Electronic Supplementary Material (ESI) for Green Chemistry.
This journal is © The Royal Society of Chemistry 2023

Supplementary information for

Repurposing the cellulase workhorse *Trichoderma reesei* as a ROBUST chassis for efficient terpene production

Meili Xiao[1, 2], Yinmei Wang[1, 2], Yan Wang[1], Xing Yan[1], Zhihua Zhu[1], Ernuo Tian[1,2], Chengshuai Yang[1], Erdong Ma[1,2], Gen Zou[1, 3, *], Zhihua Zhou[1, 2, *], Pingping Wang[1,*]

[1] CAS-Key Laboratory of Synthetic Biology, CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai, 200032, China

[2] University of the Chinese Academy of Sciences, Beijing, 100049, China

[3] Shanghai Key Laboratory of Agricultural Genetics and Breeding, Institute of Edible Fungi, Shanghai Academy of Agriculture Science, Shanghai, 201403, China

These authors contributed equally: Meili Xiao, Yinmei Wang

Email address: ppwang@cemps.ac.cn, zhouzhihua@cemps.ac.cn, zougen@sibs.ac.cn

Supplementary methods

General

Oligonucleotide primers synthesis and sequencing were performed by Biosune (shanghai, China). All the fragments for cloning were amplified by the PrimeSTAR HS DNA polymerase kit (TaKaRa, Japan). Cas9 protein tagged with a nuclear localization signal was purchased from Novoprotein, Inc. (Shanghai, China). Templates for guide RNA transcription were generated by oligo-extension using I5 polymerase (Tsingke, Beijing, China). Target sequences were designed in silico using Cas-Designer and Cas-OFFinder (http://www.rgenome.net/cas-offinder/) to reduce off-target. Guide RNAs were in vitro transcribed through runoff reactions using the T7 RNA polymerase (New England BioLabs) according to the manufacturer's protocol.

The GC analyses were performed with GC-2010 Pro (Shimadzu, Kyoto, Japan) equipped with a TG-5MS capillary column (30 m×0.25 mm×0.25 μm film thickness; Thermo Scientific) and a flame ionization detector (FID). The GC-MS analyses were conducted with an Agilent 7890B GC system coupled to an Agilent 5977B MSD fitted with an HP-5MS capillary column (30 m×0.25 mm×0.25 μm film thickness; Agilent). The program parameters of metabolites were the same as GC in GC-MS analysis. The HPLC analyses were performed with a Shimadzu LC20A system and a photo-diode detector (PDA) using a Boltimate Core-Shell C_{18} Column (2.1 mm×100mm, 2.7 μm; Welch Material, Maryland, America). A gradient of elution (A, water; B, acetonitrile) was used with the following program: 0-2 min (45% B), 2-15 min (45%-100% B), 15-27 min (100% B), 27-30 min (45% B) and the flow rate was maintained at 0.45 mL min^{-1}. The triterpenoid products were detected at 203 nm. The HPLC-MS analyses were carried out with an Agilent LC1290-MS/QTOF6545 system. The column and program were same to those in HPLC.

Extraction, analyses, and purification of terpenoids

To analyze the 1,8-cineole (**2**) from transformant harboring *hyp3*, 10% (v/v) *n*-dodecane was added into the fermentation broth. After 4-days of growth, the dodecane layer diluted at 1:100 in ethyl acetate for GC and GC-MS analysis. The GC program was as follows： each sample was injected into the column at 60 °C and hold for 3 min; the column temperature ramped to 280 °C at a rate of 15 °C min^{-1} and hold for 5 min. The temperature of inlet and FID detector were kept at 300 °C. The injection volume was 1 μL in split ratio of 10. The flow rate of nitrogen carrier gas was 1.0 mL min^{-1}.

The extraction and analysis of aristolochene (**3**) are the same as 1,8-cineole (**2**). For the purification of **3**, 1 L of the culture was extracted with equal *n*-hexane overnight at room temperature. The extract was concentrated and then subjected to silica gel column chromatography, *n*-hexane as the elution solvent, to yield a colorless liquid.

The extraction and purification of fusicoccadiene (**4**) are the same as aristolochene (**3**), except for the dodecane layer diluted at 1:100 in *n*-hexane for GC and GC-MS analysis. The GC program was as follows：each sample was injected into the column at 60 °C and hold for 2 min; the column temperature ramped to 150 °C at a rate of 30 °C min^{-1}, with increased at 10 °C min^{-1} to 180°C, then increased to 280 °C at 30 °C min^{-1} and hold for 5 min. The temperature of inlet and FID detector were kept at 300°C. The injection volume was 2 μL in split ratio of 10. The flow rate of nitrogen carrier gas was 1.0 mL min^{-1}.

The extraction and analysis of stellata-2,6,19-triene (**5**) are the same as ophiobolin F (**1**). For the purification of **5**, 1 L of the culture was extracted with equal ethyl acetate. The extract was reextracted by n-hexane and concentrated. The residues were subjected to silica-gel column chromatography, with elution using n-hexane, to yield a white powder.

For the analysis of protosta-17(20)Z,24-dien-3β-ol (**6**) from transformant, 500 μL fermented broth after mycelium disruption was extracted with 1mL ethyl acetate twice. The extract concentrated under reduced pressure was resuspended by 100 μL ethyl acetate, and then subjected to HPLC and LC-MS analysis. For the purification of **6**, the extract from 2 L culture of transformant was subjected to silica gel column chromatography with stepwise elution of petroleum ether and ethyl acetate (50:1, 30:1, 20:1, 10:1, and 0:1, v/v) to yield 5 fractions. Fraction 3 was further purified by preparative thin layer chromatography (TLC) to obtain **6** as white powder.

The extraction of taxadiene (**7**) are the same as aristolochene (**3**), except for the dodecane layer was extract for direct injection into GC and GC-MS. The GC program was as follows：each sample was injected into the column at 150 °C and hold for 2min; the column temperature ramped to 180 °C at a rate of 10 °C min^{-1} and hold for 10min, with increased at 3 °C min^{-1} to 210 °C, then increased to 250 °C at 10°C min^{-1} and hold

for 5min. The temperature of inlet and FID detector were kept at 270 °C. The injection volume was 2 μL in split ratio of 10. The flow rate of nitrogen carrier gas was 1.0 mL min^{-1}.

Supplementary figures

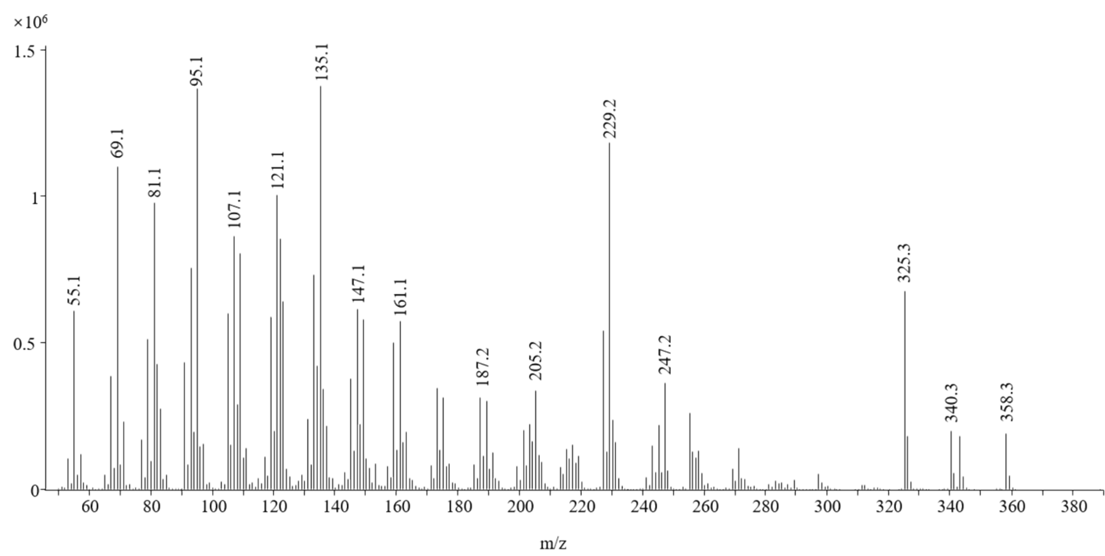


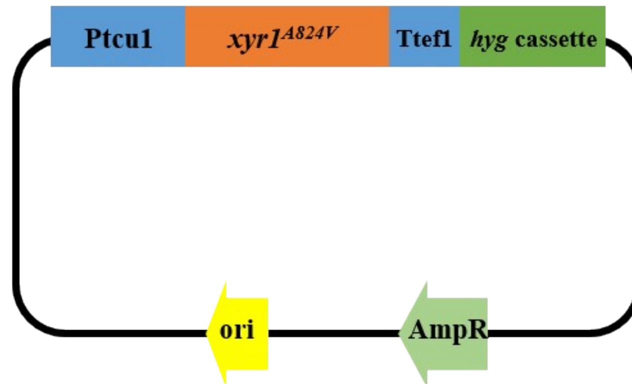
Figure S1 MS spectrum of **1** from the transformant.

a

b

Figure S2 NMR spectrum of **1**. (a) ^{1}H NMR spectrum in $CDCl_3$ at 400MHz; (b) ^{13}C NMR spectrum in $CDCl_3$ at 125MHz.

