Supporting Information for

Genetic Characterization of Neosartorin Biosynthesis Provides Insight into Heterodimeric Natural Product Generation

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Supplementary Materials and Methods

General

Solvents and chemicals were purchased from Sigma-Aldrich, VWR International, or Fisher Scientific International, Inc., unless noted otherwise. Oligonucleotide primers (Table S2) were purchased from Integrated DNA Technologies Inc. PCR was performed using a T100™ Thermal Cycler (BIO-RAD) with the PfuX7 DNA polymerase[1] or Phire Plant Direct PCR Master Mix (Thermo Scientific). Flash chromatography and preparative HPLC were performed using an Isolera flash purification system (Biotage) and a Waters 600 controller with a 996 photodiode array detector (Waters), respectively. NMR spectra were obtained with Bruker AVANCE 800 MHz spectrometers at NMR Center • DTU, and chemical shifts were recorded with reference to solvent signals (^{1}H NMR: $CDCl_3$ 7.26 ppm, DMSO-d_6 2.49 ppm, acetone-d_6 2.05 ppm; ^{13}C NMR: $CDCl_3$ 77.0 ppm, DMSO-d_6 39.5 ppm, acetone-d_6 29.9 ppm). LC-MS samples were injected into a Dionex Ultimate 3000 UHPLC system (Thermo Scientific) - a maXis 3G QTOF orthogonal mass spectrometer (Bruker Daltonics), using electrospray ionization with a Kinetex C_{18} column (2.1 i.d. x 100 mm; Phenomenex).

Strains

Aspergillus novofumigatus IBT35034 (*pyrG-, ligD*Δ*, nvfA*Δ*::AfpyrG*) was created in our previous study[2] and is available from the IBT Culture Collection at the Department of Biotechnology and Biomedicine, Technical University of Denmark. Standard DNA engineering experiments were performed using *Escherichia coli* DH5α.

Construction of plasmids for fungal transformations

For the construction of plasmids for gene deletion experiments, approximately 1-kb of the 5' and 3' flanking regions of the targeted gene were amplified by PCR from the genomic DNA of *A. novofumigatus* IBT 16806 and ligated into the pU2002c vector,[3] which had been digested with *Pac*I and subsequently treated with Nt.BbvCI, by the Uracil-Specific Excision Reagent (USER) fusion method.[4] The plasmids constructed in this study and the primers used for the construction of each plasmid are listed in Table S3.

Creation of the parent strain for the gene deletion experiment

To obtain a mutant *A. novofumigatus* strain unable to synthesize novofumigatonin and *epi*-aszonalenin C, *A. novofumigatus* IBT35034 (*pyrG-, ligD*Δ*, nvfA*Δ*::AfpyrG*)[2] was cultivated on minimal media (6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 1.52 g/L KH_2PO_4, 10 g/L D-glucose, 10 mg/L thiamine, 20 g/L agar, and 1 mL/L of a trace element solution[5]) supplemented with 1.12 g/L uracil and 2.44 g/L uridine to induce the elimination of the *pyrG* marker gene (*AfpyrG*: *pyrG* from *Aspergillus flavus*) via intramolecular homologous recombination, which was further selected on agar plate containing 1.30 g/L of 5-fluoroorotic acid (5-FOA), hereby creating the *pyrG-, ligD*Δ*, nvfA*Δ strain. The *e-anaPS* gene, which is responsible for the first committed

step in the *epi*-aszonalenin C biosynthesis, was deleted as previously described[2] to generate the *pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ*::AfpyrG* strain. Finally, the *pyrG* marker gene was eliminated in the same way as described above to yield the *pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ strain, which was used for the gene deletion experiments in this study.

Gene deletion experiment

To delete each gene in the *nsr* cluster, the pU2002c-based plasmids corresponding to the targeted gene (Table S3) was used for the transformation of the *pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ strain. Transformation of *A. novofumigatus* was performed as previously described.[2] The successful deletion of targeted genes was confirmed by colony-direct PCR of each transformant (Figure S2). The mutant strains of *A. novofumigatus* constructed in this study were deposited in the IBT Culture Collection at the Department of Biotechnology and Biomedicine, Technical University of Denmark (Table S4).

LC-MS analysis of metabolites from each transformant

Mutants strains of *Aspergillus novofumigatus* were cultivated on a MEA agar plate (20 g/L malt extract (Difco), 10 g/L peptone (Difco), 20 g/L D-glucose, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, 0.005 g/L $CuSO_4 \cdot 5H_2O$, 20 g/L agar, pH 5.5) at 25 °C for 7 days, and extracted with ethyl acetate. Separation was performed with a solvent system of water containing 20 mM formic acid (solvent A) and acetonitrile containing 20 mM formic acid (solvent B), at a flow rate of 0.4 mL/min and a column temperature of 40 °C, using the following program: a linear gradient from 10:90 (solvent B/solvent A) to 100:0 for 10 min, 100:0 for the following 3 min, and a linear gradient from 100:0 to 10:90 within the following 2 min.

Isolation and purification of each metabolite

For the isolation of each metabolite, the wild type and transformants were inoculated on 60 to 200 MEA agar plates (*ca*. 1.2-4 L), respectively, and cultivated for ~14 days at 25 °C. The resulting fungal culture was extracted with ethyl acetate twice, fractionated by flash chromatography, and purified by preparative HPLC. The detailed purification procedures for each compound are described below.

Purification conditions for neosartorin (**1**):

The extract from *A. novofumigatus* mutant (*pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ*::AfpyrG*) cultivated on 60 MEA agar plates was subjected to flash chromatography with Biotage® SNAP cartridge containing ISOLUTE® DIOL (50 g) and eluted stepwise using a *n*-heptane:dichloromethane:ethyl acetate gradient. Fractions that contained **1** were further purified by reverse-phase preparative HPLC (80% aqueous acetonitrile, 5.0 mL/min) using Luna II C18 column (250 × 10 mm, 5 μm, Phenomenex) to yield 72.7 mg of a yellowish powder.

Purification conditions for moniliphenone (**6**) and 2,2,6-trihydroxy-4-methyl-6-methoxy-acyl-diphenylmethanone (**7**):

The extract from *A. novofumigatus* mutant (*pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ, *nsrK*Δ*::AfpyrG*) cultivated on 200 MEA agar plates was subjected to flash chromatography with Biotage® SNAP cartridge containing ISOLUTE® DIOL (50 g) and eluted stepwise using a *n*-heptane:dichloromethane:ethyl acetate gradient. Approximately one-third of the fraction that contained **6** were further purified by reverse-phase preparative HPLC (40% aqueous acetonitrile, 5.0 mL/min) using Luna II C18 column (250 × 10 mm, 5 µm, Phenomenex) to yield 25.4 mg of a yellowish amorphous solid. Approximately one-third of the fraction that contained **7** were further purified by reverse-phase preparative HPLC (40% aqueous acetonitrile, 5.0 mL/min) using Luna II C18 column (250 × 10 mm, 5 µm, Phenomenex) to yield 12.4 mg of a yellowish amorphous solid.

Purification conditions for blennolide C (**8**) and 5-acetylblennolide A (**9**):

The extract from *A. novofumigatus* mutant (*pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ, *nsrP*Δ*::AfpyrG*) cultivated on 60 MEA agar plates was subjected to flash chromatography with Biotage® SNAP cartridge containing ISOLUTE® DIOL (50 g) and eluted stepwise using a *n*-heptane:dichloromethane:ethyl acetate gradient. Fractions that contained **8** were further purified by reverse-phase preparative HPLC (55% aqueous acetonitrile, 5.0 mL/min) using Luna II C18 column (250 × 10 mm, 5 µm, Phenomenex) to yield 1.5 mg of a yellowish solid. Fractions that contained **9** were further purified by reverse-phase preparative HPLC (70% aqueous acetonitrile, 5.0 mL/min) using Luna II C18 column (250 × 10 mm, 5 µm, Phenomenex) to yield 1.2 mg of a yellowish solid.

Purification conditions for deacetylneosartorin (**10**) and novofumigatin A (**11**):

The extract from *A. novofumigatus* mutant (*pyrG-, ligD*Δ*, nvfA*Δ, *e-anaPS*Δ, *nsrL*Δ*::AfpyrG*) cultivated on 60 MEA agar plates was subjected to flash chromatography with Biotage® SNAP cartridge containing ISOLUTE® DIOL (50 g) and eluted stepwise using a *n*-heptane:dichloromethane:ethyl acetate gradient. Fractions that contained **10** and **11** were further purified by reverse-phase preparative HPLC (65% aqueous acetonitrile, 5.0 mL/min) using Luna II C18 column (250 × 10 mm, 5 µm, Phenomenex) to yield 42.0 and 1.3 mg of a yellowish powder, respectively.

Screening for anti-infective properties of the obtained compounds

Anti-infective screening of compounds **1** and **10** was performed by Foundación Medina (Granada, Spain).

Analytical data

*Neosartorin (**1**).* Yellowish powder; $[\alpha]^{20}_{D}$ -222.7 (*c* 0.88, MeOH); for ^{1}H and ^{13}C NMR data see Table S5 and Figures S3 and S4; HR-ESI-MS found *m/z* 681.1819 $[M + H]^+$ (calcd 681.1814 for $C_{34}H_{33}O_{15}$). The NMR data are in good agreement with the reported data.[6]

*Moniliphenone (**6**).* Yellowish amorphous solid; for NMR data see Table S6 and Figures S5 to S6; HR-ESI-MS found *m/z* 325.0684 $[M + Na]^+$ (calcd 325.0683 for $C_{16}H_{14}O_6Na$). The NMR data are in good agreement with the reported data.[7]

*2,2',6'-Trihydroxy-4-methyl-6-methoxy-acyl-diphenylmethanone (**7**).* Yellow powder; for NMR data see Table S7 and Figures S7 to S8; HR-ESI-MS found *m/z* 325.0685 $[M + Na]^+$ (calcd 325.0683 for $C_{16}H_{14}O_6Na$). The NMR data are in good agreement with the reported data.[8]

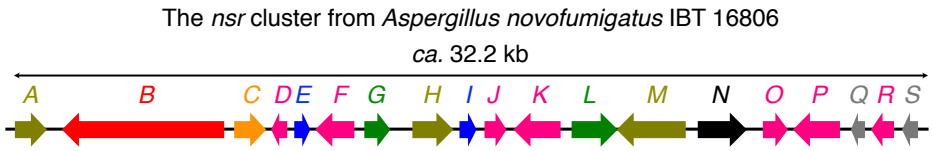
*Blennolide C (**8**).* Yellowish solid; $[\alpha]^{20}_D$ +114.4 (*c* 0.13, MeOH); for NMR data see Table S8 and Figures S9 to S10; HR-ESI-MS found *m/z* 321.0970 $[M + H]^+$ (calcd 321.0969 for $C_{16}H_{17}O_7$). The NMR data are in good agreement with the reported data.[9]

*5-Acetylblennolide A (**9**).* Yellowish solid; $[\alpha]^{20}_D$ +142.3 (*c* 0.18, MeOH); for NMR data see Table S9 and Figures S11 to S16; HR-ESI-MS found *m/z* 363.1078 $[M + H]^+$ (calcd 363.1074 for $C_{18}H_{19}O_8$).

*Deacetylneosartorin (**10**).* Yellowish powder; $[\alpha]^{20}_D$ -154.1 (*c* 0.68, MeOH); for NMR data see Table S10 and Figures S17 to S22; HR-ESI-MS found *m/z* 639.1716 $[M + H]^+$ (calcd 639.1708 for $C_{32}H_{31}O_{14}$).

*Novofumigatin A (**11**).* Yellowish powder; $[\alpha]^{20}_D$ -132.0 (*c* 0.15, MeOH); for ^1H and ^{13}C NMR data see Table S11 and Figures S23 and S28; HR-ESI-MS found *m/z* 639.1709 $[M + H]^+$ (calcd 639.1708 for $C_{32}H_{31}O_{14}$).

Table S1. Annotation of each protein of the *nsr* cluster.



Gene	Protein ID	Amino acids (base pairs)	Protein homologue in the *mdp* pathway and similarity/identity (%)	Protein homologue in the *cla* pathway and similarity/identity (%)	Protein homologue in other pathways and similarity/identity (%)	Proposed function
nsrA	372038	378 (7131)	MdpE (50/31)	ClaE (46/25)		C6 transcription factor
nsrB	487776	1792 (5723)	MdpG (75/62)	ClaG (69/53)		polyketide synthase
nsrC	460891	322 (1134)	MdpF (77/64)	ClaF (71/57)		thioesterase
nsrD	373009	143 (552)	MdpH2 (52/39)	-		anthrone oxygenase
nsrE	372388	151 (586)	MdpH1 (76/62)	ClaH (90/73)		decarboxylase
nsrF	512934	433 (1370)	MdpL (59/43)	ClaL (55/37)		Baeyer-Villiger monooxygenase
nsrG	512933	261 (924)	-	-	GedG, *Aspergillus terreus* (58/44)	methyltransferase
nsrH	512932	444 (1433)	MdpA (54/39)	ClaA (53/35)		transcriptional coactivator
nsrI	451670	162 (603)	MdpB (71/59)	ClaB (77/62)		dehydratase
nsrJ	460884	264 (795)	MdpC (87/75)	ClaC (93/85)		short chain dehydrogenase/reductase
nsrK	460883	504 (1664)	MdpD (69/52)	-		FAD-dependent monooxygenase
nsrL	512929	487 (1640)	-	-	AdrG, *Penicillium chrysogenum* (50/33)	acetyltransferase
nsrM	512928	716 (2447)	-	-	VrtR1, *Penicillium aethiopicum* (43/27)	transcription factor
nsrN	373375	506 (1741)	-	-	AflT, *Aspergillus flavus* (65/50)	efflux pump
nsrO	421366	290 (873)	-	-		short chain dehydrogenase/reductase
nsrP	372675	515 (1663)	-	ClaM (58/42)		cytochrome P450 monooxygenase
nsrQ	407796	151 (511)	-	-	AusJ, *Aspergillus nidulans* 43/26	unknown
nsrR	371720	265 (798)	MdpK (75/58)	ClaK (76/55)		short chain dehydrogenase/reductase
nsrS	442171	150 (574)	-	-	AusH, *Aspergillus nidulans* 46/24	unknown

Note: Protein IDs are as designated in JGI database.

Table S2. Primers used in this study.

