Supporting Information

Molecular Genetic Characterization of Terreic Acid Pathway in *Aspergillus terreus*

Chun-Jun Guo,[a] Wei-Wen Sun,[a] Kenneth S. Bruno, Clay C. C. Wang

Correspondence should be addressed to C.C.C.W. (clayw@usc.edu).
[a] Equally contributed to the project.

Table of Contents

Supplemental methods

Strains and molecular manipulations

Primers used in this study are listed in Table S1. *A. terreus* strains used in this study are listed in Table S2. All targeted genes were individually replaced by the *A. fumigatus pyrG* gene (*AfpyrG*) in the *A. terreus kusA-*, *pyrG-* strain. The construction of double joint fusion PCR products, protoplast generation, and transformation were carried out according to previous procedures.[1] Diagnostic PCR of the mutant strains was carried out by using the external primers from the first round of fusion PCR. The difference in size between the gene replaced by the resistant marker and the native gene allowed us to determine if the transformants carried correct gene replacement. When the targeted gene size was similar to the marker *AfpyrG*, an internal primer that specifically binds to *AfpyrG* was used with an outside primer. When the targeted gene was successfully replaced by *AfpyrG*, a PCR fragment of around 4 kb could be amplified from the mutant strains (Figure S2 and S3).

Fermentation and LC-MS analysis

A. terreus NIH 2624 was cultivated at 30 °C on YAG plates at 10×10^6 spores per plate (D = 10 cm). After 6 days, agar was chopped into small pieces and extracted by 80 ml 1:1 CH_2Cl_2/MeOH. The extract was evaporated *in vacuo* to yield a water residue, which was suspended in 25 ml H_2O and partitioned with ethyl acetate (EtOAc, 25 ml × 2). The EtOAc layer was discarded. The pH of the water layer was then adjusted to around 2 by 6M HCl and partitioned with 25ml EtOAc. The EtOAc layer was evaporated *in vacuo*, re-dissolved in 1 ml of 20% DMSO in MeOH and a portion (10μl) was examined by high performance liquid chromatography–photodiode array detection–mass spectrometry (HPLC–DAD–MS) analysis. HPLC–MS was carried out using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with an RP C18 column (Alltech Prevail C18 3 mm 2.1 × 100 mm) at a flow rate of 125 μl/min. The condition for MS analysis was carried out as previously described.[1]

Isolation of secondary metabolites

For scale up, *A. terreus* wild type and knock-outs were cultivated at 30 °C on YAG plates (D = 15 cm, ~50 ml per plate, total volume = 2 liter) for 6 days. Agar was chopped into small pieces and then soaked in 2 L of 1:1 CH_2Cl_2/MeOH for 24 hrs. After filtration, the crude extract was evaporated *in vacuo* to yield a residue, which was then suspended in 500 ml water and partitioned with EtOAc (500 ml) three times. The EtOAc layer was discarded. The pH of the water layer was then adjusted to around 2 and extracted with EtOAc (500 ml × 2). The combined EtOAc layer was evaporated *in vacuo* to a crude extract.

Further purification was carried on by gradient HPLC on a C18 reverse phase column [Phenomenex Luna 5μm C18, 250 × 10 mm] with a flow rate of 5.0 ml/min and measured by a UV detector at 254 nm. The gradient system was MeCN (solvent B)

and 5% MeCN/H_2O (solvent A) both containing 0.05% TFA.

The gradient condition for HPLC analysis of crude extract from WT was 0-2 min 100-80% A, 2-7 min 80-66% A, 7-27 min 66-54.8% A, 27-29 min 54.8-0% A, 29-31 min 0-100% A, 31-33 min 100% A. Compound **1** (28 mg/L) was eluted at about 5 min. The gradient condition for HPLC analysis of crude extracts from ATEG_06272.1Δ and ATEG_06274.1Δ was 0-5 min 100-80% A, 5-20 min 80-60% A, 20-25 min 60-0% A, 25-30 min 0-100% A, 30-35 min 100% A. Compounds **2** (16 mg/L) and **3** (5 mg/L) were eluted at 16.0 min and 6.5 min, respectively. The gradient condition for HPLC analysis of crude extract from ATEG_06277.1Δ was 0-5 min 100-80% A, 5-10 min 80% A, 10-12 min 80-0% A, 12-14 min 0% A, 14-16 min 0-100% A, 16-18 min 100% A. Compound **4** (7.5 mg/L) was eluted at 9.0 min.

Feeding compound 5 to *atX*Δ

The 3-methylcatechol (**5**) was bought from Sigma Aldrich. It was dissolved in DMSO and added to YAG medium when the medium's temperature dropped to around 50 °C after autoclaving. The *atX*Δ mutant was cultivated at 30 °C on YAG containing 0.2 mg/ml compound **5**. The WT strain and the *atX*Δ mutant were also cultivated on ordinary YAG as control. After 6 days cultivation, the followed extraction and LC-MS analysis were similar as described above.

Spectral data of compounds

HRESIMS spectra were obtained on an Agilent Technologies 1200 series high-resolution mass spectrometer. NMR spectra were collected on a Varian Mercury Plus 400 spectrometer.

Terreic acid (1), 6-methylsalicylic acid (2), terremutin (3) and (2E, 4Z)-2-methyl-2, 4-hexadienedioic acid (4). For UV-Vis and ESIMS spectra, see Figure S1; For NMR spectra, see Table S3, S4, S5 and S6 respectively. The molecular formula of compound **4** was determined by HRESIMS to be $C_7H_7O_4$ $[M - H]^-$ (*m/z* 155.0341, calcd for $C_7H_7O_4$ 155.0350). The NMR data of compounds **1, 2, 3** were in good agreement with the published data. [2,3,4]

Supplemental references

1.Guo, C.-J.; Knox, B. P.; Chiang, Y.-M.; Lo, H.-C.; Sanchez, J. F.; Lee, K.-H.; Oakley, B. R.; Bruno, K. S.; Wang, C. C. C. *Org. Lett.* **2012**, *14*, 5684.
2. Findlay, J. A.; Radics, L. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2071.
3. Fujii, I.; Ono, Y.; Tada, H.; Gomi, K.; Ebizuka, Y.; Sankawa, U. *Mol. Gen. Genet.* **1996**, *253*, 1.
4. Dewi, R. T.; Tachibana, S.; Itoh, K. *J. of Microb. Biochem. Technol.* **2012,** *04*, 10.

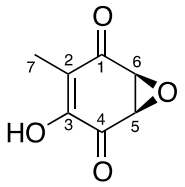
Table S1. Primers used in this study

primer	Sequence (5′→3′)
ATEG_06270.1P1	GGC GAT TGG TCT TTA ATC TG
ATEG_06270.1P2	AGA CAT GGC GTC TAT CTC GT
ATEG_06270.1P3	CGA AGA GGG TGA AGA GCA TTG GGA TCA CAG TAT TCG CCA GT
ATEG_06270.1P4	CAT CAG TGC CTC CTC TCA GAC AGA CAA CAA CCA TCA GGT CGT G
ATEG_06270.1P5	GAT TCG ACG GTG AAG AAC TC
ATEG_06270.1P6	GCC TCT ATG GTC GAA CAA TC
ATEG_06271.1P1	CCA CCA CAG TGT GAA ACC TA
ATEG_06271.1P2	CAA ATG CAA GAC TCC TGT GA
ATEG_06271.1P3	CGA AGA GGG TGA AGA GCA TTG GGG TTG TTA TCA CTG CTG CT
ATEG_06271.1P4	CAT CAG TGC CTC CTC TCA GAC AGG CTA TGG CAG ACT ACA GAA C
ATEG_06271.1P5	GTC ATG ACC TAT CCC ATT GA
ATEG_06271.1P6	CCT TAT ACC GAT GGA TGT CG
ATEG_06272.1P1	CCA GAG TTA CGA CGA TTT C
ATEG_06272.1P2	GTC CTT CTC AAC CTG TGT AT
ATEG_06272.1P3	CGA AGA GGG TGA AGA GCA TTG TAG GTC ACA CAC AAG ACA CT
ATEG_06272.1P4	CAT CAG TGC CTC CTC TCA GAC AGC CAT AAA GCT TCC AGA AGA G
ATEG_06272.1P5	GGG ACA CTA CAC GAC TGT AT
ATEG_06272.1P6	GTA TGT CAC AGG GAA CAA TG
ATEG_06273.1P1	GGT CTC ATT CTC GAC TTT GC
ATEG_06273.1P2	ATT CTC GAC TTT GCT GAG GA
ATEG_06273.1P3	CGA AGA GGG TGA AGA GCA TTG ACA ATG ATA TCG CGA TCC TG
ATEG_06273.1P4	CAT CAG TGC CTC CTC TCA GAC AGC GTT ACG ACG CTC AAG TTA G
ATEG_06273.1P5	GGT ACC AAA CAG CTT CGT CT
ATEG_06273.1P6	CCG AAA GAG GTA CCA AAC AG
ATEG_06274.1P1	TGT ACC TCA TTC GTC CCA TT
ATEG_06274.1P2	CCA TTC CAA TAG GGT TGC TA
ATEG_06274.1P3	CGA AGA GGG TGA AGA GCA TTG TCC CAG TAT GGA GAA CTC GT
ATEG_06274.1P4	CAT CAG TGC CTC CTC TCA GAC AGA ACT TAT GGG GCT TTC TGA
ATEG_06274.1P5	ACA TCT GGT GGG ATG GTT T
ATEG_06274.1P6	ACC CAC ACT GAC CTG GAG TT
ATEG_06275.1P1	CGC CTA TCT CTC CCC TTA GT
ATEG_06275.1P2	CGG ATG TCT AAA AGG AGC AG
ATEG_06275.1P3	CGA AGA GGG TGA AGA GCA TTG ATG CCT GAA GAG GAG AGA TG
ATEG_06275.1P4	CAT CAG TGC CTC CTC TCA GAC AGG CTG ACG GTT TCT CCT TAT G
ATEG_06275.1P5	TTT CTG AGG TGA TGG GCT AC
ATEG_06275.1P6	CTG TTC GAT GAG TGT GGC TA
ATEG_06276.1P1	ATG CCT GAA GAG GAG AGA TG
ATEG_06276.1P2	GCG TCG CCT CTA CAT CTT AT
ATEG_06276.1P3	CGA AGA GGG TGA AGA GCA TTG AGG ATG GCC TAA GCA CGT A
ATEG_06276.1P4	CAT CAG TGC CTC CTC TCA GAC AGC CAA ATG GAT TGA GTG TGT G
ATEG_06276.1P5	GAA GTG GCT ATC AGC TTT CG
ATEG_06276.1P6	GTG CCT TGC ATA CAG GAA GT