A



B

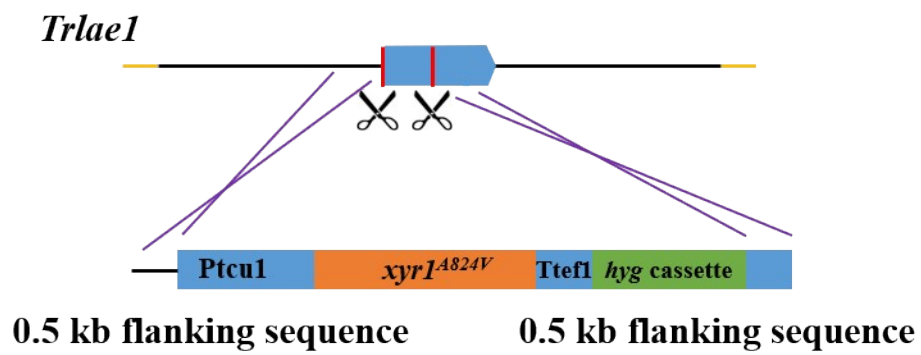


Figure S3 The schematic diagram for a strain overexpressing the cellulase production regulator $xyr1^{A824V}$ (A, OExyr1/MC3-Δ10-TrAcOS) and simultaneously deleting the secondary metabolism regulator *lae1* (B, Δlae1:: xyr1/MC3-Δ10-TrAcOS) construction in *T.reesei*.

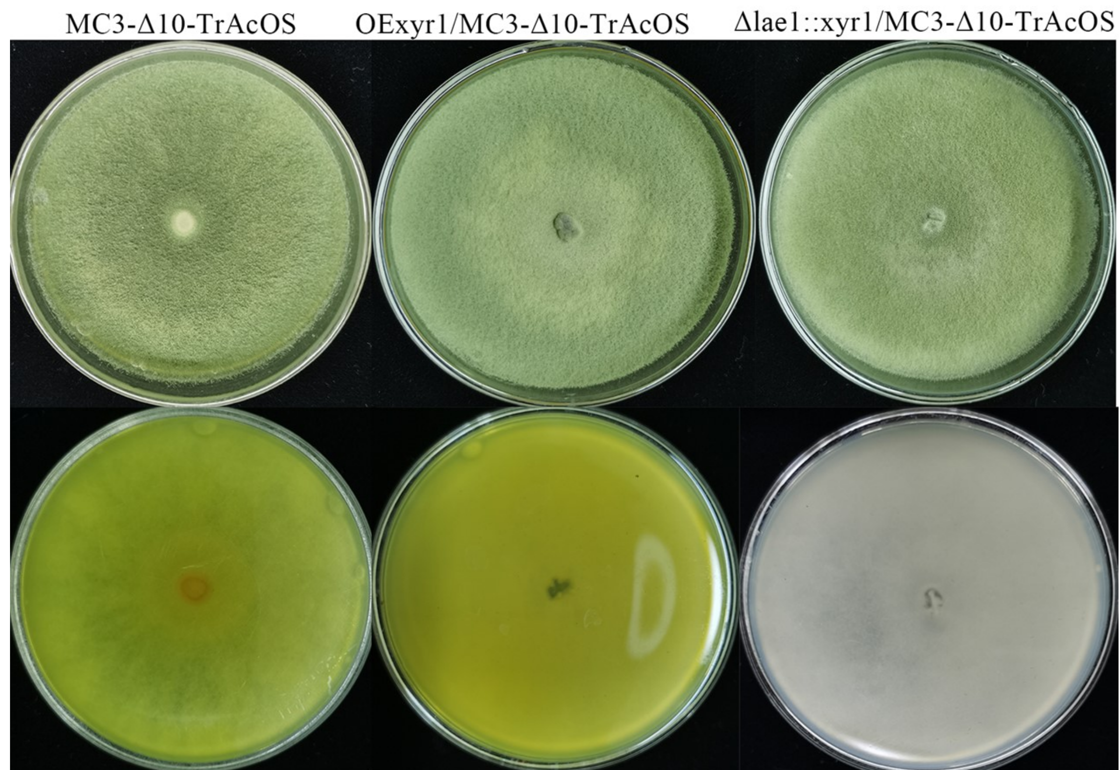


Figure S4 Phenotype of *T. reesei* MC3-Δ10-TrAcOS, OExyr1/MC3-Δ10-TrAcOS and Δlae1::xyr1/MC3-Δ10-TrAcOS strains on PDA medium.

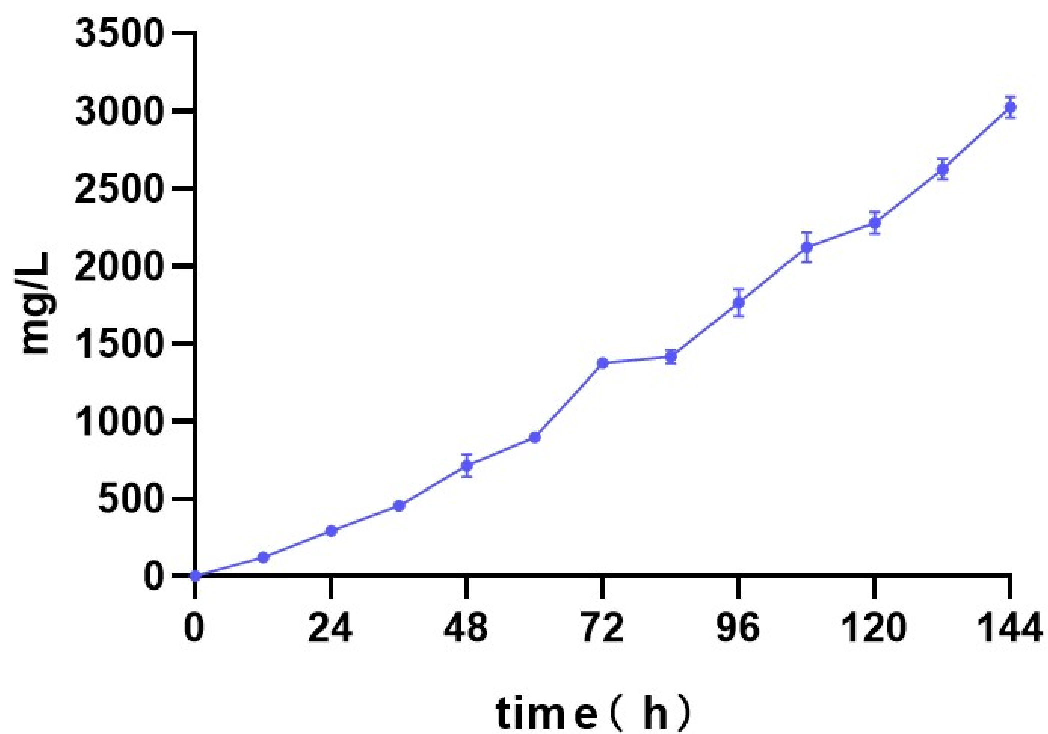


Figure S5 The time-course of ophiobolin F (**1**) production in the fermenter. The strain Δlae1::xyr1/ MC3-Δ10-TrAcOS provided with 1% lactose and 3% glucose as initial carbon source. After 48 h, 0.56 g/h sugar (lactose : glucose = 1:3) was supplemented in fed-batch system. With the accumulated production, the yield of ophiobolin F (**1**) reached 3.02 g/L on 144 h.