Primer	Sequence (5' to 3')	Note
nsrB-5'-F	GGGTTTAAUCCACGTGCTGAGATGCTCAATAGCTG	
nsrB-5'-R	GGACTTAAUATCCTACCTTCATCTTGGCAGGATCC	
nsrB-3'-F	GGCATTAAUGAGAGGCGTCAAGCTTTCATCCAGG	
nsrB-3'-R	GGTCTTAAUCAAAGTGCCATGCTATCTGCGGCTGC	
nsrF-5'-F	GGGTTTAAUGCATCACGTTGGTAGCGAAGACCATATC	
nsrF-5'-R	GGACTTAAUGGGTTCCCCATCTTCATACGCCCTC	
nsrF-3'-F	GGCATTAAUTGCCCAGATCTGTTATGATTCCACCG	
nsrF-3'-R	GGTCTTAAUGCCTCATTTTCCCTTGCCTCATATCC	
nsrG-5'-F	GGGTTTAAUCCCGAAGCCAAGATGGATGATCGGG	
nsrG-5'-R	GGACTTAAUGCGGTCGAAAAAGGAGTCAGGTACTC	
nsrG-3'-F	GGCATTAAUTAGACGATCTTCTCGCGGATGGGAAAC	
nsrG-3'-R	GGTCTTAAUTGCTTGCTGGGTGTTTCCACTTCCAG	
nsrK-5'-F	GGGTTTAAUCATGGAAGGAGACGACGTGTAGGCC	
nsrK-5'-R	GGACTTAAUCCCGACCACATCATGGCCTTTCCTC	
nsrK-3'-F	GGCATTAAUTTGTGCACTGGACACTGGAGGGAATC	
nsrK-3'-R	GGTCTTAAUCAACTACGCCAACTCTGCCGAGCAC	
nsrL-5'-F	GGGTTTAAUGCGCTTTTCTAATGGATCCGTCCGTC	
nsrL-5'-R	GGACTTAAUCGTCGAAGAAGGGCAATTCGACCACC	
nsrL-3'-F	GGCATTAAUGCGTGACAGTGCACATGCTGAGCTTC	
nsrL-3'-R	GGTCTTAAUCCACTTTATGACAATCTGGACGACGC	
nsrO-5'-F	GGGTTTAAUGGATCGGGTACCAGATCCTATACGGT	
nsrO-5'-R	GGACTTAAUAGAGACATCTTGTCGTGTATGGTTGTCC	
nsrO-3'-F	GGCATTAAUGCAAGATAGGAGGTTCCTCACAATCGG	
nsrO-3'-R	GGTCTTAAUCCTGCTCGACTACAACGACAAAGTGG	
nsrP-5'-F	GGGTTTAAUCGCCCATATCCCCTATAGCCATGCC	
nsrP-5'-R	GGACTTAAUCCATCACGGCAGGTAAGTGTAGTCTG	
nsrP-3'-F	GGCATTAAUGGGACTGTCTTACCCTCTGCTTACTG	
nsrP-3'-R	GGTCTTAAUCGCCAAGCACCATGGCTTTAACGGTC	
nsrB-check-F	GTTTGCAGCCATGGCTCTGCATTTG	P1 in Figure S2
nsrF-check-F	AATTGATGCGCGATAGCGGCCATGG	P1 in Figure S2
nsrG-check-F	AAGACGTCGATCTTCATGGGATCGTG	P1 in Figure S2
nsrK-check-F	AGGCAGCATGTATCCAGTTCAGCG	P1 in Figure S2
nsrL-check-F	GCGACGACTGCACAGAACTAAGTATC	P1 in Figure S2
nsrO-check-F	TGCTCACCACGTTCGAGGTGGATAC	P1 in Figure S2
nsrP-check-F	CTGTCCATGATGATCCATCTTCCCG	P1 in Figure S2
deletion-check-R	ATCAGCTACCGAGCAACTTCTACATCACAG	P2 in Figure S2

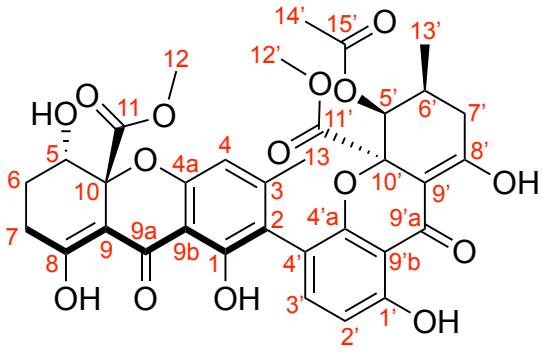
Table S3. Plasmids constructed in this study and PCR conditions for the amplification of the inserts for the plasmid constructions.

Plasmid	Inserts	Primer 1	Primer 2	PCR Template
pU2002c-nsrB	5' flanking region of *nsrB*	nsrB-5'-F	nsrB-5'-R	gDNA
	3' flanking region of *nsrB*	nsrB-3'-F	nsrB-3'-R	
pU2002c-nsrF	5' flanking region of *nsrF*	nsrF-5'-F	nsrF-5'-R	gDNA
	3' flanking region of *nsrF*	nsrF-3'-F	nsrF-3'-R	
pU2002c-nsrG	5' flanking region of *nsrG*	nsrG-5'-F	nsrG-5'-R	gDNA
	3' flanking region of *nsrG*	nsrG-3'-F	nsrG-3'-R	
pU2002c-nsrK	5' flanking region of *nsrK*	nsrK-5'-F	nsrK-5'-R	gDNA
	3' flanking region of nsrK	nsrK-3'-F	nsrK-3'-R	
pU2002c-nsrL	5' flanking region of *nsrL*	nsrL-5'-F	nsrL-5'-R	gDNA
	3' flanking region of *nsrL*	nsrL-3'-F	nsrL-3'-R	
pU2002c-nsrO	5' flanking region of *nsrO*	nsrO-5'-F	nsrO-5'-R	gDNA
	3' flanking region of *nsrO*	nsrO-3'-F	nsrO-3'-R	
pU2002c-nsrP	5' flanking region of *nsrP*	nsrP-5'-F	nsrP-5'-R	gDNA
	3' flanking region of *nsrP*	nsrP-3'-F	nsrP-3'-R	

Table S4. Mutant strains of *Aspergillus novofumigatus* constructed in this study.

Strain	Genotype
IBT35050	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ
IBT35053	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrB* Δ*::AfpyrG*
IBT35054	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrF* Δ*::AfpyrG*
IBT35055	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrG* Δ*::AfpyrG*
IBT35052	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrK* Δ*::AfpyrG*
IBT35051	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrL* Δ*::AfpyrG*
IBT35057	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrO* Δ*::AfpyrG*
IBT35056	*pyrG-, ligD* Δ*, nvfA* Δ*, e-anaPS* Δ*, nsrP* Δ*::AfpyrG*

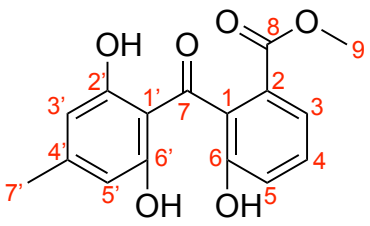
Table S5. NMR data for neosartorin (**1**).



	^{13}C	^{1}H		
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity
1	159.8			
2	118.6			
3	148.5			
4	108.9	6.48	1H	s
4a	156.8			
5	67.0	4.36	1H	dd (*J* = 4.4, 2.0 Hz)
6	23.1	2.16 (α)	1H	ddd (*J* = 14.7, 5.9, 3.9 Hz)
		1.98 (β)	1H	dddd (*J* = 14.7, 11.3, 6.9, 2.0 Hz)
7	24.4	2.84 (α)	1H	ddd (*J* = 18.6, 11.7, 6.9 Hz)
		2.39 (β)	1H	m
8	178.7			
9	100.5			
9a	187.4			
9b	104.8			
10	83.9			
11	171.2			
12	53.6	3.79	3H	s
13	21.1	2.08	3H	s
1'	161.7			
2'	110.3	6.59	1H	d (*J* = 8.3 Hz)
3'	139.9	7.15	1H	d (*J* = 8.3 Hz)
4'	114.6			
4'a	155.5			
5'	69.5	5.28	1H	d (*J* = 1.5 Hz)
6'	27.8	2.31	1H	m
7'	32.8	2.37	2H	m
8'	177.7			
9'	100.2			
9'a	187.8			
9'b	106.8			
10'	82.1			
11'	170.6			
12'	53.3	3.65	3H	s
13'	17.1	0.92	3H	d (*J* = 6.4 Hz)
14'	20.3	1.93	3H	s
15'	169.1			
1-OH		11.54	1H	s
8-OH		13.91	1H	brs
1'-OH		11.38	1H	brs
8'-OH		13.79	1H	brs

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in $CDCl_3$)

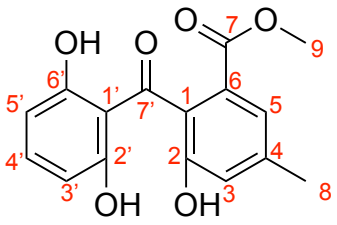
Table S6. NMR data for moniliphenone (**6**).



	^{13}C	^{1}H		
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity
1	134.4			
2	129.2			
3	121.5	7.49	1H	brs
4	129.4	7.28	1H	brs
5	120.8	7.12	1H	brs
6	154.3			
7	200.9			
8	166.9			
9	52.2	3.69	3H	s
1'	110.4			
2'	162.9			
3'	108.8	6.19	1H	s
4'	148.5			
5'	108.8	6.19	1H	s
6'	162.9			
7'	22.0	2.20	3H	s

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in acetone-d_6)

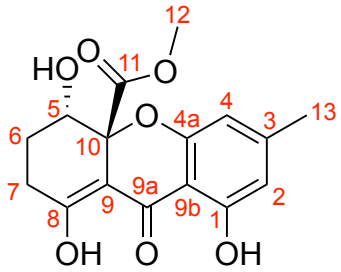
Table S7. NMR data for 2,2’,6’-Trihydroxy-4-methyl-6-methoxy-acyl-diphenylmethanone (**7**).



	^{13}C	^{1}H		
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity
1	130.5			
2	153.3			
3	120.4	6.88	1H	brs
4	138.4			
5	120.2	7.20	1H	brs
6	127.3			
7	165.9			
8	20.7	2.29	3H	s
9	51.9	3.64	3H	s
1'	111.0			
2'	161.7			
3'	106.8	6.24	1H	d (*J* = 8.3 Hz)
4'	136.2	7.21	1H	t (*J* = 8.3 Hz)
5'	106.8	6.24	1H	d (*J* = 8.3 Hz)
6'	161.7			
7'	201.0			
2-OH		9.69	1H	brs
2'-OH		11.43	1H	brs
6'-OH		11.43	1H	brs

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in DMSO-d_6)

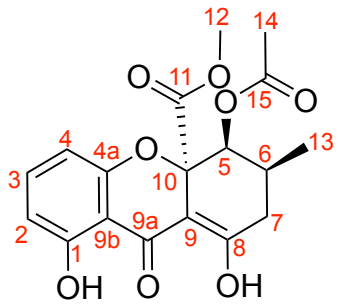
Table S6. NMR data for blennolide C (**8**).



	^{13}C	^{1}H		
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity
1	161.9			
2	111.7	6.38	1H	s
3	149.9			
4	108.7	6.35	1H	s
4a	157.6			
5	67.0	4.31	1H	brs
6	23.1	2.14 (α)	1H	ddd (*J* = 14.7, 6.4, 4.4 Hz)
		1.95 (β)	1H	m
7	24.3	2.82 (α)	1H	ddd (*J* = 19.1, 11.3, 7.3 Hz)
		2.38 (β)	1H	dd (*J* = 19.1, 6.9 Hz)
8	179.1			
9	100.1			
9a	186.9			
9b	104.9			
10	83.8			
11	171.2			
12	53.4	3.70	3H	s
13	22.5	2.29	3H	s
1-OH		11.28	1H	s
5-OH		2.66	1H	brs
8-OH		14.04	1H	s

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in $CDCl_3$)

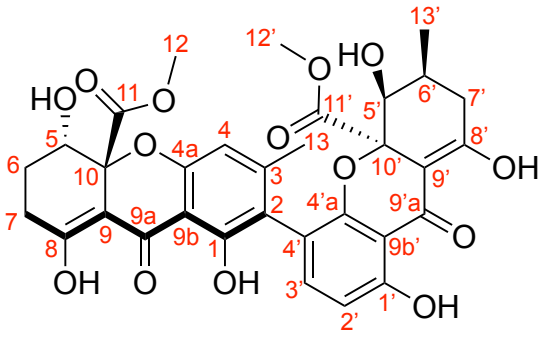
Table S9. NMR data for 5-acetylblennolide A (**9**).



	^{13}C	^{1}H					
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity	HMBC correlation	COSY correlation	NOESY correlation
1	161.9						
2	110.8	6.52	1H	d (*J* = 8.3 Hz)	1, 4, 9b	H-3	H-3
3	137.8	7.30	1H	t (*J* = 8.3 Hz)	1, 4a	H-2, H-4	H-2, H-4
4	108.0	6.44	1H	d (*J* = 8.3 Hz)	2, 4a, 9b	H-3	H-3
4a	157.9						
5	70.7	5.62	1H	brs	9, 10, 15		H-6, H-13
6	28.0	2.26	1H	m		H-15	H-5, H-12, H-13
7	33.1	2.47 (α)	1H	dd (*J* = 18.6, 5.9 Hz)	5, 6, 8, 9		H-13
		2.38 (β)	1H	dd (*J* = 18.6, 11.3 Hz)	6, 8, 9, 13		H-13
8	178.2						
9	100.7						
9a	187.5						
9b	106.9						
10	82.6						
11	170.7						
12	53.5	3.70	3H	s	11		H-6
13	20.8	1.03	3H	d (*J* = 6.4 Hz)	5, 6, 7	H-6	H-5, H-6, H-7α, H-7β, H-14
14	17.3	2.11	3H	s	15		H-13
15	170.2						
1-OH		11.29	1H	s	1, 2, 9b		
8-OH		14.03	1H	brs			

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in $CDCl_3$)

Table S10. NMR data for deacetylneosartorin (**10**).

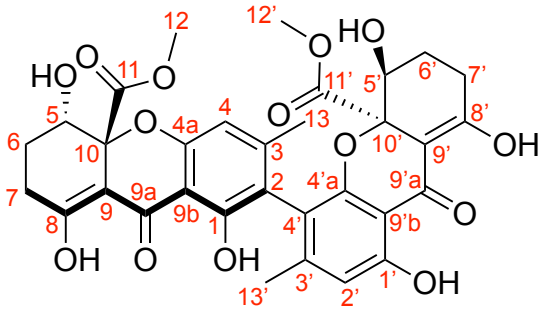


	^{13}C	^{1}H					
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity	HMBC correlation	COSY correlation	NOESY correlation
1	159.6						
2	119.2						
3	148.7						
4	109.5	6.52	1H	s	2, 4a, 9a, 9b, 13		H-13
4a	156.6						
5	67.1	4.34	1H	dd (*J* = 3.9, 2.4 Hz)	7, 9, 10	H-6α, H-6β	H-6α, H-6β
6	23.0	2.16 (α)	1H	ddd (*J* = 14.9, 7.0, 3.9 Hz)	5, 7, 8, 10	H-5, H-7α	H-5, H-7α
		1.98 (β)	1H	m	7	H-5, H-7α, H-7β	H-5, H-12
7	24.4	2.83 (α)	1H	ddd (*J* = 18.8, 11.7, 7.0 Hz)	6, 8, 9, 11	H-6α, H-6β	H-6α
		2.42 (β)	1H	dd (*J* = 19.6, 7.0 Hz)	5, 6, 8, 9	H-6β	H-6β
8	179.5						
9	99.8						
9a	186.8						
9b	104.7						
10	83.9						
11	171.3						
12	53.5	3.79	3H	s	11		H-6β, H-7β
13	21.5	2.12	3H	s	2, 3, 4		H-4, H-3', H-12'
1'	162.0						
2'	110.7	6.61	1H	d (*J* = 8.6 Hz)	1', 4', 9'a, 9'b	H-3'	
3'	140.2	7.11	1H	d (*J* = 8.6 Hz)	2, 1', 4'a	H-2'	H-13
4'	114.3						
4'a	155.0						
5'	70.7	3.90	1H	d (*J* = 1.6 Hz)	6', 7', 9', 9'a, 10', 11', 13'	H-6'	H-6', H-13'
6'	28.4	2.22	1H	m	7', 13'	H-5', H-7'α, H-7'β, H-13'	H-7'α, H-12', H-13'
7'	32.5	2.37 (α)	1H	dd (*J* = 18.8, 6.3 Hz)	5', 6', 8', 9'	H-6'	H-6', H-13'
		2.50 (β)	1H	dd (*J* = 18.8, 11.0 Hz)	6', 8', 9', 11', 13'	H-6'	H-13'
8'	179.3						
9'	99.3						
9'a	187.6						
9'b	106.7						
10'	84.0						
11'	170.7						
12'	53.1	3.64	3H	s	11'		H-13, H-6'
13'	17.5	1.13	3H	d (*J* = 6.3 Hz)	5', 6', 7'	H-6'	H-5, H-6, H-7α, H-7β
1-OH		11.72	1H	s	1, 2, 9b		
5-OH		2.69*	1H	brs			
8-OH		13.84†	1H	brs			
1'-OH		11.58	1H	s	1', 2', 9'b		
5'-OH		2.79*	1H	brs			
8'-OH		14.01†	1H	brs			

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in $CDCl_3$)

Note: The carbons indicated by * and † are interchangeable, respectively.