ATEG_06277.1P1	CCA AAT GGA TTG AGT GTG TG
ATEG_06277.1P2	GTG TGT GTA TGC CTT CGA GA
ATEG_06277.1P3	CGA AGA GGG TGA AGA GCA TTG GTG CCT TGC ATA CAG GAA GT
ATEG_06277.1P4	CAT CAG TGC CTC CTC TCA GAC AGG AGT TGG GTG GAA GAT TGA G
ATEG_06277.1P5	GTC CTC CTG GAA CTG CTC TA
ATEG_06277.1P6	AGG AAA CGT CAG TCC CTT CT
ATEG_06278.1P1	AGA GTC ACC ACA AGC GAC AT
ATEG_06278.1P2	CCG ATT GGT TAA GAA TGG AC
ATEG_06278.1P3	CGA AGA GGG TGA AGA GCA TTG GGC TGG TTG AAC AAT AGC AG
ATEG_06278.1P4	CAT CAG TGC CTC CTC TCA GAC AGG TCG TTC TTA TCG CAA CCA T
ATEG_06278.1P5	CAT CTC CCG AAG CAC TCT AC
ATEG_06278.1P6	GCA AGC TGC CTT ACA TCA AT
ATEG_06279.1P1	TCT GCG TCA GCT ATG GT CTA
ATEG_06279.1P2	ATC CCT GGA AAA CTG TTG G
ATEG_06279.1P3	CGA AGA GGG TGA AGA GCA TTG GCA GTC AAT GAG TTG CAA TG
ATEG_06279.1P4	CAT CAG TGC CTC CTC TCA GAC AGA CGG CTG AAT TAG TTC GTG T
ATEG_06279.1P5	GAC TAG TGA TCG GCA GGA TG
ATEG_06279.1P6	AAG CCG AGG TAA TGA TGC T
ATEG_06280.1P1	TAT CGG CAA AGA CAA GAT CG
ATEG_06280.1P2	GTA CGA GGT CAT GTC TGG TG
ATEG_06280.1P3	CGA AGA GGG TGA AGA GCA TTG CCG CCA ATA CTC TGT CTT TC
ATEG_06280.1P4	CAT CAG TGC CTC CTC TCA GAC AGT AGA CGT GCC AAA CTG TGA C
ATEG_06280.1P5	CAG AGG GTA TCG GTC ATG TT
ATEG_06280.1P6	GAG CGG TGT CCA TTG TTA CT
ATEG_06281.1P1	AGC TCG TTT ACC CCT GTT GG
ATEG_06281.1P2	CAG GCA TCA AAA GCC GAA GG
ATEG_06281.1P3	CGA AGA GGG TGA AGA GCA TTG GTT GCA CAC GTC CTC TCT CA
ATEG_06281.1P4	GCA TCA GTG CCT CCT CTC AGA CAG CTG GTG CTA GCT GGC TTC TT
ATEG_06281.1P5	GGC TCG CCA AGG AGT AAT CA
ATEG_06281.1P6	ACT CTG TAC TCC GTC CCT CC
ATEG_06282.1P1	TGC ACA CCA ACA CTC CTG TA
ATEG_06282.1P2	ACG TAG GAC CAA CCC TGA TAG T
ATEG_06282.1P3	CGA AGA GGG TGA AGA GCA TTG GTG ACT GCG GAT GGA AGT GT
ATEG_06282.1P4	GCA TCA GTG CCT CCT CTC AGA CAG GAA CCA AAC GCT GTC ACT GG
ATEG_06282.1P5	CAG CCA TAA ACT CCA CGA AGC
ATEG_06282.1P6	CTC ACA ATC ATC GGC TCC CC
Internal primer used for diagnostic PCR	
AfpyrGR	CGG GAG CAG CGT AGA TGC C

Table S2. *A. terreus* strains used in this study

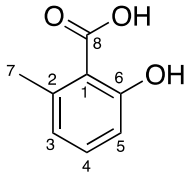
Fungal strain or Transformants	Gene mutation(s)	Genotype
Aspergillus terreus NIH2624	-	wildtype
CW6061.1, CW6061.2, CW6061.3	ATEG_06270.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06270.1*:: AfpyrG*
CW6062.1, CW6062.2	ATEG_06271.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06271.1*:: AfpyrG*
CW6063.1, CW6063.2	ATEG_06272.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06272.1*:: AfpyrG*
CW6064.1, CW6064.2, CW6064.3	ATEG_06273.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06273.1*:: AfpyrG*
CW6065.1, CW6065.2, CW6065.3	ATEG_06274.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06274.1*:: AfpyrG*
CW6066.1, CW6066.2	ATEG_06275.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06275.1*:: AfpyrG*
CW6067.1, CW6067.2, CW6067.3	ATEG_06276.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06276.1*:: AfpyrG*
CW6068.1, CW6068.2, CW6068.3	ATEG_06277.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06277.1*:: AfpyrG*
CW6069.1, CW6069.2, CW6069.3	ATEG_06278.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06278.1*:: AfpyrG*
CW6070.2, CW6070.2, CW6070.3	ATEG_06279.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06279.1*:: AfpyrG*
CW6071.1, CW6071.2, CW6071.3	ATEG_06280.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06280.1*:: AfpyrG*
CW6072.1, CW6072.2	ATEG_06281.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06281.1*:: AfpyrG*
CW6073.1, CW6073.2, CW6073.3	ATEG_06282.1Δ	*nkuA:: hph*; *pyrG-*, ATEG_06282.1*:: AfpyrG*



1

Table S3. ^{1}H and ^{13}C NMR data for compound **1**.
(400 MHz and 100 MHz in acetone-d_6)

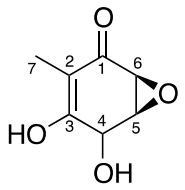
Position	δ H (*J* in Hz)	δ C
1		188.7, C
2		119.9, C
3		154.4, C
4		192.2, C
5	3.97, d (4.0)	52.9, CH
6	3.87, d (4.0)	54.7, CH
7	1.83, s	8.7, CH_3



2

Table S4. ^{1}H and ^{13}C NMR data for compound **2**.
(400 MHz and 100 MHz in acetone-d_6)

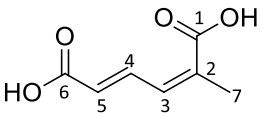
Position	δ H (*J* in Hz)	δ C
1		113.2, C
2		164.2, C
3	6.73, d (7.6)	116.1, CH
4	7.29, t (8.0)	135.0, CH
5	6.78, d (8.8)	123.5, CH
6		142.6, C
7	2.56, s	24.0, CH_3
8		174.2, C
8-OH	11.2, br s	



3

Table S5. ^{1}H and ^{13}C NMR data for compound **3**
(400 MHz and 100 MHz in acetone-d_6)

Position	δ H (*J* in Hz)	δ C
1		Not Identified
2		108.9, C
3		164.1, C
4	4.63, s	66.0, CH
5	3.66, dd (3.2, 1.2)	52.3, CH
6	3.37, dd (3.6, 1.2)	55.3, CH
7	1.64, s	7.5, CH_3



4

Table S6. ^{1}H and ^{13}C NMR data for compound **4**.
(400 MHz and 100 MHz in acetone-d_6)

Position	δ H (*J* in Hz)	δ C	HMBC[a]	COSY	NOESY
1		168.4, C			
2		136.1, C			
3	6.67, d (12.0)	136.3, CH	1, 5, 7	H-4, H-5	H-4, H_3-7
4	8.22, dd (15.2, 11.6)	141.5, CH	3, 5, 6	H-3, H-5	H-3, H-5
5	6.06, d (15.6)	126.6, CH	2, 3, 4, 6	H-3, H-5	H-4
6		168.0, C			
7	2.10, s	21.4, CH_3	1, 2, 3		H-3

[a]HMBC correlations are from proton(s) to the indicated carbon.

Table S7. AtX (ATEG_06275.1) homologs with sequence identity over 40%.

