Electronic Supplementary Information (ESI)

Genetic dereplication driven discovery of a tricinoloniol acid biosynthetic pathway in *Trichoderma hypoxylon*

Huan Liu[a, b], Yu-Han Pu[c], Jin-Wei Ren[b], Er-Wei Li[b], Li-Xia Guo[c] and Wen-Bing Yin[a, b, *]

[a]School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, 230027, PR China;

[b]State Key Laboratory of Mycology and CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, Chaoyang District, Beijing, 100101, PR. China;

[c]Environmental and Resources Institute, Chongqing Technology and Business University, Chongqing, 400067, PR China

Corresponding author: Wen-Bing Yin, State Key Laboratory of Mycology and CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, NO.1 Beichen West Road, Chaoyang District, Beijing, 100101, PR. China

E-mail: yinwb@im.ac.cn

Tel.: 86-10-64806170; Fax: 86-10-64806170

ORCID ID: Wen-Bing Yin: 0000-0002-9184-3198

Content

1. Supplementary methods

2. Supplementary tables

Table S1. Plasmids and strains used in this study

Table S2. Primers used in this study

Table S3. ^{1}H and ^{13}C NMR data for compound **4**

3. Supplementary figures

Fig. S1. The UV spectra of compounds **1**-**3**

Fig. S2. The IR spectra of compounds **1**-**3**

Fig. S3. The HR- ESI-MS spectra of the compounds **1**-**4**

Fig. S4. 1D and 2D NMR spectra of compound **1** in $CDCl_3$

Fig. S5. 1D and 2D NMR spectra of compound **2** in $CDCl_3$

Fig. S6. 1D and 2D NMR spectra of compound **3** in $CDCl_3$

Fig. S7. ^{1}H and ^{13}C NMR spectra of compound **4** in $CDCl_3$

Fig. S8. Compounds related to compounds **1**−**3** in previous studies

Fig. S9. Phylogenetic analysis terpene synthase from *T. hypoxylon* with STC5 in *F. graminearum*.

Fig. S10. Gene deletion of *traA* in *T. hypoxylon*.

4. Supplementary references

1. Supplementary materials and methods

Accession number

Sequences used in this study have been deposited in the GenBank database, and the TRA accession numbers was given as MT300195.

Fungal strains, media, and culture conditions

The fungal strains used in this study are listed in Table S1. *T. hypoxylon* used in this study was deposited in the China General Microbiological Culture Collection Center (CGMCC 3.17906, Beijing, China) and isolated from the stroma of *Hypoxylon anthochroum* in Thailand[1]. Δ*Thtri5* mutant was available in our previous study[2]. *T. hypoxylon* and its transformants were initially grown at 25°C on potato dextrose agar (PDA) or potato dextrose broth (PDB) media in the presence of appropriate antibiotics as required. *Escherichia coli* DH5α was cultured in liquid Luria-Bertani (LB) medium for plasmid extraction or on solid LB medium with 1.5 % agar (*w/v*). For selection of recombinant *E. coli* strains, 100 μg/mL of ampicillin were supplied in the media.

Construction of deletion cassette and genetic manipulation

The oligonucleotide sequences for PCR amplification primers were given in Table S2. For the creation of *traA* deletion strain, approximately 1.6 kb sequences locatedupstream and downstream the target gene *traA* were amplified from genomic DNA of *T. hypoxylon* using designated primer pairs *traA*-QC-5F F/R and *traA*-QC-3F F/R (Table S2). These two fragments were subsequently inserted into pUCH2-8 containing a hygromycin (*hph*) resistance gene to construct the plasmid pYHL94 using the Quick-Change method described previously[3]. Transformation was performed using PEG-mediated protoplast transformation described in a previous study[2]. Transformants (TYHL61) were verified using three pairs of designated primers *traA*-SCR F/*hph*-SCR R, *hph*-SCR F/*traA*-SCR R as well as *traA*-RT F/R, and selected twice using hygromycin to obtain mitotic stability (Table S2)[4].

RNA extraction and transcriptomic analysis

Δ*Thtri5* and the wild type (WT) strains were cultivated on rice media containing 60 g rice and 90 mL water at 25°C for 7 d. The total RNA from the mycelia of Δ*Thtri5* and WT was isolated using TRIZOL reagent (Invitrogen, USA) following the manufacturer's protocol. Three independent biological replicates were performed to establish the cDNA libraries. The libraries were sequenced using HiSeq X Ten platform (Annoroad Tech Co., Ltd, China). To acquire clean reads with high quality, the raw reads were firstly cleaned by removing adaptor fragments. The clean reads and reference sequence were

mapped by using HISZT2, and the mapped number was calculated by the HTSeq-count. The differentially expressed genes in *tri* and *tra* clusters were measured by using DESeq2 and identified with *P* value ≤0.05 and a log2 Foldchange ≥2 between wild type and Δ*Thtri5* strains.

Chemical analysis methods and equipment overview

HPLC analysis was conducted with a Waters HPLC system (Waters e2695, Waters 2998, Photodiode Array Detector) using an ODS column (C18, 250_4.6 mm, aters Pak, 5 μm). Water with 0.1% (*v/v*) formic acid (A) and acetonitrile with 0.1% (*v/v*) formic acid (B) were used as solvents at the flow rate of 1 mL/min. The substances were eluted with a linear gradient from 5–100% B in 30 min, washed with 100% (*v/v*) solvent B for 5 min and equilibrated with 5% (*v/v*) solvent B for 5 min. UV absorptions at 236 nm were illustrated. Semi-preparative HPLC was performed on the same equipment with an ODS column (C18, 9.4 × 250 mm, aters Pak, 5 μm) column and the flow rate of 2.5 mL/min.

UV spectra were tested using a Shimadzu UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR data (^{1}H, ^{13}C, HMBC, HSQC and ^{1}H-^{1}H COSY) were collected on a Bruker Avance-500 spectrometer using TMS as internal standard and chemical shifts were recorded as δ values. IR spectra were measured on a Thermo Nicolet Nexus 470 FT-IR spectrometer (Thermo Fisher Scientific, Massachusetts, USA). HR-ESI-MS detected on an Agilent Accurate-Mass-QTOF LC/MS 6520 instrument. Spectra were processed with MestReNova 6.1.0 (Metrelab).

Large-scale fermentation and isolation of secondary metabolites

To isolate the abolished metabolites in Δ*Thtri5* strain, the WT strain was cultivated in 500 mL-flasks containing 60 g rice media (a total 3 kg) with 90 mL water at 25℃ for 14 d. The culture was cut into pieces and extracted with ethyl acetate by sonication for 60 min. The extraction was evaporated under reduced pressure to yield a crude extract (7.2 g).

Subsequently, the crude extracts were separated by octadecyl silane chemically bonded silica (ODS) medium performance liquid chromatography (MPLC) (10% to 60% acetonitrile in H_2O for 300 min). The fractions containing the target compounds were combined and concentrated to give 5 fractions (Fr.1 to Fr.5). For further purification, semi-preparative HPLC was carried out. Fr.4 was then prepared by HPLC (55% acetonitrile in H_2O) to obtain Fr.4-2 (**1**; 4.8 mg; t_R 26.3 min), Fr.4-1 (**2**; 6.5 mg; t_R 24.2 min), and Fr.4-3 (**3**; 3.9 mg; t_R 36.7 min). Fr.5 was purified by HPLC (42% acetonitrile in H_2O) to obtain Fr.5-1 (**4**; 5.3 mg; t_R 38.1 min). The purified compounds were analyzed by LC-MS and the structures were identified by ^{1}H, ^{13}C, HMBC, HSQC and ^{1}H-^{1}H COSY spectra.

Compound (**1**): yellow oiliness; $[\alpha]_D^{25}$ -3.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 236 nm (Fig. S1); IR (MeOH) ν_{max}: 3419, 2953, 1716, 1663, 1225, 1041 cm^{-1} (Fig. S2); HR-ESI-MS *m/z* 255.1959 $[M+H]^+$ (Calcd. for $C_{15}H_{26}O_3$, 255.1955) (Fig. S3); for ^1H and ^{13}C NMR data, see Table 1.

Compound (**2**): yellow oiliness; $[\alpha]_D^{25}$ -6.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 236 nm (Fig. S1); IR (MeOH) ν_{max}: 3411, 2871, 1731, 1459, 1208, 642 cm^{-1} (Fig. S2); HR-ESI-MS *m/z* 255.1960 $[M+H]^+$ (Calcd. for $C_{15}H_{26}O_3$, 255.1955) (Fig. S3); for ^1H and ^{13}C NMR data, see Table 1.

Compound (**3**): yellow oiliness; $[\alpha]_D^{25}$ +7.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 236 nm (Fig. S1); IR (MeOH) ν_{max}: 3398, 2962, 1722, 1379, 1031 cm^{-1} (Fig. S2); HR-ESI-MS *m/z* 255.1953 $[M+H]^+$ (Calcd. for $C_{15}H_{26}O_3$, 255.1955) (Fig. S3); for ^1H and ^{13}C NMR data, see Table 2.

Preparation of the (*R*)-and (*S*)-MTPA derivatives of 1, **2** and **3**

Compounds **1**, **2** and **3** (1.0 mg) were dissolved in pyridine-d_5 for reaction with the agent (*R*)-(-)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (10 μL) at room temperature to obtain the (*S*)-MTPA ester derivatives of **1**, **2** and **3**. The (*R*)-MTPA ester derivatives of **1, 2** and **3** were prepared under the similar reaction condition as mentioned above [5]. The (*R*)-and (*S*)-MTPA derivatives of **1**, **2** and **3** NMR data were shown in supporting information.