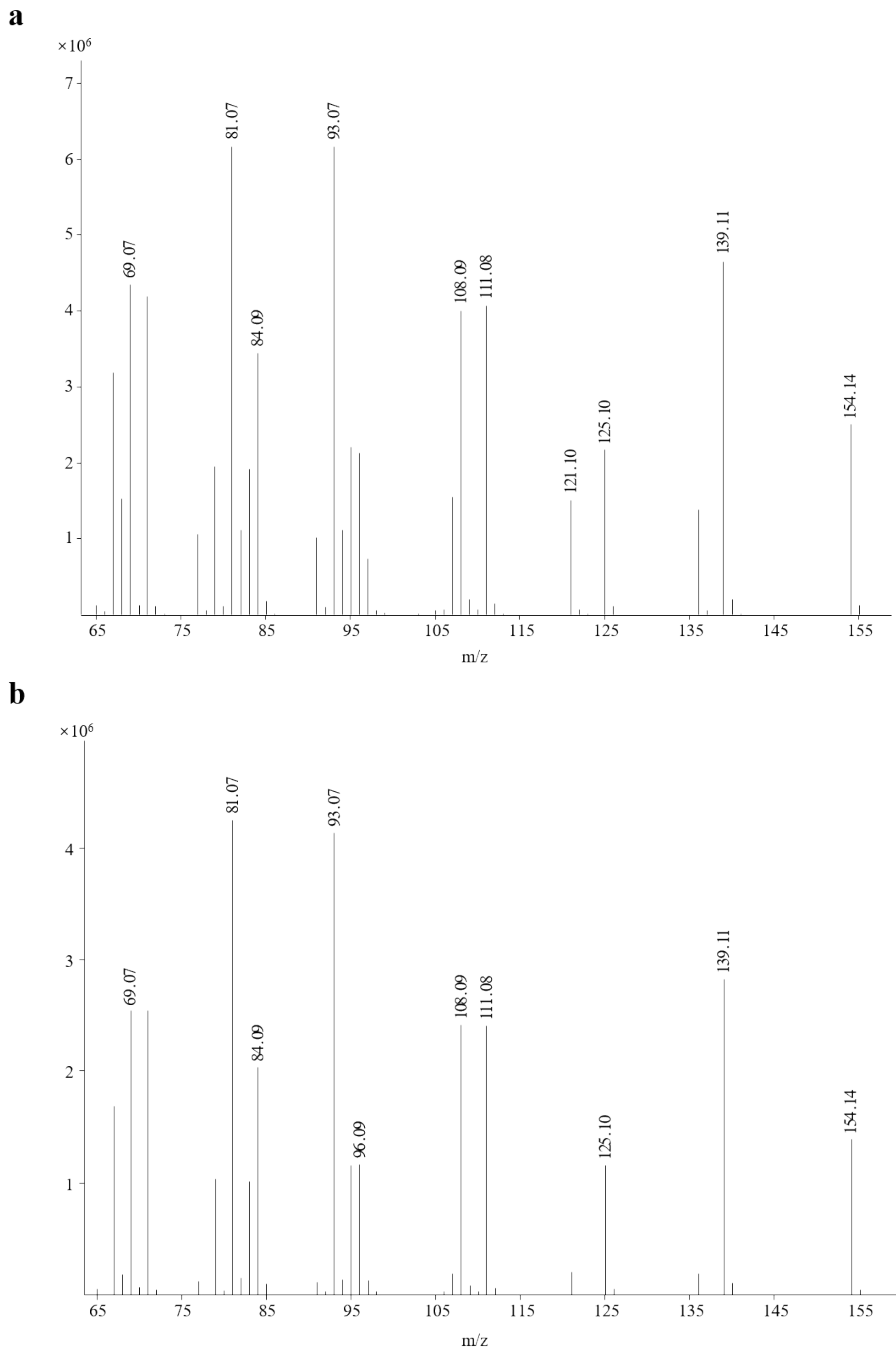


Figure S6 GC-MS analysis of **2** and authentic sample. (a) MS spectrum of **2** from transformant；(b) MS spectrum of authentic **2**.

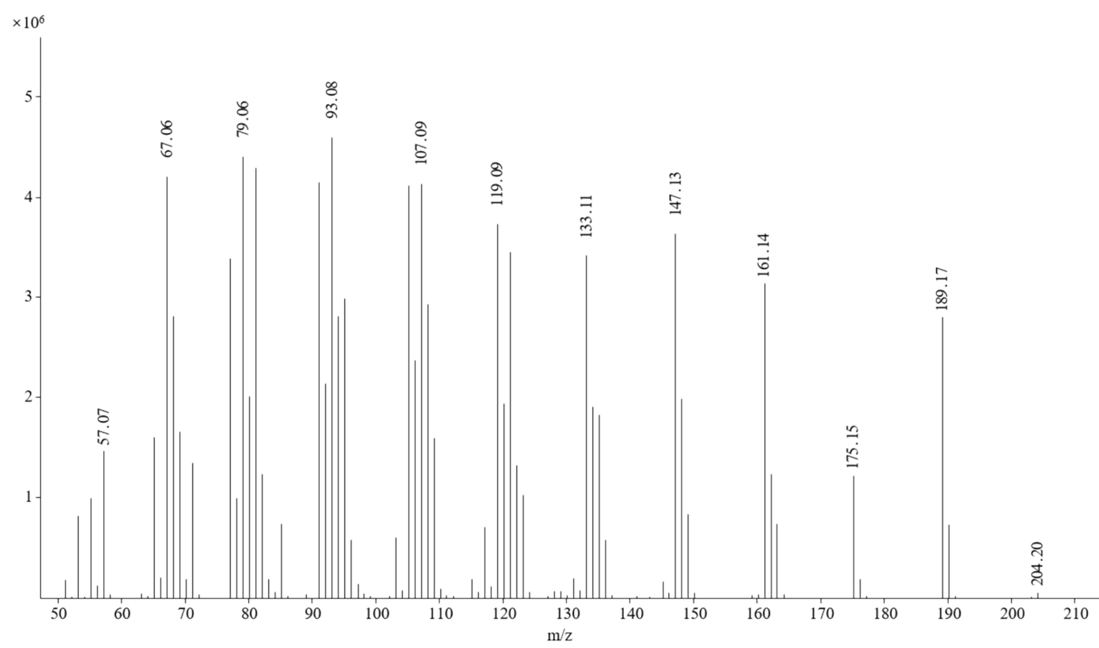
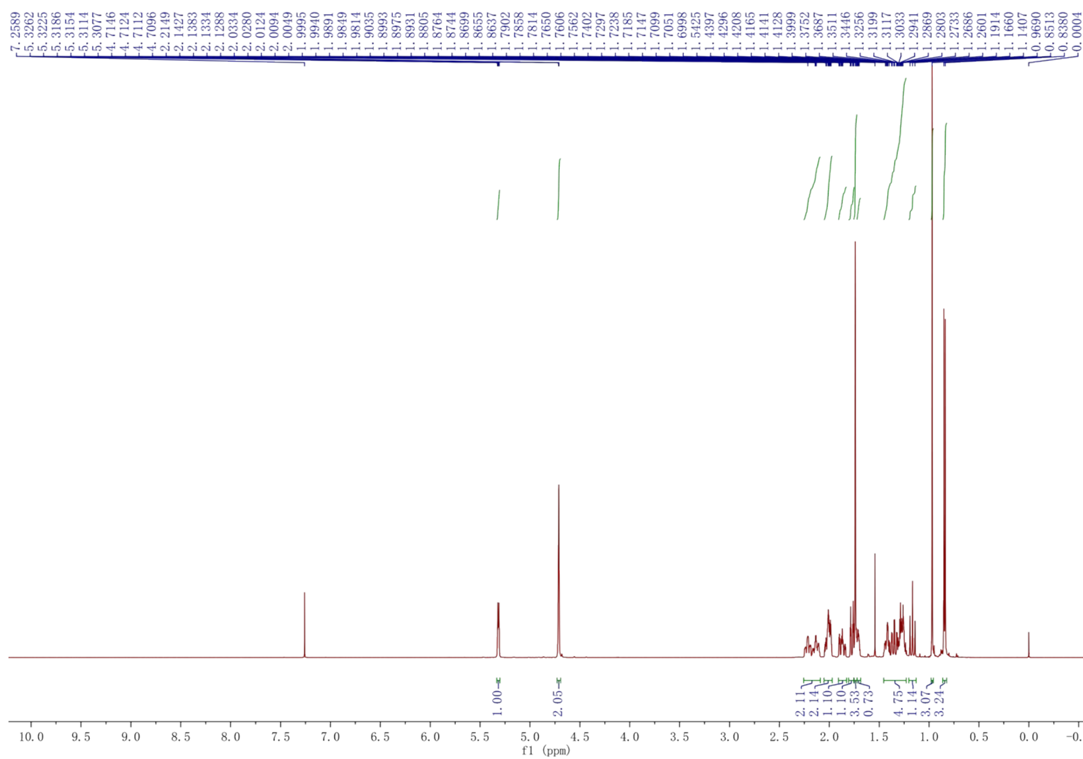


Figure S7 MS spectra of **3**.