Seq ID	Species	Amino acids	Identity/Similarity (%)
gb|AAK48943.1|AF360398_1	*Byssochlamys nivea*	1778	64/77
gb|ADF47133.1|	*Penicillium griseofulvum*	1774	63/75
ref|XP_002564832.1|	*Penicillium chrysogenum Wisconsin 54-1255*	1776	63/75
gb|EOD48890.1|	*Neofusicoccum parvum UCRNP2*	1796	63/75
ref|XP_001273093.1|	*Aspergillus clavatus NRRL 1*	1720	64/76
emb|CDM36380.1|	*Penicillium roqueforti*	1776	62/76
gb|EKG18983.1|	*Macrophomina phaseolina MS6*	1754	63/75
gb|AAC23536.1|	*Aspergillus parasiticus*	1766	60/75
gb|EIT77765.1|	*Aspergillus oryzae 3.042*	1766	60/75
dbj|BAE65442.1|	*Aspergillus oryzae RIB40*	1766	60/74
gb|EOD47055.1|	*Neofusicoccum parvum UCRNP2*	1779	57/73
gb|AFP89390.1|	*Cladosporium phlei*	1792	57/72
ref|XP_002849666.1|	*Arthroderma otae CBS 113480*	1773	56/72
gb|ERF77015.1|	*Endocarpon pusillum Z07020*	1801	56/71
ref|XP_003015543.1|	*Arthroderma benhamiae CBS 112371*	1776	55/71
ref|XP_003169413.1|	*Arthroderma gypseum CBS 118893*	1776	55/71
ref|XP_003019112.1|	*Trichophyton verrucosum HKI 0517*	1747	55/71
ref|XP_003233709.1|	*Trichophyton rubrum CBS 118892*	1774	55/71
gb|EKG12695.1|	*Macrophomina phaseolina MS6*	1738	55/71
gb|AAX35547.1|	*Glarea lozoyensis*	1791	55/70
gb|EYE91246.1|	*Aspergillus ruber CBS 135680*	1782	55/70
gb|ETS75984.1|	*Pestalotiopsis fici W106-1*	1796	54/71
ref|XP_001791162.1|	*Phaeosphaeria nodorum SN15*	1720	54/71
gb|EGX43547.1|	*Arthrobotrys oligospora ATCC 24927*	1761	53/69
gb|AAS98200.1|	*Aspergillus ochraceus*	1766	50/67

ref|XP_001826575.2|	*Aspergillus oryzae RIB40*	1701	59/74
ref|XP_001402408.2|	*Aspergillus niger CBS 513.88*	1787	48/64
gb|EHA22196.1|	*Aspergillus niger ATCC 1015*	1779	48/64
ref|XP_002560460.1|	*Penicillium chrysogenum Wisconsin 54-1255*	1783	47/63
gb|AAB49684.1|	*Penicillium griseofulvum*	1783	47/64
gb|EKV11531.1|	*Penicillium digitatum PHI26*	1785	47/63
gb|EGD94108.1|	*Trichophyton tonsurans CBS 112818*	1685	55/72
gb|EGE06642.1|	*Trichophyton equinum CBS 127.97*	1579	53/71
gb|AAR90279.1|	*Bipolaris maydis*	1766	41/59
ref|WP_003879650.1|	*Mycobacterium fortuitum*	1723	42/58
ref|YP_001071753.1|	*Mycobacterium sp. JLS*	1704	43/57
ref|YP_640635.1|	*Mycobacterium sp. MCS*	1704	42/57
ref|WP_003928522.1|	*Mycobacterium vaccae*	1712	42/57
emb|CDO33136.1|	*Mycobacterium vulneris*	1716	42/57
ref|YP_004522781.1|	*Mycobacterium sp. JDM601*	1718	42/58
gb|ACN64831.1|	*Streptomyces diastatochromogenes*	1739	43/59
ref|WP_005628551.1|	*Mycobacterium hassiacum*	1712	42/57
ref|YP_004076616.1|	*Mycobacterium gilvum Spyr1*	1735	42/57
ref|YP_954639.1|	*Mycobacterium vanbaalenii PYR-1*	1744	43/57
ref|YP_001133955.1|	*Mycobacterium gilvum PYR-GCK*	1698	42/57
ref|YP_008908204.1|	*Mycobacterium neoaurum VKM Ac-1815D*	1721	42/57
ref|YP_005002033.1|	*Mycobacterium rhodesiae NBB3*	1725	41/57
ref|WP_003926867.1|	*Mycobacterium thermoresistibile*	1758	41/56
ref|WP_003086619.1|	*Amycolatopsis vancoresmycina*	1742	42/57
gb|AAZ77673.1|	*Streptomyces antibioticus*	1756	41/56

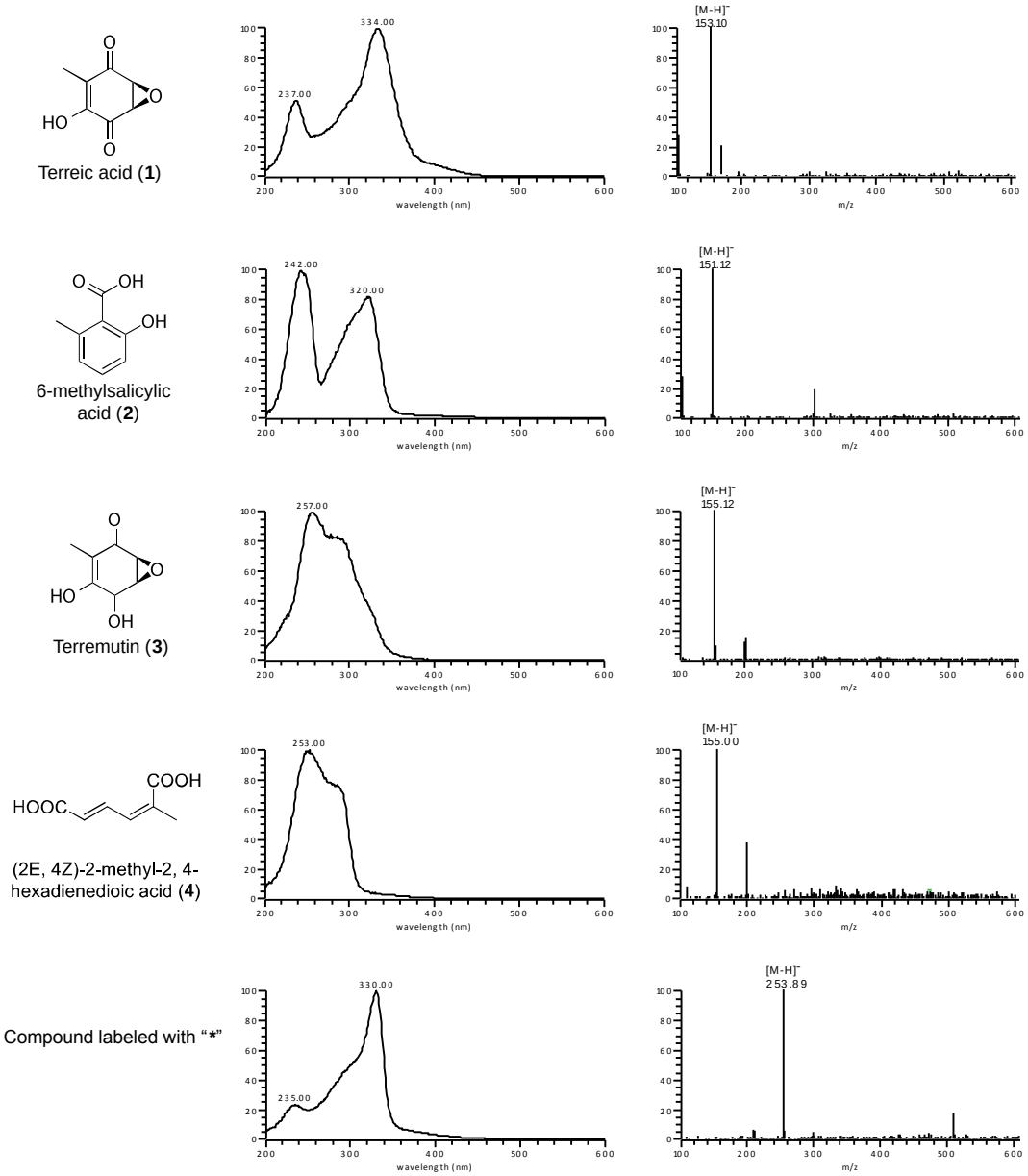


Figure S1. UV-Vis and ESIMS spectra of compounds **1** to **4** and unrelated compound (*****). The molecular formula of the compound labeled with "*" was determined by HRESIMS to be $C_{12}H_{16}NO_5$ $[M - H]^-$ (*m/z* 254.1035, calcd for $C_{12}H_{16}NO_5$ 254.1034). The compound labeled with "*" is not terreic acid (**1**) pathway related based on two evidence: (1). It can be detected in the *atX*Δ strain; (2). The HRESIMS predicted molecular formula suggests that the compound contains one nitrogen, but there is no nitrogen-bearing group identified in terreic acid (**1**) or any of the proposed intermediates in its pathway.