Table S11. NMR data for novofumigatin A (**11**).



	^{13}C	^{1}H					
position	δ (ppm)	δ (ppm)	intensitiy	multiplicity	HMBC correlation	COSY correlation	NOESY correlation
1	159.5						
2	117.5						
3	149.4						
4	109.2	6.52	1H	s	2, 4a, 9a, 9b, 13		H-13
4a	156.8						
5	67.1	4.37	1H	brs	7, 9, 10	H-6α, H-6β, 5-OH	H-6α, H-6β, 5-OH
6	23.1	2.17 (α)	1H	ddd (*J* = 14.7, 6.9, 3.9 Hz)	5, 7, 8, 10	H-5, H-7α	H-5, H-7α
		1.98 (β)	1H	m		H-5, H-7α, H-7β	H-5, H-7β
7	24.4	2.84 (α)	1H	ddd (*J* = 18.6, 11.3, 7.3 Hz)	6, 8, 9	H-6α, H-6β	H-6α
		2.41 (β)	1H	dd (*J* = 19.1, 6.9 Hz)	5, 6, 8, 9	H-6β	H-6β
8	179.3						
9	99.9						
9a	186.9						
9b	104.8						
10	83.8						
11	171.2						
12	53.4	3.76	3H	s	11		
13	20.71	2.05	3H	s	2, 3, 4		H-4, H-12'
1'	161.4						
2'	112.1	6.54	1H	s	1', 4', 4'a, 9'a, 9'b, 13'		H-13'
3'	149.5						
4'	114.2						
4'a	155.0						
5'	66.5	4.05	1H	dd (*J* = 3.9, 2.0 Hz)	7', 9', 10'	H-6'α, H-6'β, 5'-OH	H-6'α, H-6'β, 5'-OH
6'	23.0	1.98 (α)	1H	m		H-5', H-7'α, H-7'β	H-5', H-7'α
		2.07 (β)	1H	ddd (*J* = 14.7, 6.9, 4.4 Hz)	5', 7', 8', 10'	H-5', H-7'β	H-5', H-7'β
7'	24.3	2.34 (α)	1H	dd (*J* = 19.1, 6.4 Hz)	6', 8', 9'	H-6'α	H-6'α
		2.78 (β)	1H	ddd (*J* = 18.6, 11.3, 7.3 Hz)	5', 6', 8', 9'	H-6'α, H-6'β	H-6'β
8'	178.6						
9'	99.3						
9'a	187.2						
9'b	104.9						
10'	83.2						
11'	170.7						
12'	53.1	3.65	3H	s	11'		H-13
13'	20.68	1.96	3H	s	2', 3', 4'		H-2'
1-OH		11.56	1H	s	1, 2, 9b		
5-OH		2.68	1H	brs	5, 6, 10	H-5	H-5
8-OH		14.00	1H	s	7, 8, 9		
1'-OH		11.47	1H	s	1', 2', 9'b		
5'-OH		2.64	1H	brs	5', 6'	H-5'	H-5'
8'-OH		13.92	1H	s	7', 8', 9'		

^{1}H NMR: 800 MHz, ^{13}C NMR: 200 MHz (in $CDCl_3$)

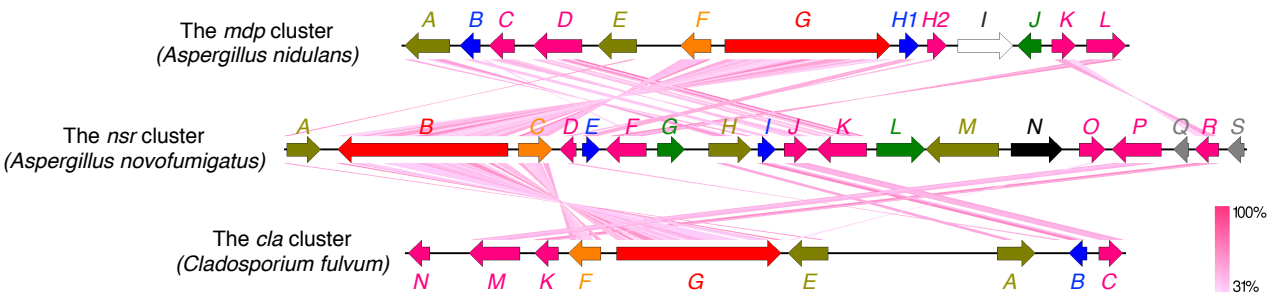


Figure S1. Tblastx comparison of the *nsr* cluster with the *mdp* cluster and the *cla* cluster. The sequence comparison was illustrated using Easyfig.[10]

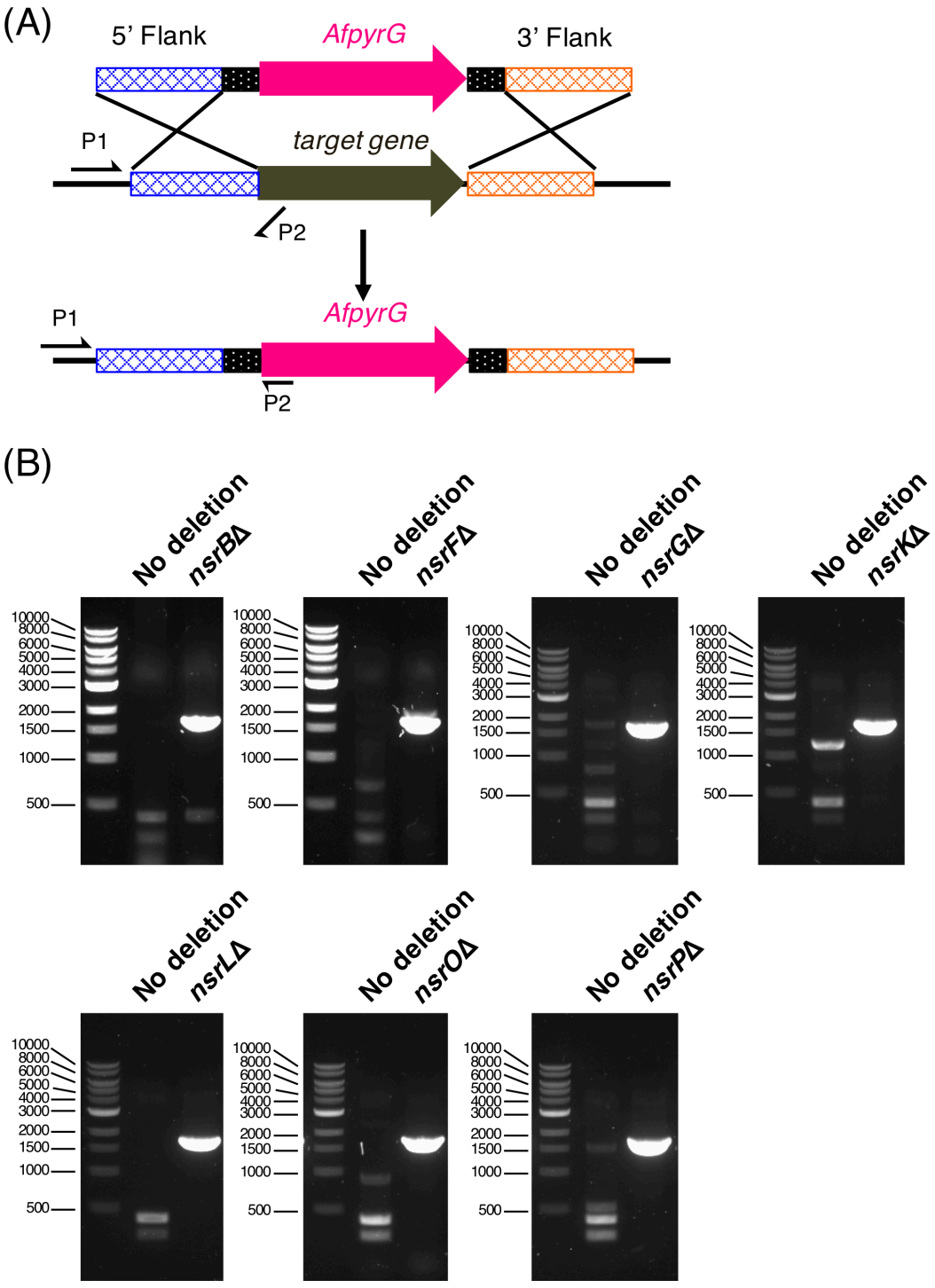


Figure S2. Deletion of the genes in the *nsr* cluster. (A) Procedure for the deletion of each biosynthetic gene using *AfpyrG* as a selection marker. (B) Result of diagnostic PCR performed with the two primers P1, binding outside of the 5' flanking region of each targeted gene used for the homologous recombination, and P2, binding to the promoter region of *AfpyrG* (see Table S2 for the primer sequences). Since P2 does not bind to the wild type genome, a band (~1700 bp) is only amplified upon successful deletion of targeted gene.

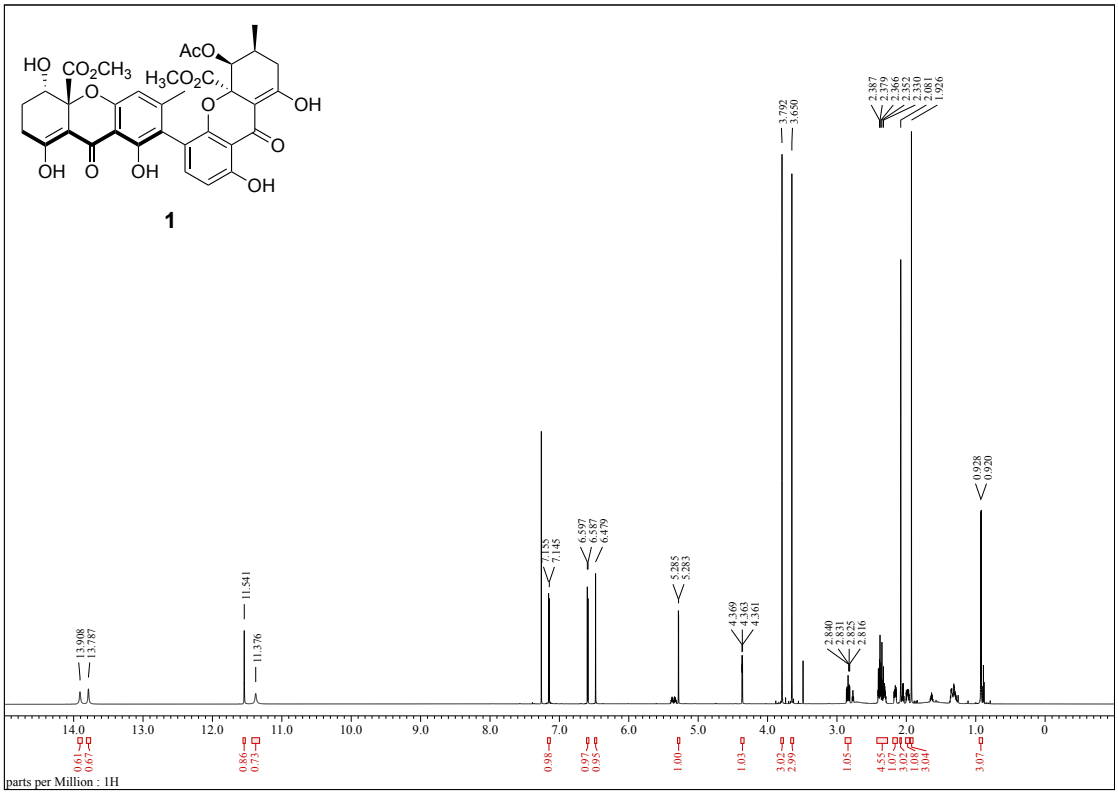


Figure S3. ^{1}H NMR spectrum of neosartorin (**1**).

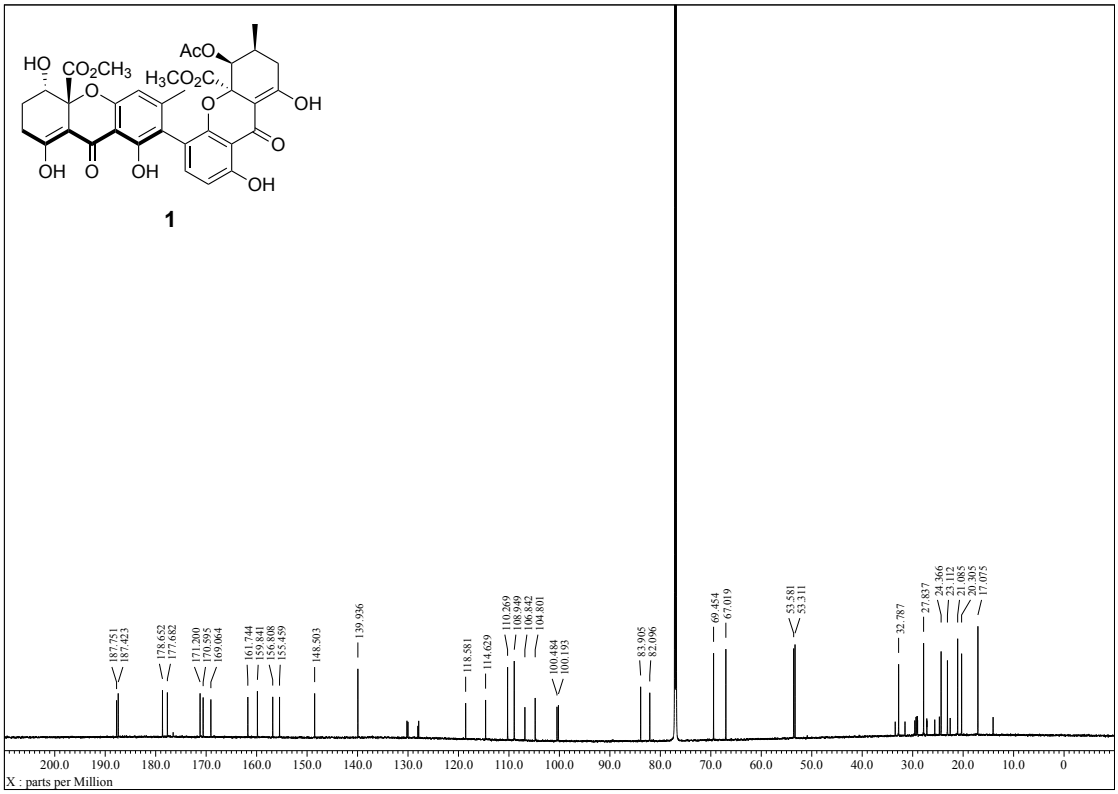


Figure S4. ^{13}C NMR spectrum of neosartorin (**1**).