a



b

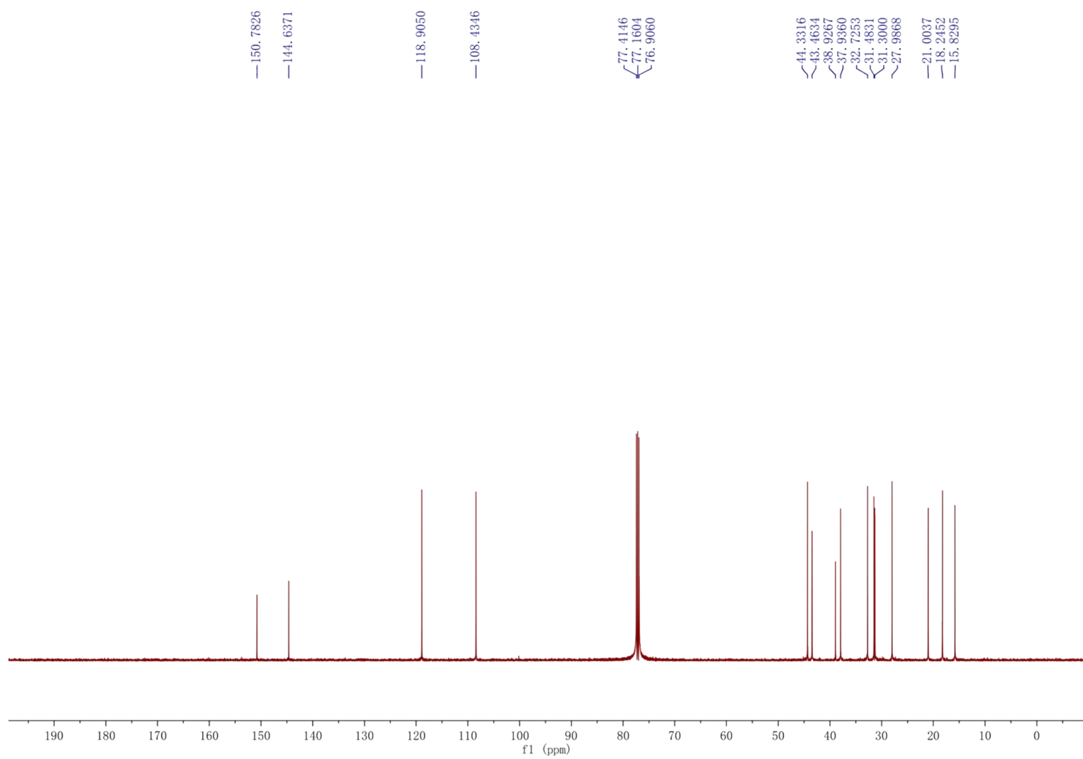


Figure S8 NMR spectrum of **3**. (a) ^{1}H NMR spectrum in $CDCl_3$ at 500MHz; (b) ^{13}C NMR spectrum in $CDCl_3$ at 125MHz.

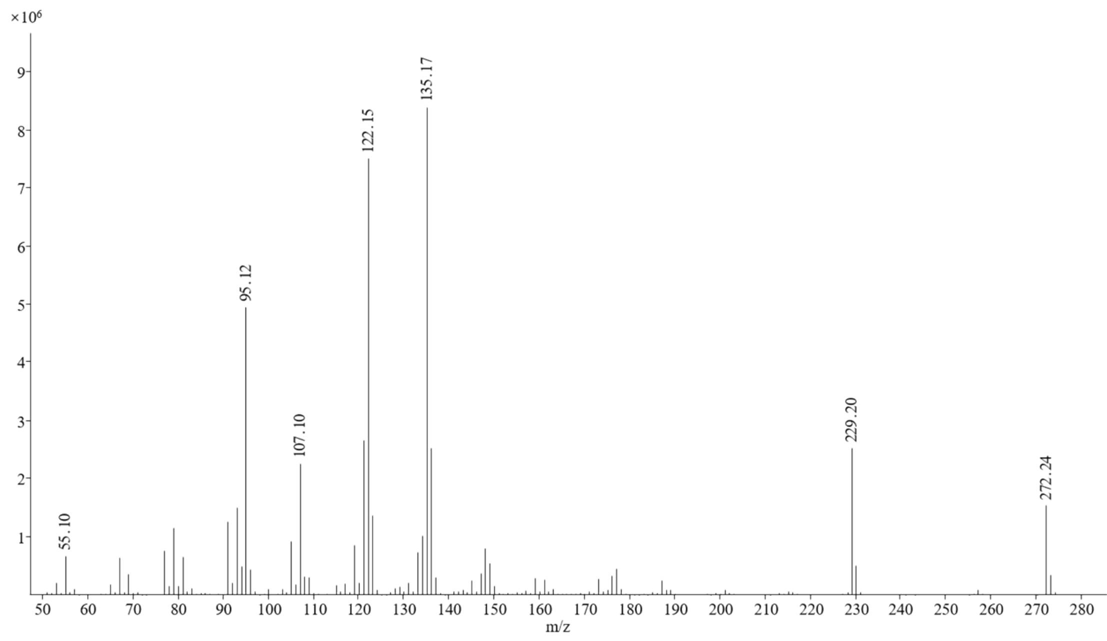
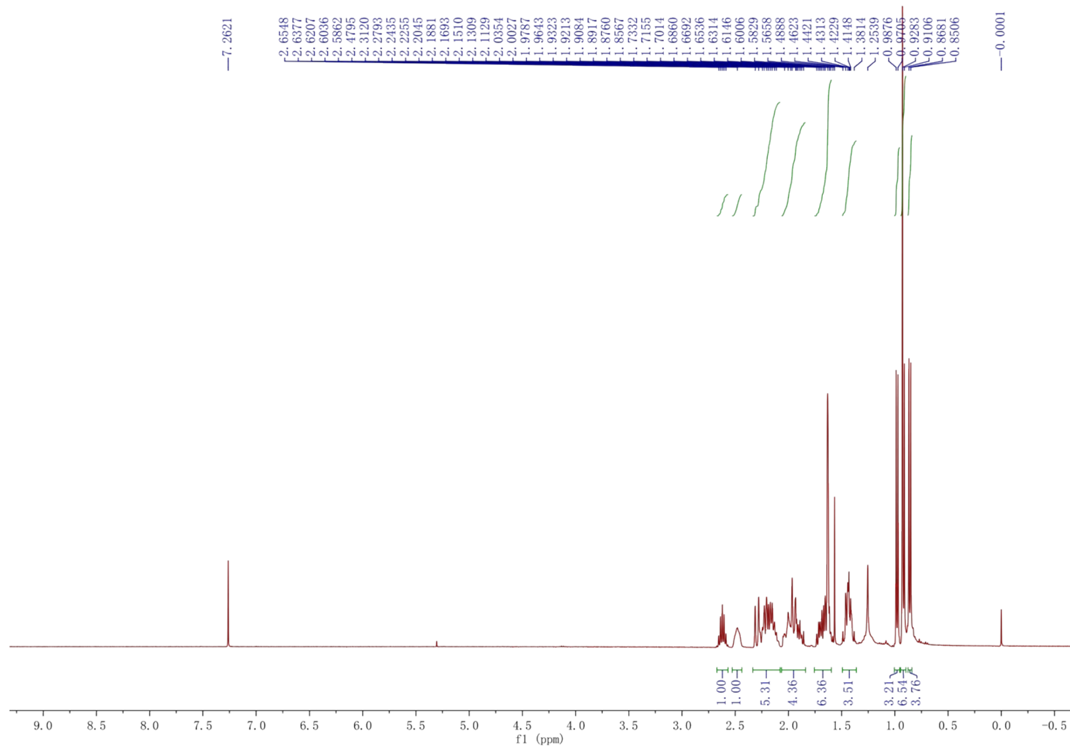


Figure S9 MS spectra of **4**.