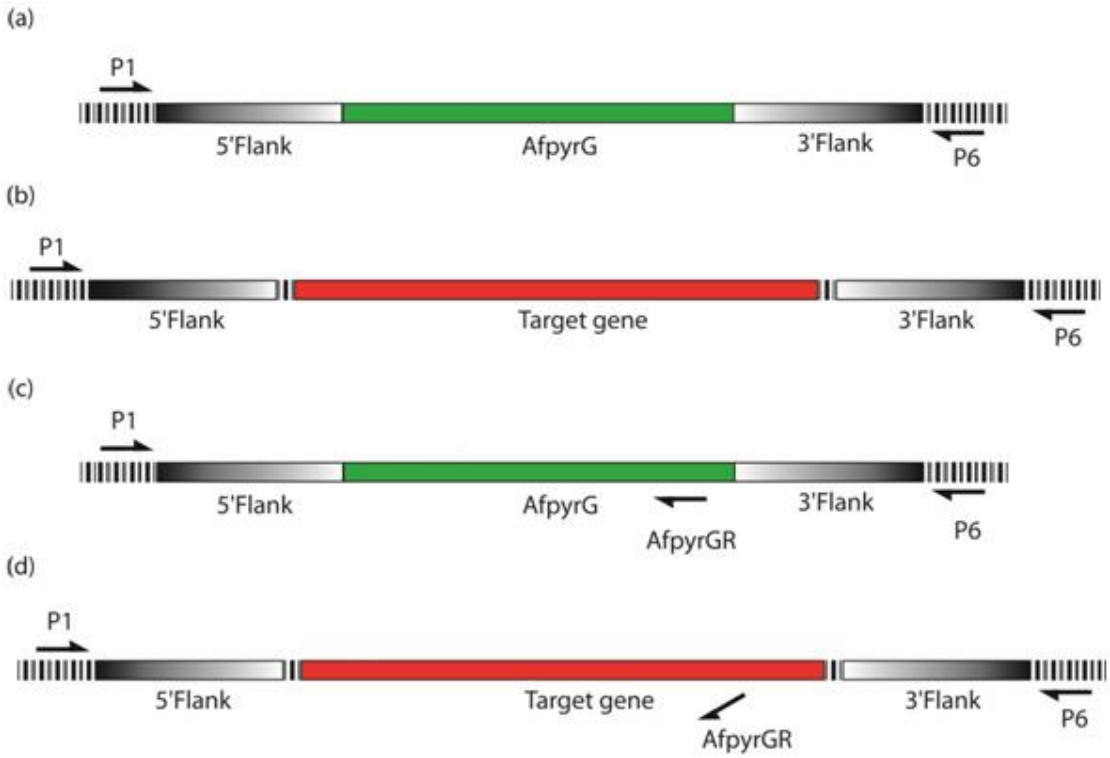


Figure S2. Schematic of the diagnostic PCR strategy.
We used two redundant strategies to determine if the target gene had been deleted by replacement with *AfpyrG*. In one strategy, DNA from transformants is amplified with two primers, P1 from the chromosomal region just outside of the 5' flank of the transforming DNA fragment and P6 from just outside of the 3' flank. If the target gene is different in size from the *AfpyrG* gene, which was used as a selectable marker for transformation, the PCR fragment amplified from a correct transformant (a) will be different in size from the fragment amplified if the target gene is intact (b). In some instances the target gene and the *AfpyrG* cassette will be of comparable size and a second strategy is applied. In the second strategy, P1 or P6 are used with internal primers specific to the *AfpyrG* cassette. For example, if the target gene has been replaced by the *AfpyrG* gene (c), P1 and AfpyrGR will amplify a fragment of a predictable size. If the target gene has not been replaced (d), the AfpyrGR primer will not anneal and there will be no specific amplification.

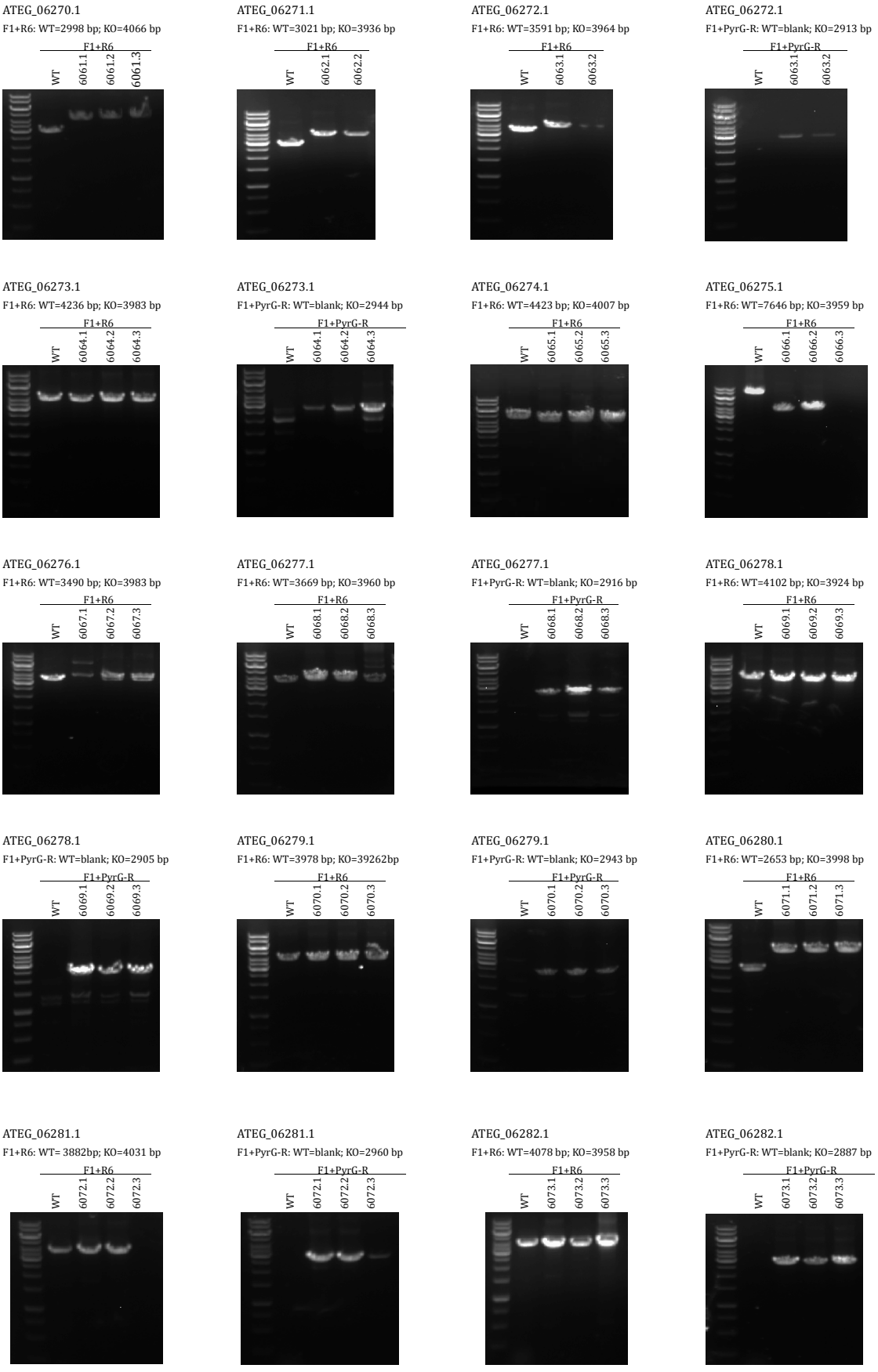


Figure S3. Results of diagnostic PCR for all the gene deletion strains.

