Electronic Supplementary Information

Elucidation of ustilaginoidins biosynthesis reveals an unrecognised class of ene-reductase

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General information

Reagents

All reagents and solvents were purchased from Sigma and Fisher. The solvents used for LC-MS and HPLC were chromatographic grade. Molecular biology kits were used according to the manufacturer's protocols. All enzymes were purchased from NEB, Sigma and Takara.

Media

All media were prepared using deionised water and autoclaved at 121°C for 20 min except for YPAD (115°C for 20 min).

LC-MS

LC-MS was performed on a QTOF 6520 mass spectrometer (Agilent) coupled to a HPLC system with a Phenomenex C18 column (i.d., 150 mm × 2.0 mm, 3 µm) eluting at 0.25 mL/min. Mass detector operated simultaneously in ESI^+ and ESI^- modes between 50 and 1400 *m/z*. Solvents were **A**: H_2O containing 0.1% formic acid, and **B**: acetonitrile containing 0.1% formic acid. A gradient elution was performed as follows over 19.5 min:

Time (min)	**% B**
0	30
10.0	90
13.0	90
13.1	30
19.5	30

HPLC-DAD analysis

HPLC analysis was performed on a Shimadzu instrument equipping with a SPD-M20A photodiode array detector using a Phenomenex C18 column (i.d., 250 mm × 4.6 mm, 5 µm). Solvents were **A**: H_2O (containing 0.02‰ oxalic acid), and **B**: methanol.

Conditions 1 and 2: A gradient elution was performed as follows over 40 min eluting at 1 mL/min.

Time (min)	% **B** (condition 1)	% **B** (condition 2)
0	70	60
5.0	70	60
25.0	100	100
35.0	100	100
35.1	70	60
40	70	60

Conditions 3, 4 and 5: A gradient elution was performed as follows over 65 min eluting at 0.5 mL/min.

Time (min)	% **B** (condition 3)	% **B** (condition 4)	% **B** (condition 5)
0	70	60	70
5.0	70	60	70
45.0	90	100	100
65.0	90	100	100

HRMS and NMR

HRESIMS was obtained using a QTOF 6520 mass spectrometer (Agilent) coupled to a HPLC system. ^{1}H, ^{13}C, and 2D NMR spectra were recorded using the Bruker 500 NMR spectrometer. Chemical shifts were expressed in δ (ppm) referencing to the solvent residual peaks, and coupling constants (J) in Hertz.

Experimental Procedures

Strains, plasmids and culture conditions

Ustilaginoidea virens P1 and knockout vector pmCAS9:tRp-gRNA (see Tables S3 and S4) were used for gene knockout, which were generously provided by Prof. Jin-Rong Xu (Purdue University). The transformants and wild-type strain were grown on PSA plates at 28°C. For conidia production, the strain was cultured in 100 mL YTD medium at 28°C for 5 days with 175 rpm shaking. For conidia germination, the strain was cultured in 100 mL PSB medium for 16-20 h.
Aspergillus oryzae NSAR1 and expression vectors pTYGSarg/met (see Tables S3 and S4) were used for heterologous expression, which were generously provided by Prof. Russell Cox (Leibniz University of Hannover). The transformants and wild-type strain were grown on DPY plates at 28°C. For shaking culture, the strain was cultured in 100 mL DPY medium at 28°C with 150 rpm shaking.
Saccharomyces cerevisiae CEN.PK (see Table S3) was used for yeast recombination. The strain was cultured on YPAD plates at 30°C.
Escherichia coli DH5α which was used for heat shock transformation, was cultured in LB at 37°C. Ampicillin (100 μg/mL) was added to the LB agar plates for selection.

Bioinformatics

Gene cluster was predicted with antiSMASH[1]. Domain prediction was conducted with the NCBI Conserved Domain Database[2].

Amplification of the homologous knockout fragment

Genomic DNA was isolated from *Ustilaginoidea virens* P1 with CTAB method[3]. ~1 kb upstream and downstream flanking sequences of the target genes were amplified using primer pairs 1F/2R and 3F/4R, respectively (Fig. S6). The selection marker G418 was amplified from vector pFL2 with primer pairs GENF/GENR. Hygromycin was amplified from vector pKOV21 with primer pairs HPHF/HPHR. Three fragments were fused via double joint PCR[4]. Primers see Table S8.

Construction of the sgRNA vectors for the gene knockout

gRNA spacers were designed with the sgRNA designer website[5]. The spacer sequences after denaturation and renaturation were cloned into the BsmBI-digested pmCas9:tRP-gRNA vector by Golden Gate Cloning[6,7]. The resulting constructs were transformed into *E. coli* and verified by sequencing.

Transformation of *Ustilaginoidea virens* P1

Homologous knockout fragment and the sgRNA vector were transformed into *Ustilaginoidea virens* P1 using PEG-mediated protoplast transformation, which were performed as described by Zheng[8]. Transformants were selected with 700 μg/mL geneticin or 200 μg /mL hygromycin.

Selection of knockout transformants

Transformants were screened by PCR with primers 5F/6R for target genes, and further verified by PCR with primer pairs 7F/G855R (7F/H855R) and G856F/8R (H856F/8R) for homologous

recombination sequences (Fig. S6, S7). Primers see Table S8.

Sequences of cloned genes

>*uvpks1*_cDNA_*Ustilaginoidea_virens*_P1

ATGGCGAACGTGTTCCAAATTGCCGTCTTCGGCGATCTGTCTGTTCCGTACCACTCGGAGCTCCGCAGACTCTTCTCTGAAAA
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>*usgD*_cDNA_*Ustilaginoidea_virens*_P1

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>*usgO*_cDNA_*Ustilaginoidea_virens*_P1

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AA

Construction of *A. oryzae* expression vectors

U. virens total RNA was extracted using Trizol reagent (Ambion) and subsequently converted to cDNA using HiScript 1st Strand cDNA Synthesis Kit (Vazyme) according to the manufacturer's instruction. The cDNA was used for amplification of ustilaginoidin biosynthetic genes by PCR (See

Table S8 for primers used in this study).

The genes *usgO*, *uvpks1*, *usgD*, *usgR*, *usgM* and *usgL* were cloned into the expression vectors pTYGSarg/met *via* yeast homologous recombination under control of the constitutive promoters PamyB, Peno, Padh or PgdpA (Fig. S8). The protocol of yeast homologous recombination was performed as described by Kahlert *et al*. 2019[9]. Constructed plasmids were extracted from yeast cells using Yeast Plasmid Miniprep kit (Coolaber, Beijing, China) and transformed into *E. coli* DH5α competent cells by heat shock transformation.

Transformation of *A. oryzae* NSAR1

Spore suspension of *A. oryzae* NSAR1 was inoculated into 100 mL DPY liquid medium and incubated overnight. Cultures were collected by filtration over sterile miracloth and suspended in 10 mL enzyme lysis buffer (10 mg/mL lysing enzyme from *Trichoderma harzianum*). The suspension was incubated for 4 h at 28°C with 90 rpm shaking. Protoplasts were collected by filtration and centrifugation (3000 × g, 5 min). The protoplast transformation and transformant selection were performed as described by Kahlert *et al*[9]. Transformants were selected on respective agar plates (CZD/S w/o arginine; CZD/S w/o arginine and w/o methionine; or CZD/S w/o methionine). For strains constructed in this study see Table S5.

Fermentation and extraction protocols

A. oryzae: Ten respective transformants were inoculated into 100 mL DPY medium and incubated for five days at 28°C with 175 rpm shaking. The cultures were homogenized using ultrasonication and separated by filtration under vacuum condition. The supernatant was extracted twice with ethyl acetate (2 × 100 mL) and the mycelia were extracted once. The combined EtOAc layers were dried over $MgSO_4$ and concentrated under vacuum at 45°C. The organic residue was dissolved in 1.5 mL methanol and analysed by LC-MS and HPLC.

U. virens: Transformants grown on PSA plates were inoculated into 100 mL PSB medium and incubated for five days at 28°C with 175 rpm shaking. 1 mL liquid culture was added to the rice solid medium, and incubated at 28°C for 1 month. The culture was extracted with EtOAc for three times and the combined EtOAc layers were concentrated under vacuum at 45°C. The organic residue was dissolved in methanol and analysed by LC-MS and HPLC.

Feeding experiment

A. oryzae transformants were inoculated into 100 mL DPY medium in 250 mL Erlenmeyer flask and grown for three days at 28°C with 150 rpm shaking. 0.1-0.2 g mycelia were harvested by filtration and resuspended with 1 mL DPY or sodium citrate buffer (100 mM, pH 6.0) in 2 mL microcentrifuge tube. The cultures were incubated with compounds (3 µL) for 48 h at 28°C with 150 rpm shaking (reaction combination see Table S6). The cultures were extracted with ethyl acetate and the solvent was evaporated under vacuum.

Expression of UsgR in *Saccharomyces cerevisiae* BY4741, and *Pichia pastoris* GS115

Yeast expression vectors PYES2-*usgR* and pPIZGM-*usgR* were transformed into *S. cerevisiae* BY4741, and *P. pastoris* GS115, respectively. The transformants were selected by PCR.

1) pYES2-*usgR* + *S. cerevisiae* BY4741: *usgR* was amplified from cDNA of *U. virens* P1 using sticky-end PCR. The product was cloned into expression vector pYES2 digested with EcoRI and XhoI. The

resulting plasmid pYES2-*usgR* was confirmed by sequencing and transformed into *S. cerevisiae* BY4741. A single colony was Inoculated into 50 mL SC-ura medium containing 2% glucose overnight at 30 °C with 200 rpm. The cells were centrifuged at 3500×g for 5 min. The pellet was resuspended with SC-ura medium containing 6% galactose to obtain an OD_{600} of 0.4. 10 μL compound **2** was added into 1 mL culture at 24 hours after galactose induction. The reaction was incubated at 28 °C for 2d and extracted with EtOAc.

2) pPIZGM-*usgR* + *P. pastoris* GS115: The plasmid pPIZGM-*usgR* was transformed into *P. pastoris* GS115 by electrotransformation. Induction expression of protein was performed according to the user manual of invitrogen (Catalog no. V190-20). The final concentration of methanol was 1% and the induction time was 3 d. The feeding experiment was the same as above.

Preparation of microsomes from *A. oryzae*-UsgR and *in vitro* enzymatic reaction.

For microsome preparation, the cell-free extract of AO-*usgR* was centrifuged at 100,000 x g for 90 min. The pellet was resuspended in 100 mM citrate buffer. To characterize the function of UsgR, the reaction was performed in 253 μL volume containing 225 μL microsomal fractions, 3 μL compound **2** (10mg/mL), and 0.5 mM NADPH or NADH. The reaction was incubated at 28 °C for 24 h. The reaction was quenched by adding EtOAc, and EtOAc extracts were evaporated and dissolved in methanol for HPLC analysis.

Preparation of UsgR-containing microsomes from recombinant yeasts and *in vitro* enzymatic reaction.

The preparation of microsomes from recombinant *S. cerevisiae* BY4741 was performed as described by Ralston *et al*.[10] The microsomal fractions were resuspended in 100 mM citrate buffer (pH 6.0).