NMR data of (*R*)-and (*S*)-MTPA derivatives for 1−3

The (*R*)-MTPA ester derivative of **1** was measured by ^1H NMR (500 MHz, pyridine-d_5): 6.1607 (1H, d, *J* = 16.0 Hz, H-5), 5.5478 (1H, dd, *J* =15.8, 9.3 Hz, H-6), 5.2905 (1H, s, H_a-12)， 5.0787 (1H, s, H_b-12), 0.8674 (3H, *J* = 6.8 Hz, H-15), 0.8349 (3H, *J* = 6.7 Hz, H-14), 1.2469 (3H, *J* = 6.5 Hz, H-11), 2.4842 (2H, m, H-2).

The (*S*)-MTPA ester derivative of **1** was measured by ^1H NMR (500 MHz, pyridine-d_5): 6.1055 (1H, d, *J* = 16.0 Hz, H-5), 5.5426 (1H, dd, *J* =15.6, 9.6 Hz, H-6), 5.2941(1H, s, H_a-12), 5.0801 (1H, s, H_b-12), 0.8330 (3H, *J* = 6.8 Hz, H-15), 0.7923 (3H, *J* = 6.7 Hz, H-14), 1.3114 (3H, *J* = 6.5 Hz, H-11), 2.4799 (2H, m, H-2).

The (*R*)-MTPA ester derivative of **2** was measured by ^1H NMR (500 MHz, pyridine-d_5): 6.0788 (1H, d, *J* = 16.0 Hz, H-5), 5.6272 (1H, dd, *J* = 15.8, 9.3 Hz, H-6), 5.2613 (1H, s, H_a-12), 5.0736(1H, s, H_b-12), 0.7763 (3H, *J* = 6.7 Hz, H-15), 0.8062 (3H, *J* = 6.8 Hz, H-14), 1.2147(3H, *J* =6.1 Hz, H-11), 2.7283 (2H, m, H-2).

The (*S*)-MTPA ester derivative of **2** was measured by ^1H NMR (500 MHz, pyridine-d_5): 6.1044 (1H, d, *J* = 16.0 Hz, H-5), 5.6733 (1H, dd, *J* =15.9, 9.5 Hz, H-6), 5.2703 (1H, s, H_a-12), 5.0866 (1H, s, H_b-

12), 0.8173 (3H, *J* = 6.5 Hz, H-15), 0.8730 (3H, *J* = 6.5 Hz, H-14), 1.1517 (3H, *J* = 6.0 Hz, H-11), 2.7409 (2H, m, H-2).

The (*R*)-MTPA ester derivative of **3** was measured by ^{1}H NMR (500 MHz, pyridine-d_5): 5.9021 (1H, d, *J* = 15.5 Hz, H-5), 5.5439 (1H, dd, *J* =15.8, 9.3 Hz, H-6), 0.8406 (3H, *J* = 6.5 Hz, H-15), 0.9835 (3H, *J* = 6.1 Hz, H-14), 1.1369 (3H, *J* = 6.5 Hz, H-11), 3.7356 (1H, *J* = 12.1, 6.0 Hz, H-10), 3.4490 (2H, m, H-2).

The (*S*)-MTPA ester derivative of **3** was measured by ^{1}H NMR (500 MHz, pyridine-d_5): 5.8980 (1H, d, *J* = 15.6 Hz, H-5), 5.4966 (1H, dd, *J* =17.5, 9.6 Hz, H-6), 0.8189 (3H, *J* = 6.8 Hz, H-15), 0.9206 (3H, *J* = 6.2 Hz, H-14), 1.1479 (3H, *J* = 6.5 Hz, H-11), 3.6924 (1H, *J* = 12.1, 6.0 Hz, H-10), 3.4280 (2H, m, H-2).

2. Supplementary tables

Table S1. Plasmids and strains used in this study

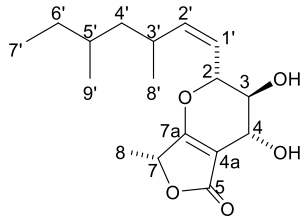
Plasmid/Strain	Description	Reference
pUCH2-8	Vector with hygromycin B (*hph*) resistance gene	6
pYHL94	*traA::hph*	This study
WT	*Trichoderma hypoxylon* (CGMCC 3.17906)	1
TYHL26	*Thtri5::neo*	2
TYHL61	*traA::hph*	This study

pXX = plasmid, TXX = original transformant

Table S2. Primers used in this study

Primer	**Sequences (5′-3′)**	
traA-QC-5F F	ctatagggcgaattggagctccaccgcCCAACTCATCACTCCAAGTAG	Up flanks' amplification for Quick change
traA-QC-5F R	gatccactagttctagagcggccgccaccGCATTCGAGCCTACACAATGG	
traA-QC-3F F	gagccggaagcataaagtgtaaagcctgGCATATTCCCCAAGACGTAG	Down flanks' amplification for Quick change
traA-QC-3F R	gtgagttagctcactcattaggcacccGTCGTACTCATAACGCAGC	
traA-SCR F	CCAACTCATCACTCCAAGTAG	Δ*traA* transformant upstream screening
hph-SCR R	GCCTATGCCTACAGCATCC	
hph-SCR F	CGTGGTCGAGCTACAAAGC	Δ*traA* transformant downstream screening
traA-SCR R	GTCGTACTCATAACGCAGC	
traA-RT F	CGAACCTCCTGATCAGCTTC	Δ*traA* transformant target screening
traA-RT R	CACATGGCATATCCTGAGAG	

Table S3. ^{1}H and ^{13}C NMR data of the compound **4**



Position	**4** acquired in $CDCl_3$	
	δ_C[a], Type	δ_H[b], (J in Hz)
2	79.6, CH	4.96, t (8.3)
3	65.3, CH	3.82, dd (7.5, 6.1)
4	72.1, CH	4.57, d (5.8)
4a	100.7, CH	-
5	172.0, C	-
7	74.3, CH	4.83, q (6.8)
7a	177.8, CH	-
8	17.4, CH_3	1.44, d (6.8)
1'	121.8, CH	5.51, dd (10.3, 7.9)
2'	145.0, CH	5.57, t (10.3, 6.8)
3'	32.2, CH	2.62, m
4'	44.4, CH_2	1.34-1.05, m
5'	30.2, CH	1.34-1.05, m
6'	30.4, CH_2	1.34-1.05, m
7'	11.4, CH_3	0.85, d (7.3)
8'	21.9, CH_3	0.98, d (6.6)
9'	18.9, CH_3	0.81, d (6.3)

[a] ^{13}C NMR data in $CDCl_3$ at 125 MHz; [b] ^{1}H NMR data in $CDCl_3$ at 500 MHz.

3. Supplementary figures

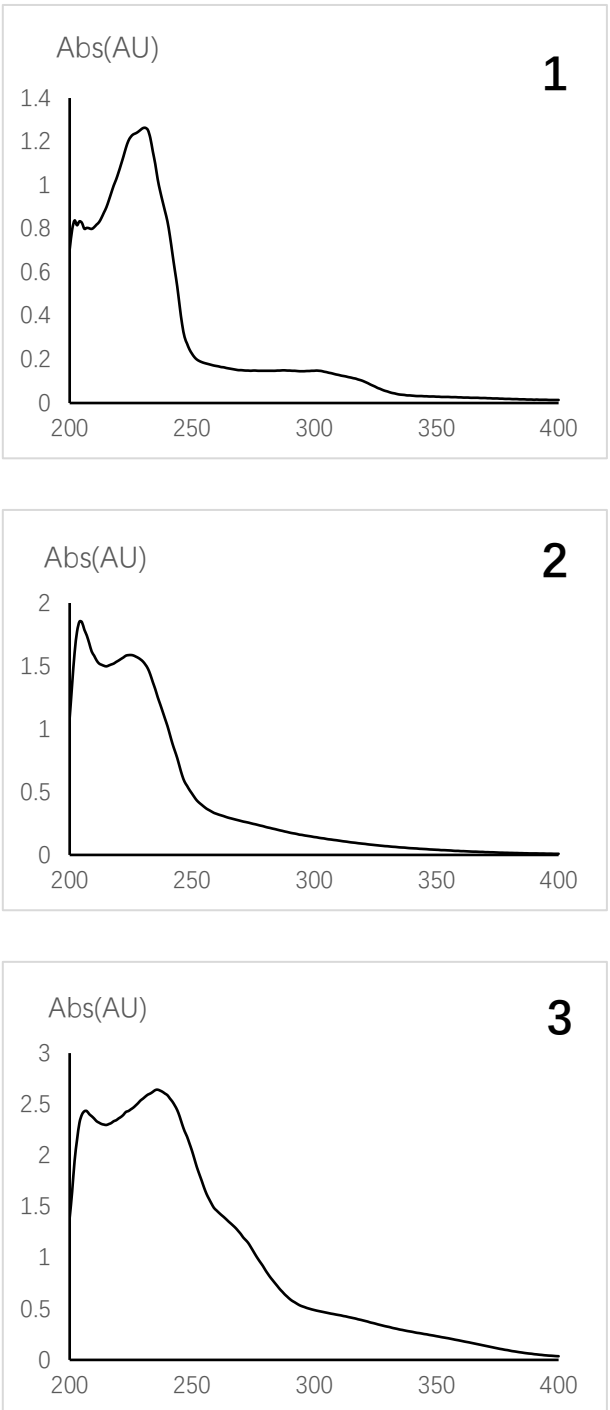
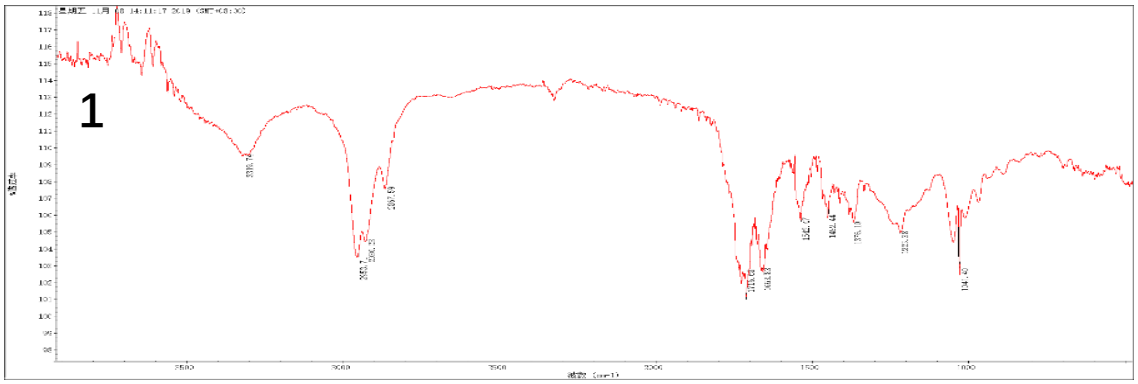
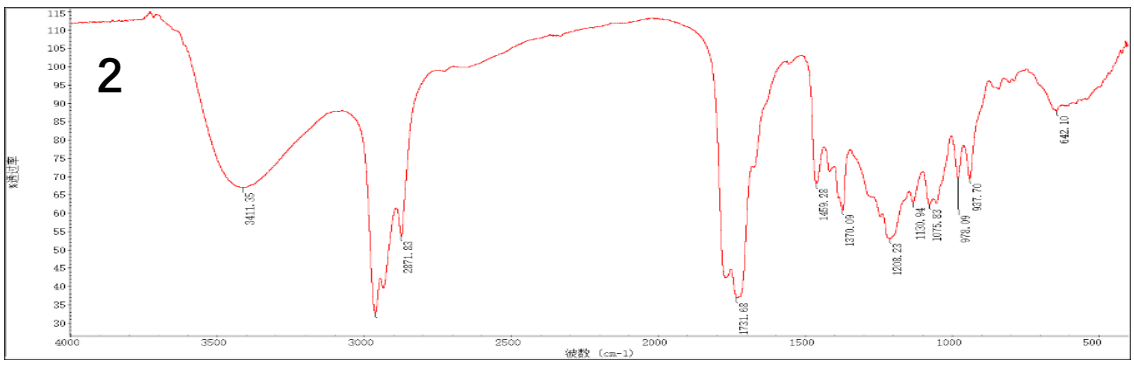