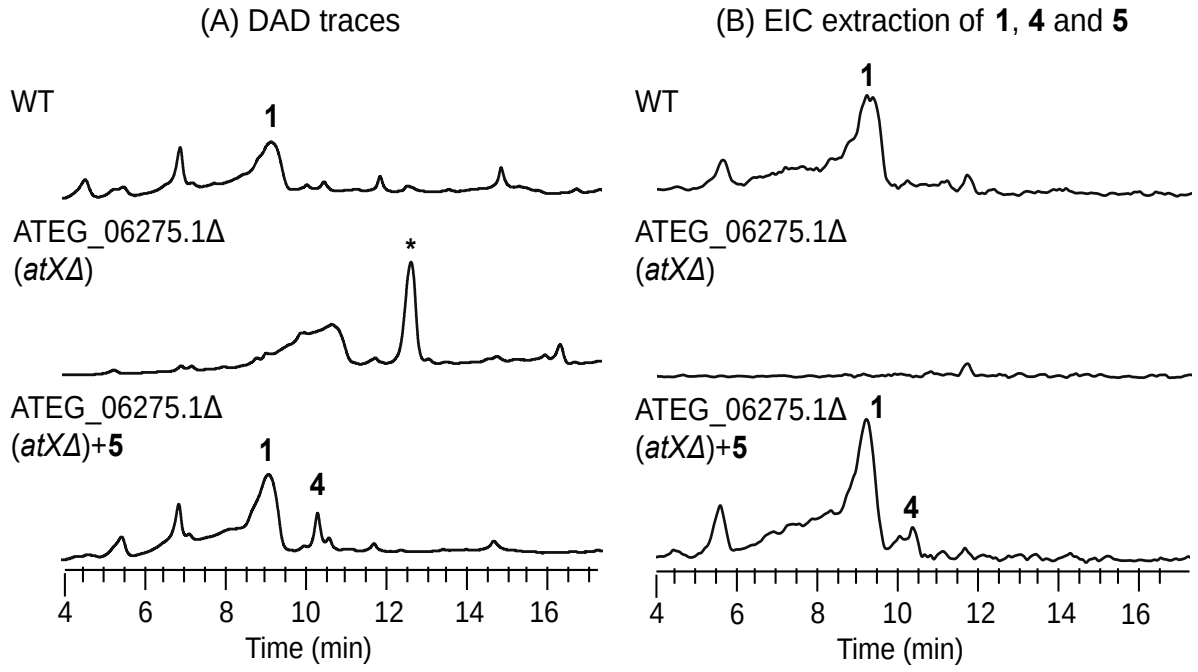
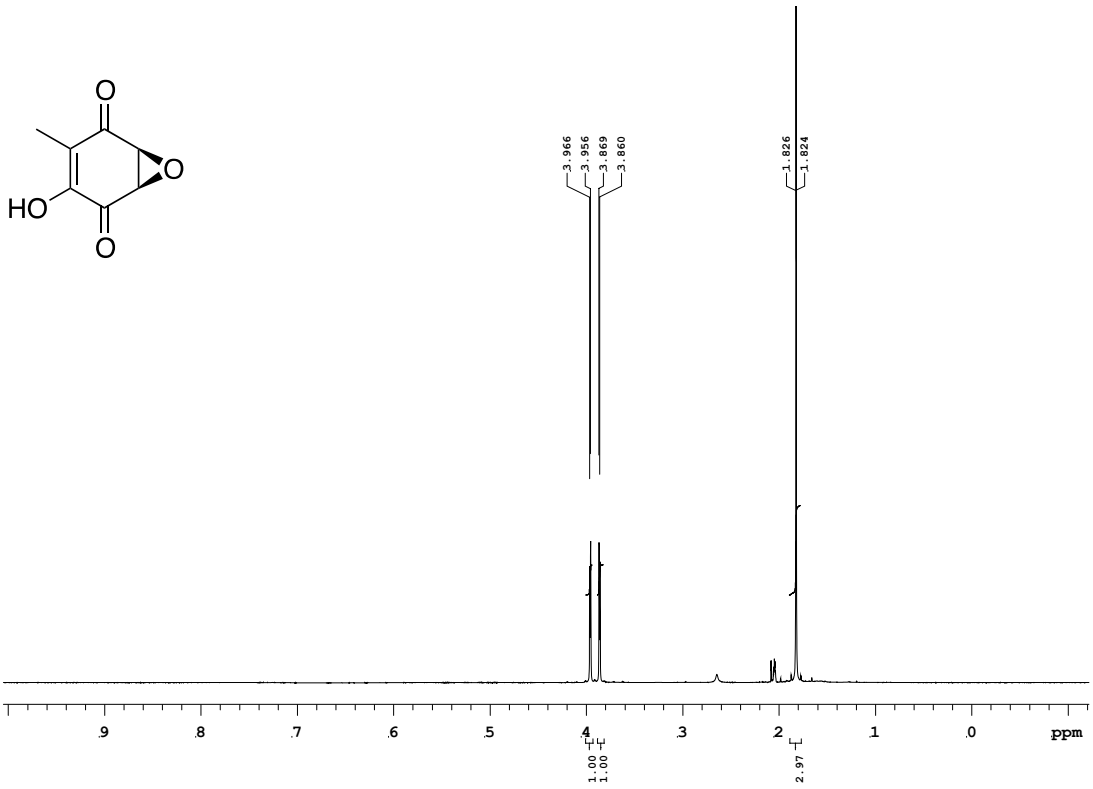
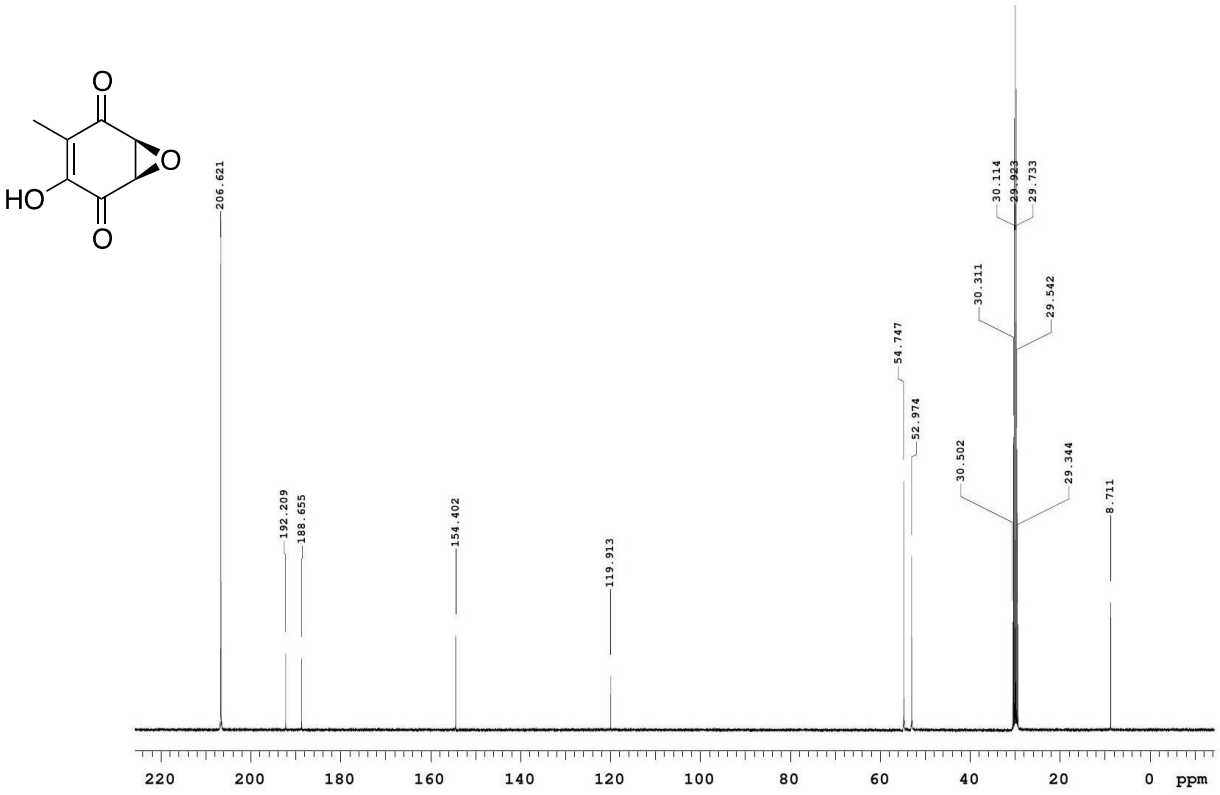


Figure S4. (A) The DAD traces of extracts from the wildtype, *atX*Δ and *atX*Δ fed with 3-methylcatechol (**5**) as detected by UV (200-600 nm). The peak labeled with "*" corresponds to the compound labeled with "*" in Figure 2. (B) EIC extraction of **1**, **4** and **5** (m/z=153, 155 and 123 $[M-H]^-$) from the negative ESI-MS trace in the profiles of the wildtype, *atX*Δ and *atX*Δ fed with 3-methylcatechol (**5**). Feeding the *atX*Δ mutant with 0.2 mg/ml 3-methylcatechol (**5**) recovers TA (**1**) production and accumulates **4**. This suggests that 3-methylcatechol (**5**) is an intermediate of TA (**1**) and compound **5** could be degraded to **4**. The retention time of 3-methylcatechol (**5**) is 12.83 min, but it was not detected probably due to complete decomposition.