The cell-free extract of recombinant *P. pastoris* GS115 was performed according to the user manual of invitrogen (Catalog no. V190-20). This fraction was centrifuged at 100,000 x g for 90 min. The pellet was resuspended in the 100 mM citrate buffer.

In vitro enzymatic reaction was performed as described for the microsomal fraction of *A. oryzae* (see above).

In vitro* enzymatic reaction assay using cell-free extract of AO-*usgL

Laccase-producing strain AO-*usgL* was incubated for 3 days in 100 mL DPY medium. Mycelia were harvested by filtration and washed once with sterile water. 0.1-0.2 g mycelia per 2 mL microcentrifuge tube was resuspended in 1 mL sodium citrate buffer (100 mM citrate buffer, pH 6.0) and disrupted by 0.4 g glass bead (0.5 mm diameter) with tissue homogenizer at 60 Hz (30 s with 1 min intermittent cooling on ice for seven times). Cellular debris was removed by centrifugation at maximum speed, 4°C for 1 hour. The supernatant was combined and used for *in vitro* assays.

The reaction mixture contains 250 μL cell-free lysate and 3 μL substrate (10 mg/mL in DMSO; except for **4**, 5 mg/mL). The reaction was carried out at 28°C, 150 rpm and incubated for 24 h. The assays were extracted with ethyl acetate for three times. The organic layer was evaporated under vacuum and dissolved in 100 μL methanol.

<u>For atropselectivity test</u>:

The strain AO-*usgL* was cultured in DPY medium for 3d at 28°C and 200 rpm. Mycelia were

harvested by filtration and washed once with water. Then mycelia were re-suspended in 100 mM citrate buffer (pH 6.0) and disrupted by blender (30 s with cooling on ice for 30 s, repeat 7 times). The cell debris were collected by centrifugation (21, 000 g). The cell-free extract (supernatant) was concentrated by centrifugal filter (10kDa protein cutoff, Millipore), then fractionated using acetone precipitation as described by Obermaier *et al*. [11] Fraction II was used for *in vitro* reaction. The precipitate was re-suspended in citrate buffer, and different volumes of the lysate were added to the reaction.

Purification of compounds 1-4, and 9

The double knockout strains Δ*usgD*/Δ*usgL*, Δ*usgR*/Δ*usgL* and Δ*usgM*/Δ*usgD* were grown on PSA medium at 28°C for 14 days. Then, several agar plugs containing mycelia were added into 100 mL PSB medium and incubated at 28°C for 7 days with 150 rpm shaking. The liquid culture was subsequently inoculated to the solid rice medium and incubated at 28°C for 1 month. The moldy rice was extracted with EtOAc for three times. The EtOAc extract was concentrated under vacuum at 45°C.

The crude extract of Δ*usgD*/Δ*usgL* was chromatographed over silica gel eluting with CH_2Cl_2, CH_2Cl_2:EtOAc (1:1, v/v) and EtOAc to give six fractions (Fr. A-F). **1** (63.4 mg) was purified from Fr. C by semi-preparative HPLC eluting with MeOH-H_2O (50:50, v/v). **3** (23.4 mg) was purified from Fr. C by semi-preparative HPLC eluting with MeOH-H_2O (65:35, v/v).

The crude extract of Δ*usgR*/Δ*usgL* was chromatographed over silica gel eluting with CH_2Cl_2, CH_2Cl_2:EtOAc (1:1, v/v) and EtOAc to give six fractions (Fr. A-F). **2** (43 mg) was purified from Fr. B by semi-preparative HPLC eluting with MeOH-H_2O (50:50, v/v). **4** (13.5 mg) was purified from Fr. B by semi-preparative HPLC eluting with MeOH-H_2O (65:35, v/v).

The crude extract of Δ*usgM*/Δ*usgD* was chromatographed over Sephadex LH-20 eluting with MeOH/CH_2Cl_2 (1:1, v/v) to obtain seven fractions (Fr. A-G). **9** (10.5 mg) was purified from Fr. D by semi-preparative HPLC eluting with MeOH-H_2O (65:35, v/v).

Characterization of compounds

The molecular formula for each compound was obtained by HR-ESIMS. Structure elucidation was performed by analysis of the UV, MS, and NMR data, and comparison with the authentic standards isolated previously by our group[12, 13] where applicable, or with the literature data.

Tables

Table S1. Blastp search of UsgR

The BLASTP search identified 97 putative enzymes (amino acid sequence identity ≥30%, coverage ≥80%, e-value ≤1e−10) from over 73 microbial species

Gene tag	Microbial species	Habitat	Genome
MGU_10267	*Metarhizium guizhouense* ARSEF 977	Insect	√
MAJ_07352	*Metarhizium majus* ARSEF 297	Insect	√
UCDDA912_g04898	*Diaporthe ampelina*	Plant	√
MYCTH_113600	*Thermothelomyces thermophilus* ATCC 42464	Terrestrial	√
MAA_08367	*Metarhizium robertsii* ARSEF 23	Insect	√
H634G_10273	*Metarhizium anisopliae* BRIP 53293	Insect	√
MAN_09376	*Metarhizium anisopliae* ARSEF 549	Insect	√
MBR_06612	*Metarhizium brunneum* ARSEF 3297	Insect	√
X797_008469	*Metarhizium robertsii* ARSEF 2575	Insect	-
MANI_018949	*Metarhizium anisopliae* E6	Insect	√
MAC_01520	*Metarhizium acridum* CQMa 102	Insect	√
NA57DRAFT_49043	*Rhizodiscina lignyota*	Plant	√
BDR25DRAFT_301033	*Lindgomyces ingoldianus*	Plant	√
V490_05392	*Pseudogymnoascus* sp. VKM F-3557	Aquatic	√
O988_01060	*Pseudogymnoascus* sp. VKM F-3808	Aquatic	√
V502_09231	*Pseudogymnoascus* sp. VKM F-4520 (FW-2644)	Aquatic	-
V497_06862	*Pseudogymnoascus* sp. VKM F-4516 (FW-969)	Aquatic	-
V495_02567	*Pseudogymnoascus* sp. VKM F-4514 (FW-929)	Aquatic	-
V496_09341	*Pseudogymnoascus* sp. VKM F-4515 (FW-2607)	Aquatic	-
V498_04060	*Pseudogymnoascus* sp. VKM F-4517 (FW-2822)	Aquatic	-
ASPZODRAFT_140511	*Penicilliopsis zonata* CBS 506.65	Terrestrial	√
W97_08614	*Coniosporium apollinis* CBS 100218	Terrestrial	√
AOQ84DRAFT_190463	*Glonium stellatum*	Terrestrial	√
PtrV1_09647	*Pyrenophora tritici-repentis* V0001	Plant	√
PTRG_10960	*Pyrenophora tritici-repentis* Pt-1C-BFP	Plant	√
SNOG_11160	*Parastagonospora nodorum* SN15	Plant	√
BDV96DRAFT_601282	*Lophiotrema nucula*	Plant	√
CC77DRAFT_1006940	*Alternaria alternata* SRC1lrK2f	Plant	√
CUC08_Gglean001050	*Alternaria* sp. MG1	Plant	√
AA0117_g2514	*Alternaria alternata* FERA 1177	Plant	√
PTTW11_06978	*Pyrenophora teres f. teres* W1-1	Plant	√
BDZ99DRAFT_458110	*Mytilinidion resinicola* CBS 304.34	Terrestrial	√
BU16DRAFT_491253	*Lophium mytilinum* CBS 269.34	Plant	√
GT037_002153	*Alternaria burnsii* CBS107.38	Plant	√
K441DRAFT_548639	*Cenococcum geophilum* 1.58	Plant	√

HRS9139_07302	*Pyrenophora teres f. teres* HRS9139	Plant	√
AG0111_0g631	*Alternaria gaisen* FERA 650	Plant	√
PTT_15456	*Pyrenophora teres f. teres* 0-1	Plant	√
AA0111_g5680	*Alternaria arborescens* FERA 675	Plant	√
EJ04DRAFT_246949	*Polyplosphaeria fusca* CBS 125425	Terrestrial	√
EV356DRAFT_448451	*Viridothelium virens*	Plant	√
EJ05DRAFT_479127	*Pseudovirgaria hyperparasitica*	Terrestrial	√
EV356DRAFT_526851	*Viridothelium virens*	Plant	√
K432DRAFT_324702	*Lepidopterella palustris* CBS 459.81	Aquatic	√
CC80DRAFT_526846	*Byssothecium circinans*	Terrestrial	√
EKO05_009025	*Ascochyta rabiei* Me14	Plant	√
P280DRAFT_503145	*Massarina eburnea* CBS 473.64	Aquatic	√
IQ06DRAFT_290454	*Stagonospora sp.* SRC1lsM3a	Plant	√
E8E11_011998	*Didymella keratinophila* 9M1	Human	√
B0A49_01576	*Cryomyces minteri* CCFEE 5187	Terrestrial	√
E8E13_003200	*Curvularia kusanoi* 30M1	Plant	√
K402DRAFT_425461	*Aulographum hederae* CBS 113979	Terrestrial	√
BDU57DRAFT_511507	*Ampelomyces quisqualis*	Plant	√
BDY21DRAFT_365434	*Lineolata rhizophorae* ATCC 16933	Aquatic	√
K491DRAFT_76618	*Lophiostoma macrostomum* CBS 122681	Plant	√
BDV97DRAFT_300995	*Delphinella strobiligena* CBS 735.71	Plant	√
GMOD_00004333	*Pyrenophora seminiperda* CCB06	Plant	√
EJ02DRAFT_452855	*Clathrospora elynae* CBS 161.51	Plant	√
B9Z65_1923	*Elsinoe australis* NL1	Plant	√
TW65_07162	*Stemphylium lycopersici* CIDEFI 216	Plant	√
EJ07DRAFT_173363	*Lizonia empirigonia*	Plant	√
EK21DRAFT_80330	*Setomelanomma holmii* CBS 110217	Plant	√
K505DRAFT_263962	*Melanomma pulvis-pyrius* CBS 109.77	Plant	√
K460DRAFT_307839	*Cucurbitaria berberidis* CBS 394.84	Plant	√
D9617_3g021860	*Elsinoe fawcettii* 53147a	Plant	√
M436DRAFT_43143	*Aureobasidium namibiae* CBS 147.97	Terrestrial	√
K458DRAFT_365768	*Lentithecium fluviatile* CBS 122367	Aquatic	√
CAC42_251	*Sphaceloma murrayae* CQ-2017a	Plant	√
T440DRAFT_447602	*Plenodomus tracheiphilus* IPT5	Plant	√
P153DRAFT_370060	*Dothidotthia symphoricarpi* CBS 119687	Plant	√
C1H76_0945	*Elsinoe australis* Hillstone_2	Plant	√
D0862_04345	*Hortaea werneckii* EXF-171	Human	√
CC86DRAFT_22383	*Ophiobolus disseminans* CBS 113818	Plant	√
M011DRAFT_469003	*Sporormia fimetaria* CBS 119925	Digestive tract	√
BTJ68_14050	*Hortaea werneckii* EXF-2000	Human	√
PV09_02259	*Verruconis gallopava* CBS 43764	Human	√
B0A54_07706	*Friedmanniomyces endolithicus* CCFEE 5311	Terrestrial	√
EKO04_010827	*Ascochyta lentis* Al4	Plant	√
K490DRAFT_65894	*Saccharata proteae* CBS 121410	Plant	√