a



b

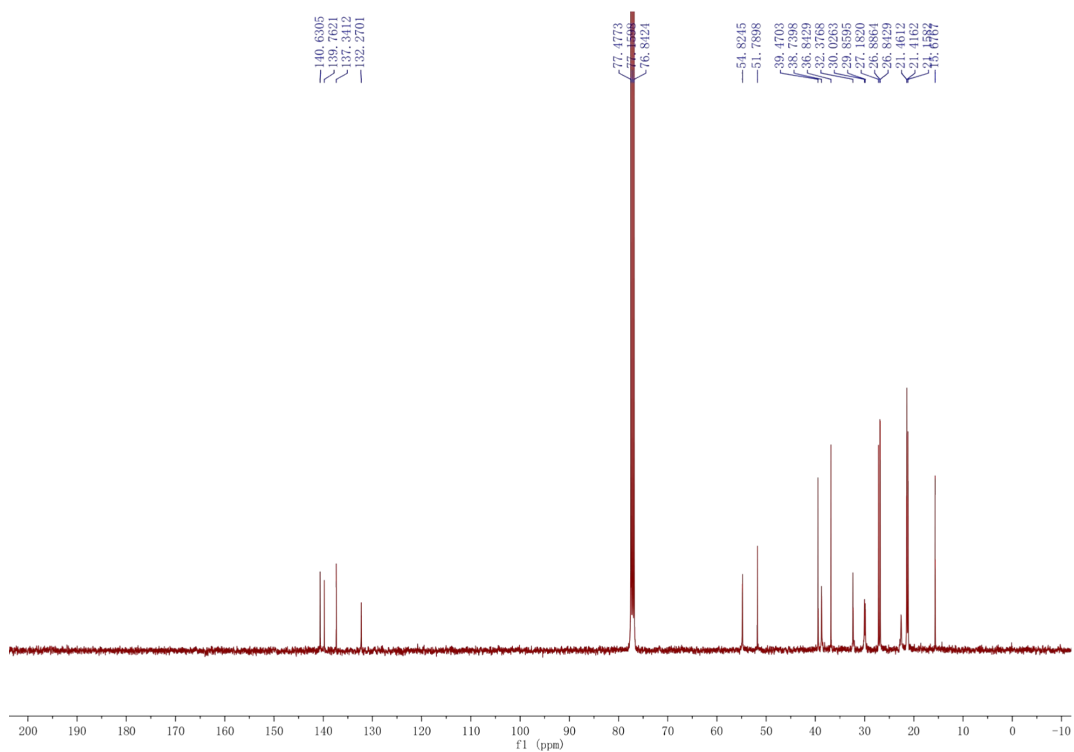


Figure S10 NMR spectrum of **4**. (a) ^{1}H NMR spectrum in $CDCl_3$ at 500MHz; (b) ^{13}C NMR spectrum in $CDCl_3$ at 125MHz.

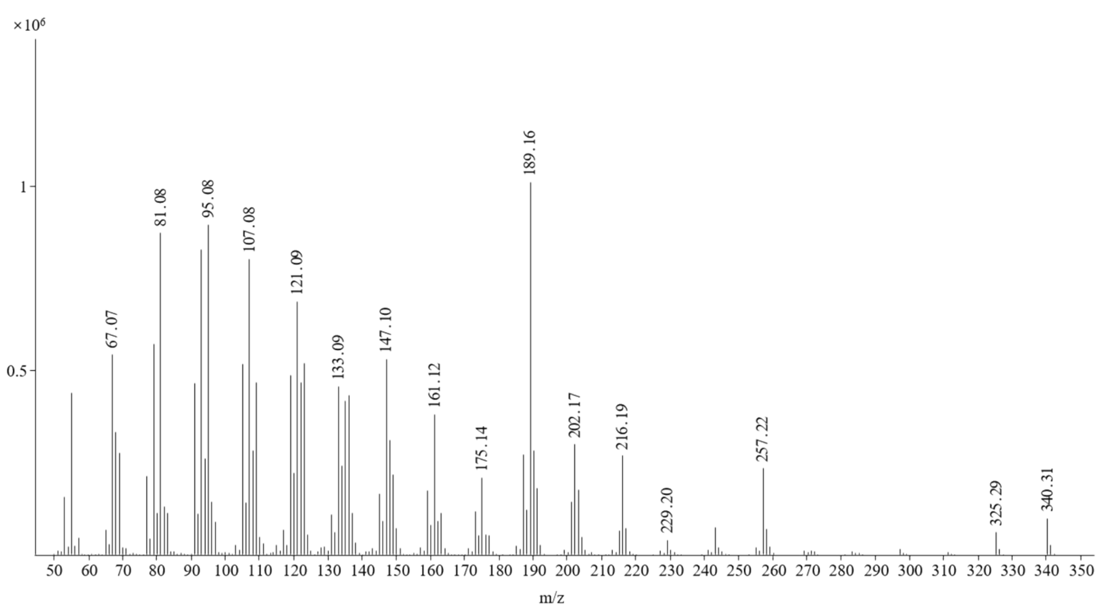
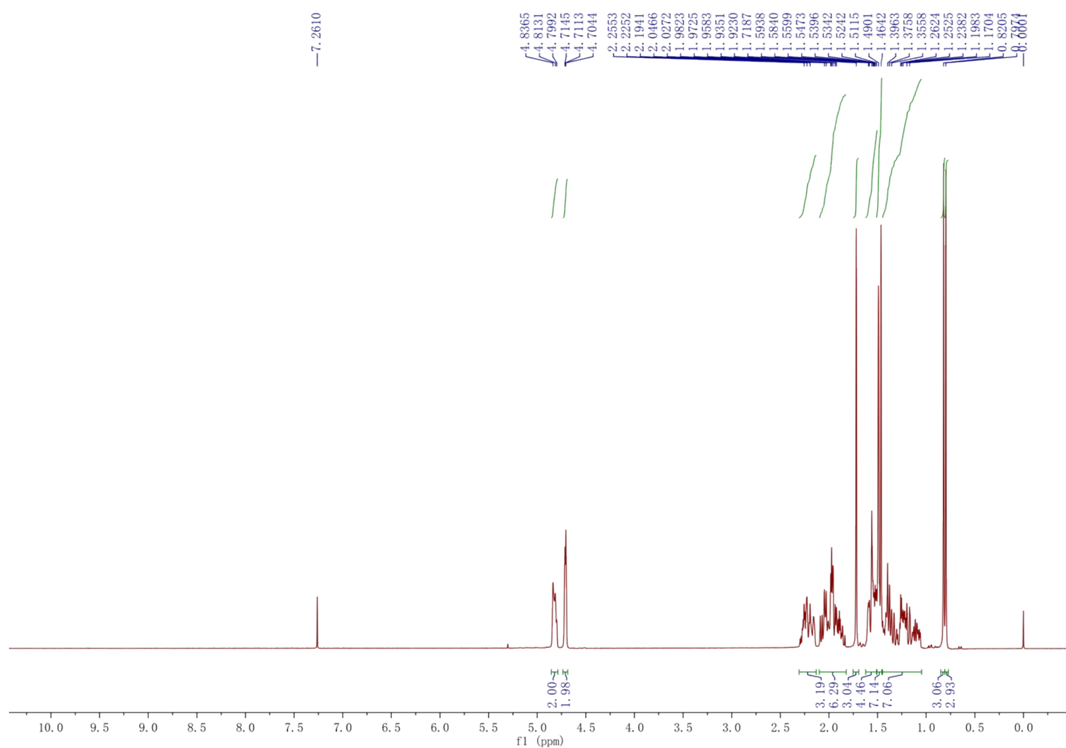


Figure S11 MS spectra of **5**.

a



b

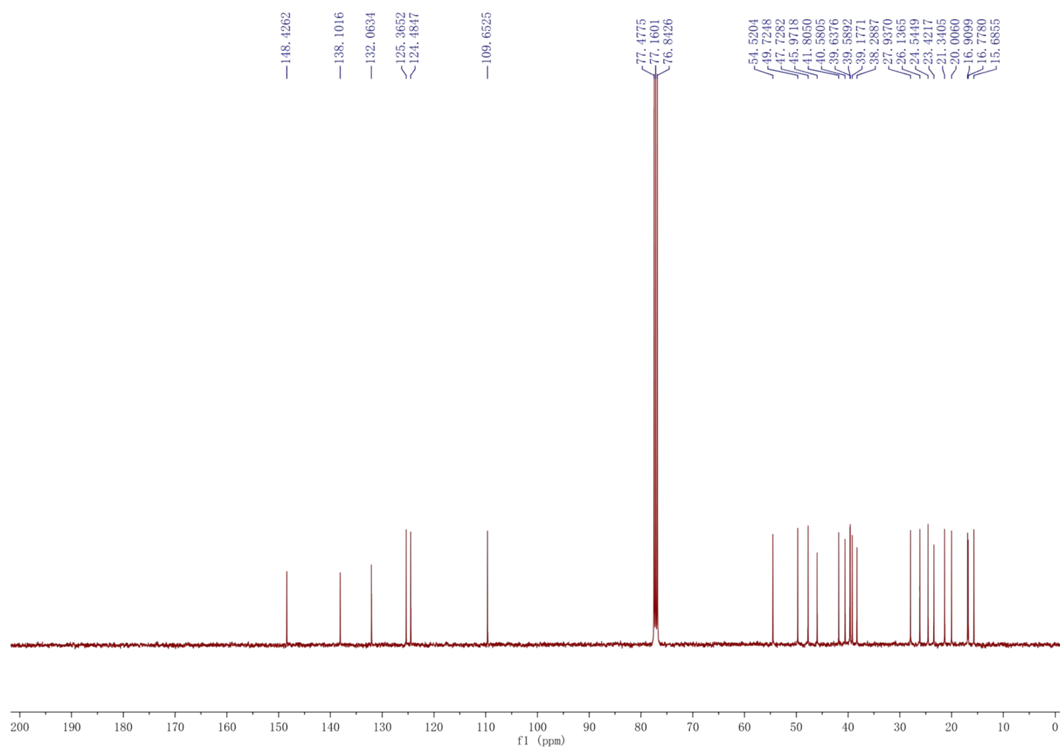


Figure S12 NMR spectrum of **5**. (A) ^{1}H NMR spectrum in $CDCl_3$ at 400MHz; (B) ^{13}C NMR spectrum in $CDCl_3$ at 100MHz.