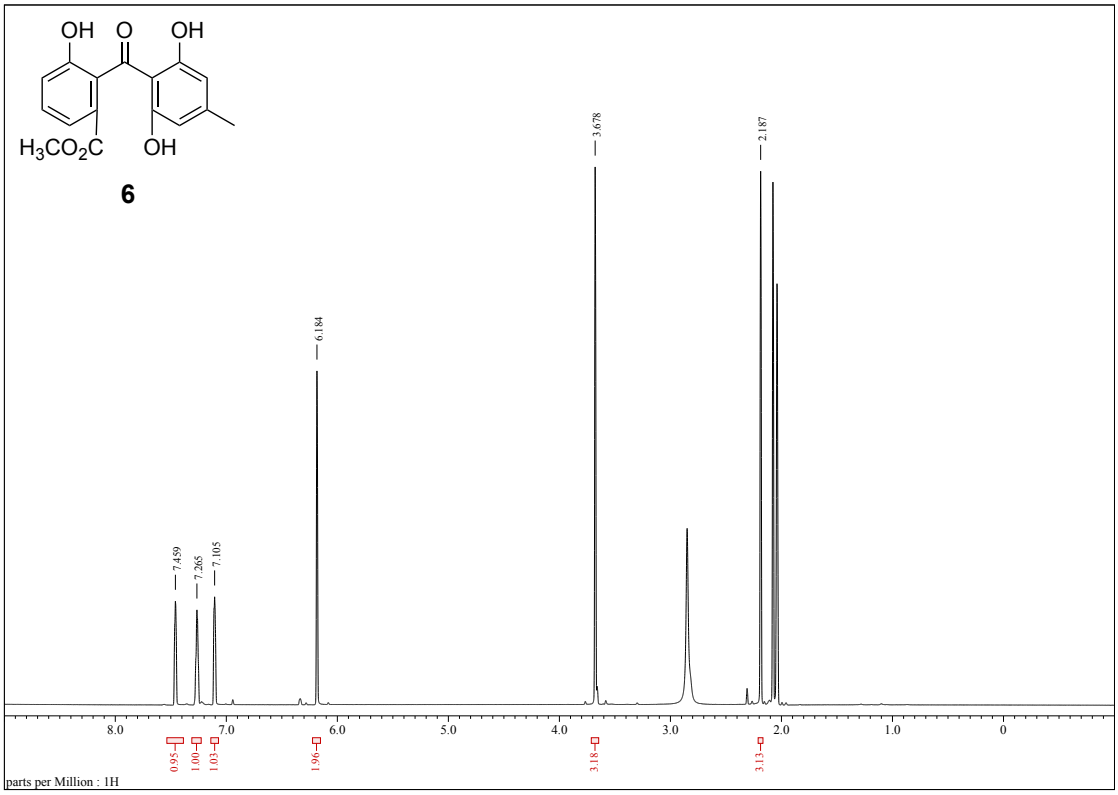


Figure S5. ^{1}H NMR spectrum of moniliphenone (**6**).

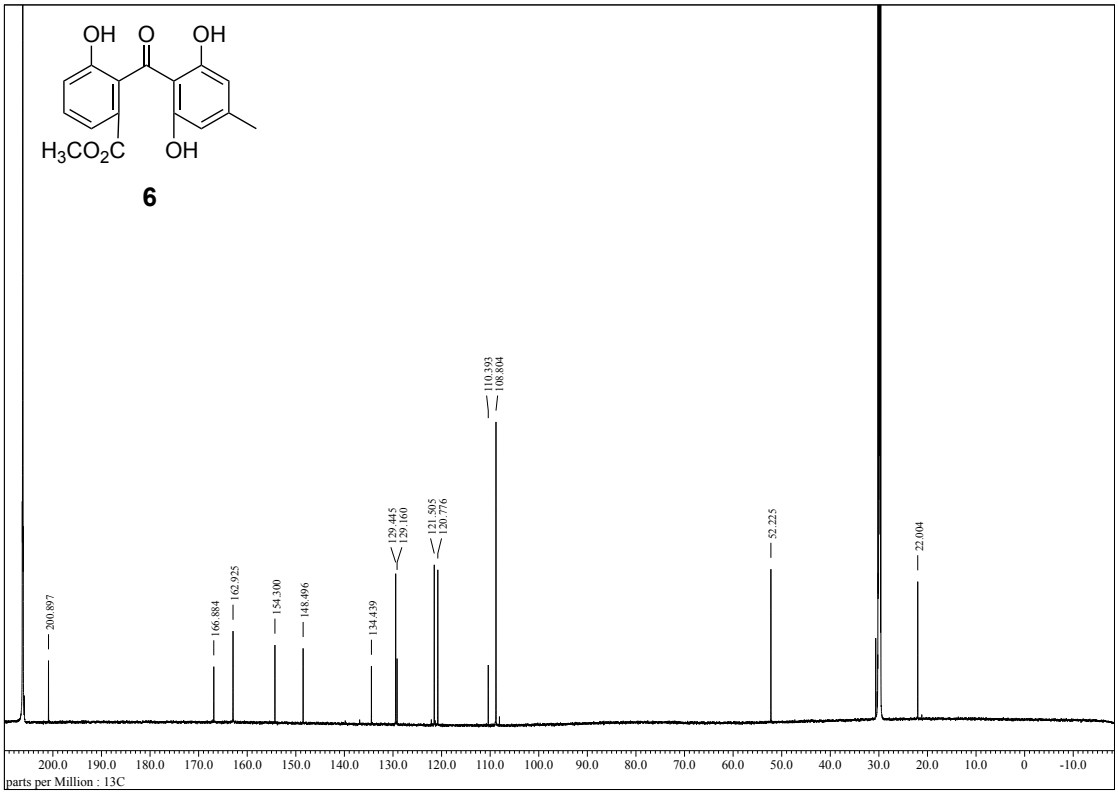


Figure S6. ^{13}C NMR spectrum of moniliphenone (**6**).

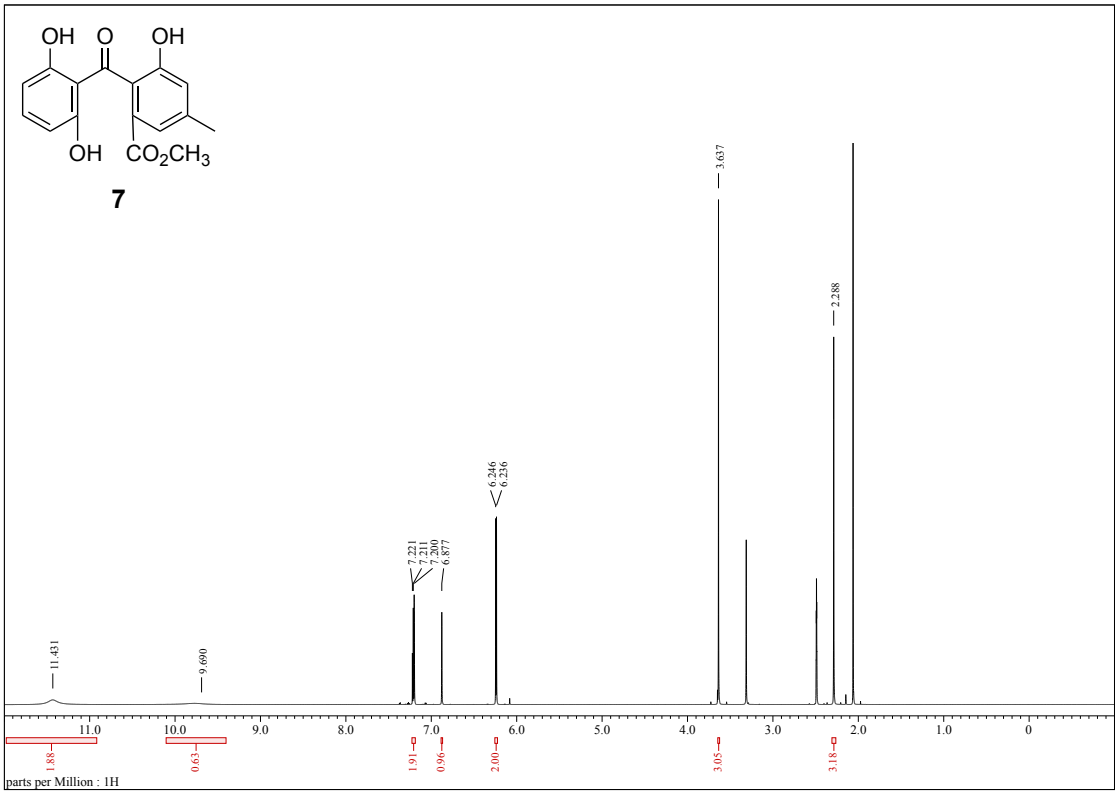


Figure S7. ^{1}H NMR spectrum of 2,2',6'-trihydroxy-4-methyl-6-methoxy-acyl-diphenylmethanone (**7**).

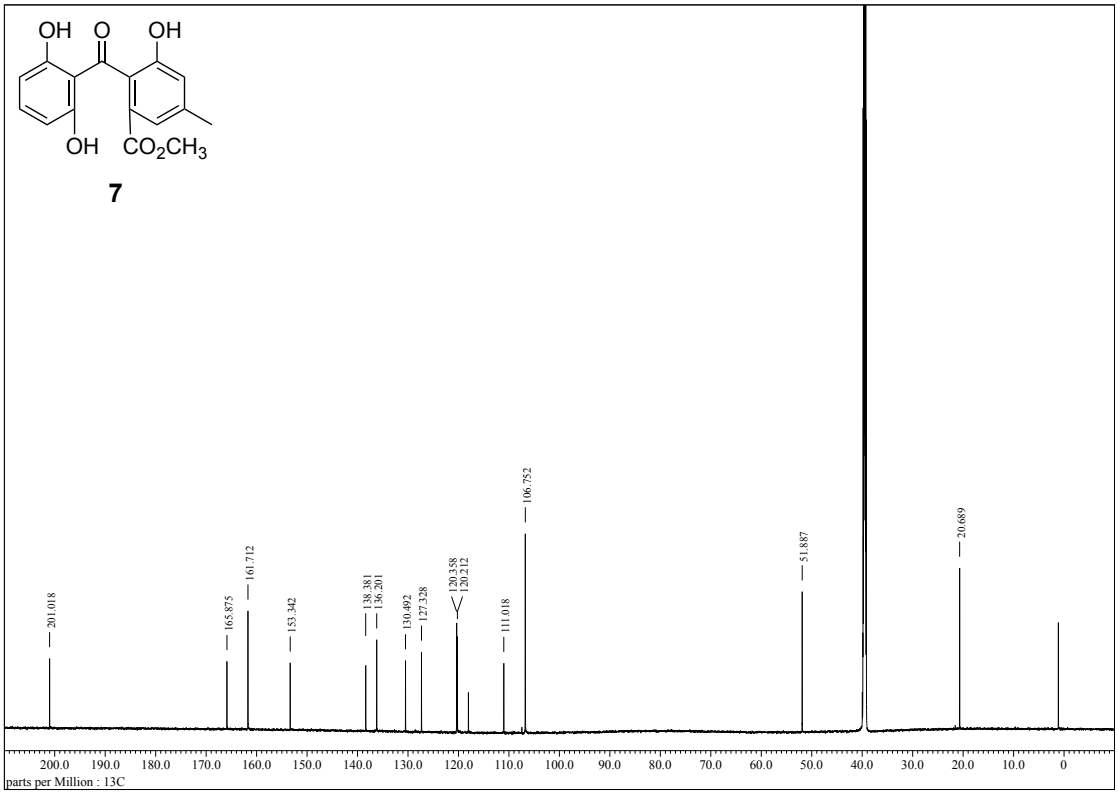


Figure S8. ^{13}C NMR spectrum of 2,2',6'-trihydroxy-4-methyl-6-methoxy-acyl-diphenylmethanone (**7**).

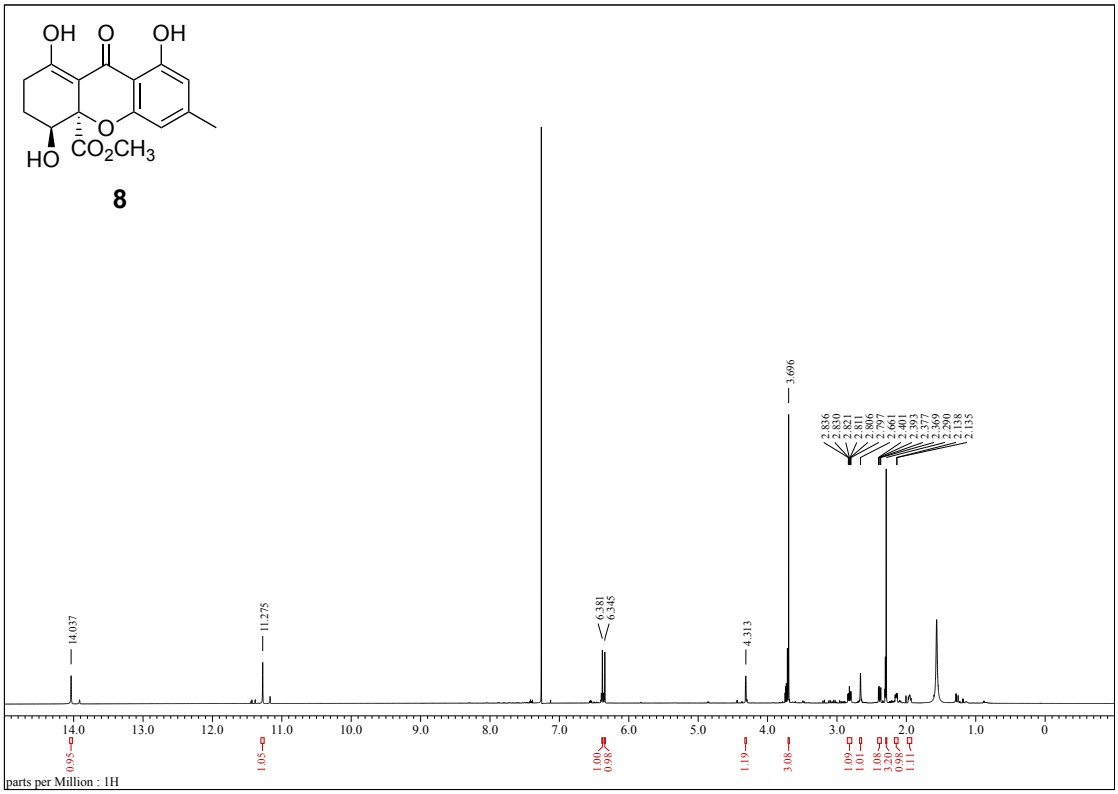


Figure S9. ^{1}H NMR spectrum of blennolide C (**8**).