E8E12_008824	*Didymella heteroderae* 28M1	Plant	√
BDW02DRAFT_20370	*Decorospora gaudefroyi* P77	Aquatic	√
BU26DRAFT_521611	*Trematosphaeria pertusa* CBS 122368	Terrestrial	√
CPB83DRAFT_860062	*Crepidotus variabilis* CBS 506.95	Terrestrial	√
D0861_05467	*Hortaea werneckii* EXF-2788	Human	√
EI97DRAFT_38179	*Westerdykella ornata* CBS 379.55	Aquatic	√
Vi05172_g5625	*Venturia inaequalis* 05/172	Plant	√
D6D05_00937	*Aureobasidium pullulans* EXF-8828	Terrestrial	√
D6D29_02830	*Aureobasidium pullulans* EXF-11991	Terrestrial	√
BDR25DRAFT_61103	*Lindgomyces ingoldianus* ATCC 200398	Aquatic	√
CPB83DRAFT_860077	*Crepidotus variabilis* CBS 506.95	Terrestrial	√
D6D28_05712	*Aureobasidium pullulans* EXF-11900	Terrestrial	√
AUEXF2481DRAFT_30567	*Aureobasidium subglaciale* EXF-2481	Aquatic	√
D6D21_07030	*Aureobasidium pullulans* EXF-10796	Terrestrial	√
D0865_02701	*Hortaea werneckii* EXF-151	Human	√
M501DRAFT_929268	*Patellaria atrata* CBS 101060	Terrestrial	√
BS50DRAFT_596352	*Corynespora cassiicola* Philippines	Plant	√
D0869_14757	*Hortaea werneckii* EXF-6656	Human	√

Table S2. NCBI accession numbers of the protein sequences from publicly available genomes

Genome (accession No.)	PKS	Dehydratase	Reductase	Methyltransferase	Laccase	Production of reduced naphtho-γ-pyrones (ref.)
Ustilaginoidea virens P1	UVPKS1	UsgD	UsgR	UsgM	UsgL	This study
Metarhizium robertsii ARSEF 2575 (JELW00000000.1)	EXU98524.1	EXU98523.1	EXU98521.1	EXU98520.1	EXU98519.1	-
M. anisopliae E6 (JNNZ01000000)	KFG79736.1	KFG79735.1	KFG79733.1	KFG79732.1	KFG79731.1	Ref [14]
M. brunneum ARSEF 3297 (AZNG00000000.1)	XP_014543084.1	XP_014543083.1	XP_014543081.1	XP_014543080.1	XP_014543079.1	-
Thermothelomyces thermophilus ATCC 42464 (GCA_000226095.1)	XP_003666434.1	XP_003666433.1	XP_003666431.1	XP_003666429.1	XP_003666430.1	-
Chaetomium olivicolor CBS 102434 (Genozymes project) [a]	The biosynthetic gene cluster (BGC) spans nucleotides 1951–40596 of scaffold 0220 as predicted by AntiSMSH (version 6.0) [1]. All these genes were found.					This species was closely related to *C. arcuatum* (producing chaetochromin)[11]
Rhizodiscina lignyota (JAADKO000000000.1)	KAF2093015.1	KAF2093014.1	KAF2093012.1		KAF2093011.1	-
Lindgomyces ingoldianus (JAAEJD000000000.1)	XP_033552105.1	XP_033552106.1	XP_033552108.1		XP_033552109.1	-
Pseudogymnoascus sp. VKM F-3557 (JPJS00000000.1)	KFX92420.1	KFX92421.1	KFX92423.1		KFX88072.1	-
Melanomma pulvis-pyrius CBS 109.77 (JAAEJI000000000.1)	KAF2790288.1	KAF2790289.1	KAF2800417.1			-
Corynespora cassiicola Philippines (GCA_003016335.1)	PSN72911.1	PSN72910.1	PSN75367.1			-
Glonium stellatum (LKAO00000000.1)	OCL14472.1	OCL14473.1	OCL02234.1			-
Cenococcum geophilum 1.58 (LKKR00000000.1)	OCK97414.1	OCK97413.1	OCK97342.1			-

[a] The genome can be found at https://gb.fungalgenomics.ca/fgb2/gbrowse/Chaol_public/

Table S3. Fungal strains used in this study

Strain	Genotype
Ustilaginoidea virens P1	wild type
Δ*uvpks1*	*uvpks1* deletion mutant of P1
Δ*usgD*	*usgD* deletion mutant of P1
Δ*usgR*	*usgR* deletion mutant of P1
Δ*usgM*	*usgM* deletion mutant of P1
Δ*usgL*	*usgL* deletion mutant of P1
Δ*usgO*	*usgO* deletion mutant of P1
Δ*usgD*/Δ*usgL*	*usgD* and *usgL* deletion mutant of P1
Δ*usgR*/Δ*usgL*	*usgR* and *usgL* deletion mutant of P1
Δ*usgM*/Δ*usgL*	*usgM* and *usgL* deletion mutant of P1
Δ*usgM*/Δ*usgD*	*usgM* and *usgD* deletion mutant of P1
Δ*usgM*/Δ*usgR*	*usgM* and *usgR* deletion mutant of P1
Δ*usgD*/Δ*usgR*	*usgD* and *usgR* deletion mutant of P1
Aspergillus oryzae NSAR1	argB-, adeA-, sC^-, niaD
Saccharomyces cerevisiae CEN.PK	MATa/α ura3-52/ura3-52 trp1-289/trp1-289 leu2-3_112/leu2-3_112 his3Δ1/his3 Δ1 MAL2-8C/MAL2-8C SUC2/SUC2
AO-*usgP*	+*uvpks1*, sC^- , niaD
AO-*usgR*	+*usgR*, sC^- , niaD
AO-*usgM*	+*usgM*, sC^- , niaD
AO-*usgL*	+*usgL*, $argB^-$, niaD
AO-*usgO*	+*usgO*, sC^- , niaD
AO-*usgPL*	+*uvpks1*, +*usgL*, niaD
AO-*usgPO*	+*uvpks1, +usgO*, sC^-, niaD
AO-*usgPR*	+*uvpks1, +usgR*, sC^-, niaD
AO-*usgPD*	+*uvpks1, +usgD*, sC^-, niaD
AO-*usgPM*	+*uvpks1, +usgM*, sC^-, niaD
AO-*usgPDL*	+*uvpks1*, +*usgD*, +*usgL*, niaD
AO-*usgPOL*	+*uvpks1*, +*usgO*, +*usgL*, niaD
AO-*usgPDM*	+*uvpks1, +usgD, +usgM*, sC^-, niaD
AO-*usgPDR*	+*uvpks1, +usgD, +usgR*, sC^-, niaD
AO-*usgPRM*	+*uvpks1, +usgR, +usgM*, sC^-, niaD
AO-*usgPDRL*	+*uvpks1, +usgD, +usgR*, +*usgL*, niaD
AO-*usgPDML*	+*uvpks1, +usgD, +usgM*, +*usgL*, niaD
AO-*usgPDRM*	+*uvpks1, +usgD, +usgR, +usgM*, sC^-, niaD
AO-*usgPDRMO*	+*uvpks1, +usgD,+usgR, +usgM*, +*usgO,*sC^-, niaD
AO-*usgPDRML*	+*uvpks1, +usgD, +usgR, +usgM*, +*usgL*, niaD

Table S4. Plasmids used in this study

Plasmid	Feature
pmCAS9:tRp-gRNA	Cas9-gRNA vector with the tRNA promoter
pmCAS9:tRp-gRNA-*uvpks1*	pmCAS9:tRp-gRNA with the *uvpks1* spacer
pmCAS9:tRp-gRNA-*usgD*	pmCAS9:tRp-gRNA with the *usgD* spacer
pmCAS9:tRp-gRNA-*usgR*	pmCAS9:tRp-gRNA with the *usgR* spacer
pmCAS9:tRp-gRNA-*usgM*	pmCAS9:tRp-gRNA with the *usgM* spacer
pmCAS9:tRp-gRNA-*usgL*	pmCAS9:tRp-gRNA with the *usgL* spacer
pmCAS9:tRp-gRNA-*usgO*	pmCAS9:tRp-gRNA with the *usgO* spacer
pTYGSarg/met	PamyB, Padh, Peno, PgdpA, Amp^R, ColE1, 2μ ori, URA3, ccdB, *argB/sC*
pTYGSarg-*usgO*	*usgO* under control of PamyB
pTYGSarg-*uvpks1*+*usgO*	*uvpks1* under control of PamyB*, usgO* under control of Padh
pTYGSarg-*uvpks1*	*uvpks1* under control of PamyB
pTYGSarg-*uvpks1*+*usgD*	*uvpks1* under control of PamyB*, usgD* under control of Padh
pTYGSarg-*usgM*	*usgM* under control of PamyB
pTYGSarg-*uvpks1*+*usgD*+*usgM*	*uvpks1* under control of PamyB*, usgD* under control of Padh*, usgM* under control of Peno
pTYGSarg-*usgR*	*usgR* under control of PamyB
pTYGSarg-*uvpks1*+*usgD*+*usgR*+*usgM*	*uvpks1* under control of PamyB*, usgD* under control of Padh*, usgR* under control of PgpdA*, usgM* under control of Peno
pTYGSmet-*usgL*	*usgL* under control of PamyB
pFL2	G418, Amp^R
pKOV21	Hyg^R, Neo^R, Kan^R, Amp^R
pET28a	T7 promoter, *lacI* (lac repressor), *N*/*C*-His tag, Kan^R
pETM10	T7 promoter, *lacI*, *N*-His tag, Kan^R, EFH
pMBP_1a	T7 promoter, *lacI*, *N*-His tag, Kan^R, MBP, EYFP, TEV
pGEX-6P-1	lac promoter, *N*-GST tag, Amp^R, *lacI*

Table S5. Overview of transformations in *A. oryzae* NSAR1 with different combinations of genes

Transformation ID	Transformed vector constructs	Genes as					
		uvpks1	*usgD*	*usgR*	*usgM*	*usgL*	*usgO*
AO-*usgP*	pTYGSarg-*uvpks1*	√					
AO-*usgR*	pTYGSarg-*usgR*			√			
AO-*usgM*	pTYGSarg-*usgM*				√		
AO-*usgL*	pTYGSarg-*usgL*					√	
AO-*usgO*	pTYGSarg-*usgO*						√
AO-*usgPD*	pTYGSarg-*uvpks1+usgD*	√	√				
AO-*usgPR*	pTYGSarg-*uvpks1+usgR*	√		√			
AO-*usgPM*	pTYGSarg-*uvpks1+usgM*	√			√		
AO-*usgPL*	pTYGSarg-*uvpks1*, pTYGSmet-*usgL*	√				√	
AO-*usgPO*	pTYGSarg-*uvpks1+usgO*	√					√
AO-*usgPDL*	pTYGSarg-*uvpks1+usgD*, pTYGSmet-*usgL*	√	√			√	
AO-*usgPOL*	pTYGSarg-*uvpks1+usgO,* pTYGSmet-*usgL*	√				√	√
AO-*usgPDM*	pTYGSarg-*uvpks1+usgD+usgM*	√	√		√		
AO-*usgPDR*	pTYGSarg-*uvpks1+usgD+usgR*	√	√	√			
AO-*usgPRM*	pTYGSarg-*uvpks1+usgR+usgM*	√		√	√		
AO-*usgPDRM*	pTYGSarg-*uvpks1+usgD+usgR+usgM*	√	√	√	√		
AO-*usgPDML*	pTYGSarg-*uvpks1+usgD+usgM*, pTYGSmet-*usgL*	√	√		√	√	
AO-*usgPDRL*	pTYGSarg-*uvpks1+usgD+usgR*, pTYGSmet-*usgL*	√	√	√		√	
AO-*usgPDRMO* [a]	pTYGSarg-*uvpks1+usgD+usgR+usgM,* pTYGSarg-*usgO*	√	√	√	√		√
AO-*usgPDRML*	pTYGSarg-*uvpks1+usgD+usgR+usgM,* pTYGSmet-*usgL*	√	√	√	√	√	

[a] AO-*usgPDRMO*: co-culture of transformants AO-*usgPDRM* and AO-*usgO*

Table S6. Strains, substrates, and media in feeding experiments.