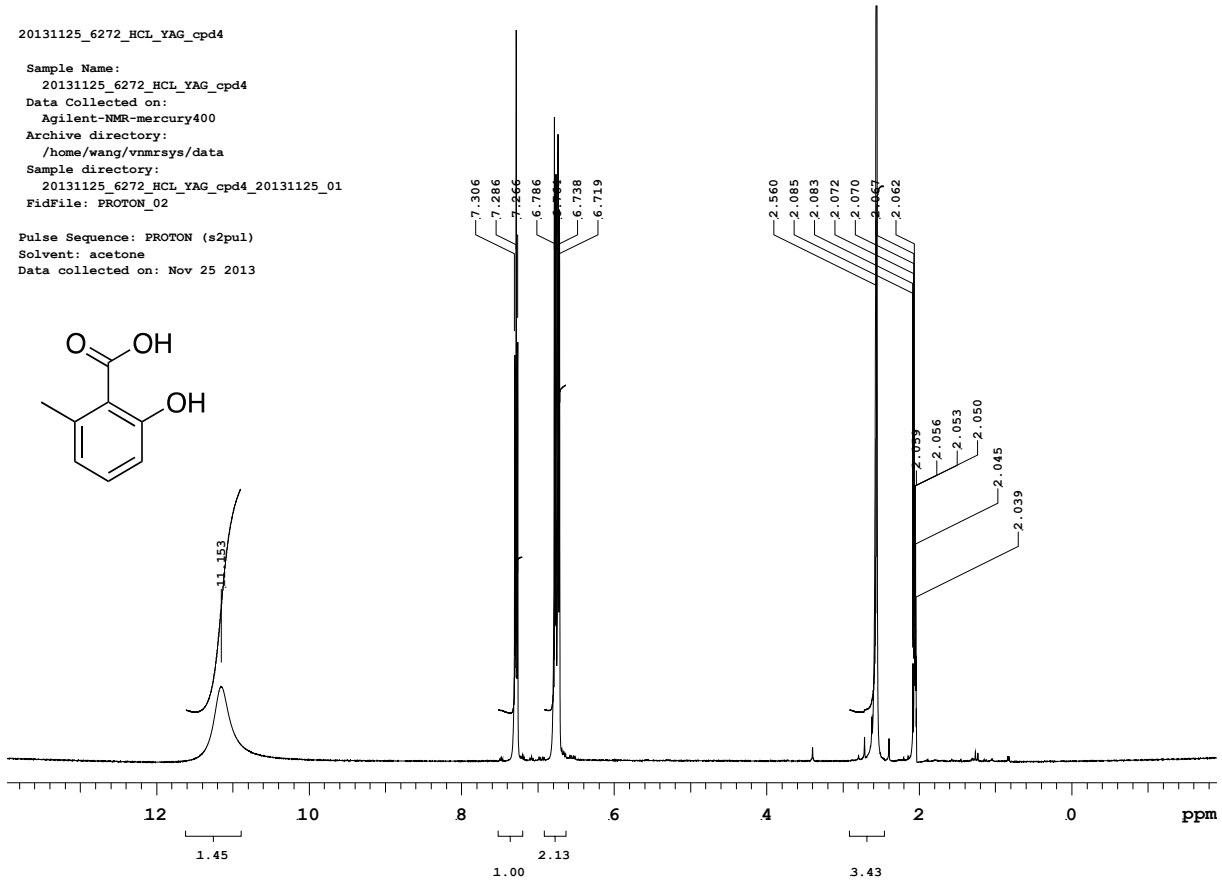


a.^{1}H NMR spectrum of TA **1**, acetone-d_6: 2.05 (m) ppm

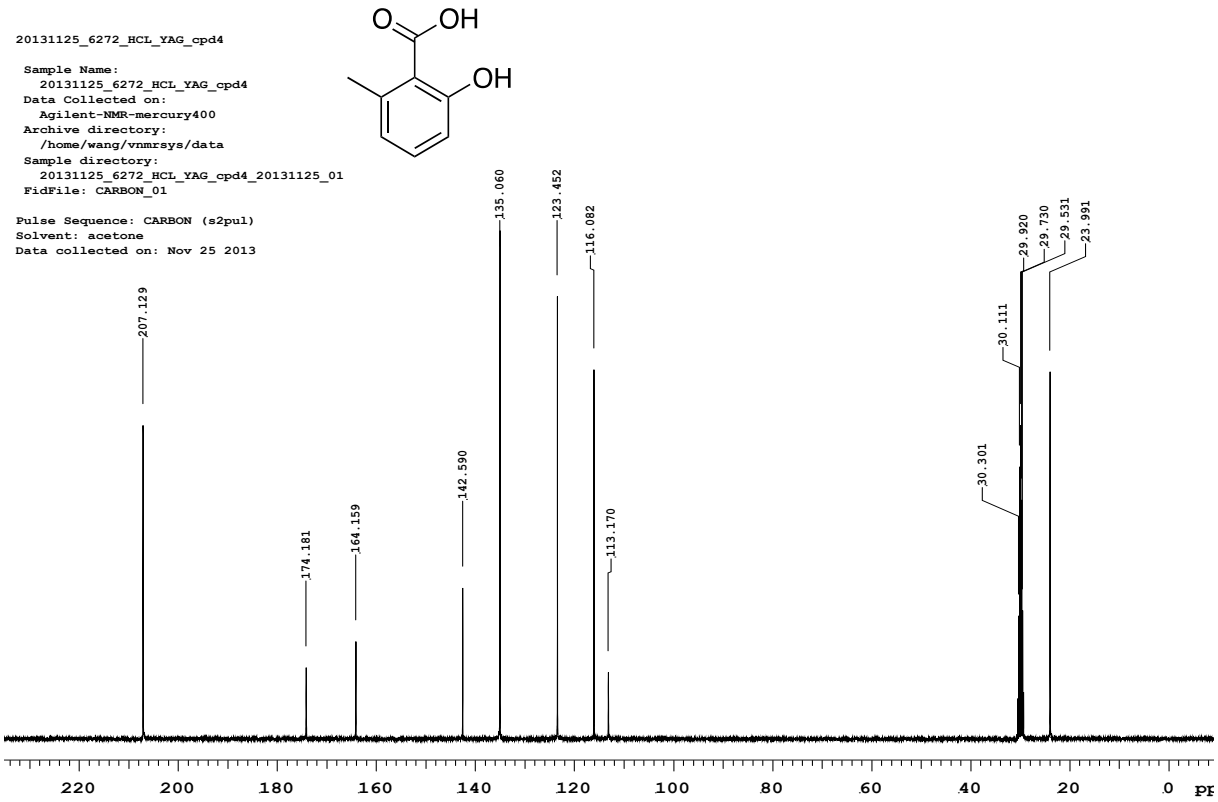


b.^{13}C NMR s pectrum of TA **1**, acetone-d_6: 206.6 (s), 29.92 (m) ppm

Figure S5. ^{1}H NMR and ^{13}C NMR spectrum of TA **1**.

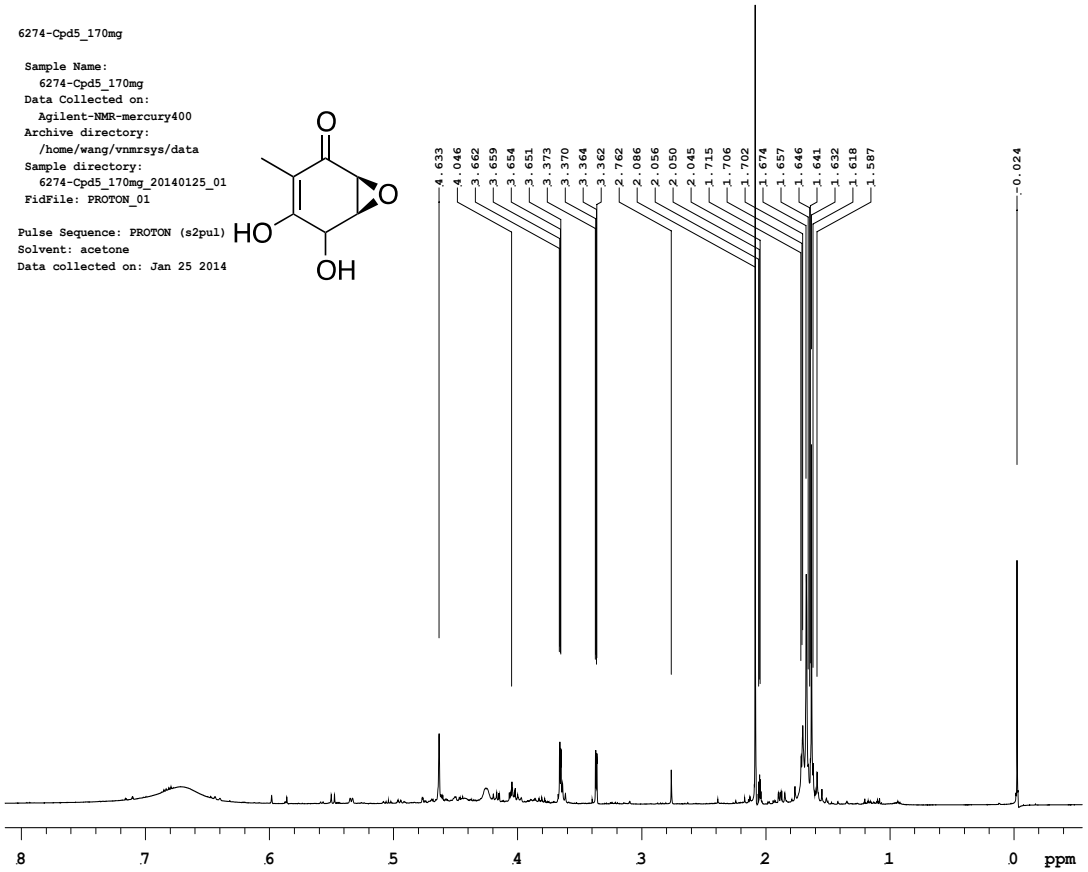


a.[1]H NMR spectrum of compound **2**, acetone-d_6: 2.05 (m) ppm

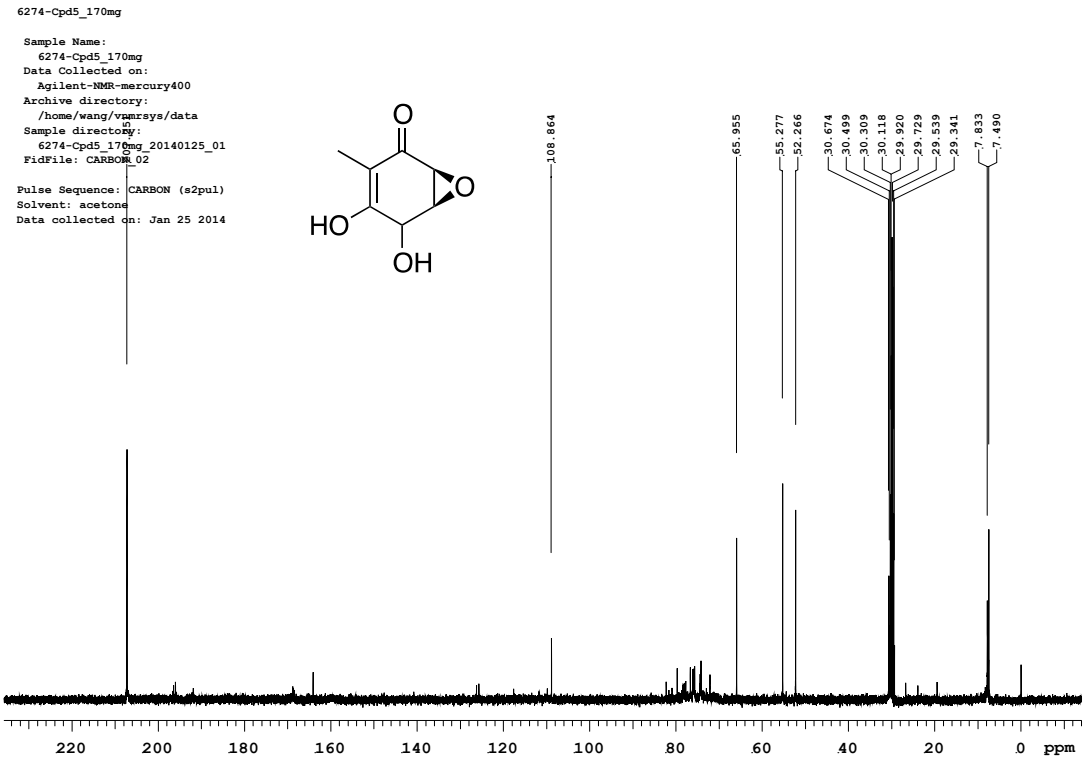


b.[13]C NMR spectrum of compound **2**, acetone-d_6: 207.1 (s), 29.92 (m) ppm

Figure S6. [1]H NMR and [13]C NMR spectrum of compound **2**.

