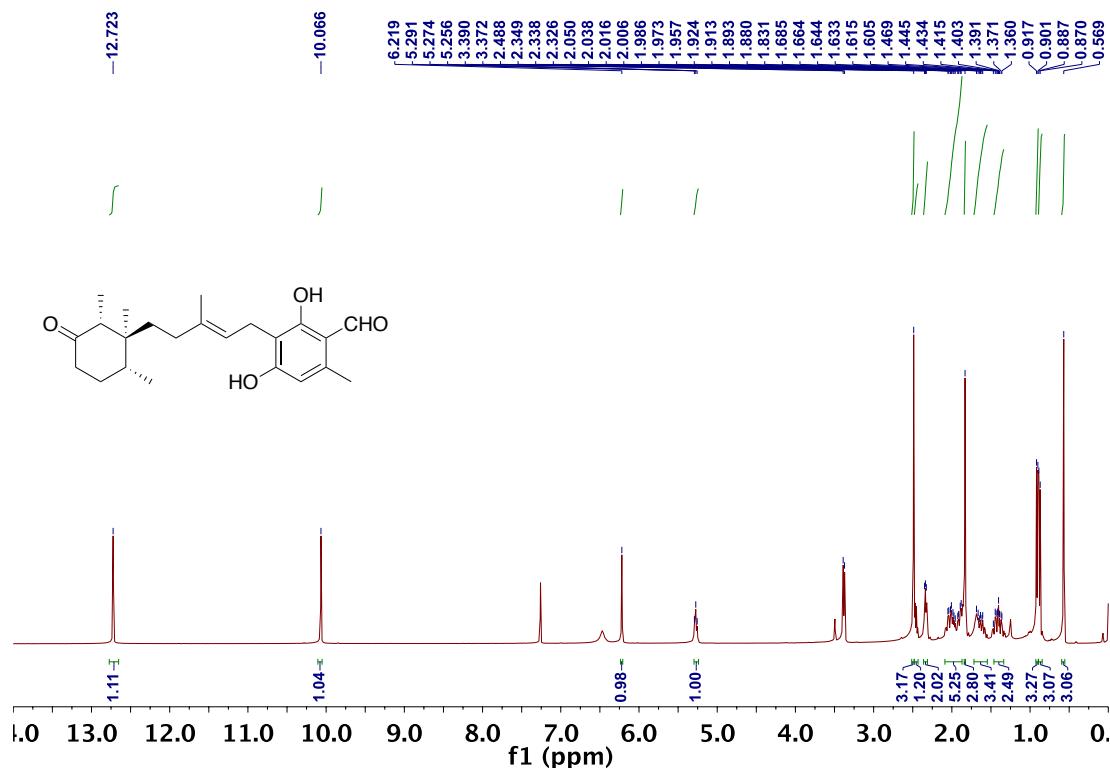


Figure S28. ^1H NMR spectrum of dechloro-12,13-dihydroascochlorin (**5**)

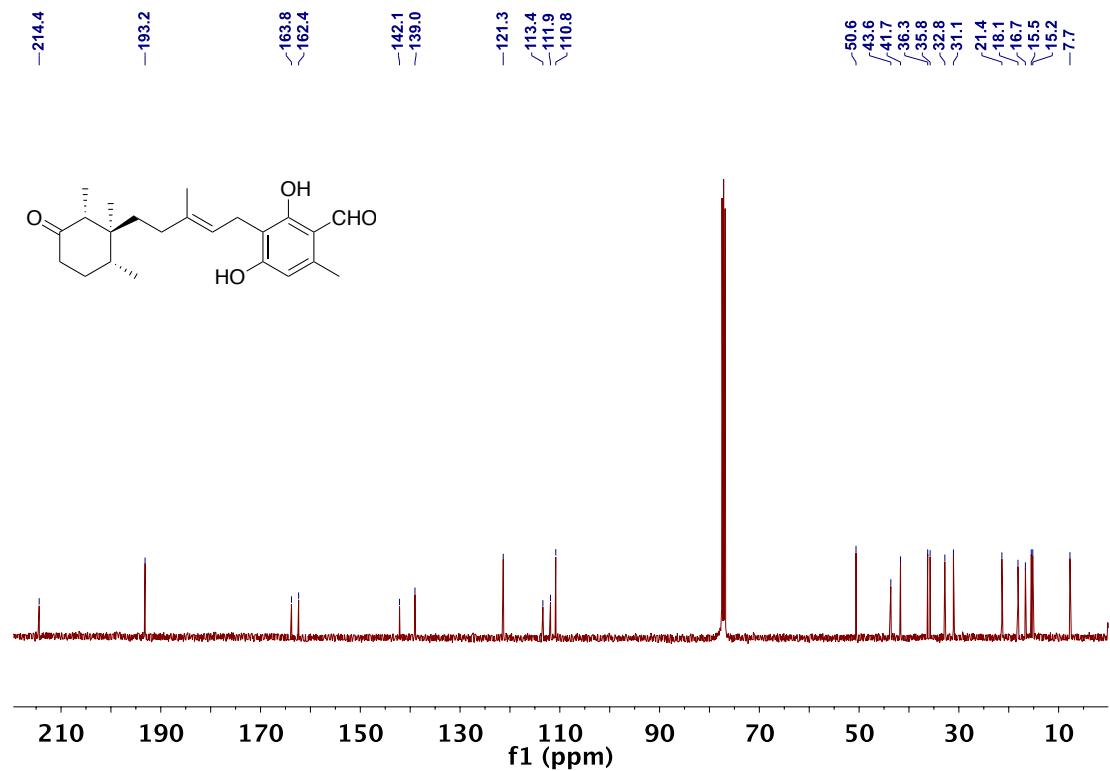


Figure S29. ^{13}C NMR spectrum of dechloro-12,13-dihydroascocochlorin (**5**)