Strain	Substrate	Medium/ Buffer
AO-*usgR*	**2** (10 mg/mL in DMSO)	DPY and citrate buffer
	4 (5 mg/mL in DMSO)	DPY and citrate buffer
	10+**15**+**21** (10 mg/mL in DMSO)	DPY
	23 (10 mg/mL in DMSO)	DPY
	24 (10 mg/mL in DMSO)	DPY
	25 (10 mg/mL in DMSO)	DPY
	26 (10 mg/mL in DMSO)	DPY
	27 (10 mg/mL in DMSO)	DPY
	28 (10 mg/mL in DMSO)	DPY
	29 (10 mg/mL in DMSO)	DPY
AO-*usgM*	**1** (10 mg/mL in DMSO)	DPY
AO-*usgO*	**2** (10 mg/mL in DMSO)	DPY
	4 (5 mg/mL in DMSO)	DPY
	14 (10 mg/mL in DMSO)	DPY
	17 (10 mg/mL in DMSO)	DPY

Table S7. Media used in this study

Medium	Component
DPY	2% Dextrin, 1% Polypeptone, 0.5% Yeast extract, 0.5% KH_2PO_4, 0.5% $MgSO_4 \cdot 6H_2O$, 1.5% Agar
LB	0.5% Yeast extract, 1% Trptone, 1% NaCl
LB Agar	0.5% Yeast extract, 1% Trptone, 1% NaCl, 1.5% Agar
YPAD	1% Yeast extract, 2% Trptone, 2% Glucose, 0.03% Adenine
YPAD Agar	1% Yeast extract, 2% Trptone, 2% Glucose, 0.03% Adenine, 1.5% Agar
PSB	200 g/L Potato, 20 g/L Sucrose
PSA	200 g/L Potato, 20 g/L Sucrose, 15 g Agar
Rice solid medium	100 g rice, 100 mL ddH_2O
YTD	0.1% Yeast extract, 0.1% Trptone, 1% Glucose
YTD Agar	0.1% Yeast extract, 0.1% Trptone, 1% Glucose, 1.5% Agar
Bottom Agar	0.3% Yeast extract, 20% Sucrose, 0.3% Casein acid hydrolysate, 1% Agar
Top Agar	0.3% Yeast extract, 20% Sucrose, 0.3% Casein acid hydrolysate, 1.5% Agar
TB_3	0.3% Yeast extract, 20% Sucrose, 0.3% Casein acid hydrolysate
SM-ura	0.67% YNB (contain ammonium sulfate), 2% Glucose, 0.077% complete supplement mixture minus uracil, 1.5% Agar
CZD/S Agar (w/o Arginine)	3.5% Czapek Dox broth, 1 M Sorbitol, 0.05% Adenine, 0.15% Methionine, 0.1% Ammonium sulfate, 1.5% Agar
CZD/S soft Agar (w/o Arginine)	3.5% Czapek Dox broth, 1 M Sorbitol, 0.05% Adenine, 0.15% Methionine, 0.1% Ammonium sulfate, 0.8% Agar
CZD/S Agar (w/o L-Methionine)	3.5% Czapek Dox broth, 1 M Sorbitol, 0.05% Adenine, 0.1% Arginine, 0.1% Ammonium sulfate, 1.5% Agar
CZD/S soft Agar (w/o L-Methionine)	3.5% Czapek Dox broth, 1 M Sorbitol, 0.05% Adenine, 0.1% Arginine, 0.1% Ammonium sulfate, 0.8% Agar
CZD/S Agar (w/o Arginine and w/o Methionine)	3.5% Czapek Dox broth, 1 M Sorbitol, 0.05% Adenine, 0.1% Ammonium sulfate, 1.5% Agar
CZD/S soft Agar (w/o Arginine and w/o Methionine)	3.5% Czapek Dox broth, 1 M Sorbitol, 0.05% Adenine, 0.1% Ammonium sulfate, 0.8% Agar

Table S8. Primers used in this study

Primer name	Sequence (5′ to 3′)	Description
AO-*usgO*-5F	TCACCGGTGTCACCTACGAAC	For screening heterologous expression transformants of *A. oryzae*
AO-*usgO*-6R	TTGGACGCCTTGGAGTTG	
AO-*uvpks1*-5F	TCCAAGAACCAGGTCTACATCA	
AO-*uvpks1*-6R	CCGTCATCTCGCCAAACT	
AO-*usgD*-5F	ATGGCCTCGCACCACCAGCT	
AO-*usgD*-6R	CTACTCCGCAGCGGACAAGCT	
AO-*usgR*-5F	AAGACTTCGTGCCCAGAG	
AO-*usgR*-6R	TGAATGCCGTGTAGTTGG	
AO-*usgM*-5F	AGTTTGCGGAATGTCTCACC	
AO-*usgM*-6R	AACAGCCACCAACCATCCA	
AO-*usgL*-5F	TACGTTTGCCAGATTCTGGAGC	
AO-*usgL*-6R	GAAGTGGCAGTGGAAAAGCC	
AO-*uvpks1*-F+PamyB	cttctctgaacaataaaccccacagcaagctccgaATGGCGAACGTGTTCCAAATTG	For cloning *uvpks1* into pTYGSarg under control of PamyB
AO-*uvpks1*-R+TamyB	catatactctccacccttcacgagctactacagatTTAGATACCCGCGCCGGTAGA	
AO-*usgD*-F+Padh	tctttcaacacaagatcccaaagtcaaaggATGGCCTCGCACCACCAGC	For cloning *usgD* into pTYGSarg under control of Padh
AO-*usgD*-R+Tagh	ttcattctatgcgttatgaacatgttccctCTACTCCGCAGCGGACAAGCTG	
AO-*usgR*-F+PgpdA	cagctaccccgcttgagcagacatcaccggATGGCCGATGCAAAGGCC	For cloning *usgR* into pTYGSarg under control of PgpdA
AO-*usgR*-R+TgpdA	acgacaatgtccatatcatcaatcatgaccTCAAAGTACGTATCGGATCATGGTAAATG	
AO-*usgR*-F+PamyB	cttctctgaacaataaaccccacagcaagctccgaATGGCCGATGCAAAGGCC	For cloning *usgR* into pTYGSarg under control of PamyB
AO-*usgR*-R+TamyB	catatactctccacccttcacgagctactacagatTCAAAGTACGTATCGGATCATGGTAAATG	
AO-*usgM*-F+Peno	cgactgaccaattccgcagctcgtcaaaggATGCGGGCTACGAACCAGCC	For cloning *usgM* into pTYGSarg under control of Peno
AO-*usgM*-R+Teno	ggttggctggtagacgtcatataatcatacTTAGTCTGTGCAGGCTACCAAAAGCTGC	
AO-*usgM*-F+PamyB	cttctctgaacaataaaccccacagcaagctccgaATGCGGGCTACGAACCAGCC	For cloning *usgM* into pTYGSarg under control of PamyB
AO-*usgM*-R+TamyB	catatactctccacccttcacgagctactacagatTTAGTCTGTGCAGGCTACCAAAAGCTGC	
AO-*usgL*-F+PamyB	cttctctgaacaataaaccccacagcaagctccgaATGACTTCTTTAACTGGTCTTGCCCTC	For cloning *usgL* into pTYGSmet under control of PamyB
AO-*usgL*-R+TamyB	catatactctccacccttcacgagctactacagatcTCAGCGTCGCAGCTCCTCG	
AO-*usgO*-F+Padh	tctttcaacacaagatcccaaagtcaaaggATGGCTCCCAGTGTCATTGTT	For cloning *usgO* into pTYGSarg under control of Padh
AO-*usgO*-R+Tadh	ttcattctatgcgttatgaacatgttccctTTAAATCTCGAGGCTGGGTTCAAC	
AO-*usgO*-F+PamyB	cttctctgaacaataaaccccacagcaagctccgaATGGCTCCCAGTGTCATTGTT	For cloning *usgO* into pTYGSarg under control of PamyB
AO-*usgO*-R+TamyB	catatactctccacccttcacgagctactacagatcTTAAATCTCGAGGCTGGG	