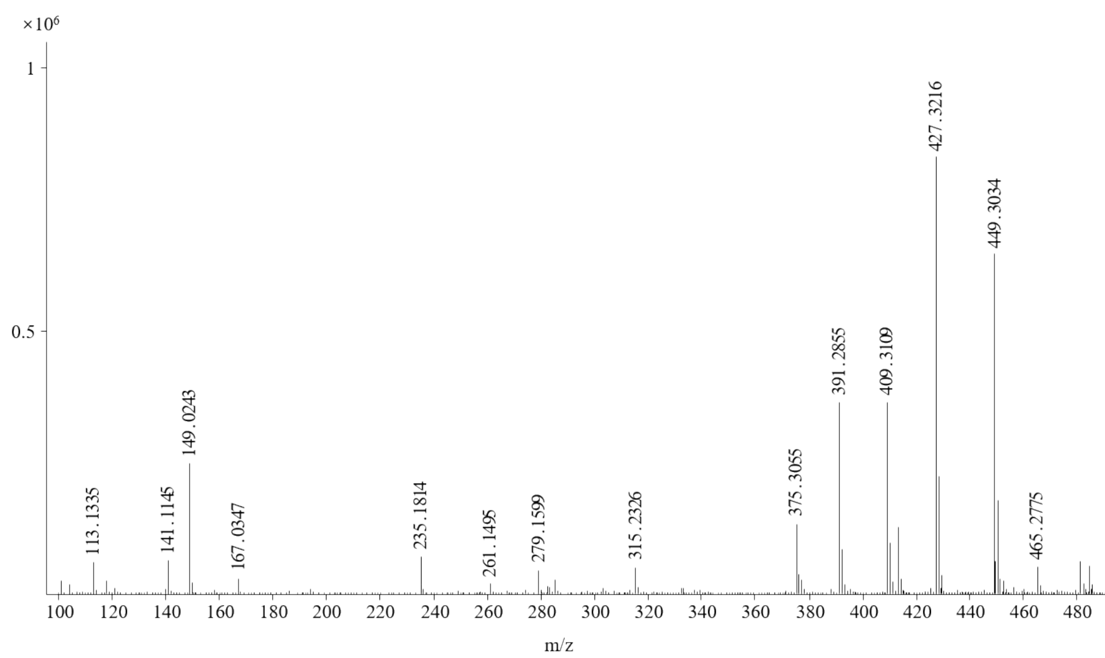
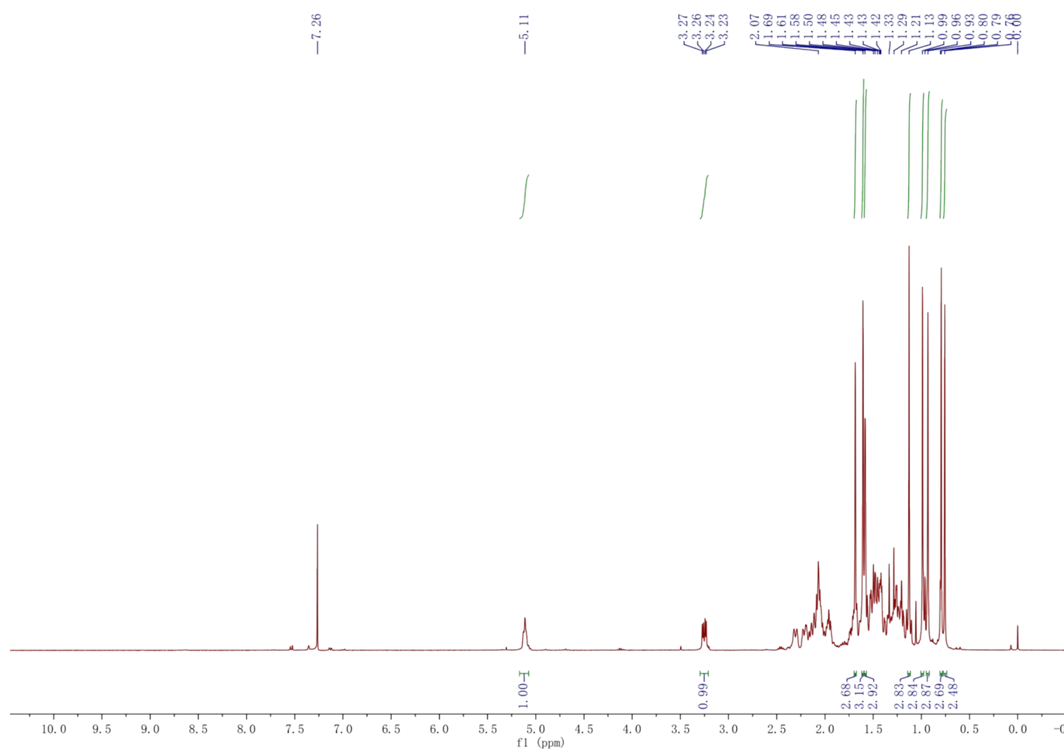


Figure S13 MS spectra of **6**.

a



b

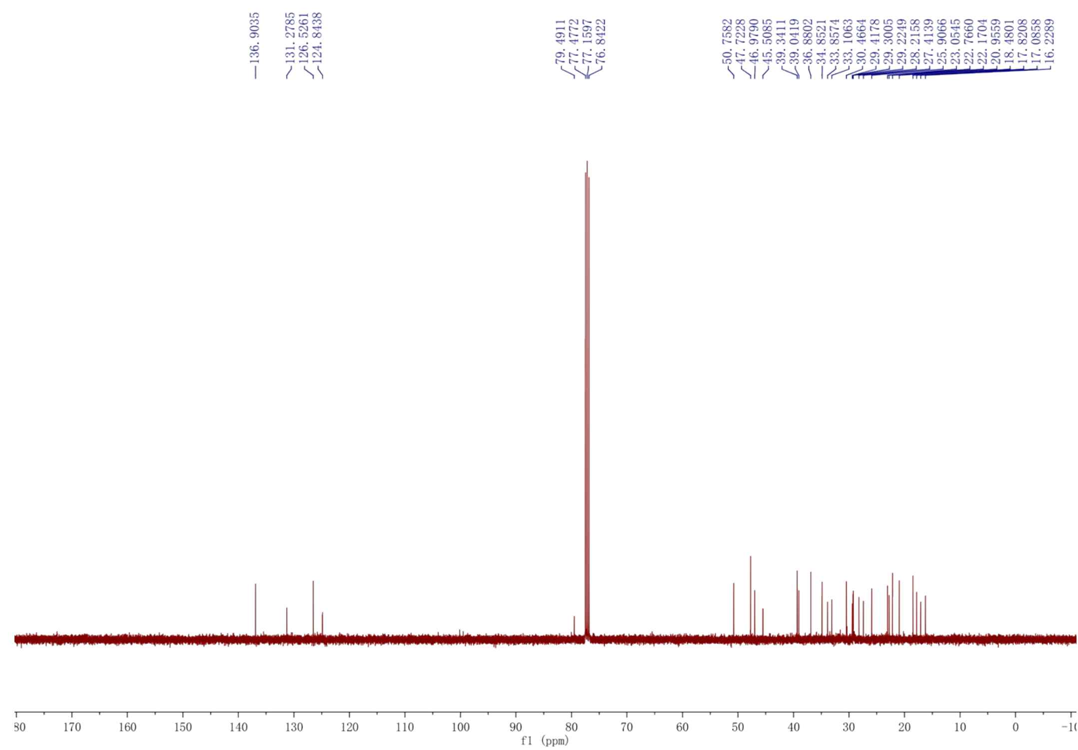
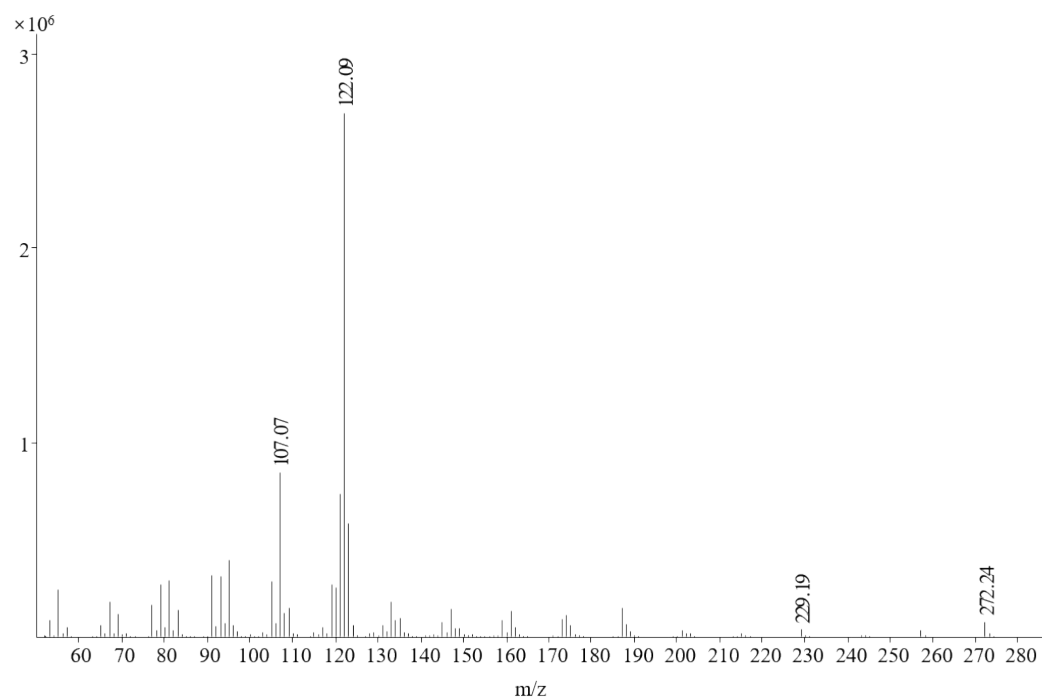