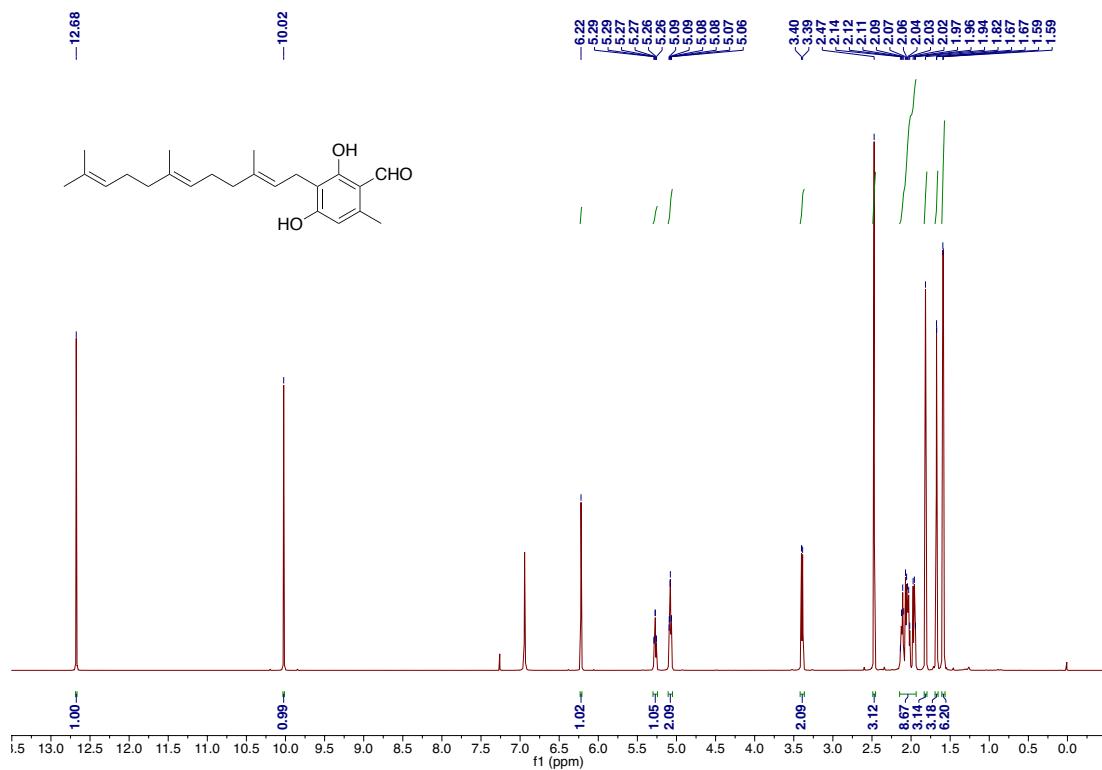


Figure S30. ¹H NMR spectrum of LL-Z1272 β (2)