Figure S1. UV spectra of compounds **1-3**





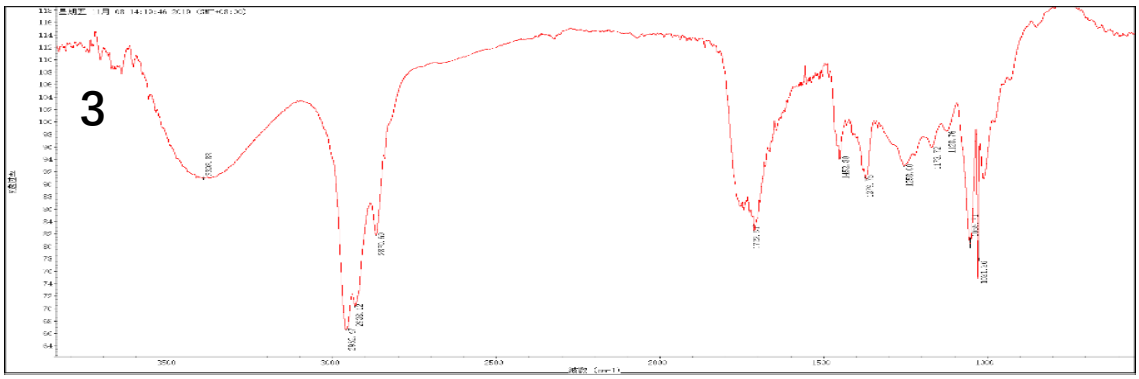


Figure S2. IR spectra of compounds **1- 3**

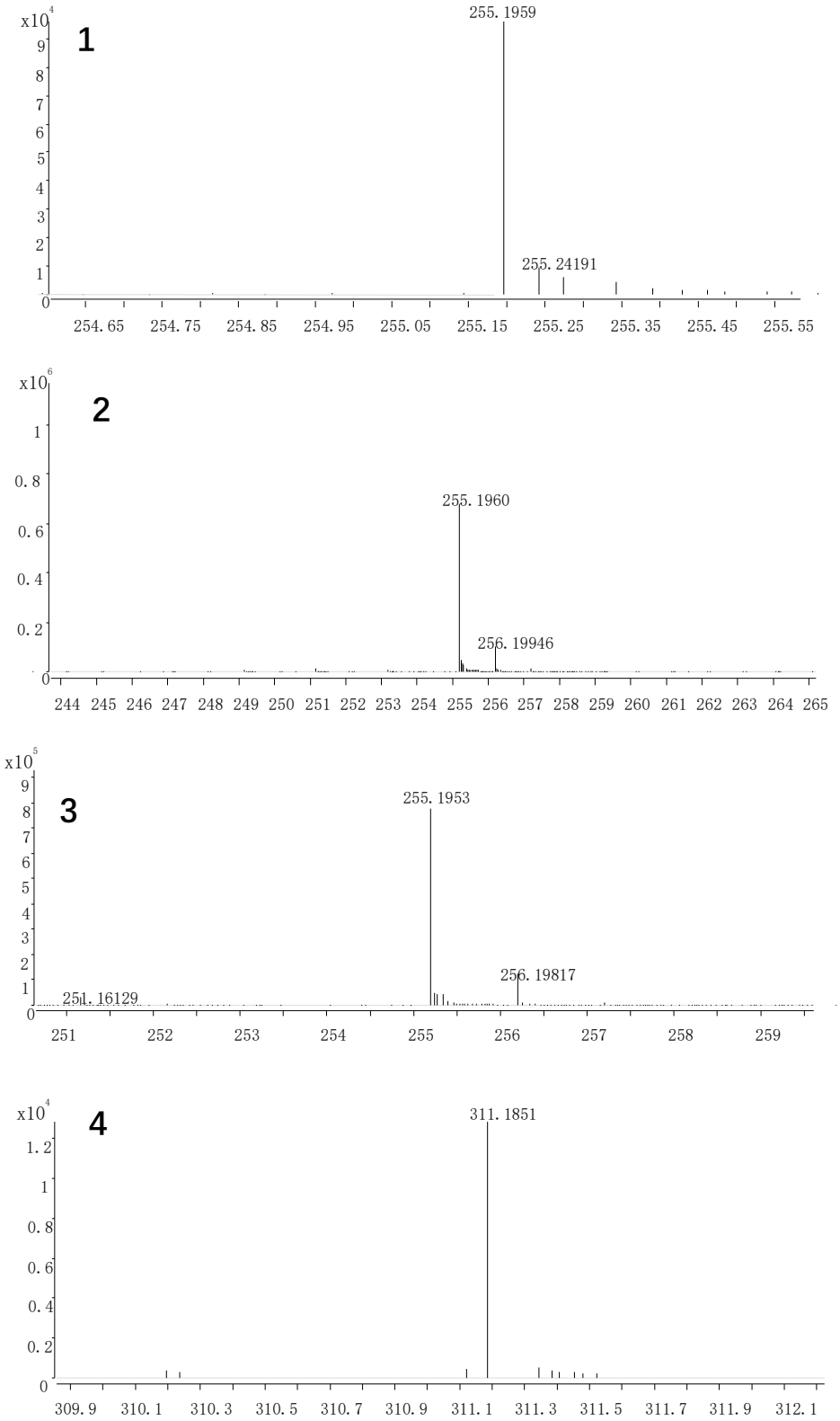


Figure S3. HR-ESI-MS data of the compounds **1-4**

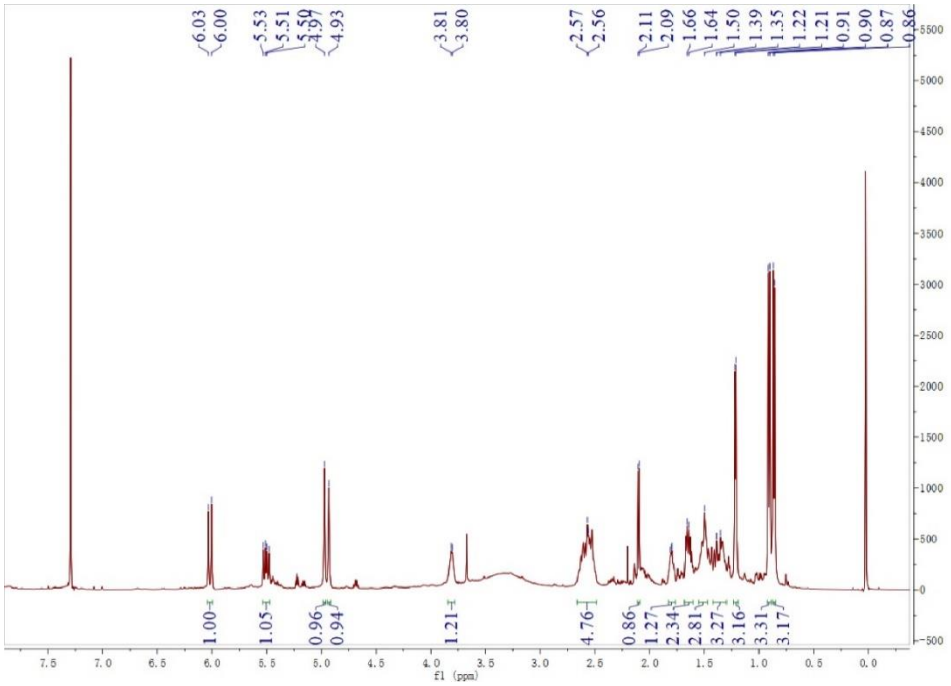


Figure S4. (a) ^{1}H NMR spectrum of compound **1** in $CDCl_3$ (500 MHz)