	TTCAAC	
KO-*usgO*-1F	GGAGCAACCAGACCATTC	For amplifying homologous knockout fragments
KO-*usgO*-2R+G418	cagatacggcagagaaatcgcaacctcAACAACAATGACACTGGGAG	
KO-*usgO*-3F+G418	gtttagattccaagtgtctactgctggcTGCCACGGAGGAGTTTGA	
KO-*usgO*-4R	GGGTCATCATTCGCTCCAT	
KO-*uvpks1*-1F	TTCTTACGGCGATGCTGGTG	
KO-*uvpks1*-2R+G418	cagatacggcagagaaatcgcaacctcAAAGCCCGATGGCACAACC	
KO-*uvpks1*-3F+G418	gtttagattccaagtgtctactgctggcCTGTTTGCCGACATCCTGG	
KO-*uvpks1*-4R	GCATCTGGACCATTACAGCAAC	
KO-*usgD*-1F	ATGGCCATCATGAGTCAATCAC	
KO-*usgD*-2R+G418	cagatacggcagagaaatcgcaacctcGGATGTTGAGGCAGAGCAG	
KO-*usgD*-2R+HPH	ttgacctccactagctccagccaagccGGATGTTGAGGCAGAGCAG	
KO-*usgD*-3F+G418	gtttagattccaagtgtctactgctggcTGCGGAGTAGTAGAATGCTCG	
KO-*usgD*-3F+HPH	gcaaaggaatagagtagatgccgaccgTGCGGAGTAGTAGAATGCTCG	
KO-*usgD*-4R	CAACTGGATCGGCTATCAGATC	
KO-*usgR*-1F	TTGCCAGCATCGTCCTTC	
KO-*usgR*-2R	cagatacggcagagaaatcgcaacctcACTTGTTCCTGGGCGAGA	
KO-*usgR*-3F	gtttagattccaagtgtctactgctggcCATGATCCGATACGTACTTTGA	
KO-*usgR*-4R	GCTTGCTTACGACTGGAGG	
KO-*usgM*-1F	TGGAGCGTTACACTGAATAGC	
KO-*usgM*-2R	cagatacggcagagaaatcgcaacctcCCGACATACCCGAACTGAT	
KO-*usgM*-3F	gtttagattccaagtgtctactgctggcGTCTTGTGGGCTGATGTGG	
KO-*usgM*-4R	CTCCCAATCGTGTGTAGTTGA	
KO-*usgL*-1F	GCTCAGGCACGAGATAAT	
KO-u*sgL*-2R+G418	cagatacggcagagaaatcgcaacctcGAGGCAAGGACAATAGGA	
KO-*usgL*-2R+HPH	ttgacctccactagctccagccaagccGAGGCAAGGACAATAGGA	
KO-*usgL*-3F+G418	gtttagattccaagtgtctactgctggcGGGCGGCGAGAGAGTAATGT	
KO-*usgL*-3F+HPH	gcaaaggaatagagtagatgccgaccgGGGCGGCGAGAGAGTAATGT	
KO-*usgL*-4R	GCGAAGGAAGGAGAAGGCAC	
KO-GEN-F	GAGGTTGCGATTTCTCTGCCGTATCTG	
KO-GEN-R	GCCAGCAGTAGACACTTGGAATCTAAAC	
KO-HPH-F	GGCTTGGCTGGAGCTAGTGGAGGTCAA	
KO-HPH-R	CGGTCGGCATCTACTCTATTCCTTTGC	
KO-*usgO*-spacerF	acctAAGTCGAGTCAAATACCCCG	For construction of sgRNA vector
KO-*usgO*-spacerR	aaacCGGGGTATTTGACTCGACTT	
KO-*uvpks1*-spacerF	acctCACGGCAGGAGGGTCTTTAT	
KO-*uvpks1*-spacerR	aaacATAAAGACCCTCCTGCCGTG	
KO-*usgD*-spacerF	acctATCCCTACTTCAAGCAGACG	
KO-*usgD*-spacerR	aaacCGTCTGCTTGAAGTAGGGAT	
KO-*usgR*-spacerF	acctTGGACTGCTACATTCAATAT	
KO-*usgR*-spacerR	aaacATATTGAATGTAGCAGTCCA	
KO-*usgM*-spacerF	acctACCATAGTAACGTATCCTCG	
KO-*usgM*-spacerR	aaacCGAGGATACGTTACTATGGT	
KO-*usgL*-spacerF	acctTGACTGGTCACGCTTCACTT	

KO-*usgL*-spacerR	aaacAAGTGAAGCGTGACCAGTCA	
KO-*usgO*-5F	GACCTGCTCTTACCGCTCAA	For screening knockout transformants
KO-*usgO*-6R	TTGGACGCCTTGGAGTTG	
KO-*uvpks1*-5F	TCCAAGAACCAGGTCTACATCA	
KO-*uvpks1*-6R	CCGTCATCTCGCCAAACT	
KO-*usgD*-5F	ACCGAAGACTACCGCAACT	
KO-*usgD*-6R	CGTCTGCTTGAAGTAGGGAT	
KO-*usgR*-5F	AAGACTTCGTGCCCAGAG	
KO-*usgR*-6R	TGAATGCCGTGTAGTTGG	
KO-*usgM*-5F	AGTTTGCGGAATGTCTCACC	
KO-*usgM*-6R	AACAGCCACCAACCATCCA	
KO-*usgL*-5F	GCTTCAGATCACGGTCCACA	
KO-*usgL*-6R	GAAGTGGCAGTGGAAAAGCC	
KO-*usgO*-7F	TCTCCTCGTCTATCGTGGGC	
KO-*usgO*-8R	TTCTGCCAGTGATGGTGAT	
KO-*uvpks1*-7F	GGATGTTGAGGCAGAGCAG	
KO-*uvpks1*-8R	TATGGCGTCTGACTGTAATGG	
KO-*usgD*-7F	TGCCACGTACGGTAGCCAAT	
KO-*usgD*-8R-1 (G418)	GCCATTGTCGGACCTCTTATCG	
KO-*usgD*-8R-2 (HPH)	GCGGCTCAAGCTGGTATCAG	
KO-*usgR*-7F	TGGCTCAGCAGACATCAGG	
KO-*usgR*-8R	TAGCCTGCACAGACTAAGTAG	
KO-*usgM*-7F	CATCTTGAGGCAGGCTTTG	
KO-*usgM*-8R	AATGTCTCGTGGACGGAT	
KO-*usgL*-7F	TACAACCGCCTCTTCCTAC	
KO-*usgL*-8R	GGCTTGTAGATTGCGTTCC	
G856F	GAATGGTCAAATCAAACTGCTAGATAT	
G855R	TGTTGGGTTTGAGCTAGGTGGG	
H856F	CCGATGGCTGTGTAGAAGTACT	
H855R	ACAAGTGGGGCTGATCTGA	

Table S9. Results of expressing UsgR$^{76-273}$ and UsgR$^{160-264}$ in different expression vectors and strains

Expression vector	Expression strain	Fused protein	Result	Expected size (kDa)
pET28a-UsgR$^{76-273}$	*E.coli* BL21 (DE3)	His-UsgR$^{76-273}$	×	23.5
pET28a-UsgR$^{160-264}$		His-UsgR$^{160-264}$	×	13.3
pETM10-UsgR$^{76-273}$		His-UsgR$^{76-273}$	×	23.5
pETM10-UsgR$^{160-264}$		His-UsgR$^{160-264}$	√	13.3
pGEX-6P-1-UsgR$^{76-273}$		GST-UsgR$^{76-273}$	×	49.0
pGEX-6P-1-UsgR$^{160-264}$		GST-UsgR$^{160-264}$	√	38.8
pMBP_1a-UsgR$^{76-273}$		MBP+His-UsgR$^{76-273}$	×	65.2
pMBP_1a-UsgR$^{160-264}$		MBP+His-UsgR$^{160-264}$	√	55
pET28a-UsgR$^{76-273}$	*E. coli* Rosetta (DE3)	His-UsgR$^{76-273}$	×	23.5
pET28a-UsgR$^{160-264}$		His-UsgR$^{160-264}$	×	13.3
pETM10-UsgR$^{76-273}$		His-UsgR$^{76-273}$	×	23.5
pETM10-UsgR$^{160-264}$		His-UsgR$^{160-264}$	√	13.3
pGEX-6P-1-UsgR$^{76-273}$		GST-UsgR$^{76-273}$	×	49.0
pGEX-6P-1-UsgR$^{160-264}$		GST-UsgR$^{160-264}$	×	38.8
pMBP_1a-UsgR$^{76-273}$		MBP+His-UsgR$^{76-273}$	×	65.2
pMBP_1a-UsgR$^{160-264}$		MBP+His-UsgR$^{160-264}$	√	55
pET28a-UsgR$^{76-273}$	*E.coli* C43	His-UsgR$^{76-273}$	×	23.5
pET28a-UsgR$^{160-264}$	(DE3)	His-UsgR$^{160-264}$	×	13.3
pETM10-UsgR$^{76-273}$		His-UsgR$^{76-273}$	×	23.5
pETM10-UsgR$^{160-264}$		His-UsgR$^{160-264}$	×	13.3
pGEX-6P-1-UsgR$^{76-273}$		GST-UsgR$^{76-273}$	×	49.0
pGEX-6P-1-UsgR$^{160-264}$		GST-UsgR$^{160-264}$	×	38.8
pMBP_1a-UsgR$^{76-273}$		MBP+His-UsgR$^{76-273}$	×	65.2
pMBP_1a-UsgR$^{160-264}$		MBP+His-UsgR$^{160-264}$	×	55
pET28a-UsgR$^{76-273}$	*E.coli* SHuffle T7	His-UsgR$^{76-273}$	×	23.5
pET28a-UsgR$^{160-264}$		His-UsgR$^{160-264}$	×	13.3
pETM10-UsgR$^{76-273}$		His-UsgR$^{76-273}$	×	23.5
pETM10-UsgR$^{160-264}$		His-UsgR$^{160-264}$	×	13.3
pGEX-6P-1-UsgR$^{76-273}$		GST-UsgR$^{76-273}$	×	49.0
pGEX-6P-1-UsgR$^{160-264}$		GST-UsgR$^{160-264}$	√	38.8
pMBP_1a-UsgR$^{76-273}$		MBP+His-UsgR$^{76-273}$	×	65.2
pMBP_1a-UsgR$^{160-264}$		MBP+His-UsgR$^{160-264}$	√	55
pET28a-UsgR$^{76-273}$	*E.coli* BL21 pLys	His-UsgR$^{76-273}$	×	23.5
pET28a-UsgR$^{160-264}$	(DE3)	His-UsgR$^{160-264}$	×	13.3
pETM10-UsgR$^{76-273}$		His-UsgR$^{76-273}$	×	23.5
pETM10-UsgR$^{160-264}$		His-UsgR$^{160-264}$	×	13.3
pGEX-6P-1-UsgR$^{76-273}$		GST-UsgR$^{76-273}$	×	49.0
pGEX-6P-1-UsgR$^{160-264}$		GST-UsgR$^{160-264}$	√	38.8
pMBP_1a-UsgR$^{76-273}$		MBP+His-UsgR$^{76-273}$	×	65.2

pMBP_1a-UsgR$^{160-264}$	MBP+His-UsgR$^{160-264}$	√	55

Figures

(A)

```
Uv_UsgM     ----------------YGNDMTEPL---KMDMLQFFRDALVQETGRHVDNDDFDALFSELG
Th_TlnC     -------------------------------MSLEKVREIFCDVTGLDADEVEEDSELDELG
Ff_pks4     ----------------------------GSASGLIQKALEIIADEIGVDISQLTDTTLLADLG
Af_PksA     -------------------------GVGVSNEKLDAVMRVVSEESGIALEELTDDSNFADMG
Cb_CTB1     AVATAVEIVKEEALTSLEELTDPSPNEIGTVWRDALKILSEESGLTDEELTDDTSFADVG
Cg_CazM     ------------------KTQKDSG---WRDITEEVRNLVAHVSGIEASEIGLDSEMADFG
                                                . .  *  .:    : : :.*

Uv_UsgM     ADPLVGVAVIERVTNSTGIEFPA--SLLRDCKSLSD-------------------------
Th_TlnC     VDTILAKELARKLSVFSGRAVES--SRILESENFIGLAHYIQSILDIGNDK----------
Ff_pks4     VDSLMSLTILGNFREELDLDIPA--AQFYEFSTVQDLKSFLGANDQDFSSSN--SEAESS
Af_PksA     IDSLSSMVIGSRFREDLGLDLGPEFSLFIDCTTVRALKDFMLGSGDAGSGSN--VEDPPP
Cb_CTB1     VDSLMSLVITSRLRDELDIDFPD-RALFEECQTIFDLRKRFSGSTESFDSTT--TKPSAG
Cg_CazM     IDSLMGMELGREVELTFKCKLDQ--AEQMEATSLRKFVAVVAKAL-FGTDQPAEVEDEAS
             *:.  :  ..       .    :   :  ..
```

(B)