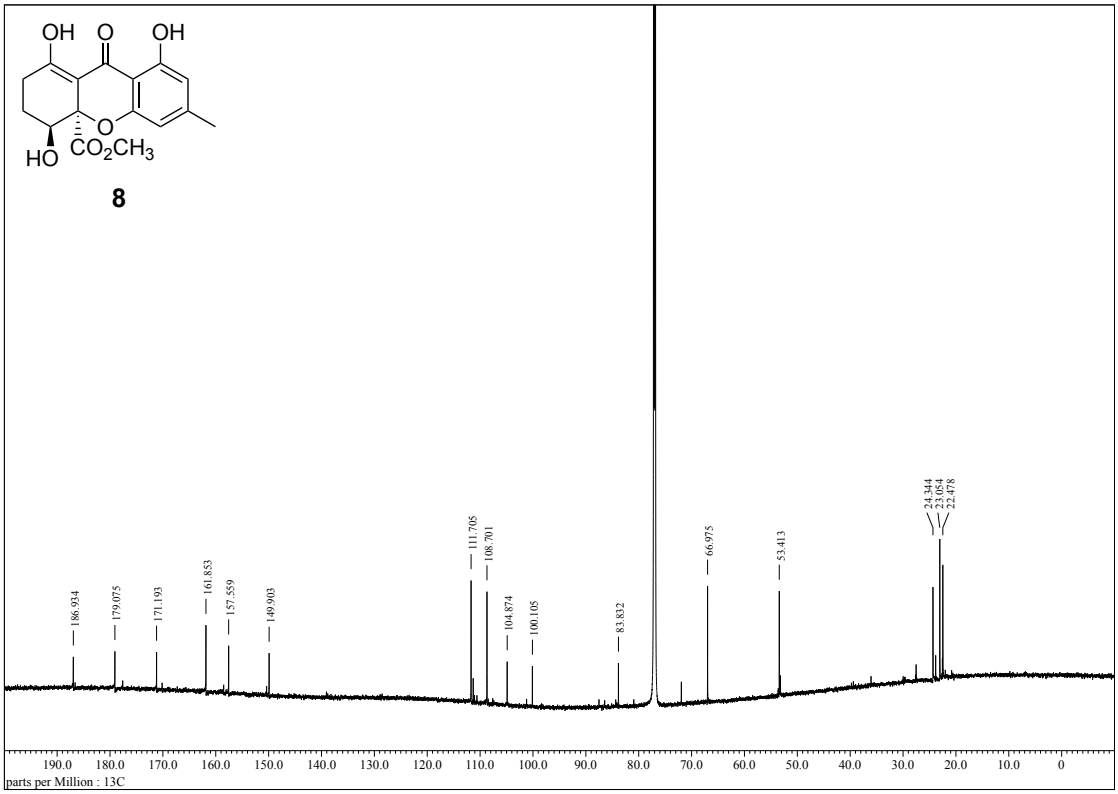


Figure S10. ^{13}C NMR spectrum of blennolide C (**8**).

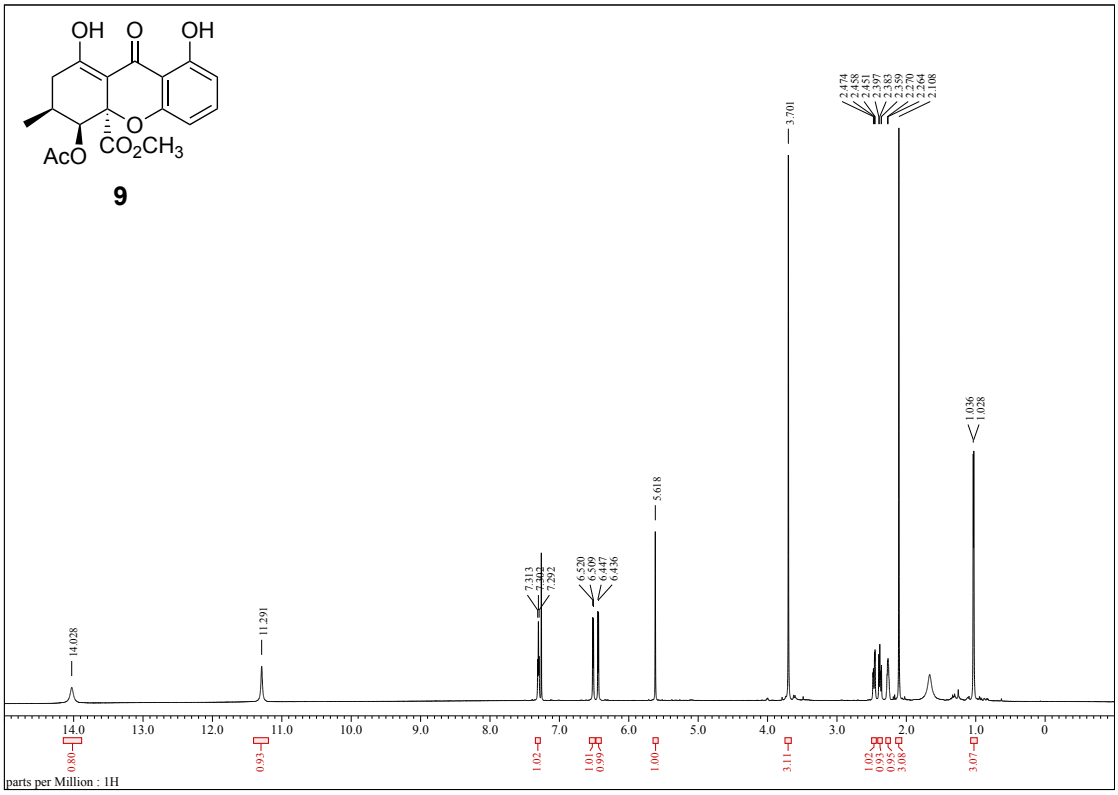


Figure S11. ^{1}H NMR spectrum of 5-acetylblennolide A (**9**).

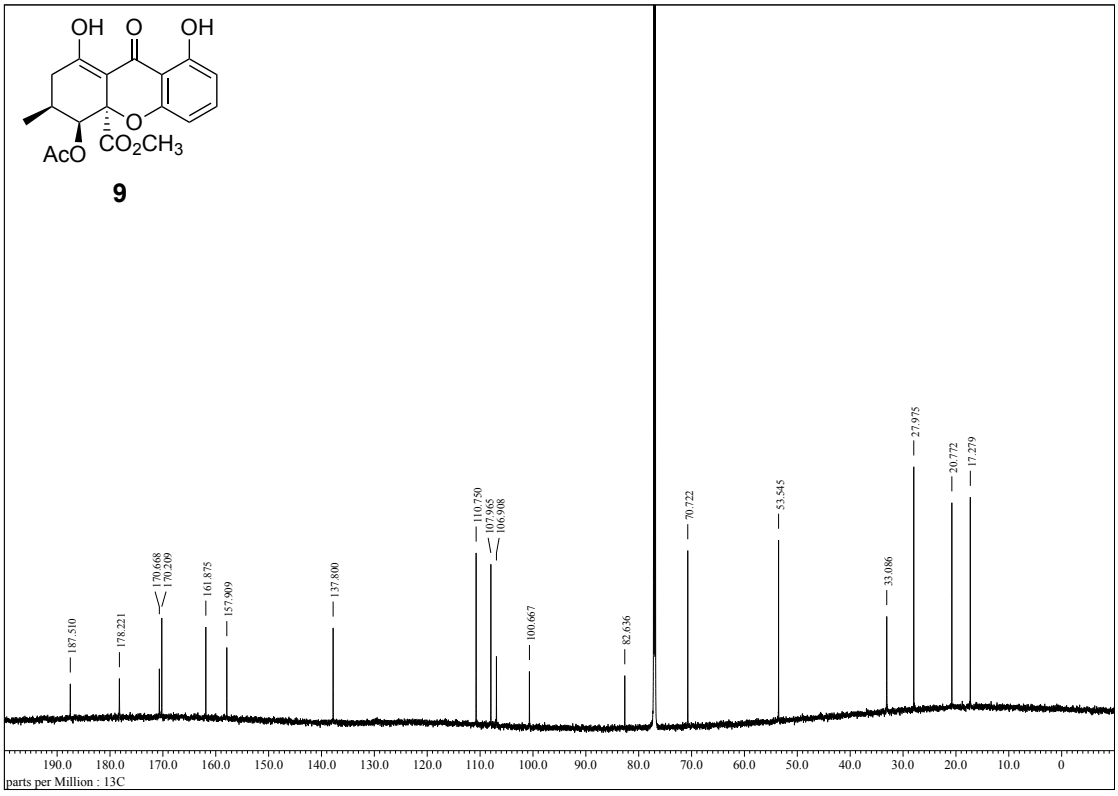


Figure S12. ^{13}C NMR spectrum of 5-acetylblennolide A (**9**).

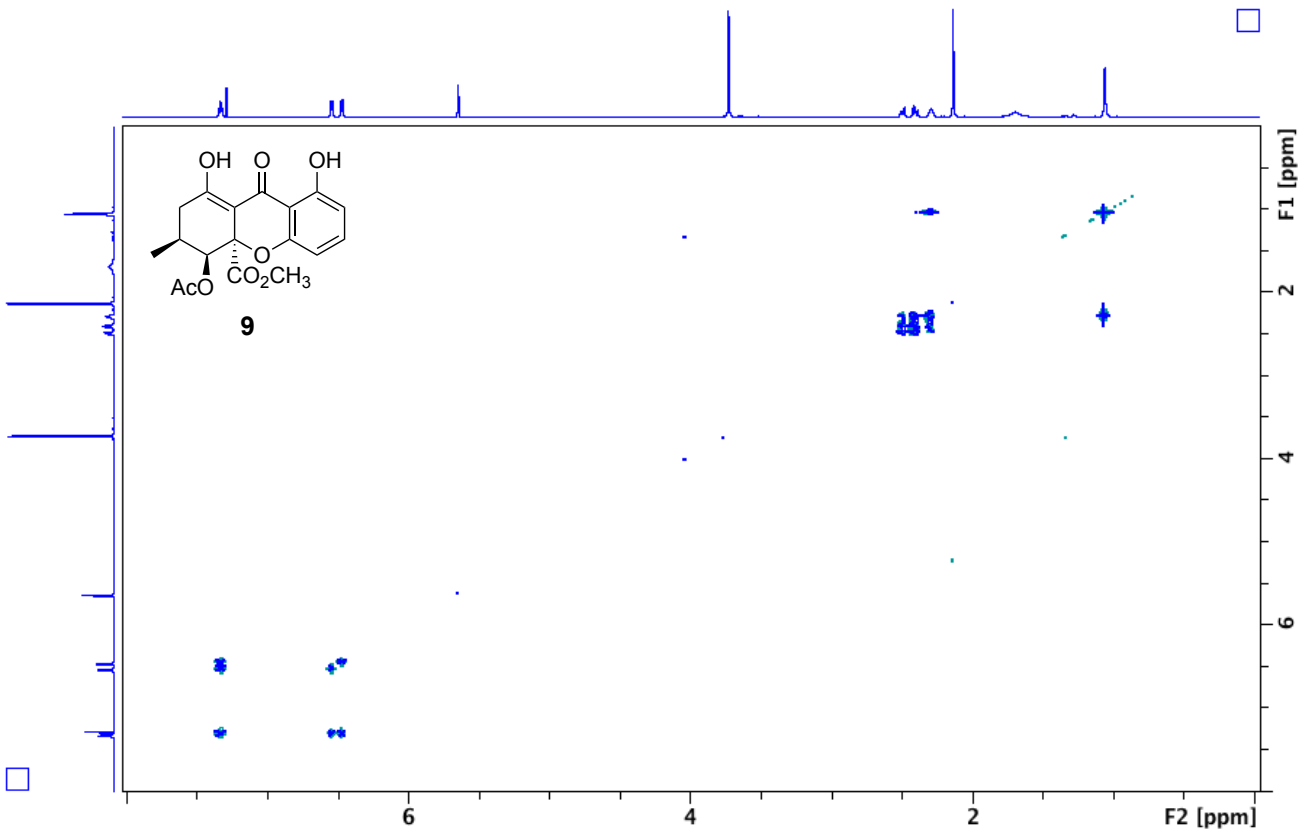


Figure S13. ^{1}H-^{1}H DQF-COSY spectrum of 5-acetylblennolide A (**9**).

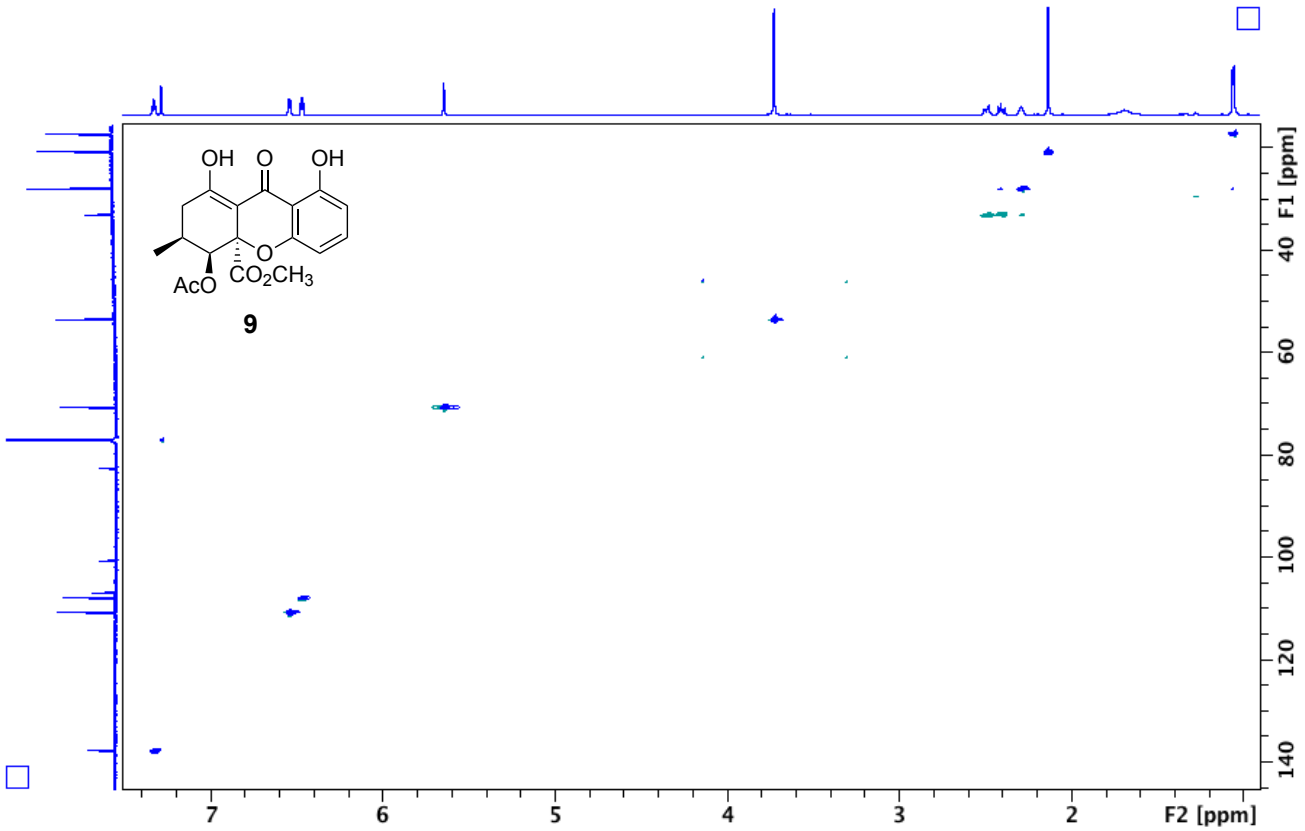


Figure S14. HSQC spectrum of 5-acetylblennolide A (**9**).