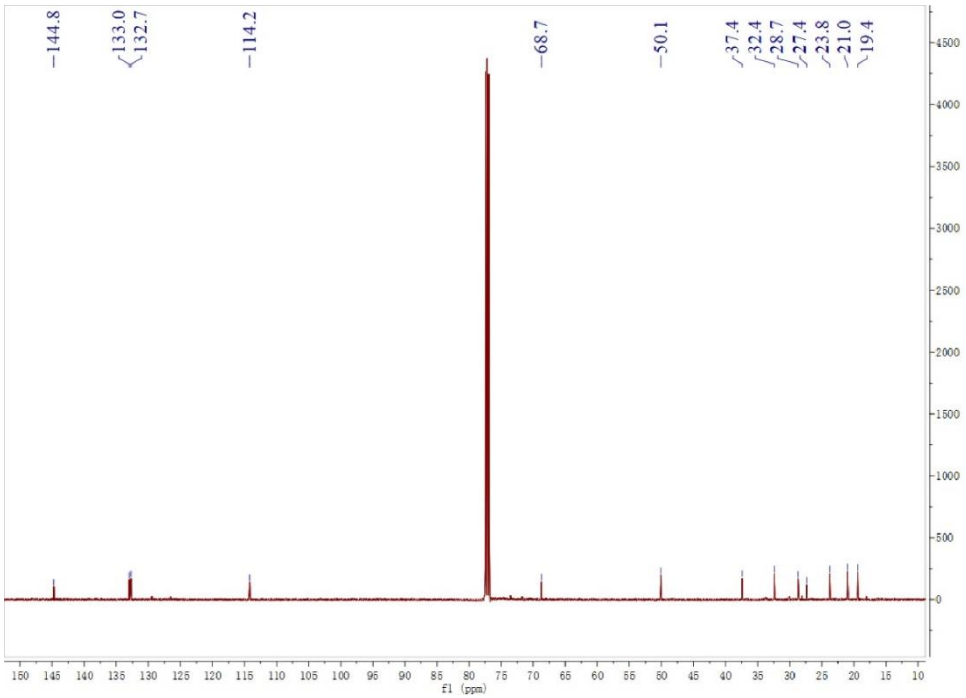


Figure S4. (b) ^{13}C NMR spectrum of compound **1** in $CDCl_3$ (125 MHz)