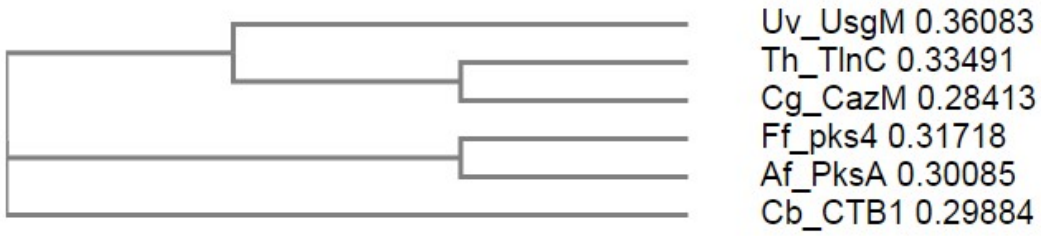
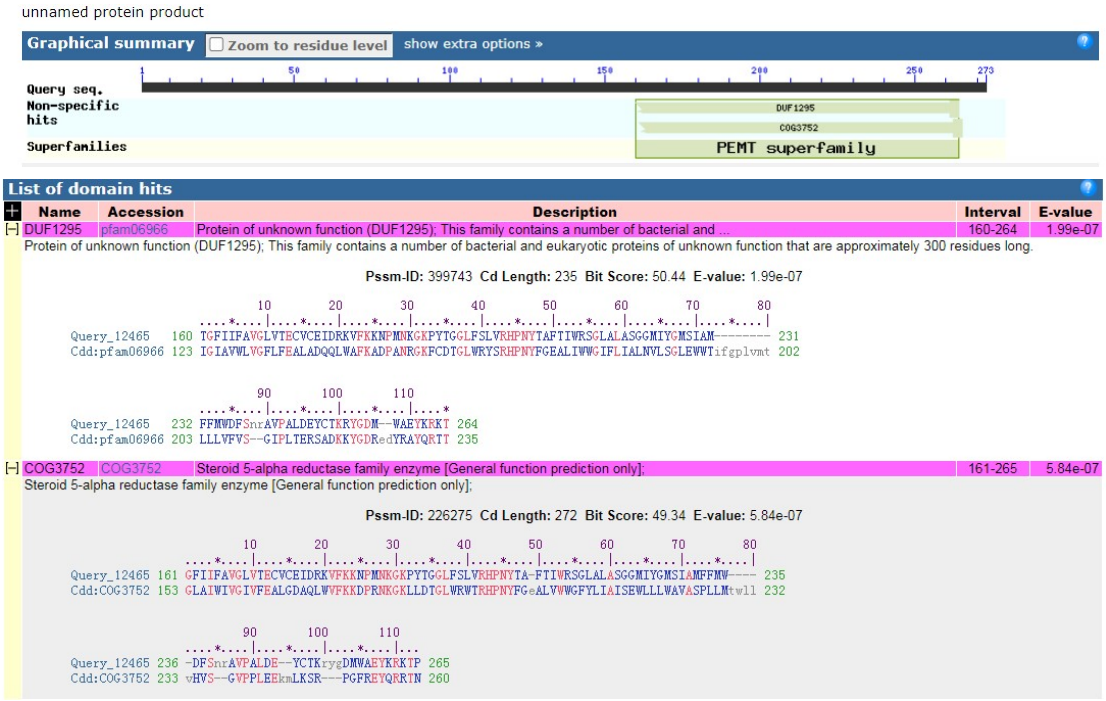


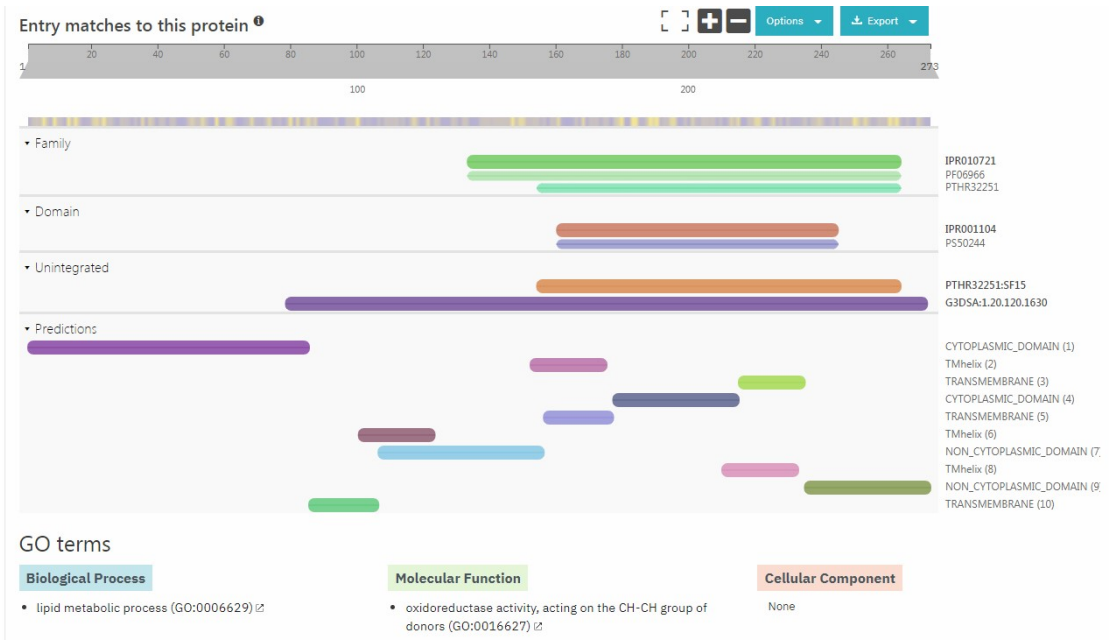
Fig. S1 Multiple sequence alignment of previously reported ACPs from nrPKS.
(A) Multiple sequence alignment, and (B) phylogenetic tree of nrPKS ACPs. The aspartate residue in DSL motif is highlighted in red, among all characterized ACPs. Uv: *Ustilaginoidea virens*, Th: *Trichoderma harzianum*, Ff: *Fusarium fujikuroi*, Af: *Aspergillus flavus*, Cb: *Cercospora beticola*, Cg: *Chaetomium globosum* CBS 148.51.

(A)



[Superfamily, PEMT] Phospholipid methyltransferase.

(B)



domain hits:

3-oxo-5-alpha-steroid 4-dehydrogenase, C-terminal, IPR001104 (*3-oxo-5_α-steroid_4-DH_C*)

Steroid 5-alpha reductase C-terminal domain profile, PS50244, (S5A_REDUCTASE)

Fig. S2 Conserved domain analysis of UsgR.

(A) CDD search, (B) Interpro search.

TMHMM result

HELP with output formats

```
# WEBSEQUENCE Length: 273
# WEBSEQUENCE Number of predicted TMHs:  3
# WEBSEQUENCE Exp number of AAs in TMHs: 93.19776
# WEBSEQUENCE Exp number, first 60 AAs:  9.84814
# WEBSEQUENCE Total prob of N-in:        0.96621
WEBSEQUENCE     TMHMM2.0        inside      1   100
WEBSEQUENCE     TMHMM2.0        TMhelix   101   123
WEBSEQUENCE     TMHMM2.0        outside   124   152
WEBSEQUENCE     TMHMM2.0        TMhelix   153   175
WEBSEQUENCE     TMHMM2.0        inside    176   210
WEBSEQUENCE     TMHMM2.0        TMhelix   211   233
WEBSEQUENCE     TMHMM2.0        outside   234   273
```

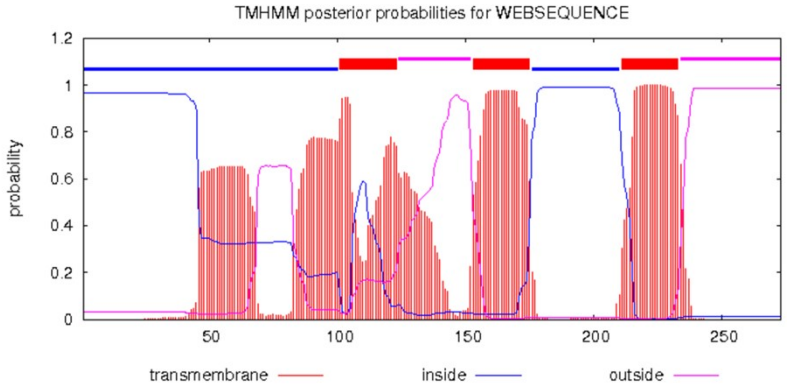


Fig. S3 TMHMM analysis of UsgR.

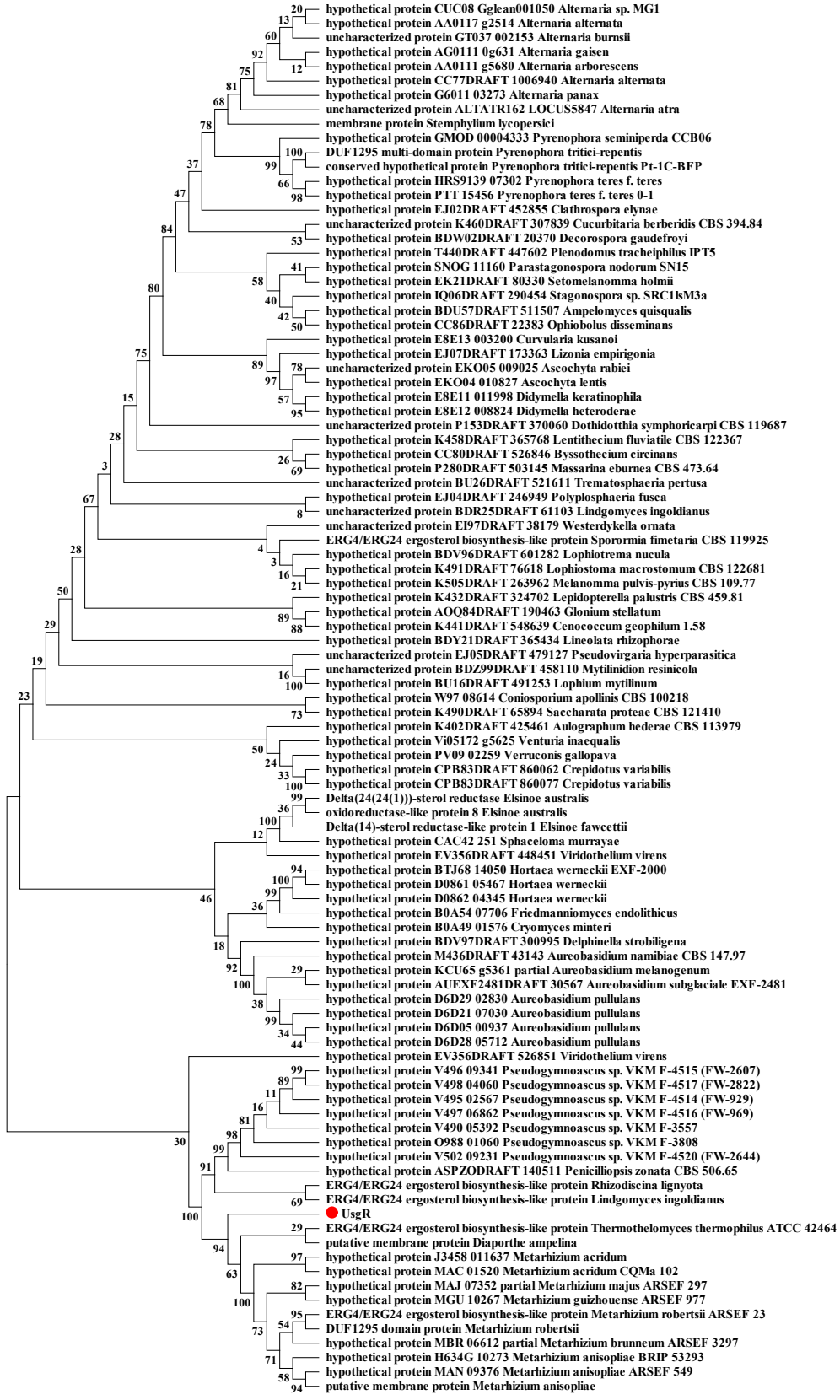


Fig. S4 Phylogenetic analysis of UsgR.

note: protein ID, see table S1. The maximum likelihood method was used with Bootstrap 1000 using the software Mega 5.