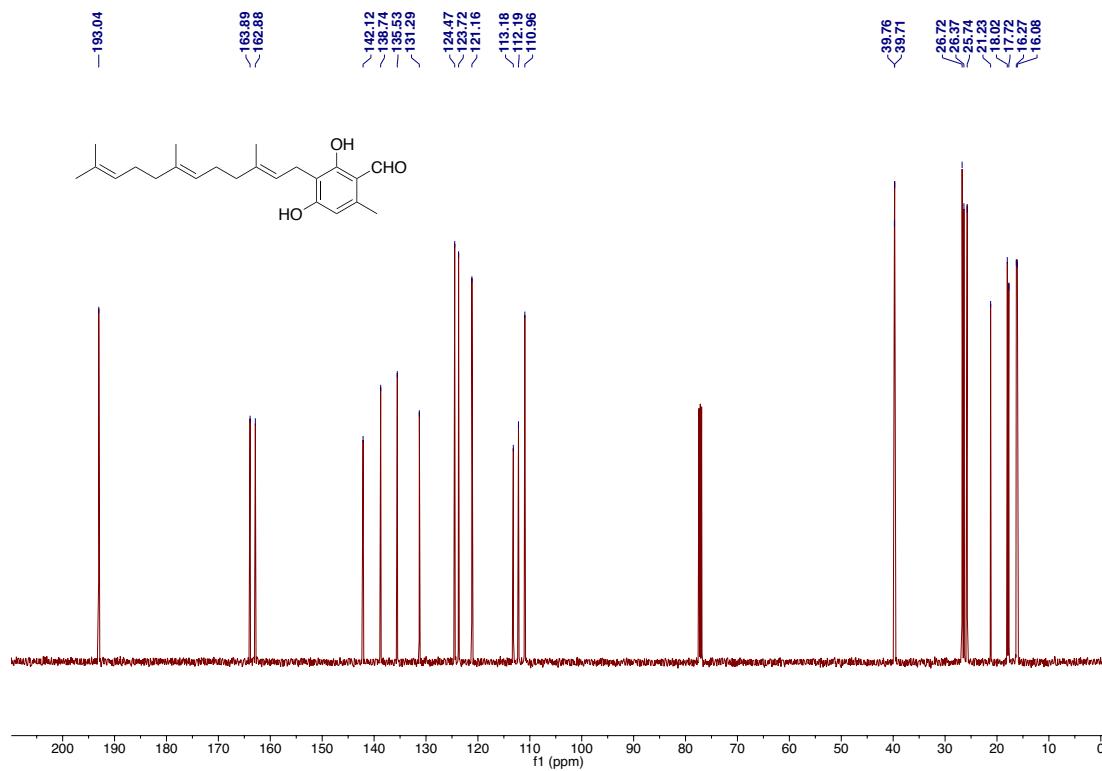


Figure S31. ¹³C NMR spectrum of LL-Z1272 β (2)

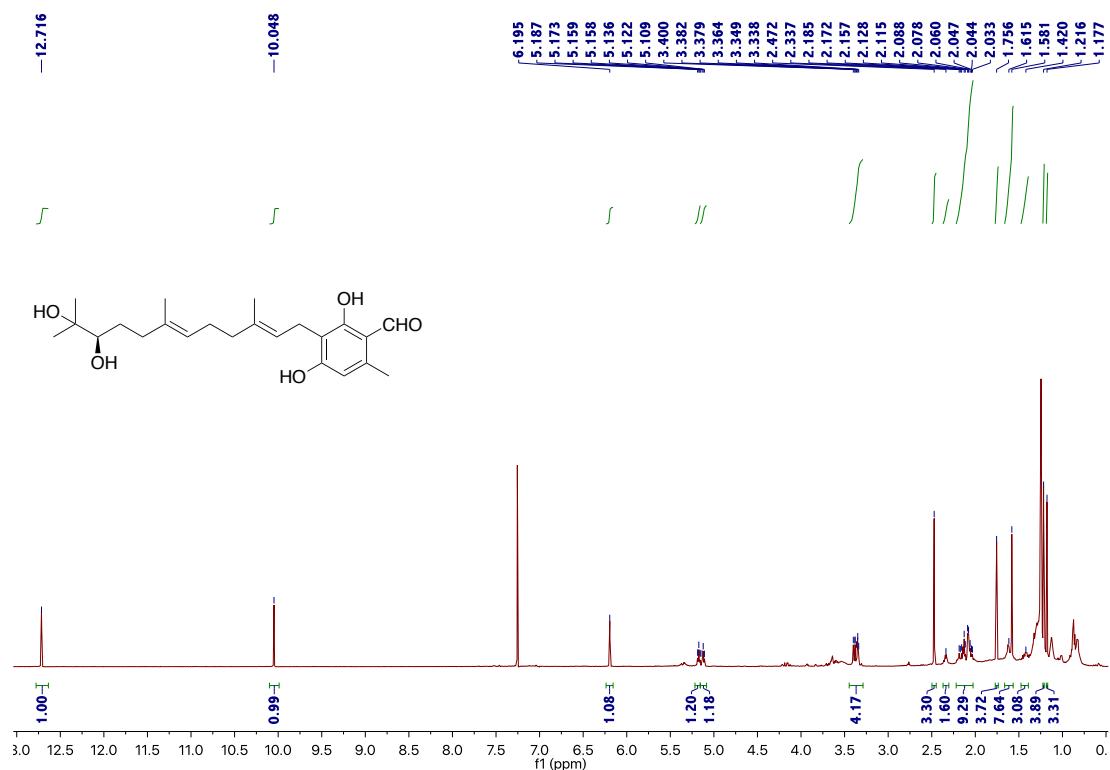


Figure S32. ¹H NMR spectrum of cylindrocarpol (**4'**)