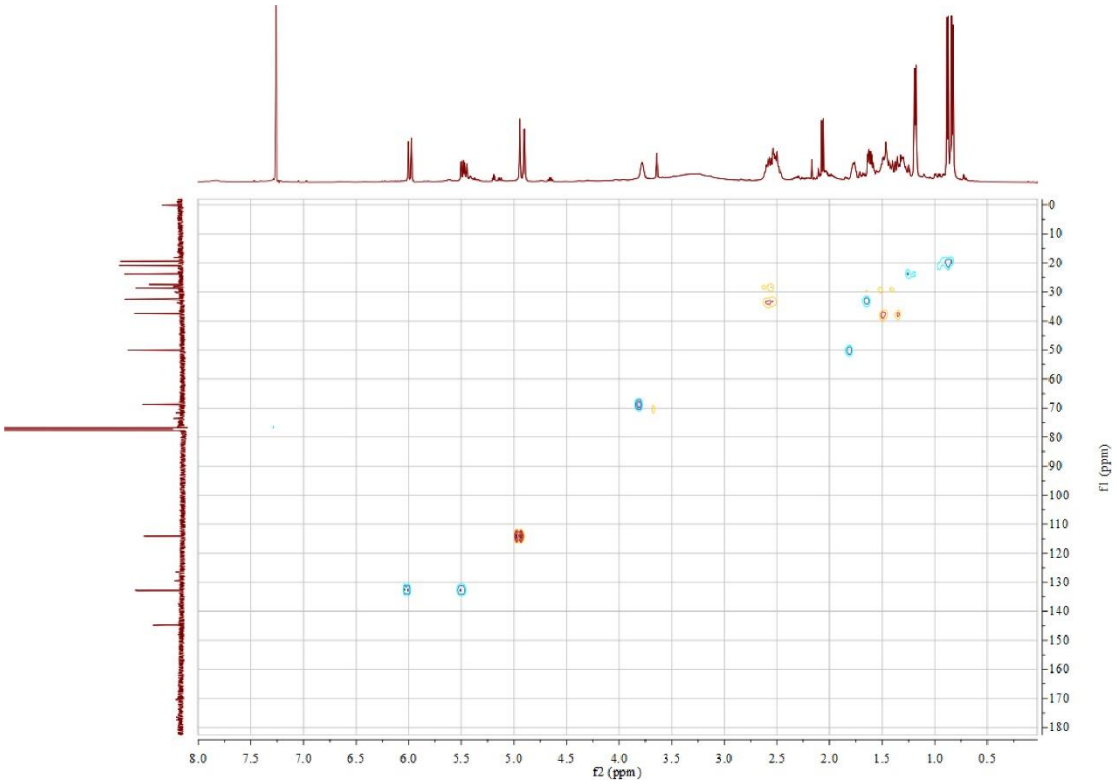


Figure S4. (c) HSQC NMR spectrum of compound **1**

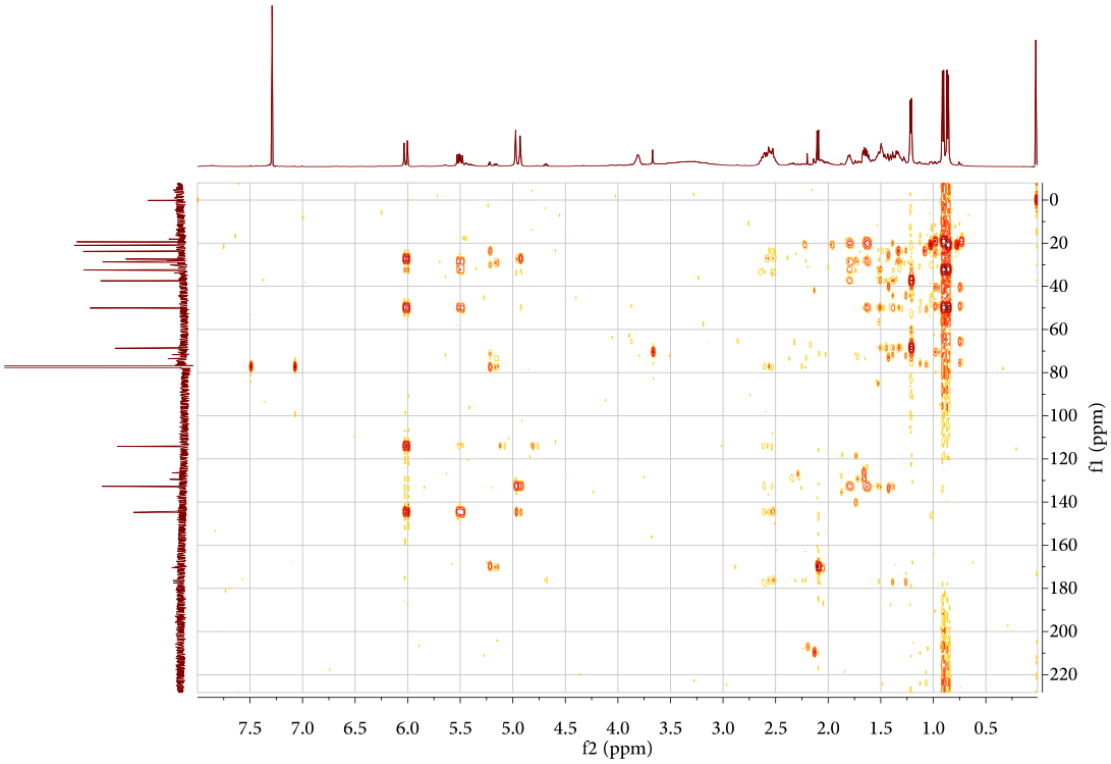


Figure S4. (d) HMBC NMR spectrum of compound **1**

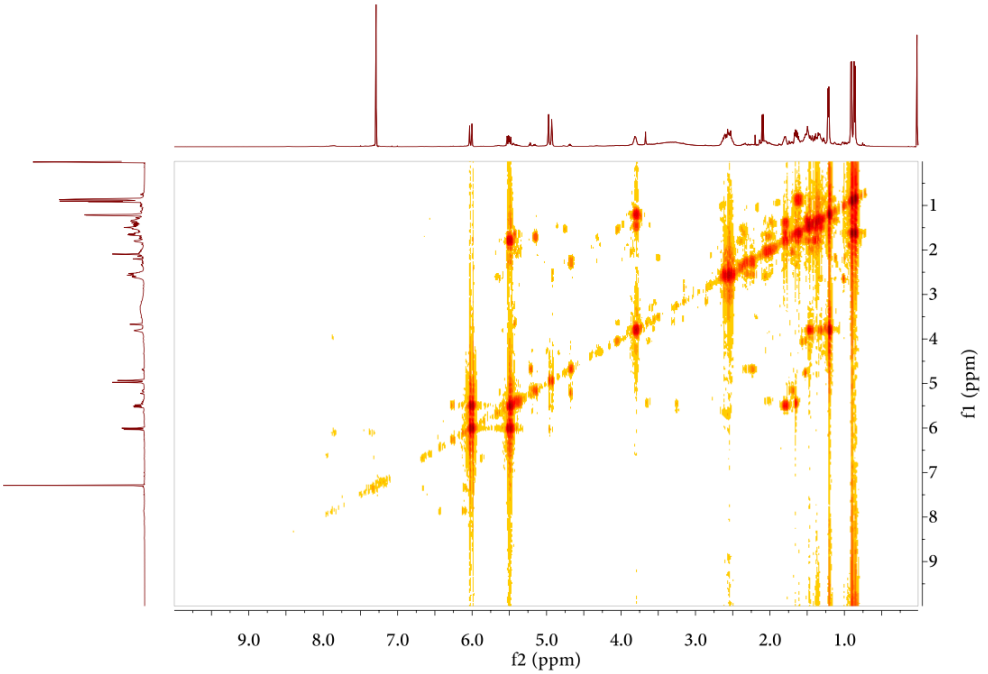


Figure S4. (e) ^{1}H-^{1}H COSY NMR spectrum of compound **1**

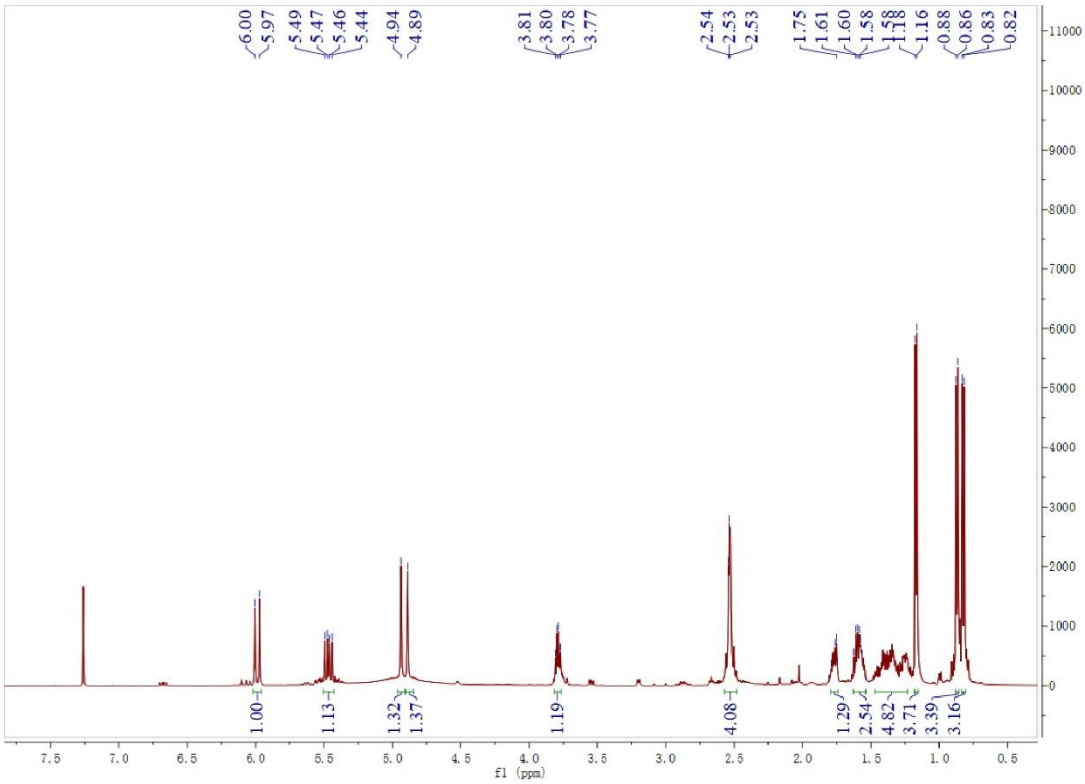


Figure S5. (a) ^{1}H NMR spectrum of compound **2** in $CDCl_3$ (500 MHz)

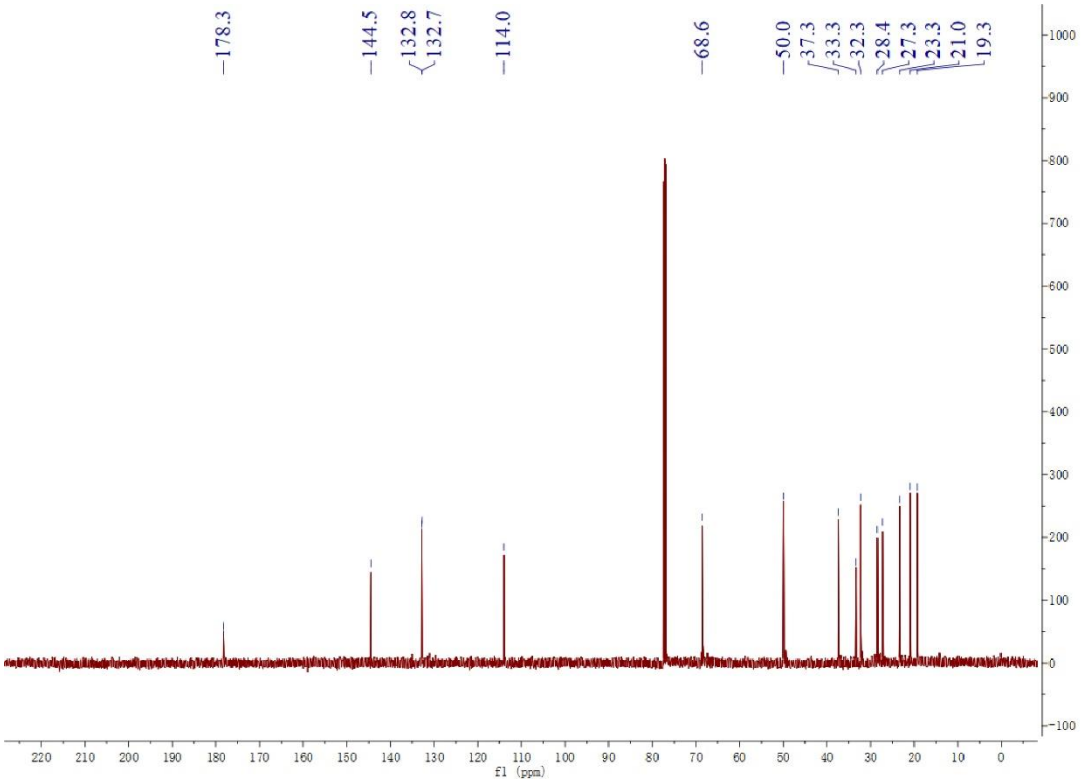


Figure S5. (b) ^{13}C NMR spectrum of compound **2** in $CDCl_3$ (125 MHz)