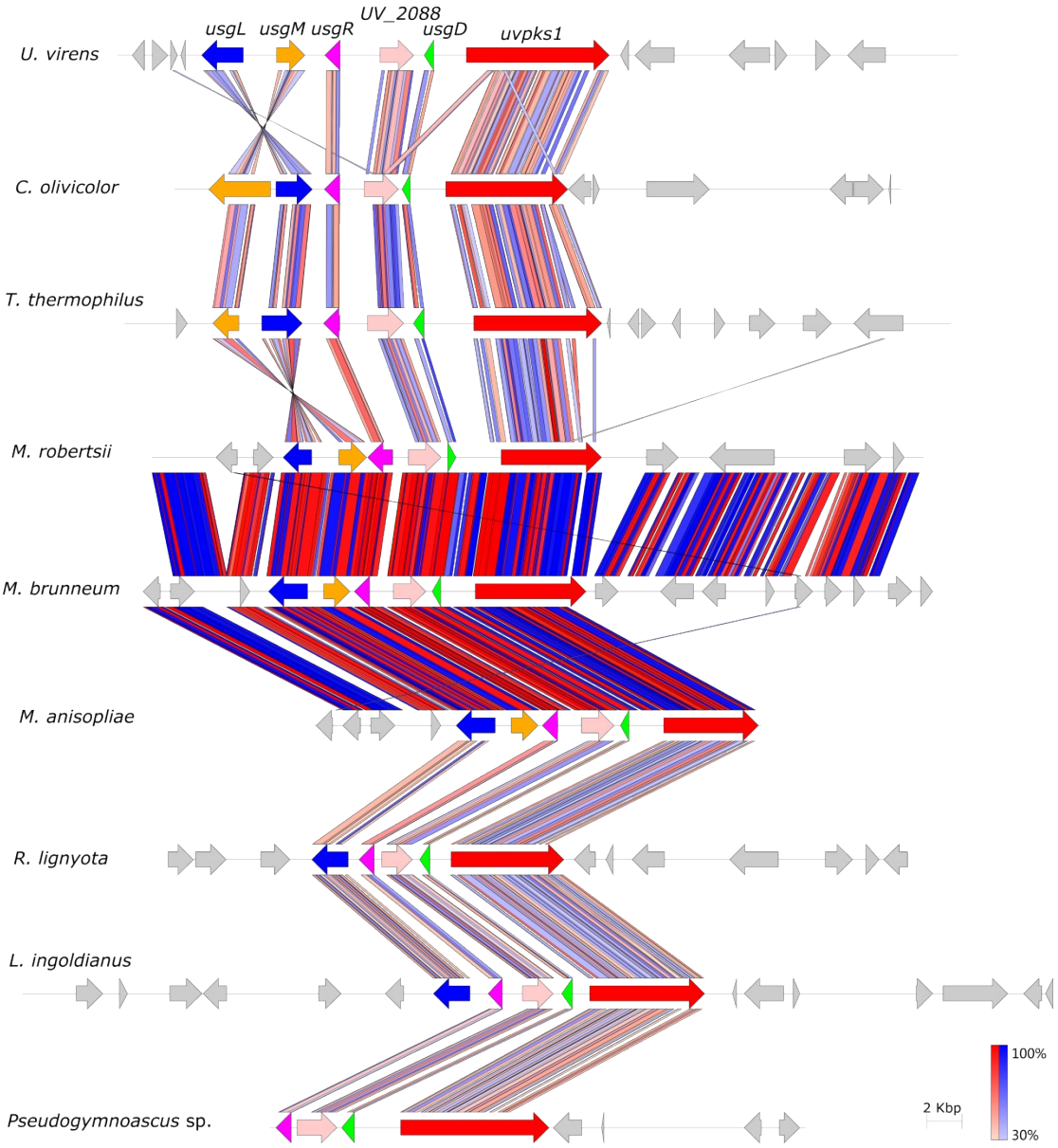


Fig. S5 Tblastx comparison of the *usg* BGC.

The illustration was created using Easyfig.[15] For genome and protein information see Table S2.

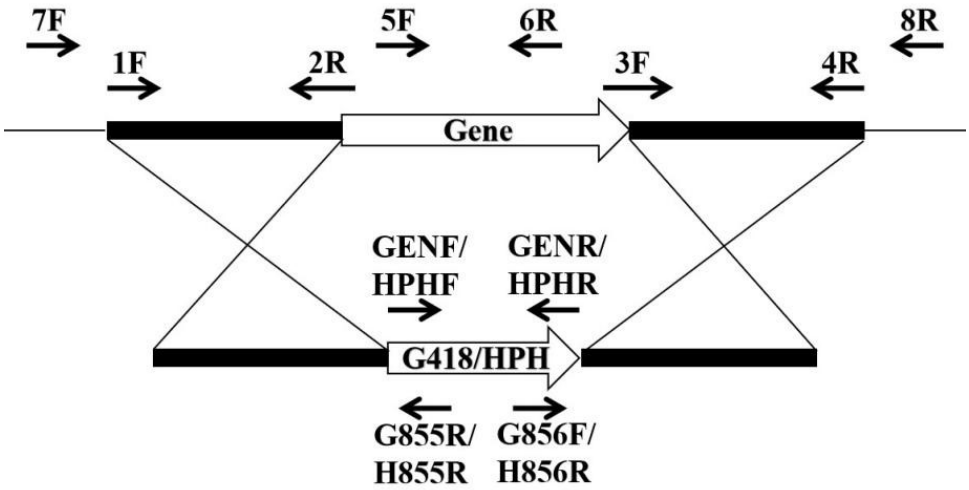


Fig. S6 Schematic of gene knockout.

The position and direction of primers used to generate and screen transformants are marked with arrows.

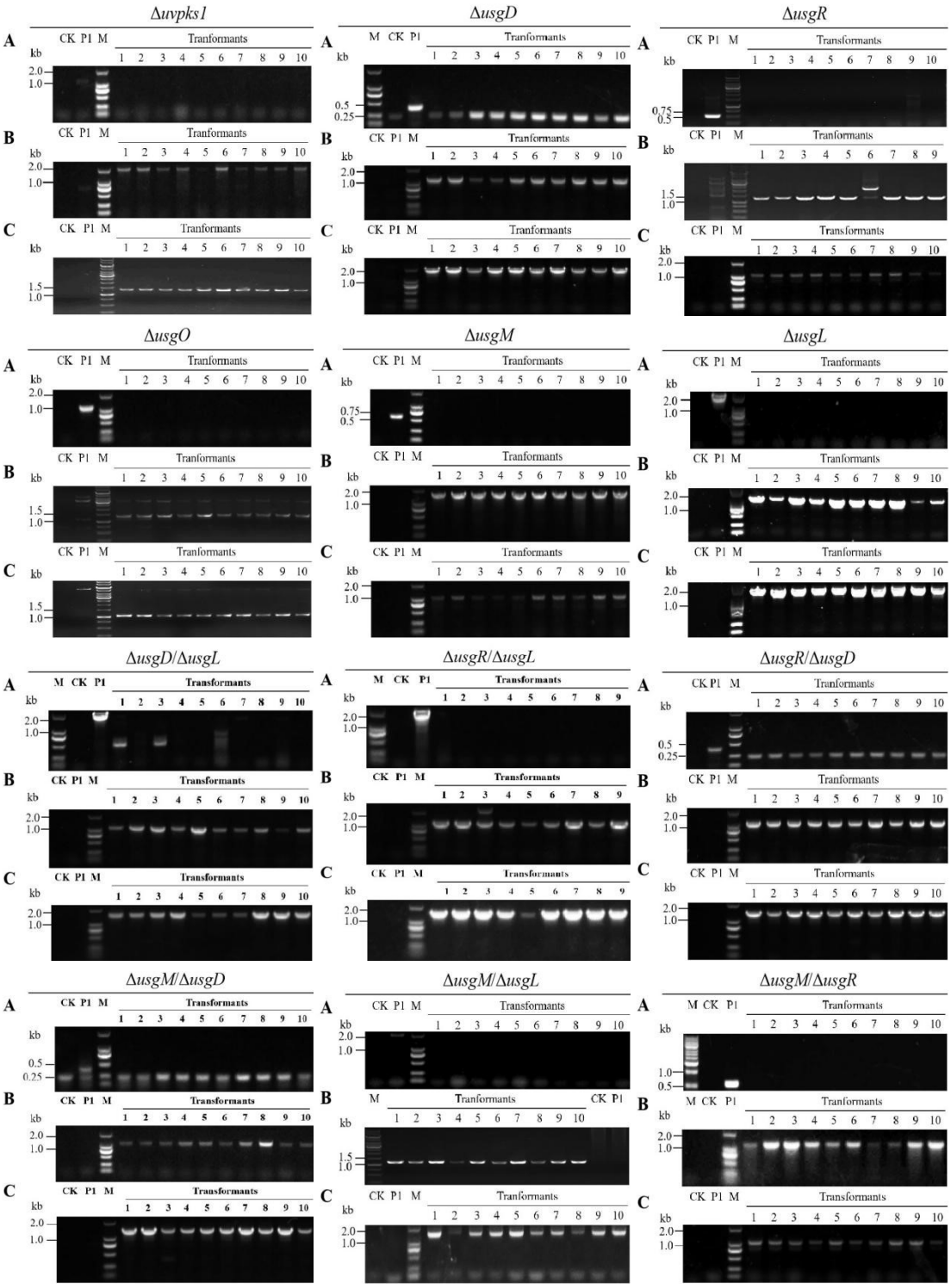


Fig. S7 Selection of knockout transformants by PCR.

A) Target gene was amplified with primer pairs 5F/6R. B) Upstream recombination sequence was amplified with primer pairs 7F/G855R or 7F/H855R. Fragment was only found in the transformants. C) Downstream recombination sequence was amplified with primer pairs G855F/8R or H855F/8R. Fragment was only found in the transformants. P1: wild-type; M: Marker; CK: blank control.