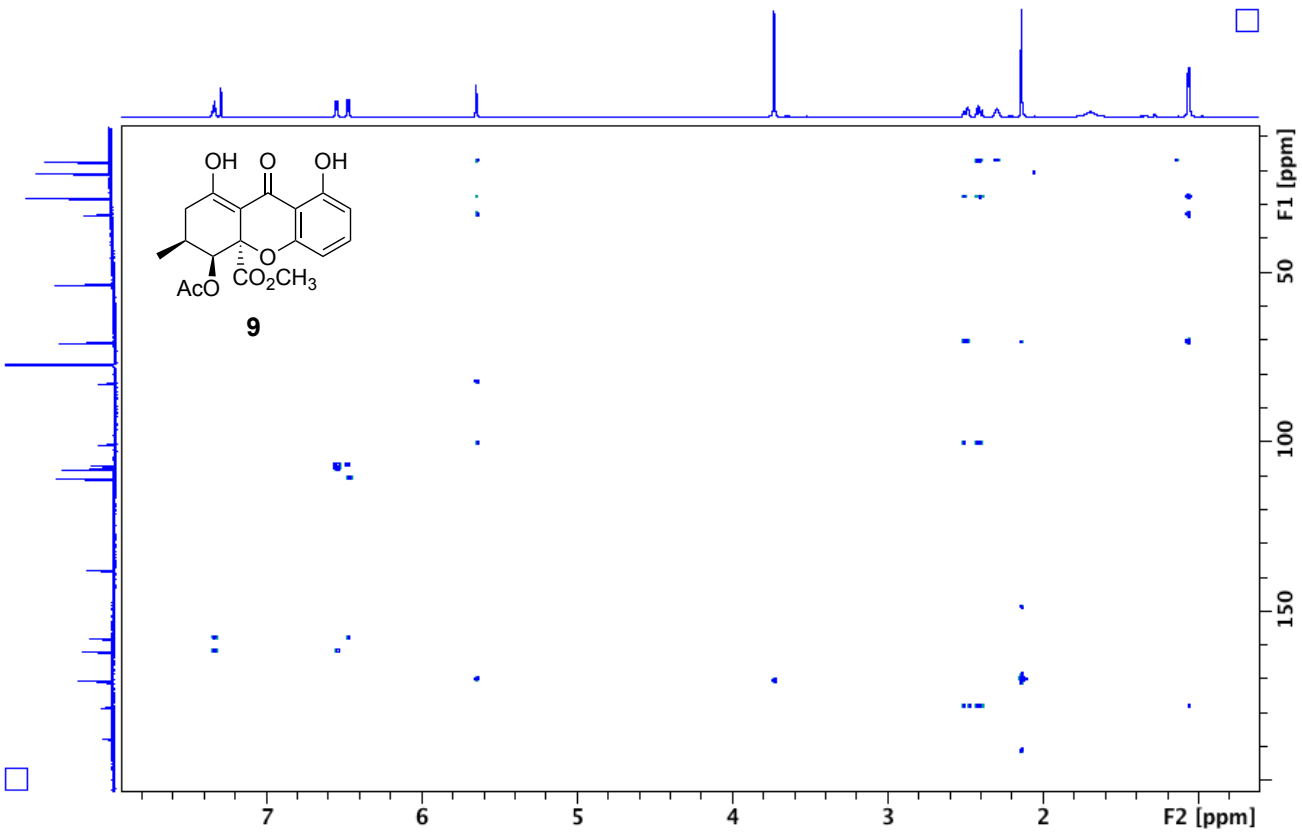


Figure S15. HMBC spectrum of 5-acetylblennolide A (**9**).

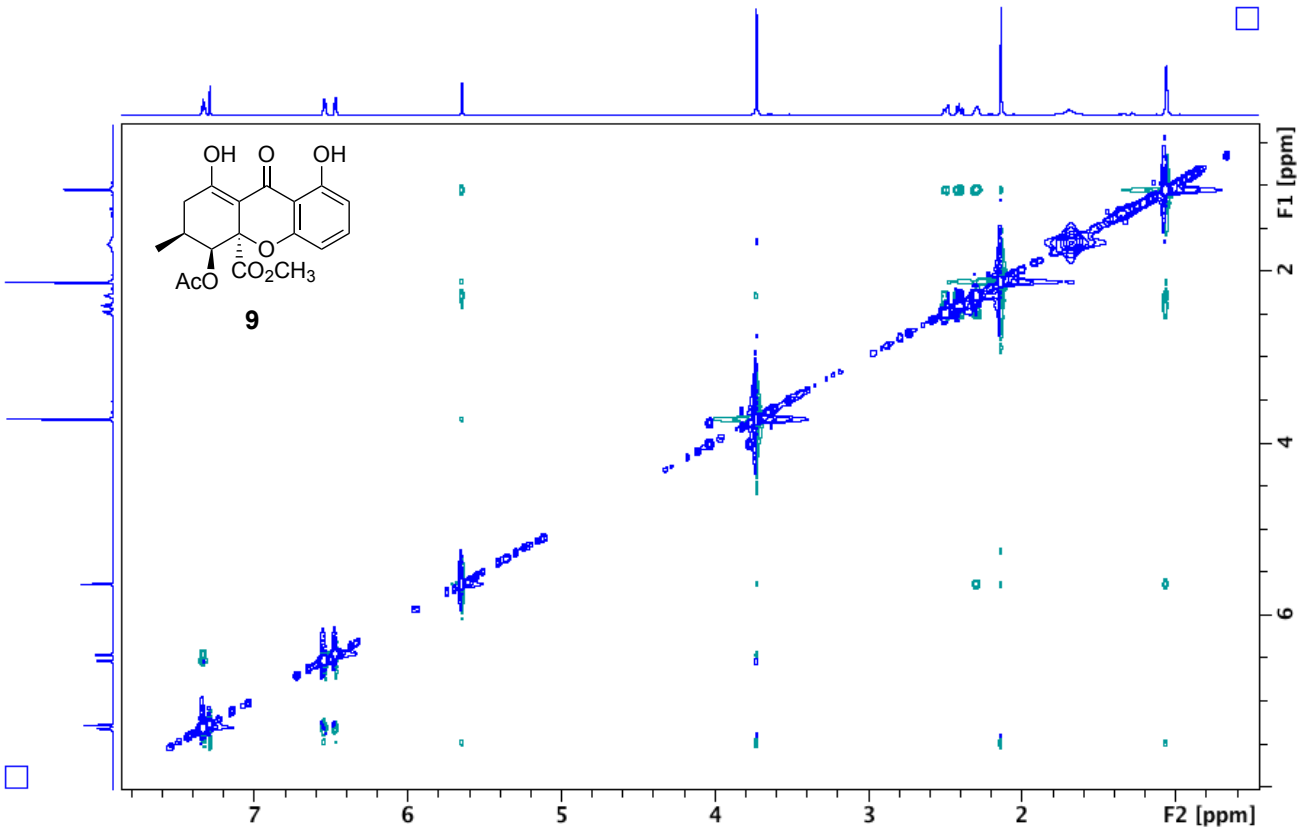


Figure S16. NOESY spectrum of 5-acetylblennolide A (**9**).

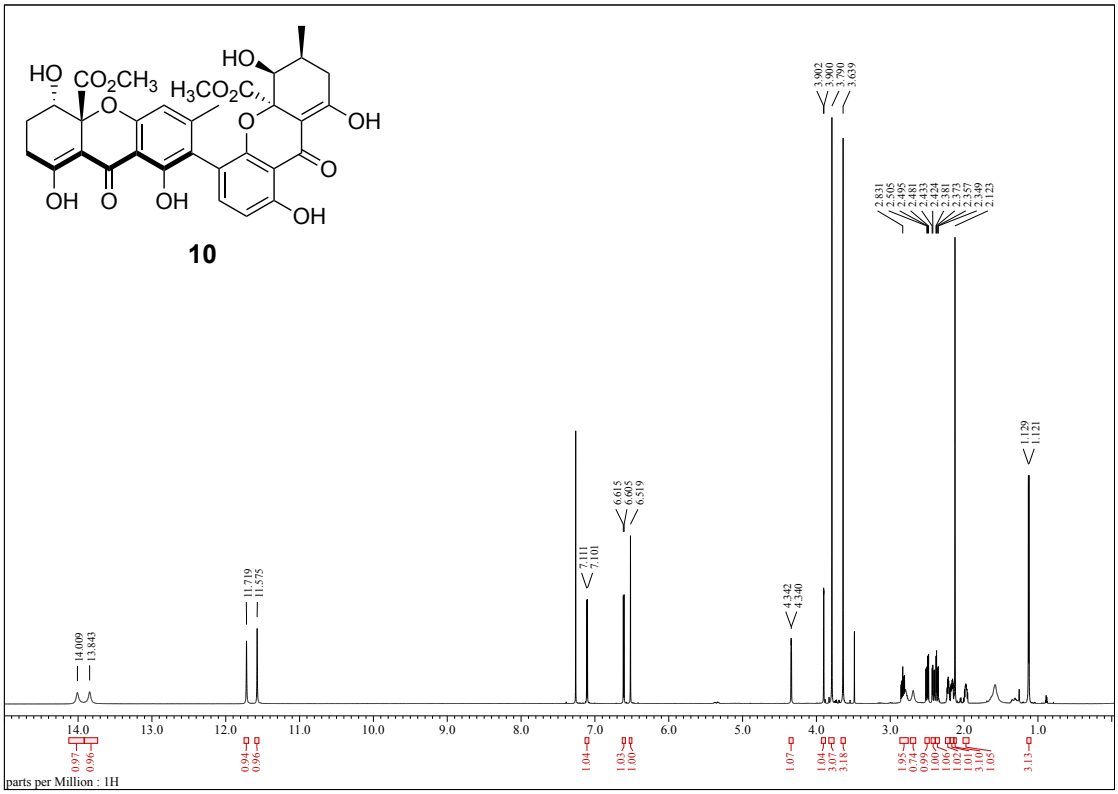


Figure S17. ^{1}H NMR spectrum of deacetylneosartorin (**10**).

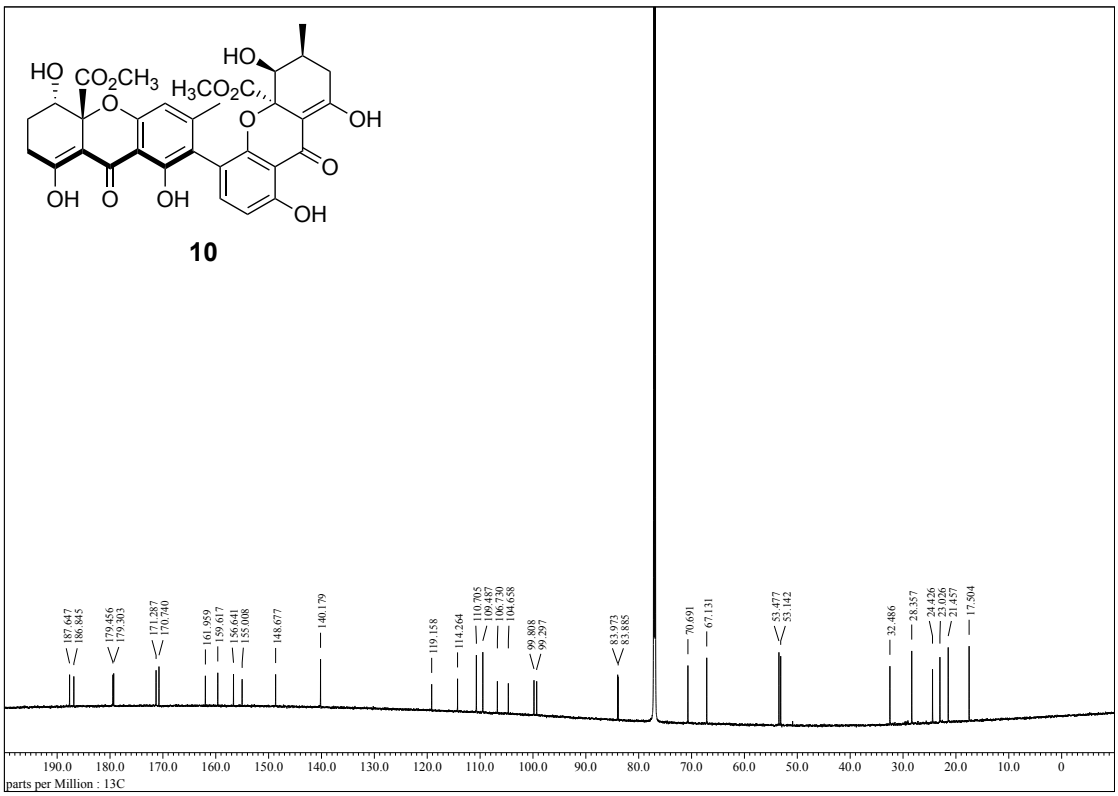


Figure S18. ^{13}C NMR spectrum of deacetylneosartorin (**10**).

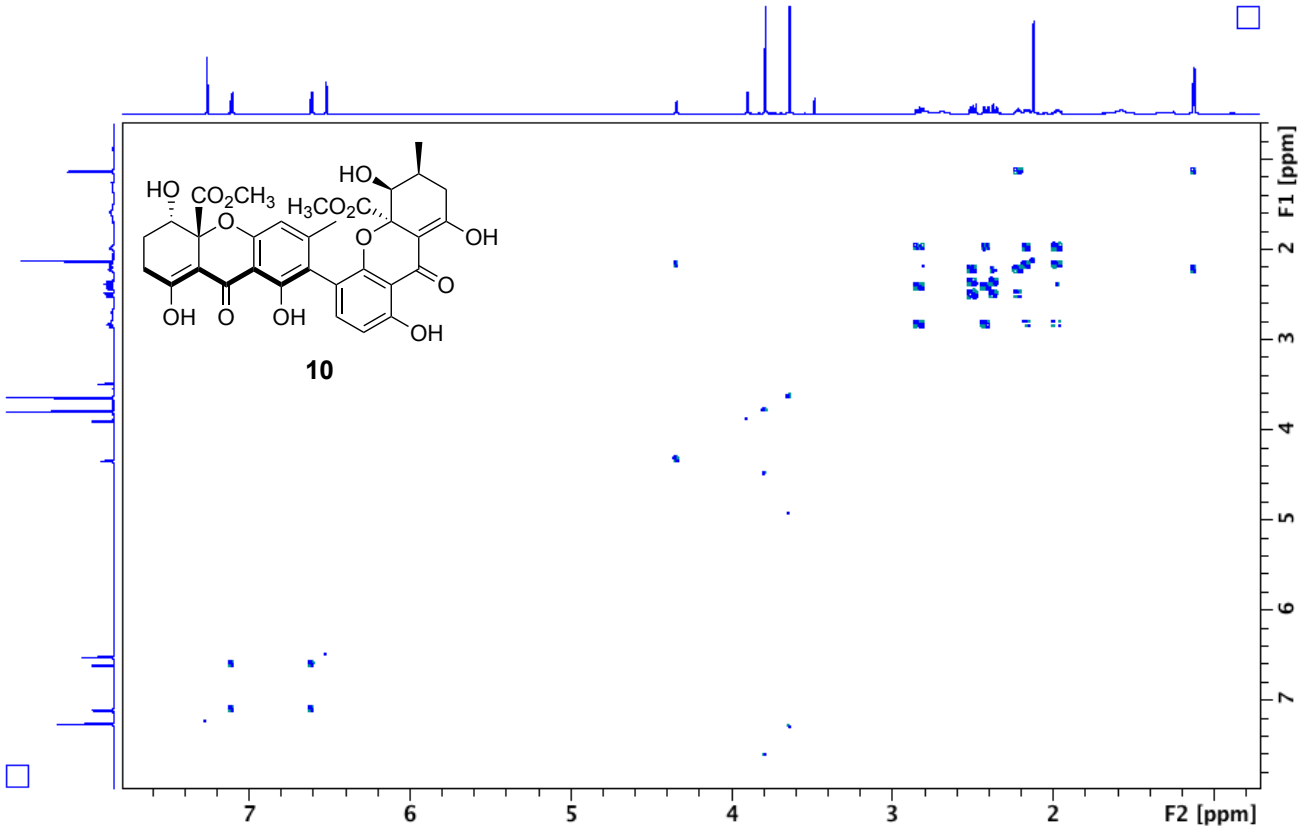


Figure S19. ^{1}H-^{1}H DQF-COSY spectrum of deacetylneosartorin (**10**).