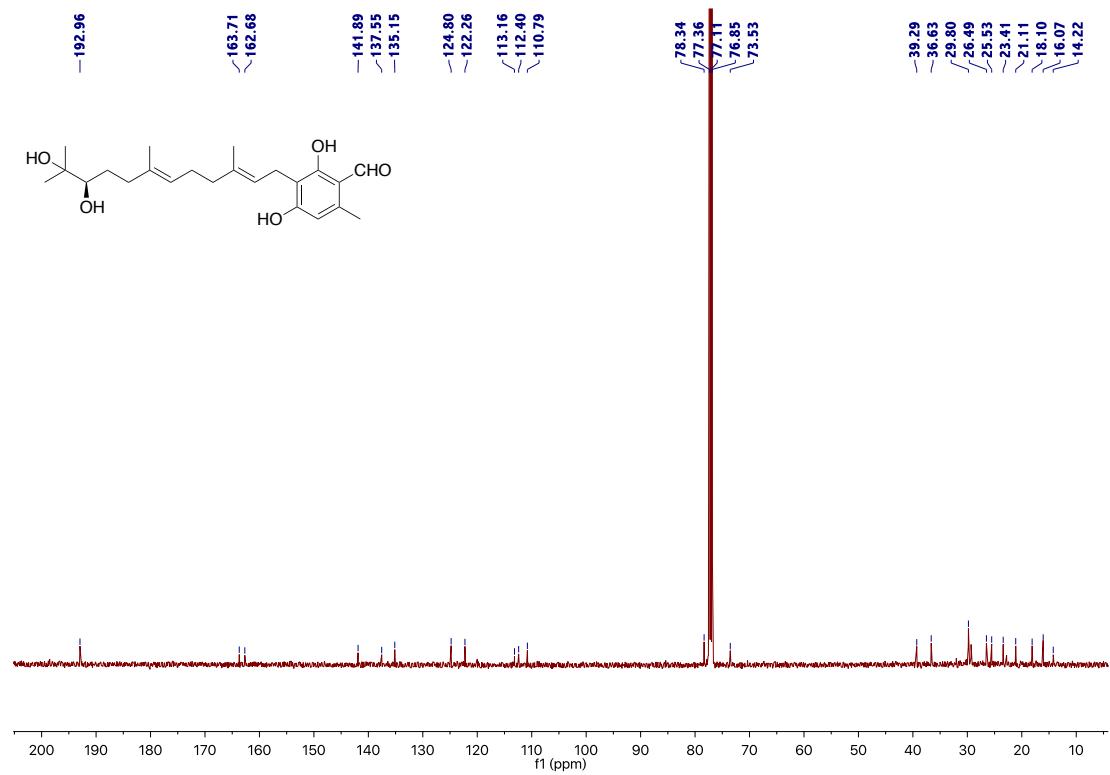


Figure S33. ¹³C NMR spectrum of cylindrocarpol (**4'**)

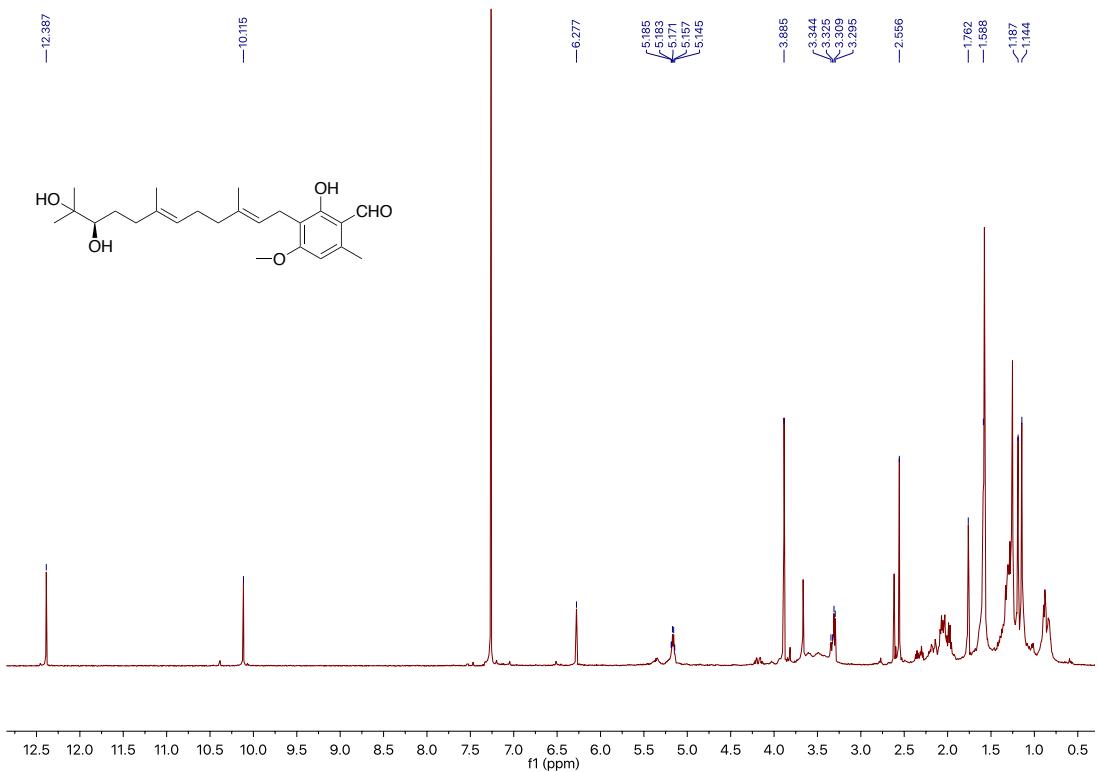


Figure S34. ^1H NMR spectrum of 4-*O*-methylated-4'