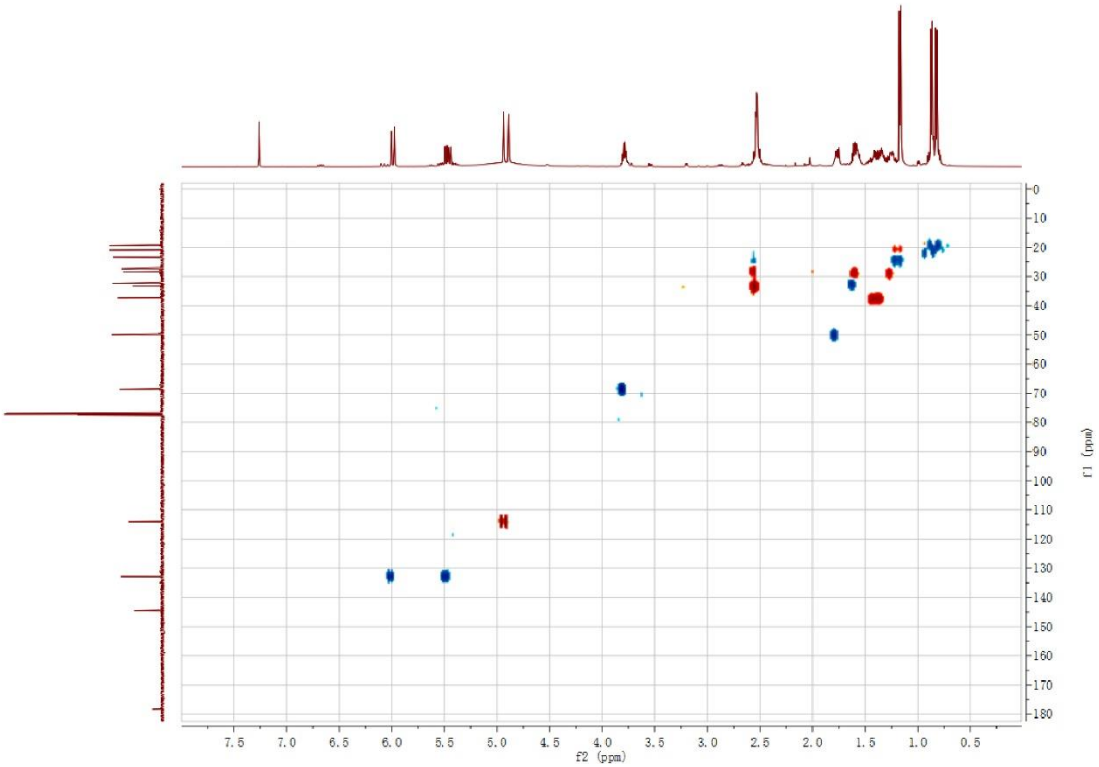


Figure S5. (c) HSQC NMR spectrum of compound **2**

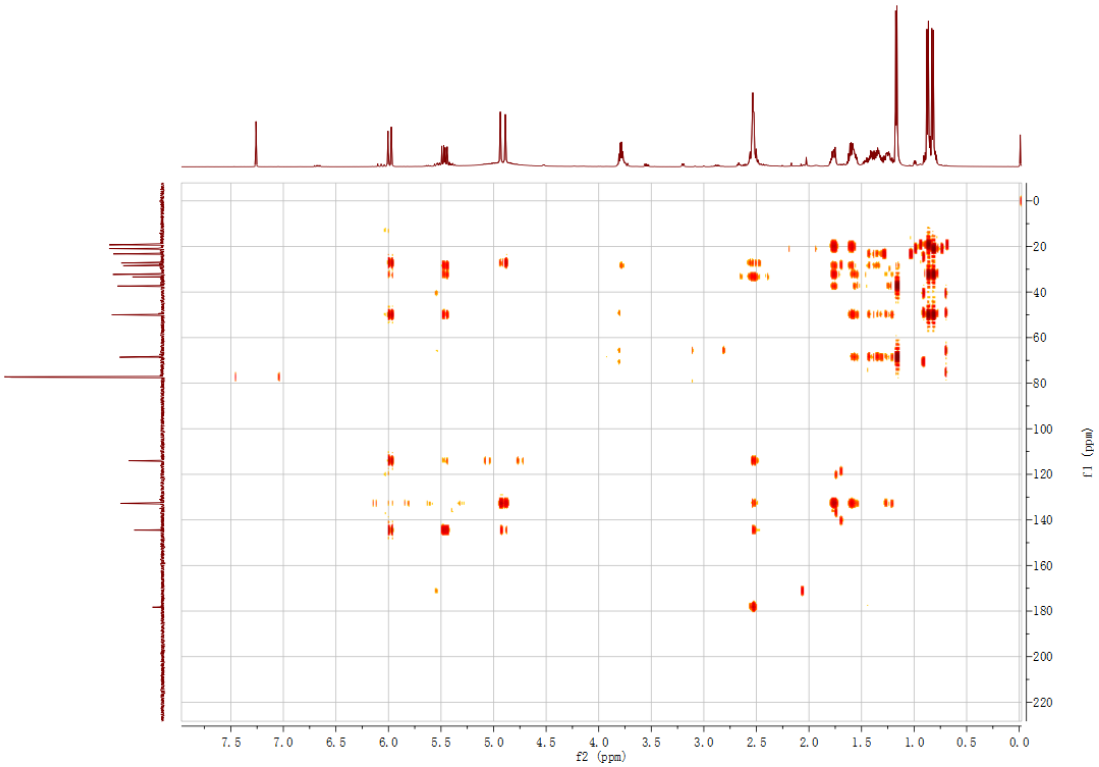


Figure S5. (d) HMBC NMR spectrum of compound **2**

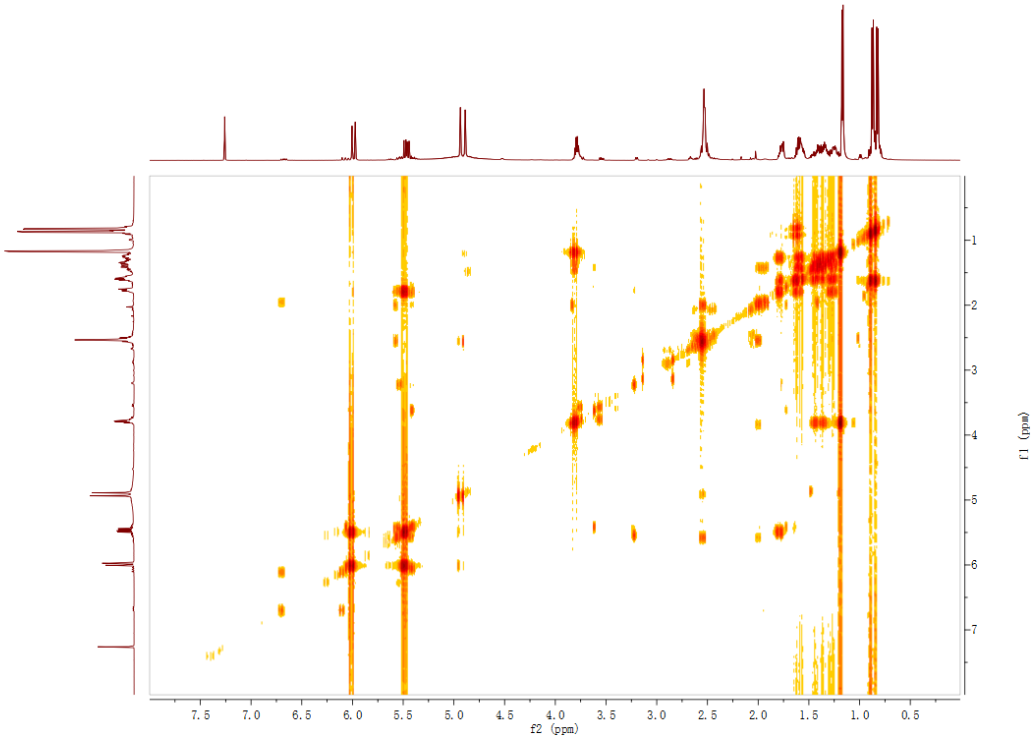


Figure S5. (e) ^{1}H-^{1}H COSY NMR spectrum of compound **2**

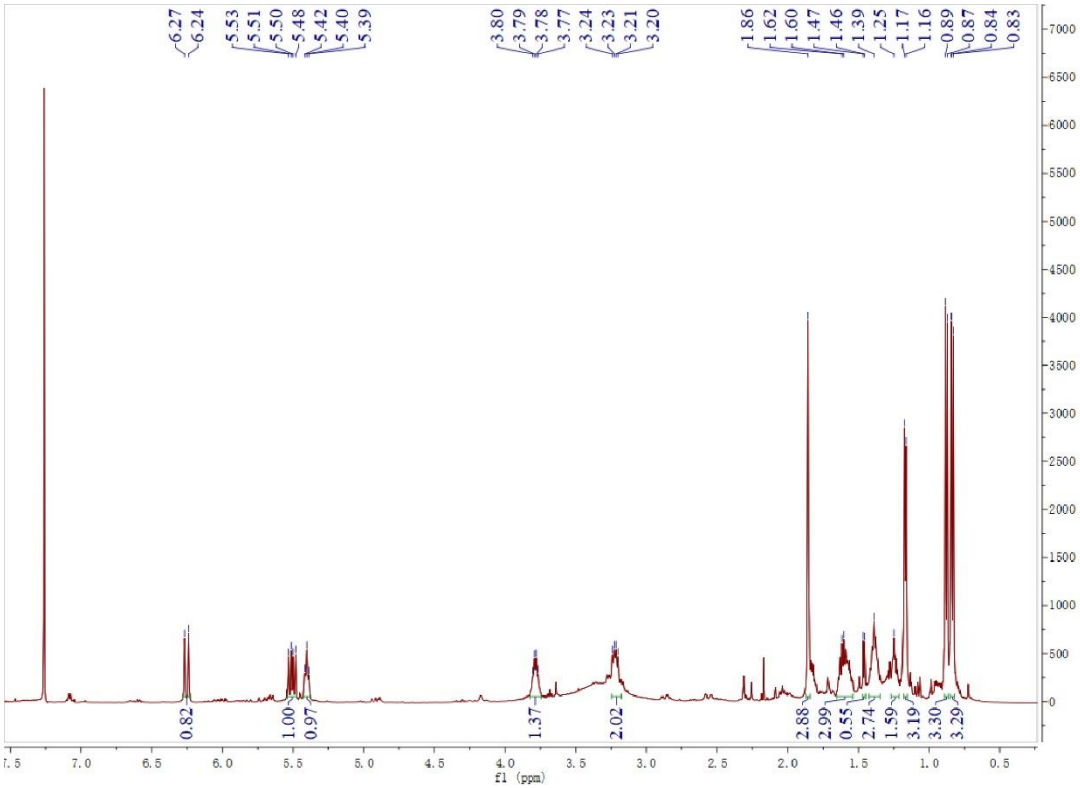


Figure S6. (a) ^{1}H NMR spectrum of compound **3** in $CDCl_3$ (500 MHz)

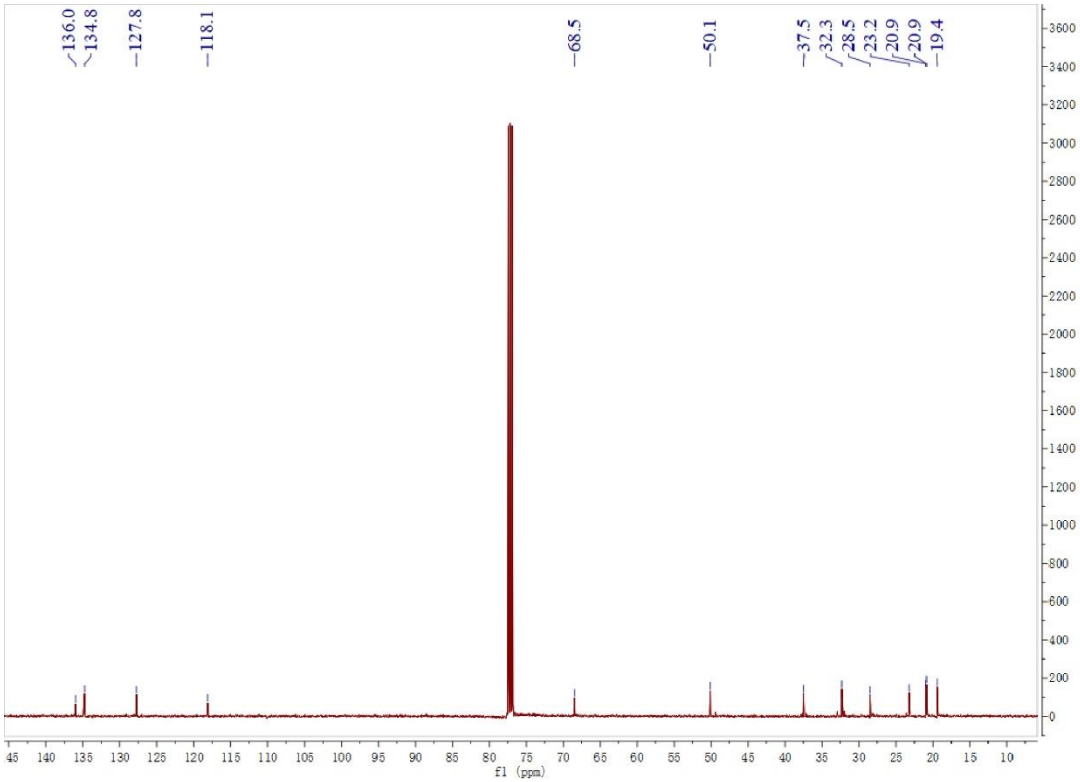


Figure S6. (b) ^{13}C NMR spectrum of compound **3** in $CDCl_3$ (125 MHz)