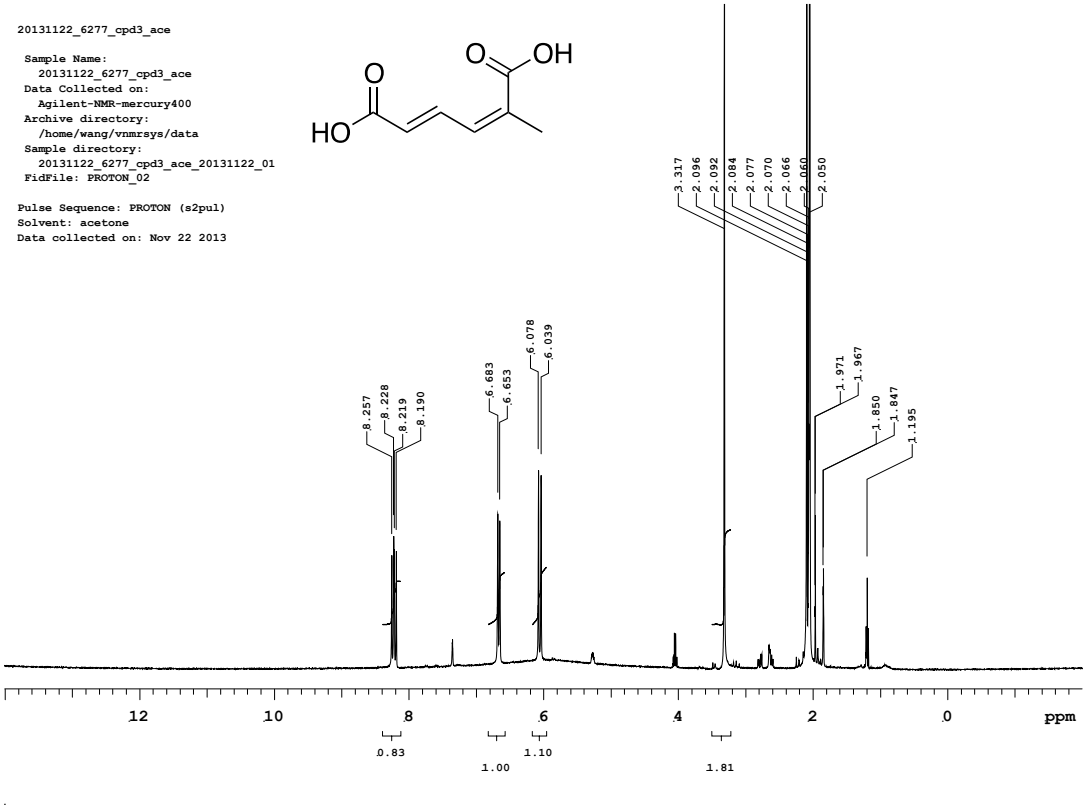


a.^{1}H NMR spectrum of compound **3**, acetone-d_6: 2.05 (m) ppm

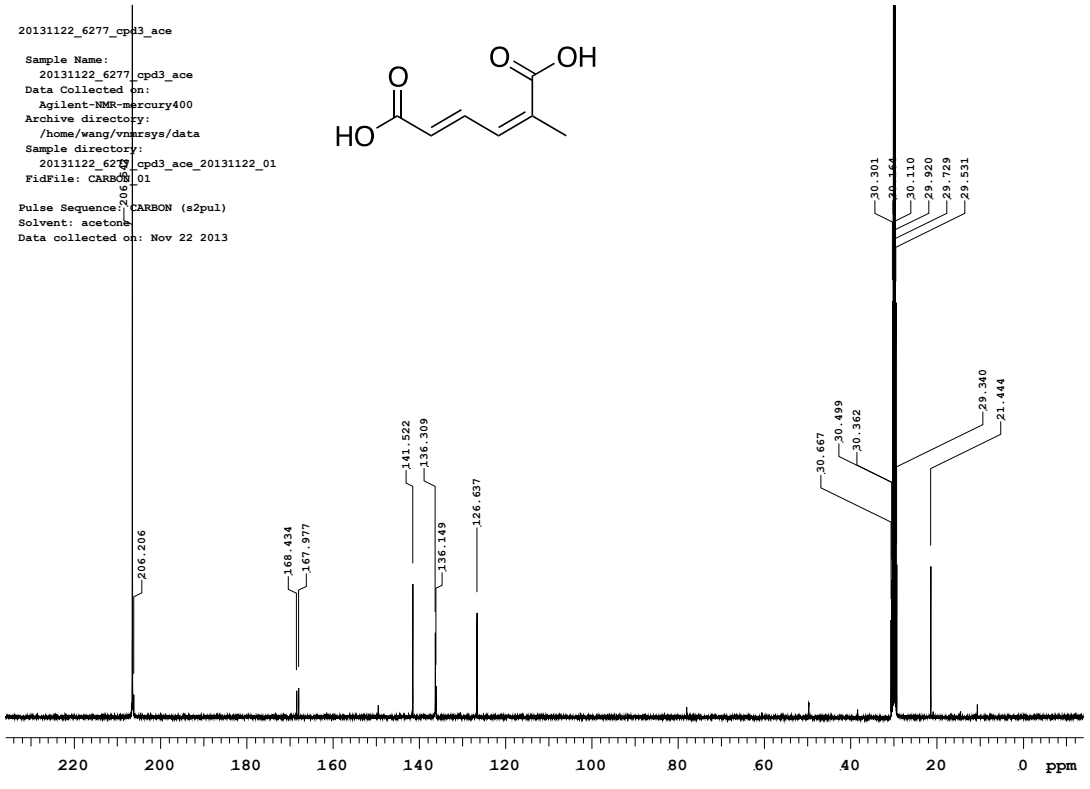


b.^{13}C NMR spectrum of compound **3**, acetone-d_6: 207.2 (s), 29.92 (m) ppm

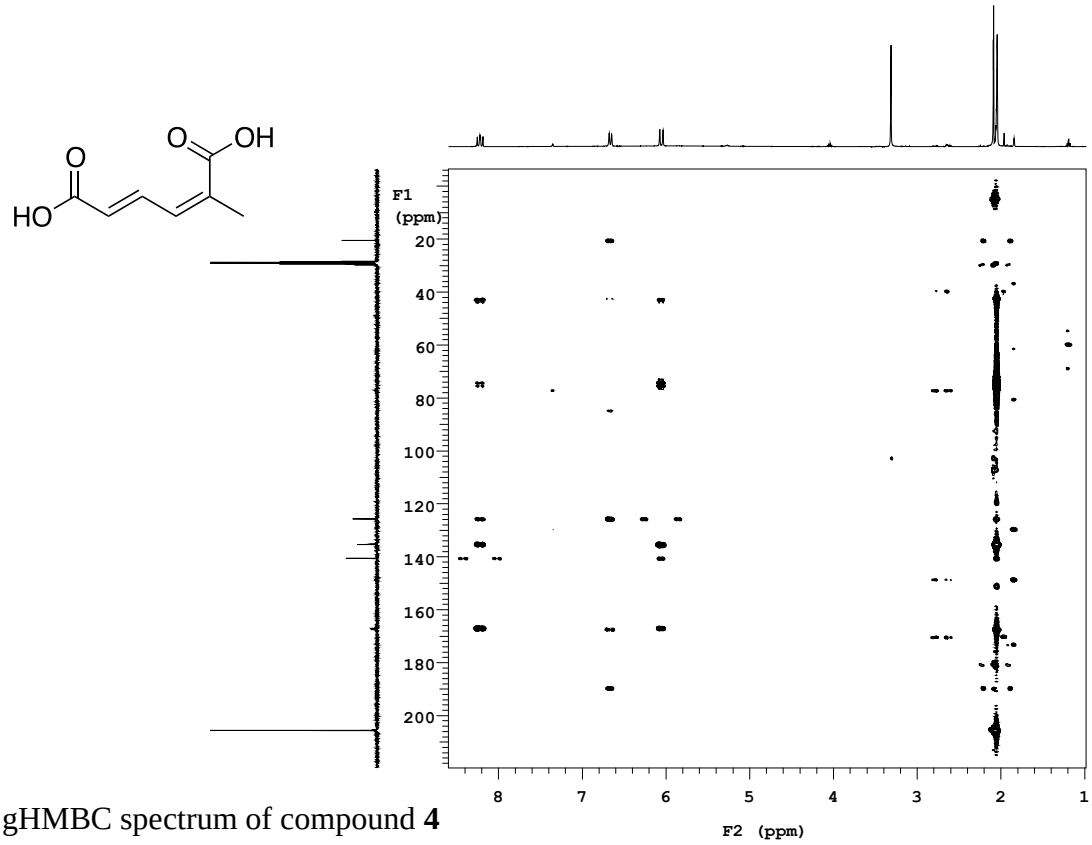
Figure S7. ^{1}H NMR and ^{13}C NMR spectrum of compound **3**.