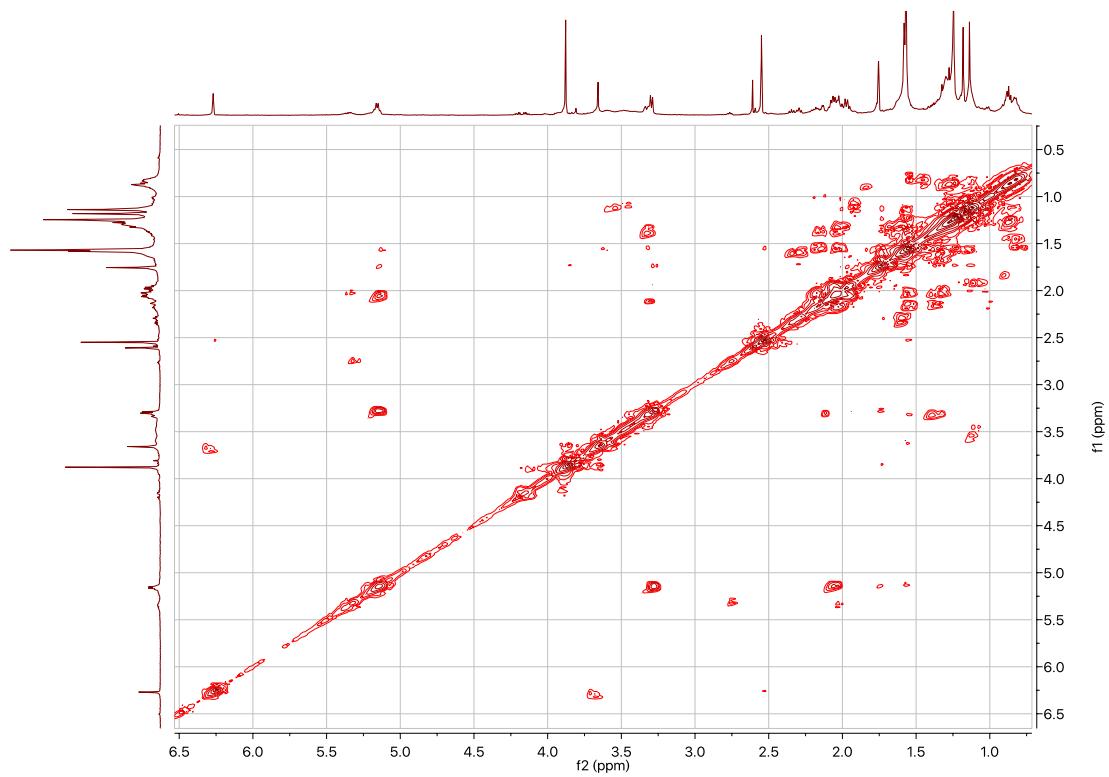


Figure S35. COSY spectrum of 4-*O*-methylated-4'

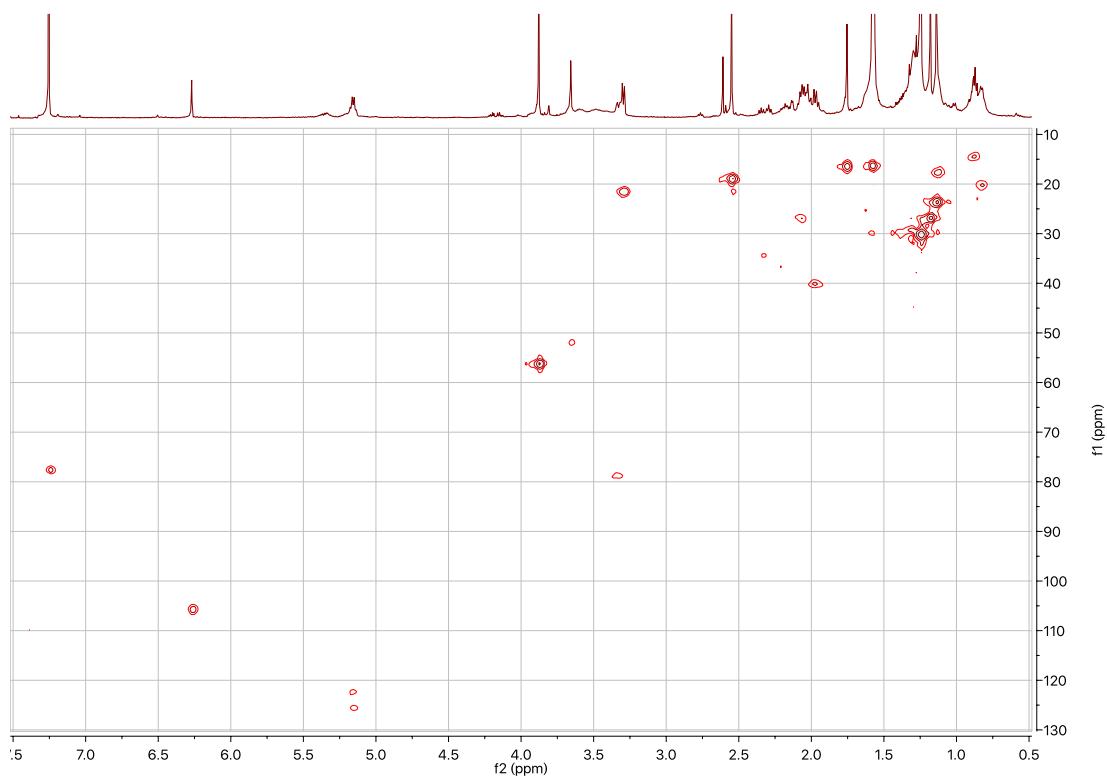


Figure S36. HMQC spectrum of 4-*O*-methylated-4'