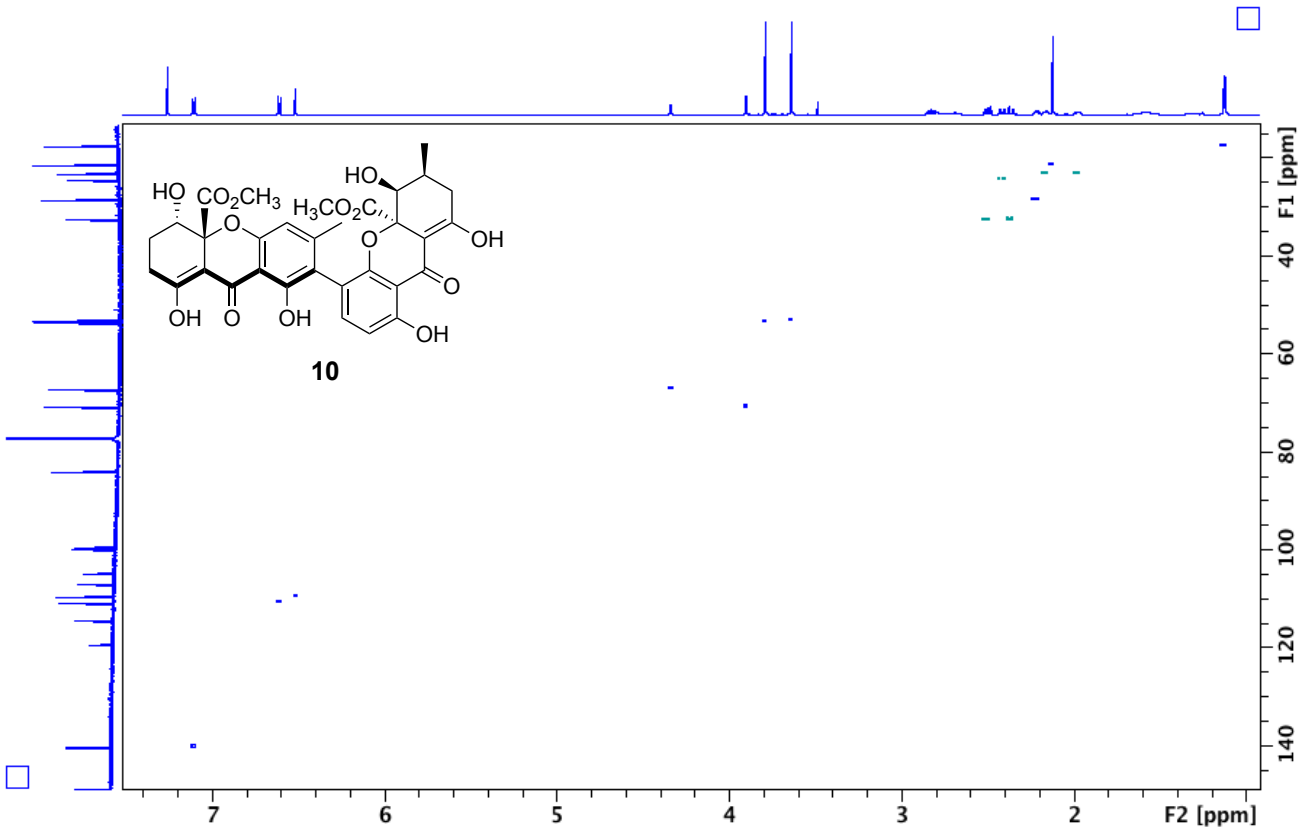


Figure S20. HSQC spectrum of deacetylneosartorin (**10**).

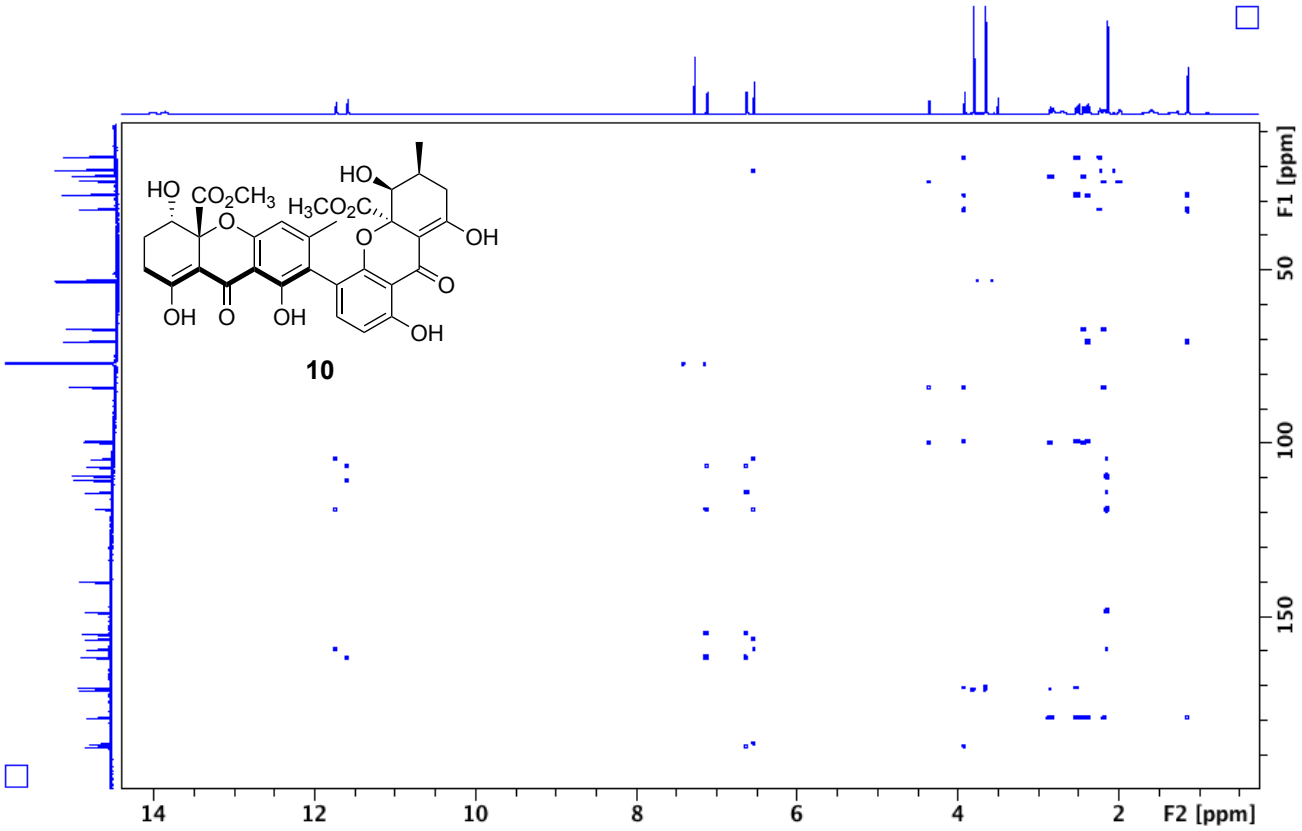


Figure S21. HMBC spectrum of deacetylneosartorin (**10**).

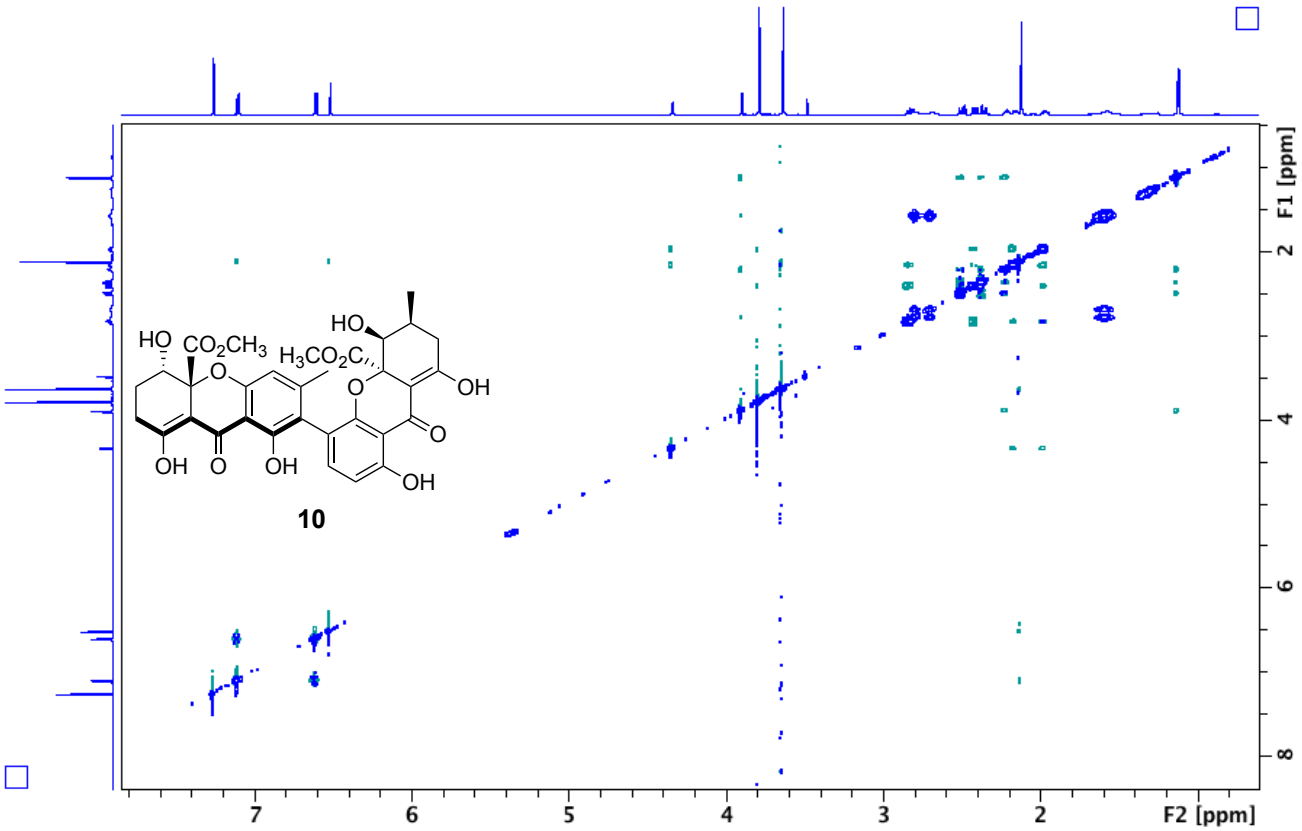


Figure S22. NOESY spectrum of deacetylneosartorin (**10**).

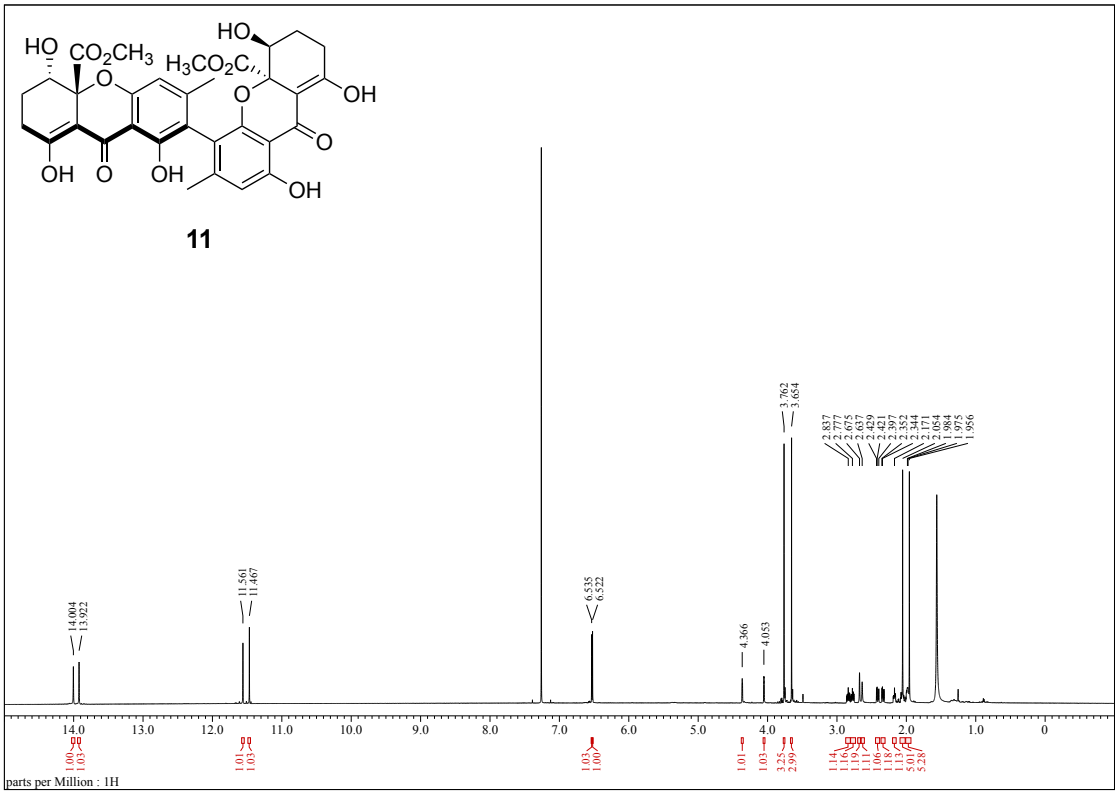


Figure S23. ^{1}H NMR spectrum of novofumigatin A (**11**).