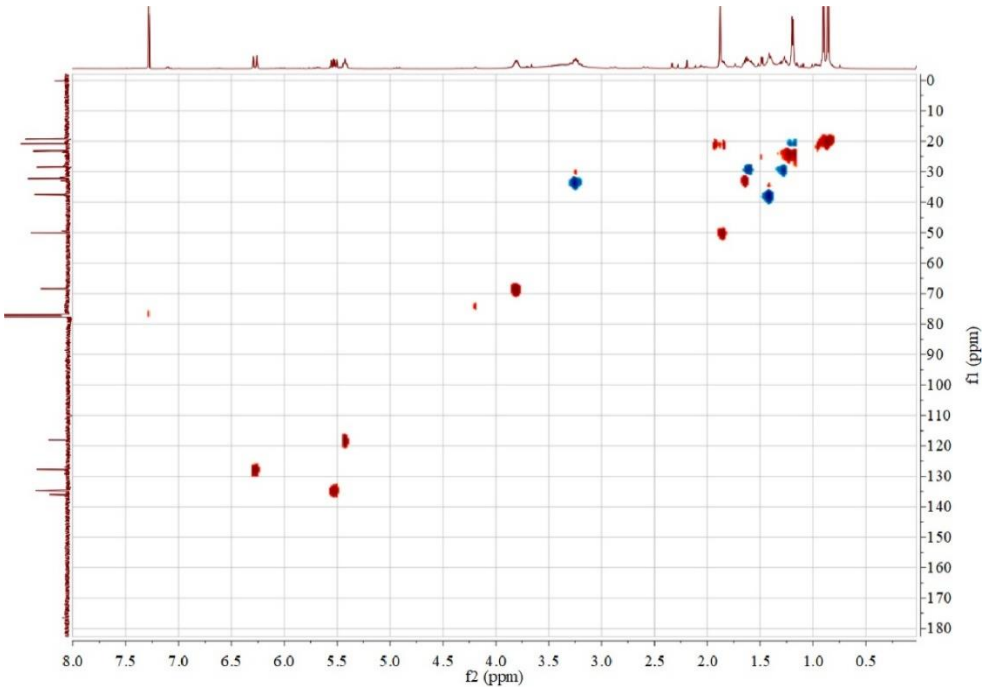


Figure S6. (c) HSQC NMR spectrum of compound **3**

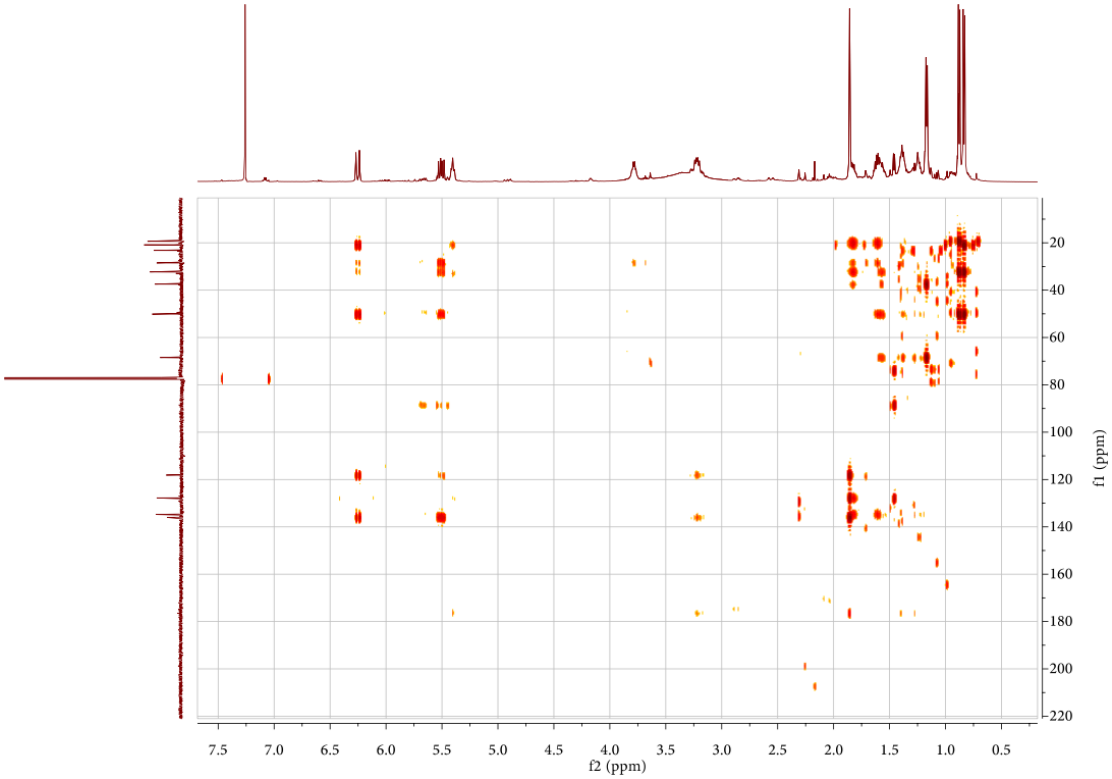


Figure S6. (d) HMBC NMR spectrum of compound **3**

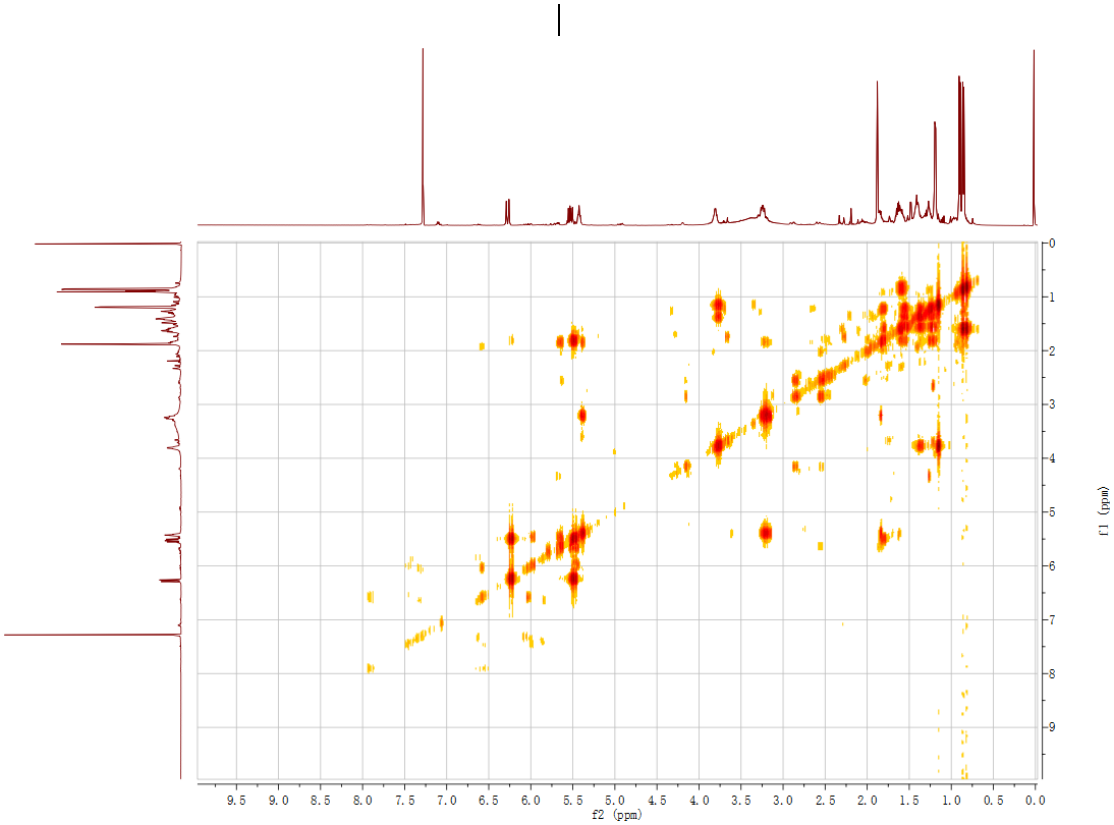


Figure S6. (e) ^{1}H-^{1}H COSY NMR spectrum of compound **3**

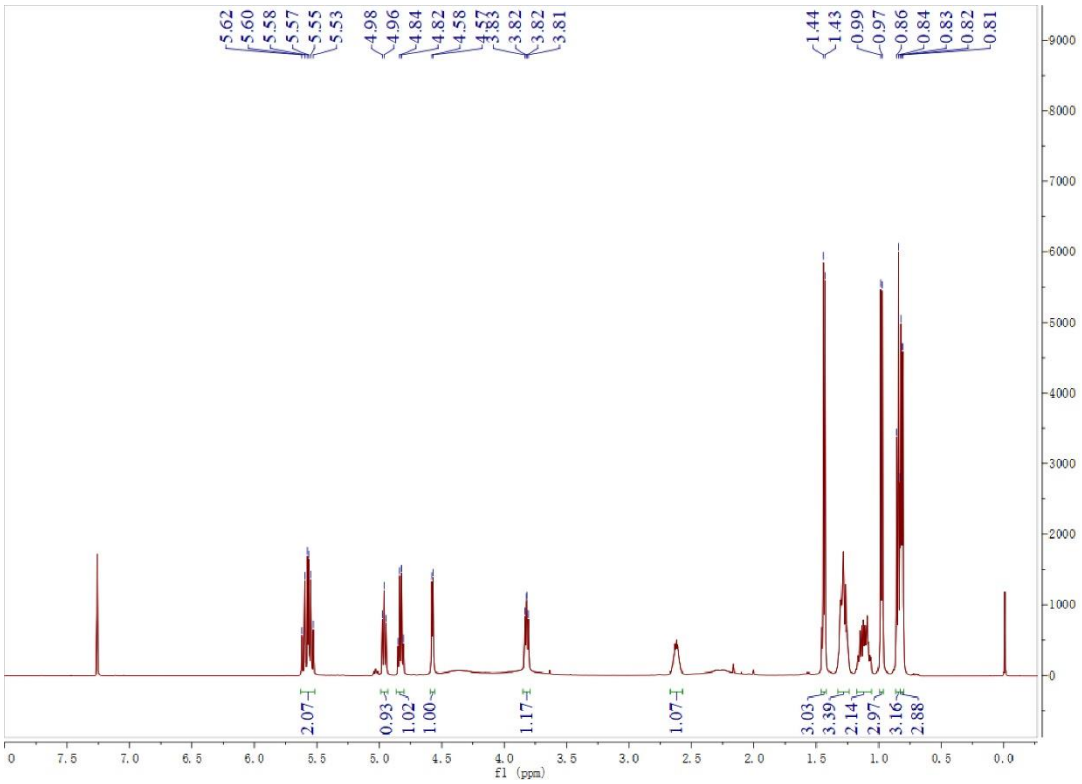


Figure S7. (a) ^{1}H NMR spectrum of compound **4** in $CDCl_3$ (500 MHz)

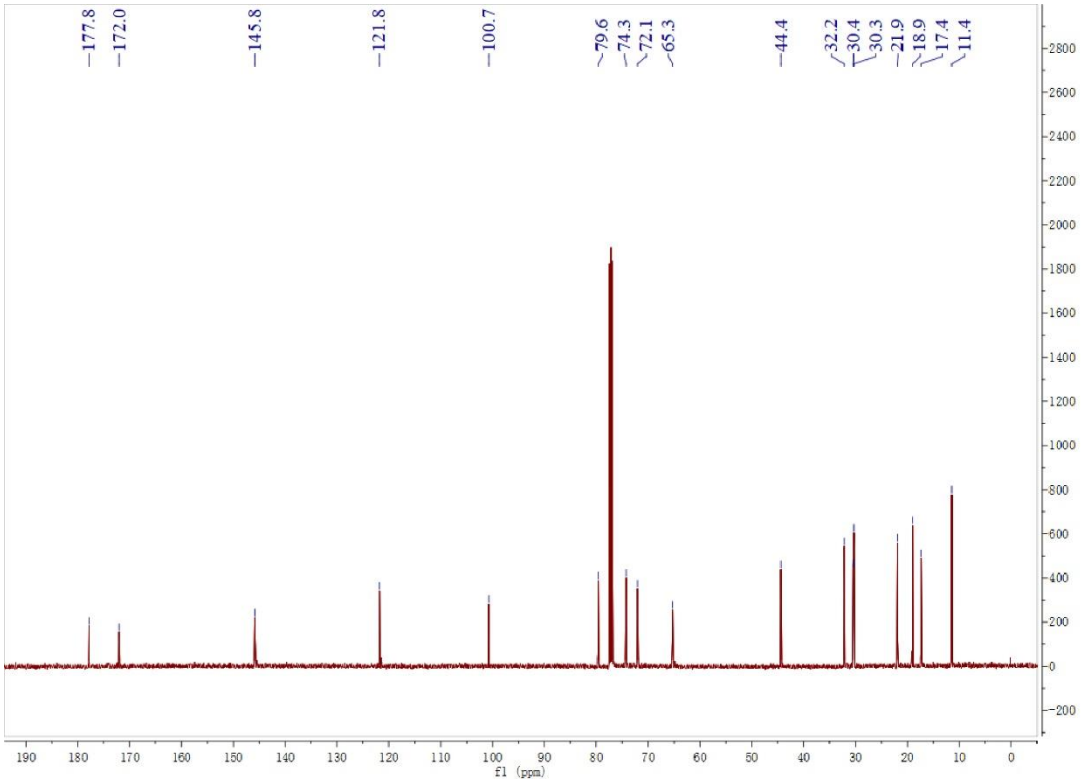
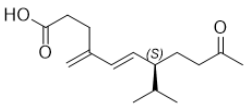
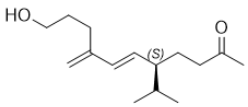


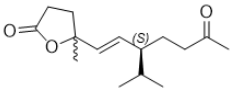
Figure S7. (b) ^{13}C NMR spectrum of compound **4** in $CDCl_3$ (125 MHz)



tricinonoic acid



FARI



5*E*,7*S*-isopropyl-4-methyl-10-oxo-undecen-4-olide

Figure S8. Compounds related to compounds **1**–**3** in previous studies[7-9]

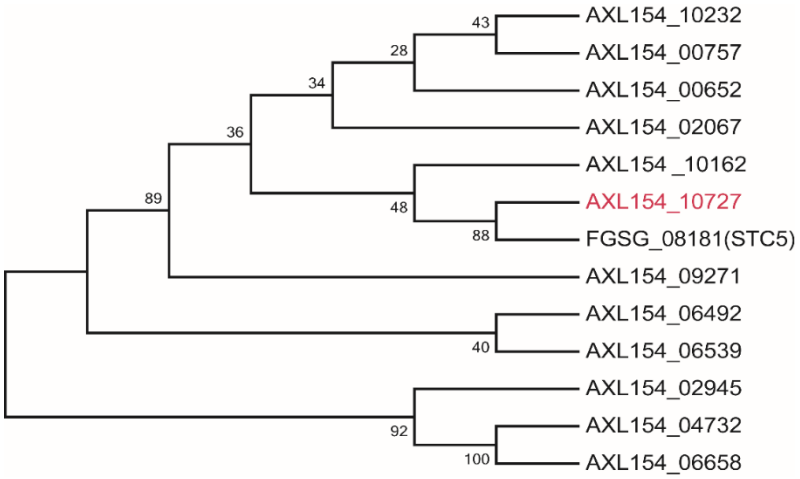


Figure S9. Phylogenetic analysis the terpene cyclases from *T. hypoxylon* with STC5 in *F. graminearum*[10]. Phylogenetic tree constructed by neighbor-joining with 1000 bootstrap replicates using MEGA 7.0. Enzyme investigated in this study is highlighted in red.

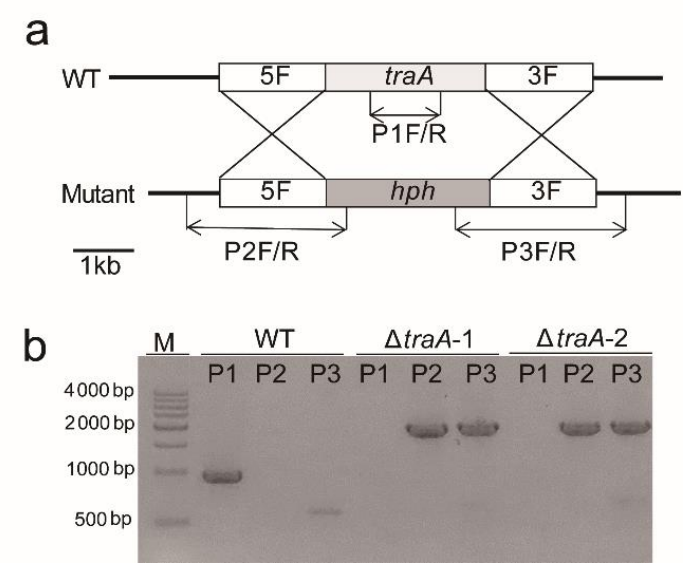