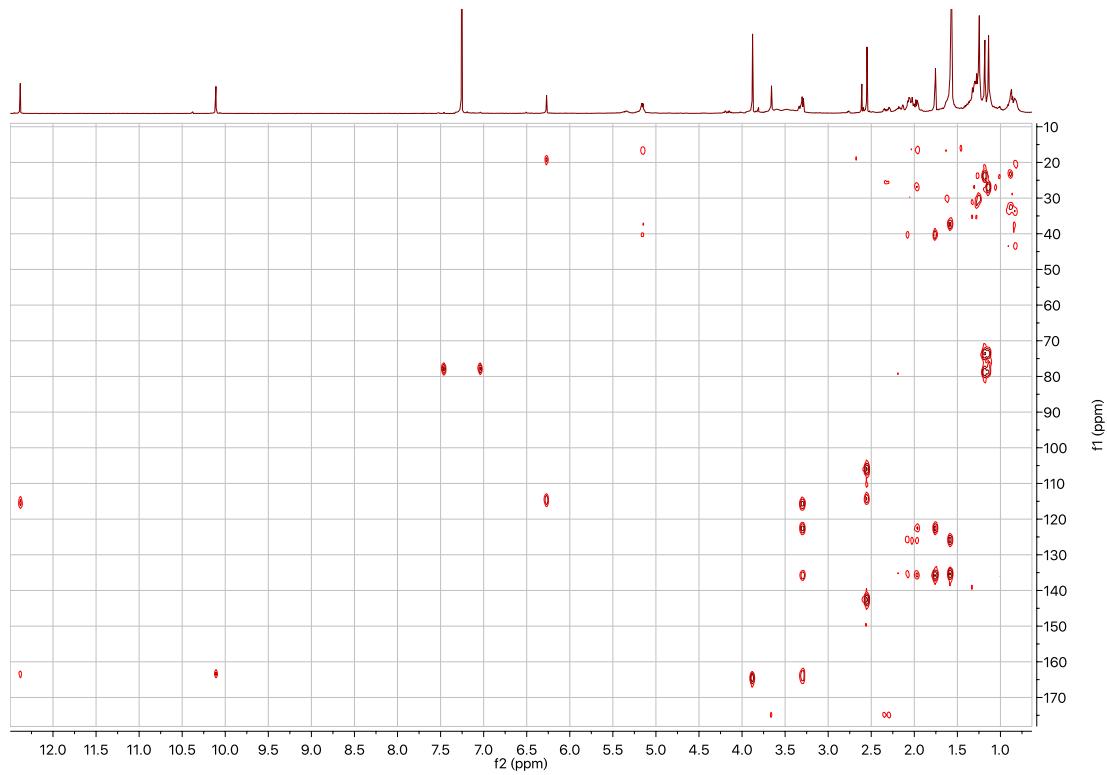


Figure S37. HMBC spectrum of 4-*O*-methylated-4'

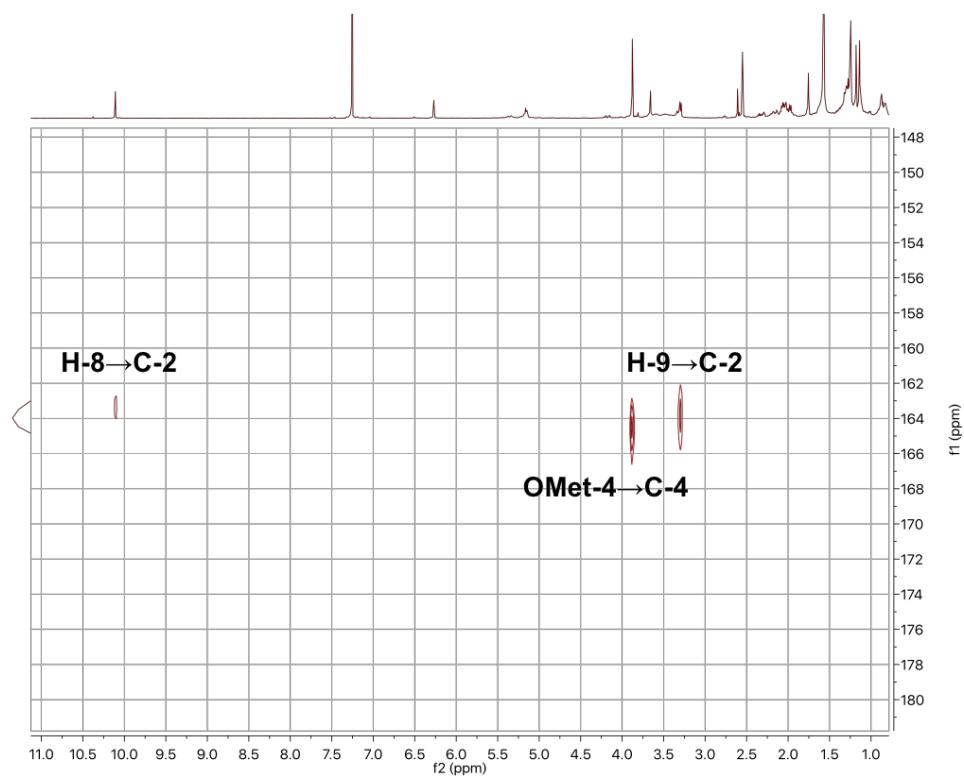


Figure S38. Key HMBC correlation of 4-*O*-methylated-4'