Figure S14 NMR spectrum of **6**. (a) ^{1}H NMR spectrum in $CDCl_3$ at 500MHz; (b) ^{13}C NMR spectrum in $CDCl_3$ at 125MHz.

a



b

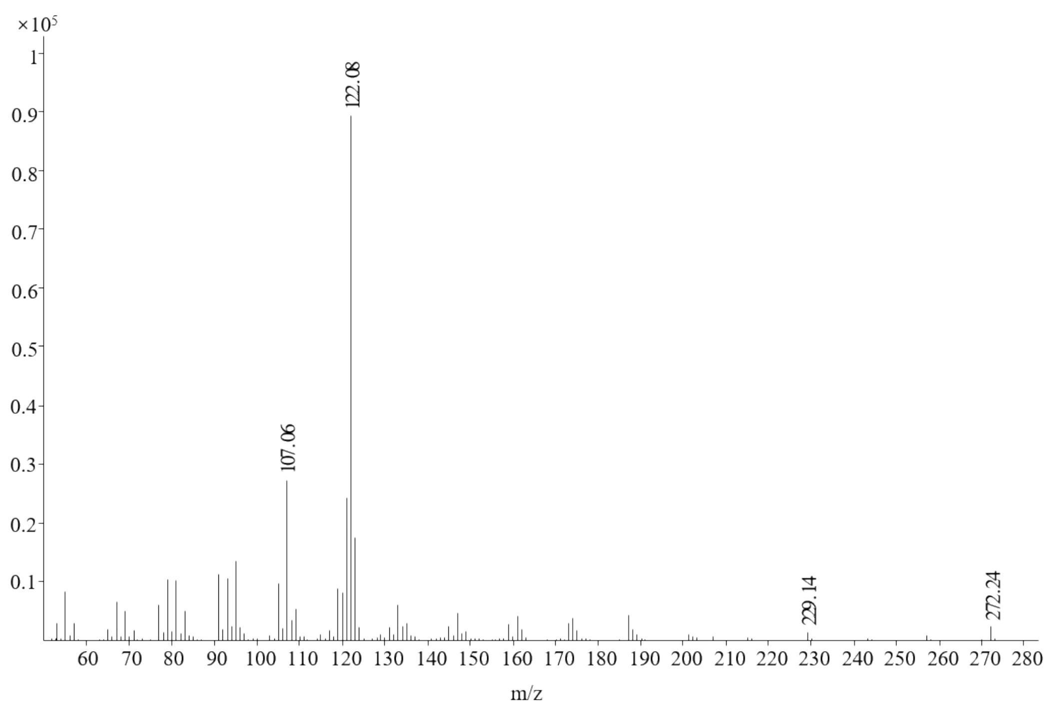


Figure S15 GC-MS analysis of **7** produced by MC3-Δ10-TAS and authentic sample. (a) MS spectrum of **7** from MC3-Δ10-TAS；(b) MS spectrum of authentic **7**.

Supplementary Tables

Table S1 Putative functions of genes in the terpenoid backbone biosynthesis pathways in *T.reesei* RutC30

Name	NCBI-Protein ID	Putative function
ACAT	ETS00561	acetyl-CoA acetyltransferase
HMGS	ETS04320	hydroxymethylglutaryl-CoA synthase
HMGR	ETS06777	hydroxymethylglutaryl-CoA reductase
MK	ETS02989	mevalonate kinase
PMK	ETS05511	phosphomevalonate kinase
MDC	ETR99754	Mevalonate pyrophosphate decarboxylase
IDI	ETS05484	isopentenyl-diphosphate delta-isomerase
GGPPS	ETR98769	geranylgeranyl pyrophosphate synthetase
FDFT	ETS00586	farnesyl-diphosphate farnesyltransferase
SQLE	ETS03457	squalene epoxidase

Table S2 Primers used in the study

Name	Sequences (5'-3')	Usage
Pcbh1-F	CTCGGTACCCGGGGATCCTCTAGAGACTGACCGGACGTGTTTTG	Amplification of *cbh1* promoter from RutC30 genome DNA
Pcbh1-R	GATGCGCAGTCCGCGGTTG	
TrAcOS-F	CAACCGCGGACTGCGCATCATGGCCTGCAAGTACAGCAC	Amplification of *TrAcOS* from synthetic gene
TrAcOS-R	ATGATGATGATGATGATGGACCTTCAGCAGCTCGAG	
Tcbh1-F	CATCATCATCATCATTAAAGCTCCGTGGCGAAAGCCTG	Amplification of *cbh1* terminator including His-tag sequence from RutC30 genome DNA
Tcbh1-R	ACACATTCCACAGAGATCTACTAATTTCCACTGTTGC	
ura5-F	AGATCTCTGTGGAATGTGTG	Amplification of *ura5* marker cassette from plasmid dDNA-lae1
ura5-R	TGCCTGCAGGTCGACGATTCTAGACAGGGCTGGTGACGGAATTTTC	
cbh1-down-F	CTCGGTACCCGGGGATCCTCTAGACCAGTGCGGCGGTATTGG	Amplification of 3′ flanking region for HDR of *T.reesei cbh1*
cbh1-down-R	TGCCTGCAGGTCGACGATTCTCATTGCGCACTATAG	
IDENcbh1-F	ATGGTCATCAAACAAAGAAC	Verification of transformants
IDENcbh1-R	GAAGATCAATGCTGTGCAAG	
hyp3-F	CAACCGCGGACTGCGCATCATGCGCCCCATCACCTGCAG	Amplification of *hyp3* from synthetic gene
hyp3-R	AATGATGATGATGATGATGGATGCCGCGGAGGCCGGGCT	
ari-F	CAACCGCGGACTGCGCATCATGAAGAAGCCCAACGGCAC	Amplification of *ari* from synthetic gene

ari-R	TAATGATGATGATGATGATGGTCGACGACGACGCTGTAG	
PaFS-F	CAACCGCGGACTGCGCATCATGGAGTTCAAGTACAGC	Amplification of *Pafs* from synthetic gene
PaFS-R	TTAATGATGATGATGATGATGGACGCGGAGCAGCTCGAG	
EvSS-F	CAACCGCGGACTGCGCATCATGGAGTACAAGTTCAGC	Amplification of *Evss* from synthetic gene
EvSS-R	TAATGATGATGATGATGATGGCCGGTCTTAAGCAGCTC	
HelA-F	CAACCGCGGACTGCGCATCATGGCCACCGACAGCAGC	Amplification of *helA* from synthetic gene
HelA-R	TAATGATGATGATGATGATGGATGGCGAGGCACTCGTTG	
TAS-F	CAACCGCGGACTGCGCATCATGAGCAGCAGCACCGGCAC	Amplification of *TAS* from synthetic gene
TAS-R	TAATGATGATGATGATGATGGACCTGGATGGGGTCAATG	
TAS-linker-R	CCCCCCCGCCAGAGCCGCCGCCGCCGGAGCCACCACCTCCGACCTGGAT GGGGTCAATG	Amplification of *TAS* from synthetic gene
PT-F	GCGGCTCTGGCGGGGGGGGCAGCGGCGGAGGAGGCTCGACCCAGCTCG AGTGGATG	Amplification of GGPPS domain from *PaFS*
LHA- Lae1-F	CCGGGGATCCTCTAGAGATATATGAATGCTGTGCTCAG	Amplification of 5' flanking of *lae1* from *T. reesei* genome served as right homologous arm
LHA- Lae1-R	AATGTAGGATTCCGCTCTGCGGGTCAATCCTTGGGAATG	
Ptcu1-F	AGAGCGGAATCCTACATTC	Amplification of *tcu1* promoter
Ptcu1-R	GGAGAGGATTGGACAACATTGTCGTATCAACCAGGTC	