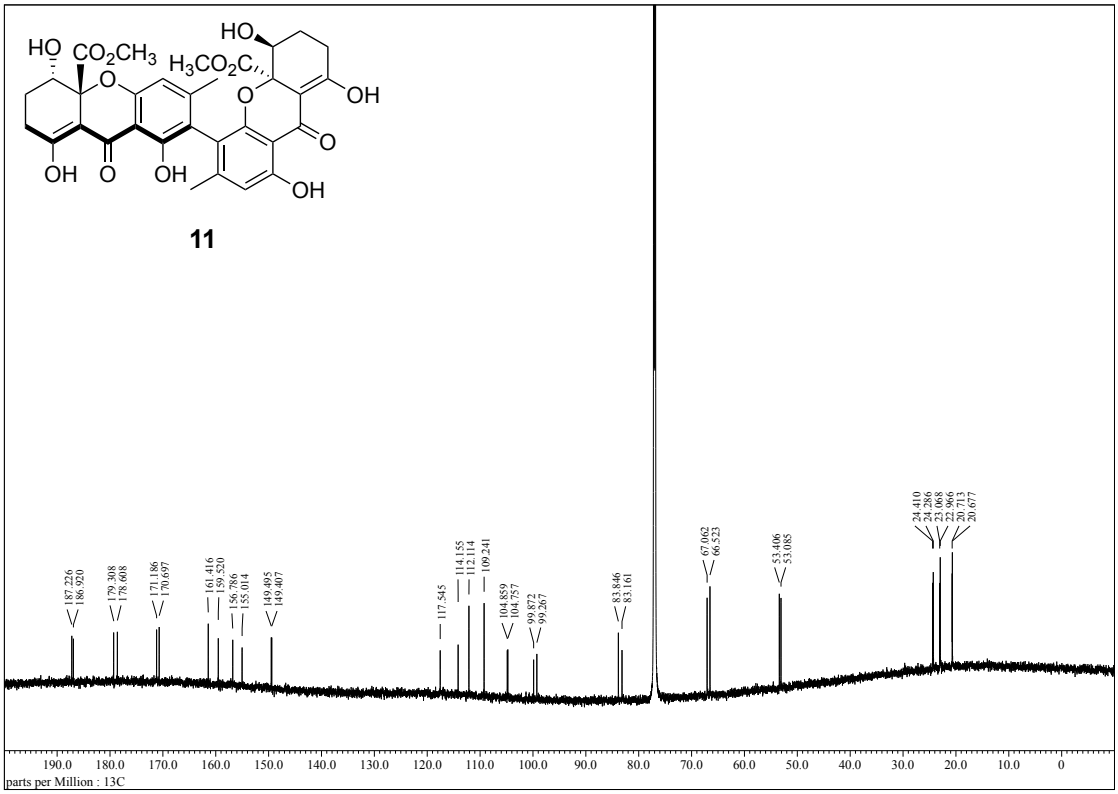


Figure S24. ^{13}C NMR spectrum of novofumigatin A (**11**).

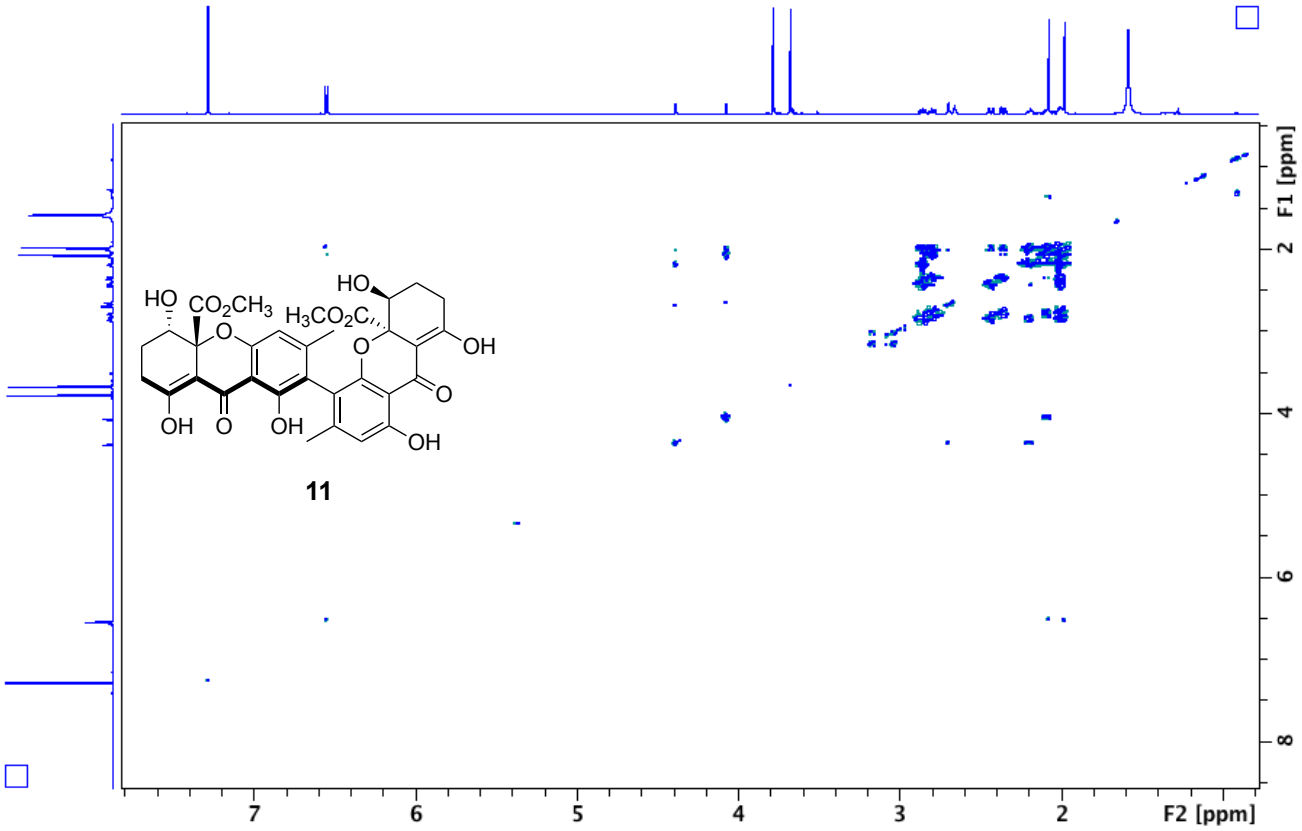


Figure S25. ^{1}H-^{1}H DQF-COSY spectrum of novofumigatin A (**11**).

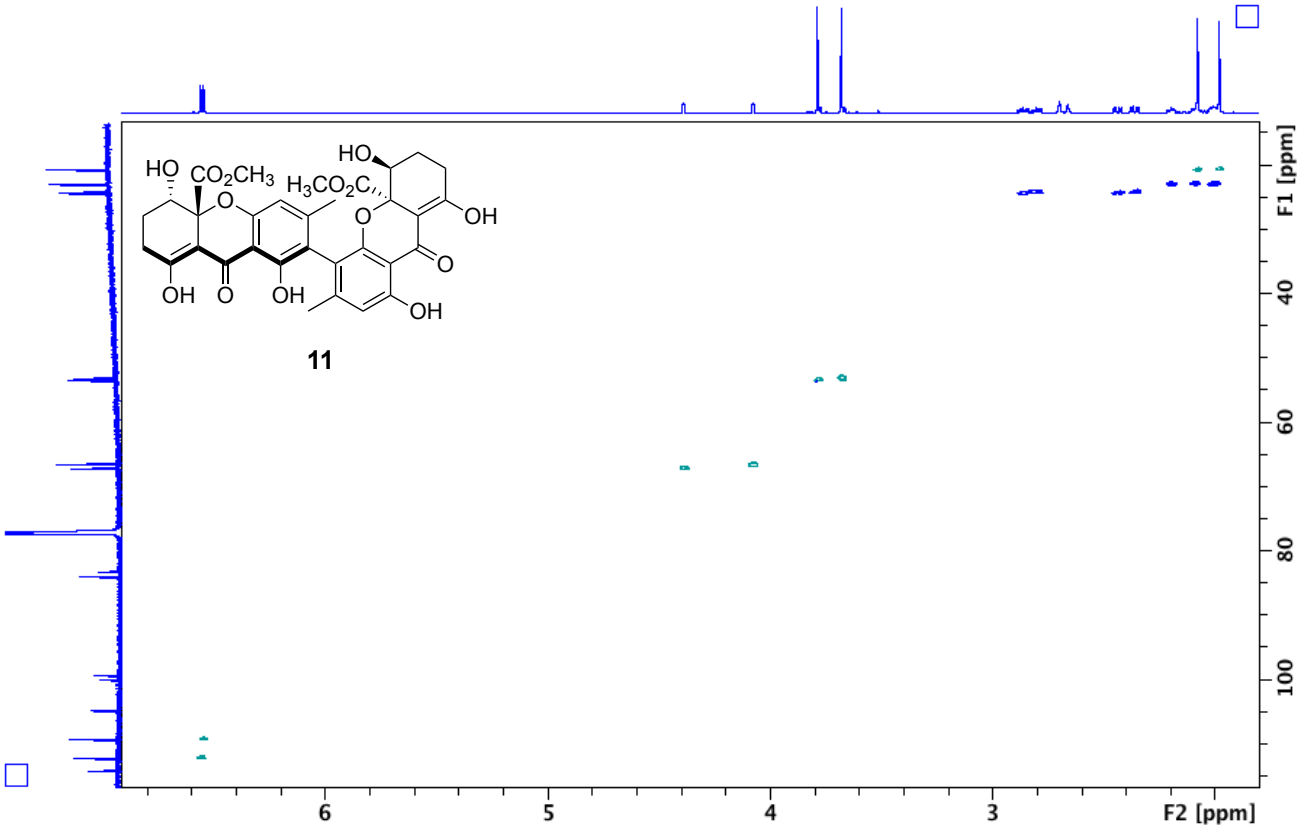


Figure S26. HSQC spectrum of novofumigatin A (**11**).

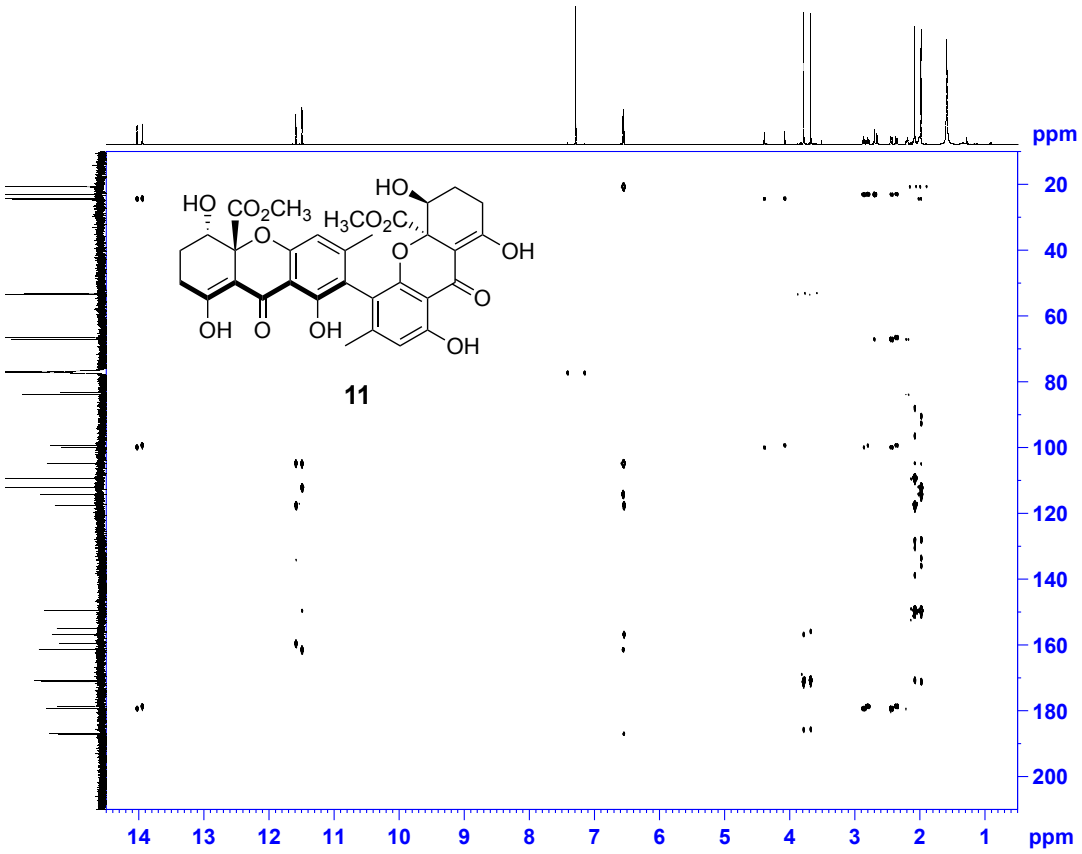


Figure S27. HMBC spectrum of novofumigatin A (**11**).

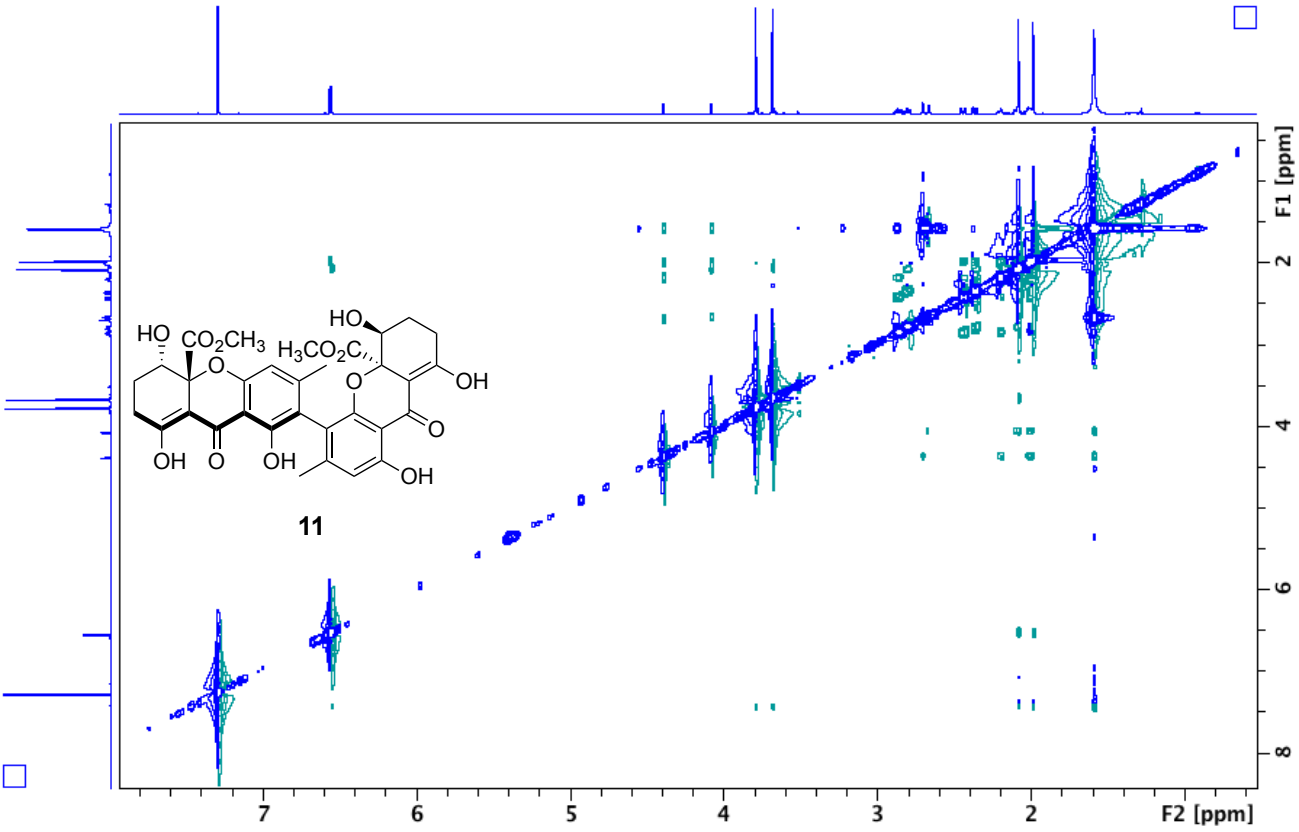


Figure S28. NOESY spectrum of novofumigatin A (**11**).

Supplementary References

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