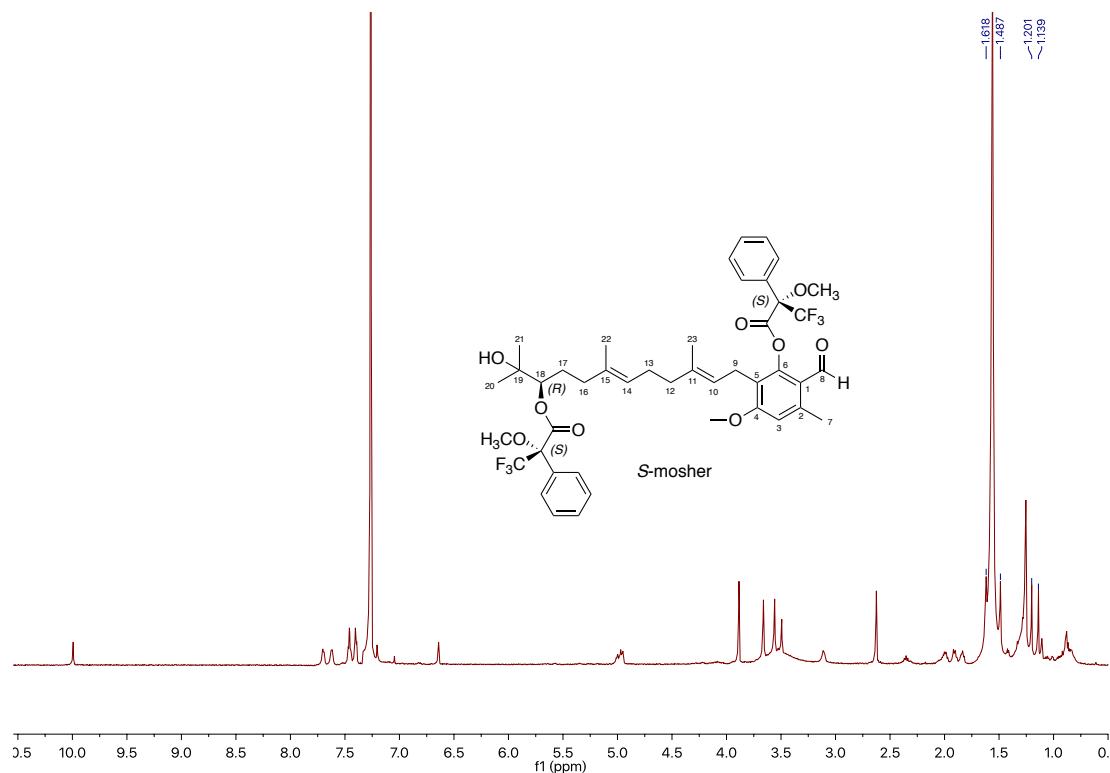


Figure S39. ¹H NMR spectra of *S*-mosher diester of 4-*O*-methylated-4'

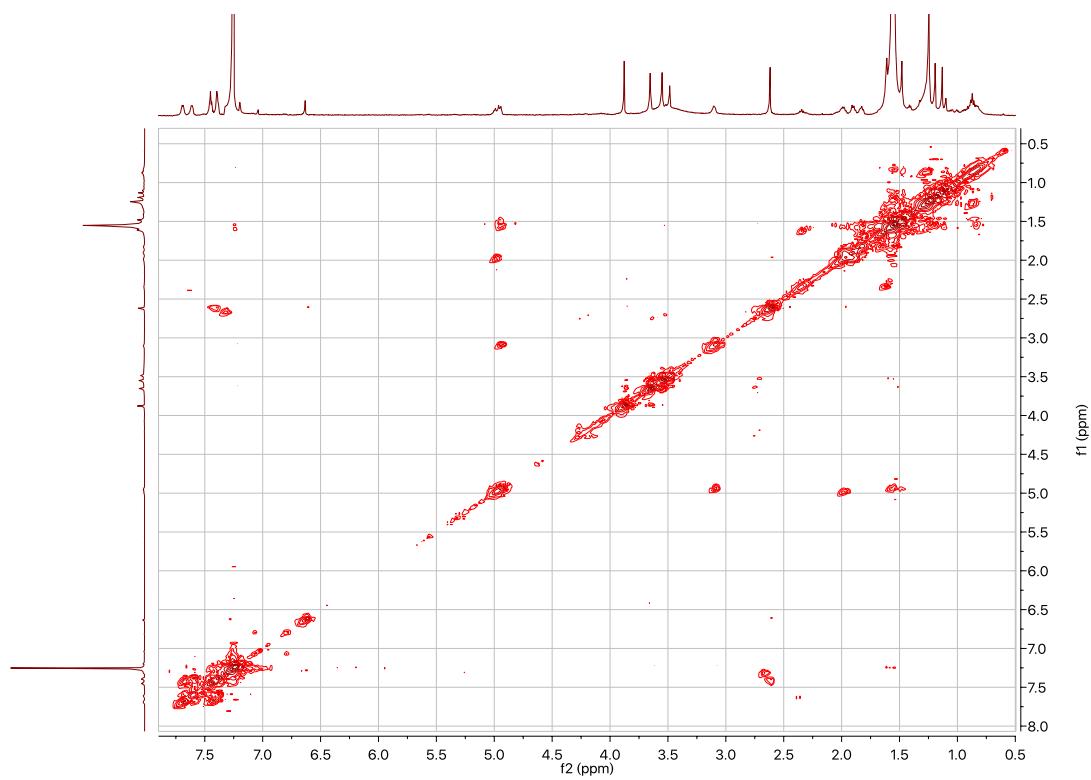


Figure S40. COSY spectrum of *S*-mosher diester of 4-*O*-methylated-4'