xyr1-F	ATGTTGTCCAATCCTCTCC	Amplification of *xyr1*^{A824V} gene
xyr1-R	TCATCACCATCACCACTAAGCGATACCCATCATCAAC	Amplification of *xyr1*^{A824V} gene
Ttef1-F	CCTCCATCATCACCATCACCACTAAGCGATACCCATCATCAACACCTGATGTTCTGGGG	Amplification of *tef1* terminator
Ttef1-R	AGTGAGGGTTAATTGCGCCAACGATTCCTCTCACGTT	
hyg-F	GCGCAATTAACCCTCACT	Amplification of hygromycin resistance cassette
hyg-R	CAGGGCTGGTGACGGAATT	
RHA- Lae1-F	ATTCCGTCACCAGCCCTGGAAAATCATGGATCTCGGCA	Amplification of 3' flanking of *lae1* from *T. reesei* genome served as left homologous arm
RHA- Lae1-R	ATGCCTGCAGGTCGACGATCTGCGGTGGTAGCGATGC	
PamyB-F	TCGGTACCTCTAGAATTGTGCAAATGCCGTAAGC	Amplification of *amyB* promoter
PamyB-R	GATGATGATGGCTAGCAAATGCCTTCTGTGGGGTTTATTG	
TamyB-F	CATCATCATCATCATCATTAAAGGGTGGAGAGTATATGATG	Amplification of *amyB* terminator including His-tag sequence
TamyB-R	TAGCCGGAGATTTGACCGAATC	
Ade-F	TTCGGTCAAATCTCCGGCTACTCATGCCGTCATGTCCAG	Amplification of wild type *AoadeA* as the selectable marker
Ade-R	TCTGCGCAACAGCATACGAG	
RHA-F	TCGTATGCTGTTGCGCAGACGGCCAAGAACAGCACTAC	Amplification of the 3′ flanking region for HDR of *AoamyB*
RHA-R	CTGCAGGTCGACGATCTCTAGACACGAGCTACTACAGATCTTG	

AoAcOS-F	AAACCCCACAGAAGGCATTTATGGCCTGCAAGTACAGCACC	Amplification of *AoAcOS* gene from synthetic gene
AoAcOS-R	TTAATGATGATGATGATGATGGACCTTCAGCAGCTCGAGCAT	
ΔamyB-FF	GGCAACAAGCATGGAGTC	verification of NSAR1-AoAcOS transformants for HDR
ΔamyB-FR	CTGCCATGTTCCACCACAG	
ΔamyB-RF	CTCATGCCGTCATGTCCAG	verification of NSAR1-AoAcOS transformants for HDR
ΔamyB-RR	GCCATTTCGTCAAGGTATAG	
OF-DN-F	CTGTCGATTCGATACTAACGCCGCCATCCAGTGTCGAGCCAGGCGCCTT TATATCATAT	Construction of yeast strain BB-ScAcOS
OF-DN-R	GACTATAATATTATGCATATAGGA	
OF-GAL1-F	TGAATCTTGTCTGTTTGGTAGCGGCTGCTTTATTTATATTGAATTTTCAA AAATTCTTA	Construction of yeast strain BB-ScAcOS
OF-GAL1-R	GGAGTCGATCAAGGTGGAGTACTTACAGGCCATTATAGTTTTTTCTCCTT GACGTTAAA	
OF-HYG-F	AATGAATCTTTCTGTCGTGCTTGAAAGATTTTCGGCTAGCTTGCCTCGTC CCCGCCGGG	Construction of yeast strain BB-ScAcOS
OF-HYG-R	TTTTGTGTCTTAATTATATGATATAAAGGCGCCTGGCTCGACACTGGAT GGCGGCGTTA	

OF-RBL-F	GGAGTTGTTGAAGGTCCACCACCATCACCACCACTGAGCGGATTGAGA GCAAATCGTTA	Construction of yeast strain BB-ScAcOS
OF-RBL-R	TCGCTGGCCGGGTGACCCGGCGGGGACGAGGCAAGCTAGCCGAAAATC TTTCAAGCACG	
OF-UP-F	TTTGCCAACAATCGAAACCAAACA	Construction of yeast strain BB-ScAcOS
OF-UP-R	TCCAAAAAAAAAGTAAGAATTTTTGAAAATTCAATATAAATAAAGCAG CCGCTACCAAA	
OF-yeAcOS-F	TACCTCTATACTTTAACGTCAAGGAGAAAAAACTATAATGGCCTGTAAG TACTCCACCT	Construction of yeast strain BB-ScAcOS
OF-yeAcOS-R	TACTTGACCTGAACTTAACGATTTGCTCTCAATCCGCTCAGTGGTGGTG ATGGTGGTGG	

Table S3 Plasmids used in the study

Plasmids	Characteristics	Source
pMD18T-TrAcOS	Plasmids containing Pcbh1-TrAcOS-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-AoAcOS	Plasmids containing PamyB-AoAcOS-TamyB cassette and *adeA* marker cassette served as donor DNA to replace the *AoamyB* gene, (*Amp*^{R})	This work
pMD18T-xyr1	Plasmids containing Ptcu1-xry1^{A824V}-Ttef1 cassette and *hph* marker gene cassette served as overexpressing *xry1*^{A824V} gene, (*Amp*^{R})	This work
dDNA-lae1::xyr1	Plasmids containing Ptcu1-xry1^{A824V}-Ttef1 cassette and *hph* marker gene cassette served as donor DNA to replace the *lae1* gene, (*Amp*^{R})	This work
pMD18T-hyp3	Plasmids containing Pcbh1-hyp3-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-ari	Plasmids containing Pcbh1-ari-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-Pafs	Plasmids containing Pcbh1-Pafs-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-Evss	Plasmids containing Pcbh1-Evss-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-helA	Plasmids containing Pcbh1-helA-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-TAS	Plasmids containing Pcbh1-TAS-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work
pMD18T-GPTAS	Plasmids containing Pcbh1-GPTAS-Tcbh1 cassette and ura5 marker gene cassette served as donor DNA to replace the *cbh1* gene, (*Amp*^{R})	This work

Table S4 Strains used in the study

name	genotype	Source
MC3-TrAcOS	*T. reesei* MC3, Δ*cbh1*::*TrAcOS*	This work
NSAR1-AoAcOS	*A.oryzae* NSAR1, Δ*amyB*::*AoAcOS*	This work
BB-ScAcOS	*S. cerevisiae* BB, *ScAcOS*	This work
MC3-Δ10	*T. reesei* MC3, Δ*cbh2*Δ*xyn2*Δ*egl1*Δ*egl2*Δ*egl3*Δ*bxl1*Δ*egl4* Δ*cip1*Δ*cel74a*Δ*bgl1*, $ura5^{-}$	This work
MC3-Δ10-TrAcOS	*T. reesei* MC3-Δ10, Δ*cbh1*::*TrAcOS*	This work
OExyr1/MC3-Δ10	*T. reesei* MC3-Δ10, $xry1^{A824V}$	This work
Δlae1::xyr1/MC3-Δ10 (ROBUST chassis)	*T. reesei* MC3-Δ10, Δ*lae1*:: $xry1^{A824V}$	This work
OExyr1/MC3-Δ10-TrAcOS	*T. reesei* OExyr1/MC3-Δ10, Δ*cbh1*:: *TrAcOS*	This work
Δlae1::xyr1/MC3-Δ10-TrAcOS	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *TrAcOS*	This work
ROBUST-HYP3	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *hyp3*	This work
ROBUST-ARI	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *ari*	This work
ROBUST-PaFS	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *Pafs*	This work
ROBUST-EvSS	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *Evss*	This work
ROBUST-HelA	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *helA*	This work
MC3-Δ10-TAS	*T. reesei* MC3-Δ10, Δ*cbh1*::*TAS*	This work
MC3-Δ10-GPTAS	*T. reesei* MC3-Δ10, Δ*cbh1*::*GPTAS*	This work
ROBUST-GPTAS	*T. reesei* Δlae1::xyr1/MC3-Δ10, Δ*cbh1*:: *GPTAS*	This work