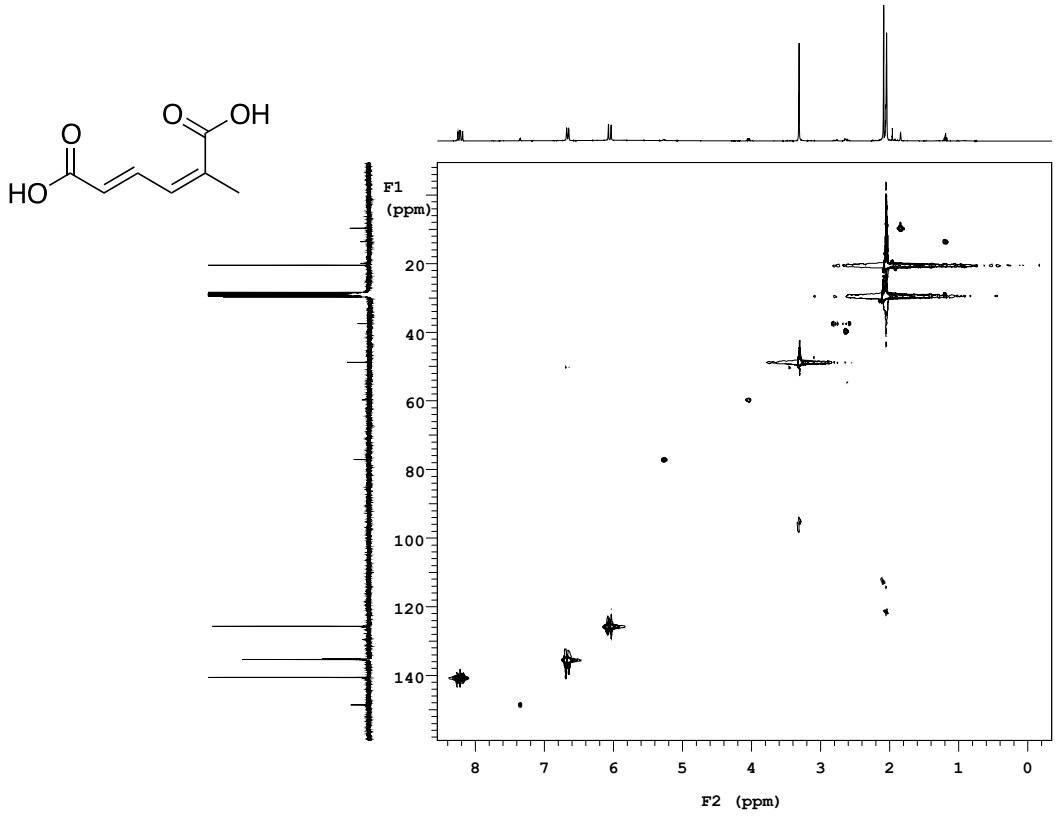
a.[1]H NMR spectrum of compound **4**, acetone-d_6: 2.05 (m) ppm



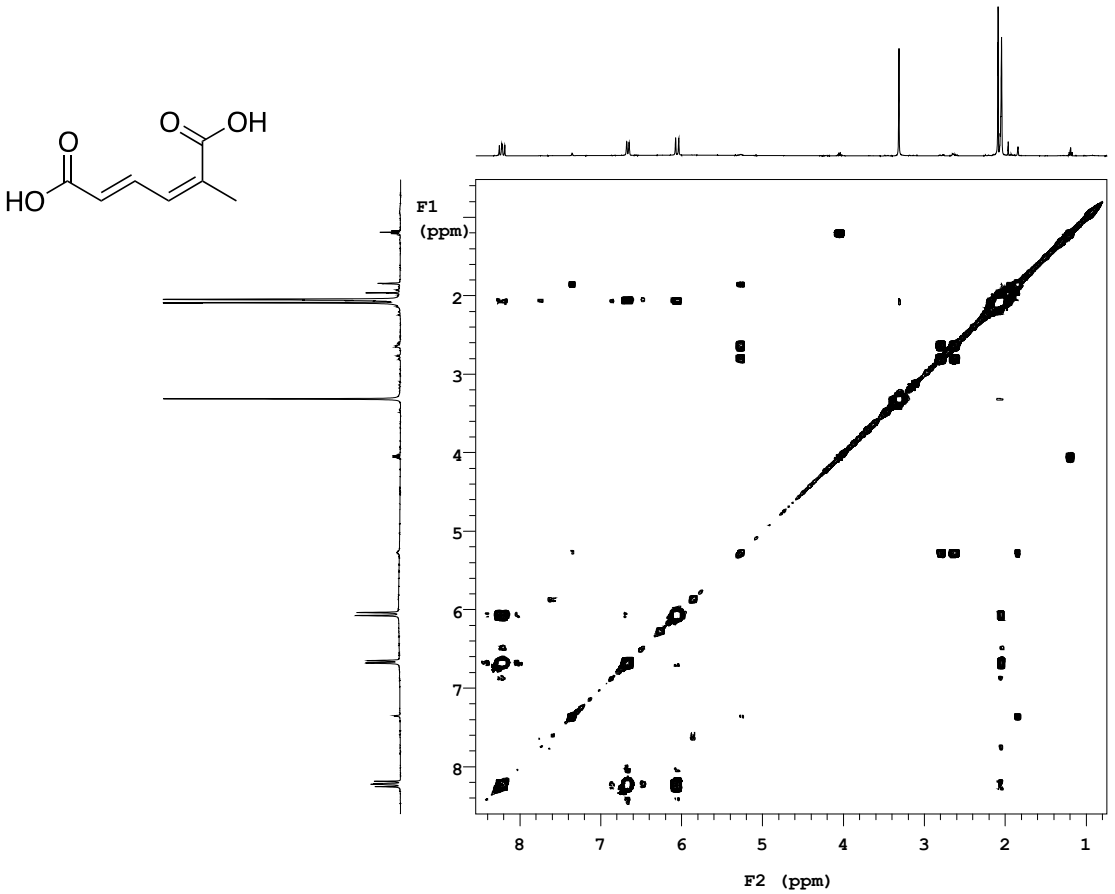
b. [13]C NMR spectrum of compound **4**, acetone-d_6: 207.1 (s), 29.92 (m) ppm



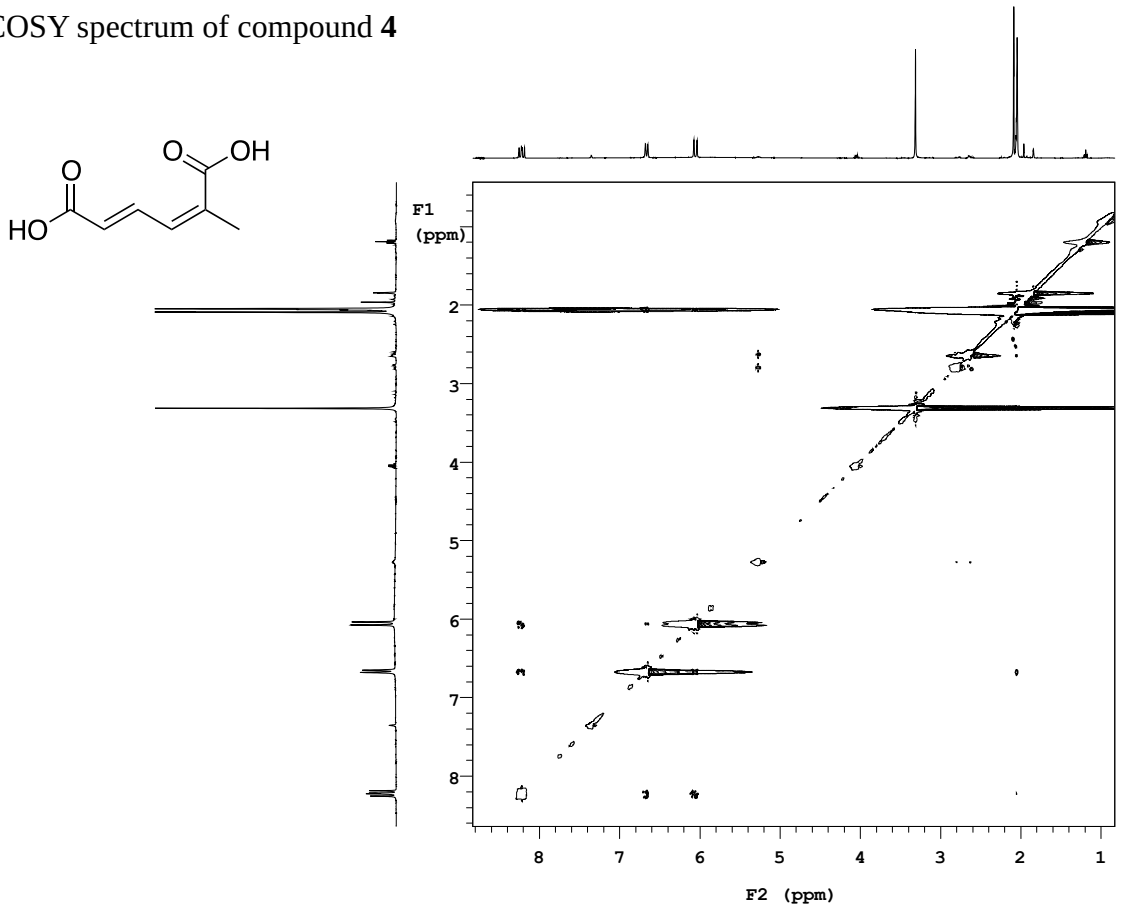
c. gHMBC spectrum of compound **4**



d. gHMQC spectrum of compound **4**



e. gCOSY spectrum of compound **4**



f. NOESY spectrum of compound **4**

Figure S8. ^{1}H NMR, ^{13}C NMR, gHMBC, gHMQC, gCOSY and NOESY spectrum of compound **4**.