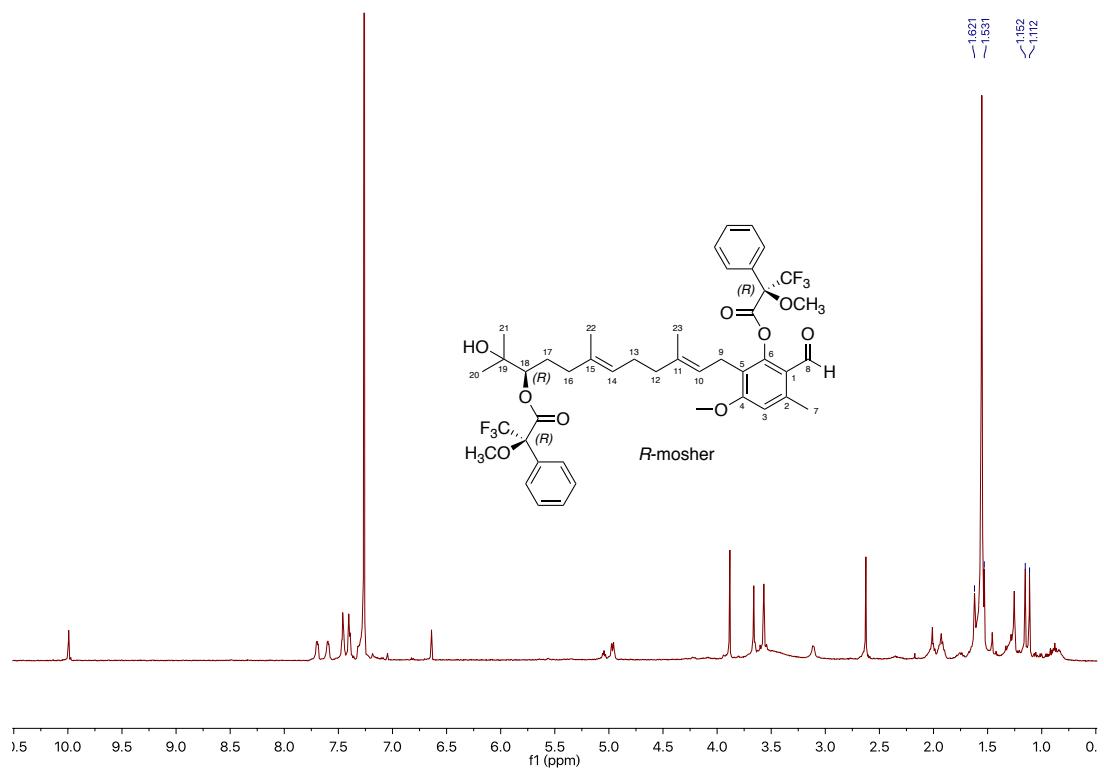


Figure S41. ¹H NMR spectrum of *R*-mosher diester of 4-*O*-methylated-4'

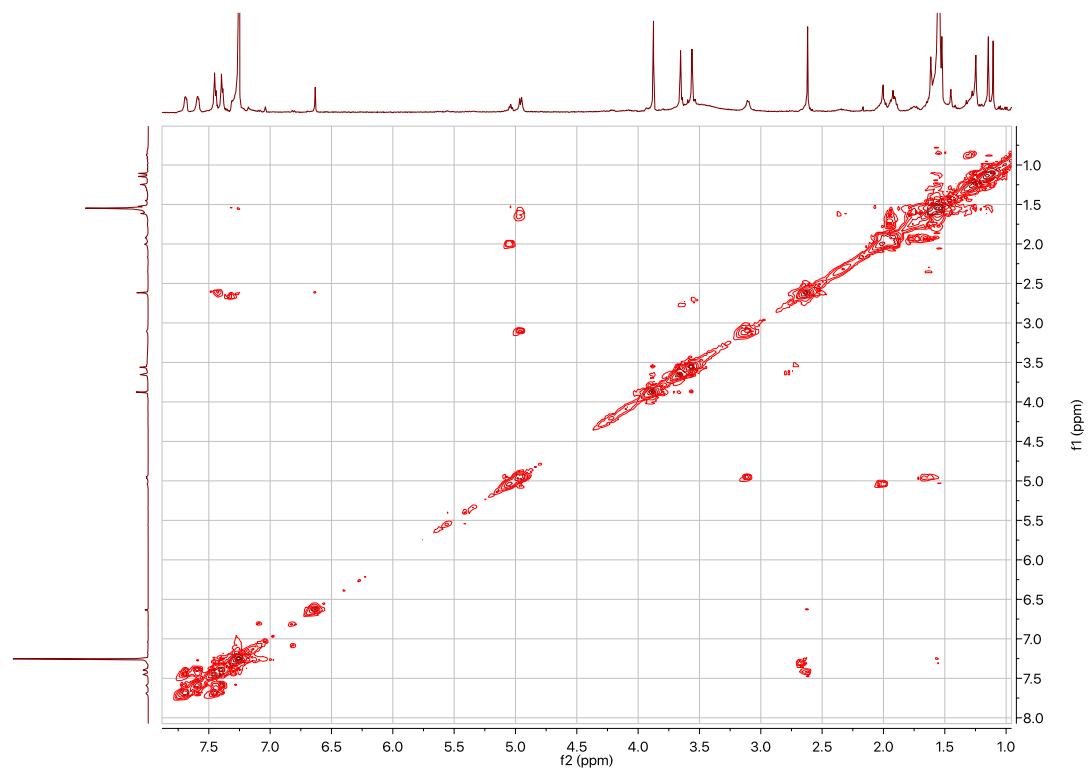


Figure S42. COSY spectrum of *R*-mosher diester of 4-*O*-methylated-4'

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