Figure S10. Gene deletion of *traA* in *T. hypoxylon*. Schematic illustration for disruption of *traA* gene by homologous recombination (a). Verification of *traA* deletion mutant by diagnostic PCR (b).

4. Supplementary references

1. J. Sun, Y. Pei, E. Li, W. Li, K. D. Hyde, W. B. Yin and X. Liu, *Sci. Rep.*, 2016, **6**, 37369-37379.
2. H. Liu, G. Wang, W. Li, X. Liu, E. Li and W. B. Yin, *Microbiology*, 2018, **164**, 769-778.
3. J. W. Bok and N. P. Keller, *Methods mole bio*, 2012, **944**, 163-174.
4. X. Wang, F. Wu, L. Liu, X. Liu, Y. Che, N. P. Keller, L. Guo and W. B. Yin, *Fungal Genet Biol*, 2015, **81**, 221-228.
5. I. Ohtani, T. Kusumi, Y. Kashman and H. Kakisawa, *J Am Chem Soc*, 1991, **113**, 4092-4096.
6. N. J. Alexander, T. M. Hohn and S. P. McCormick, *Appl. Environ. Microbiol.*, 1998, **64**, 221-225.
7. B. P. Bashyal and A. A. Leslie Gunatilaka, *Nat Prod Res*, 2010, **24**, 349-356.
8. A. J. Aasen, J. R. Hlubucek and C. R. Enzell, *Acta Chem Scand B: Org Chem Biochem*, 1975, **29**, 677-681.
9. J. Qi, L. Cheng, Y. Sun, Y. Hirata, N. Ushida, Z. Ma, H. Osada, T. Nishikawa and L. Xiang, *Angew Chem Int Edit*, 2018, **57**, 8100-8104.
10. D. A. Adpressa, L. R. Connolly, Z. M. Konkel, G. F. Neuhaus, X. L. Chang, B. R. Pierce, K. M. Smith, M. Freitag and S. Loesgen, *Fungal Genet Biol*, 2019, **132**, 103256-103270.