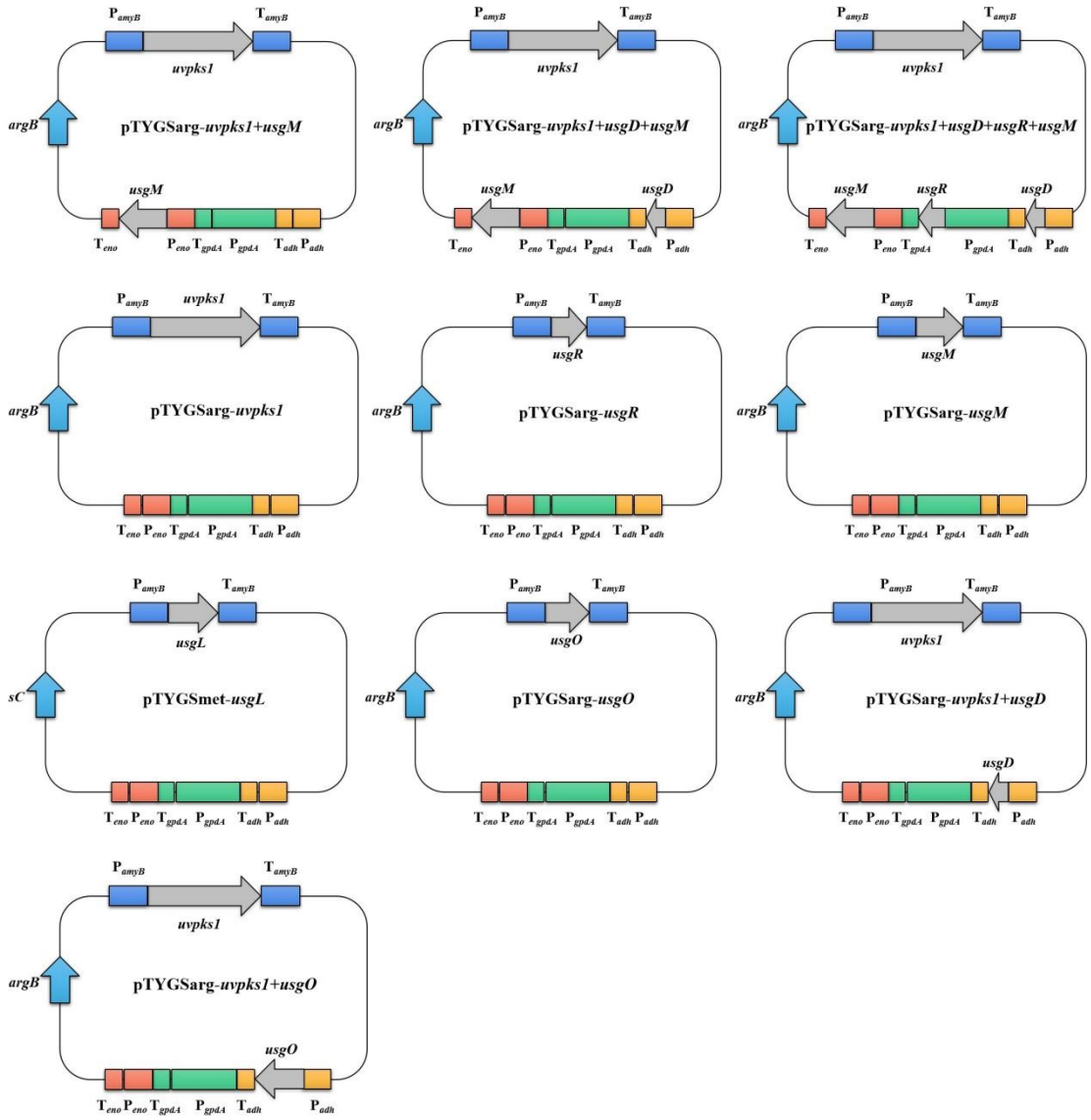
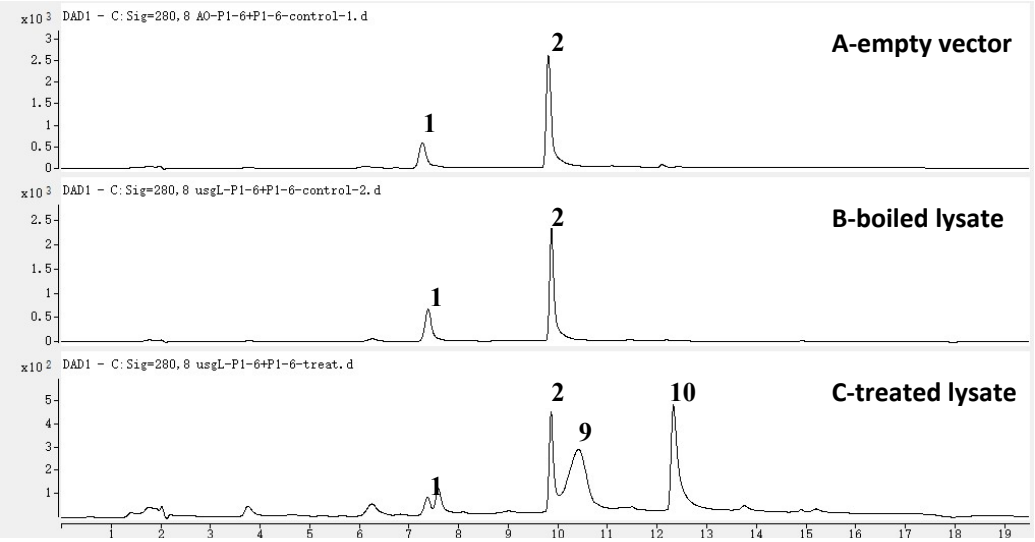
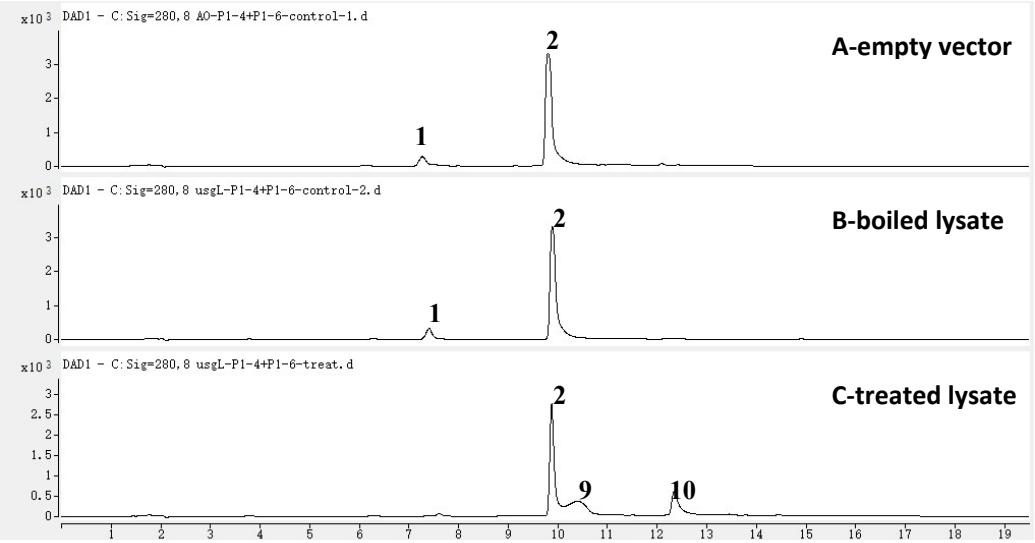


Fig. S8 Overview of *A. oryzae* heterologous expression vectors constructed in this work.

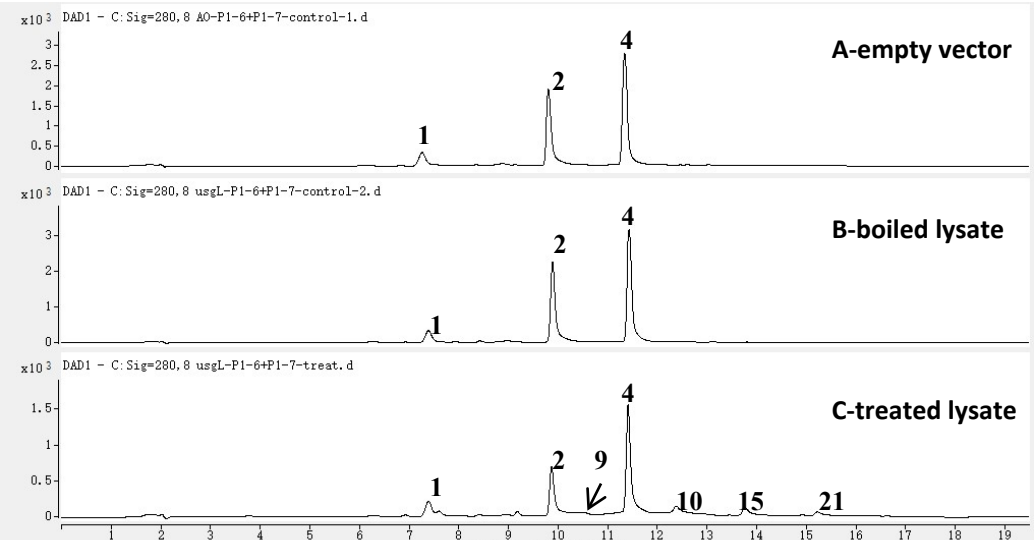
EXPL1: **1**+UsgL



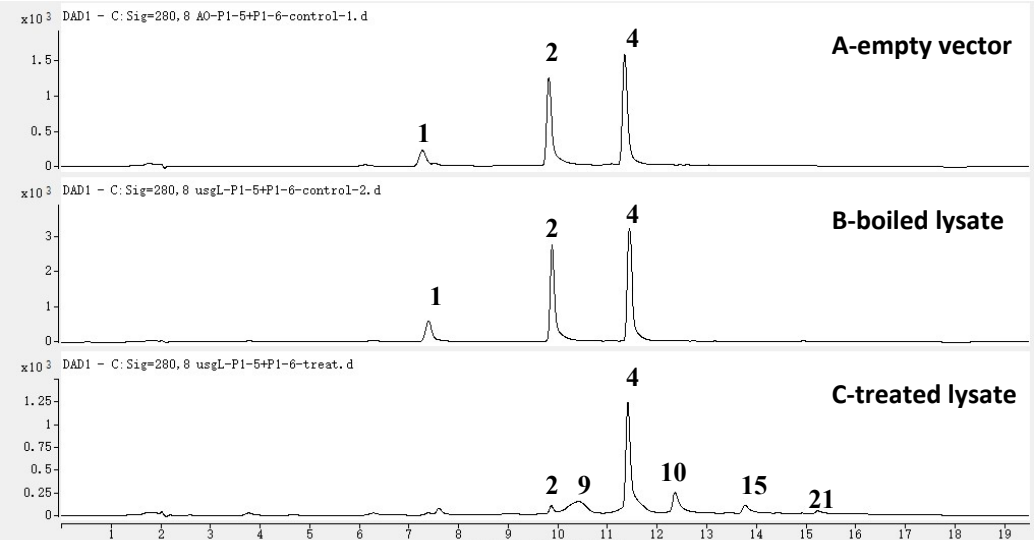
EXPL2: **1**+**2**+UsgL



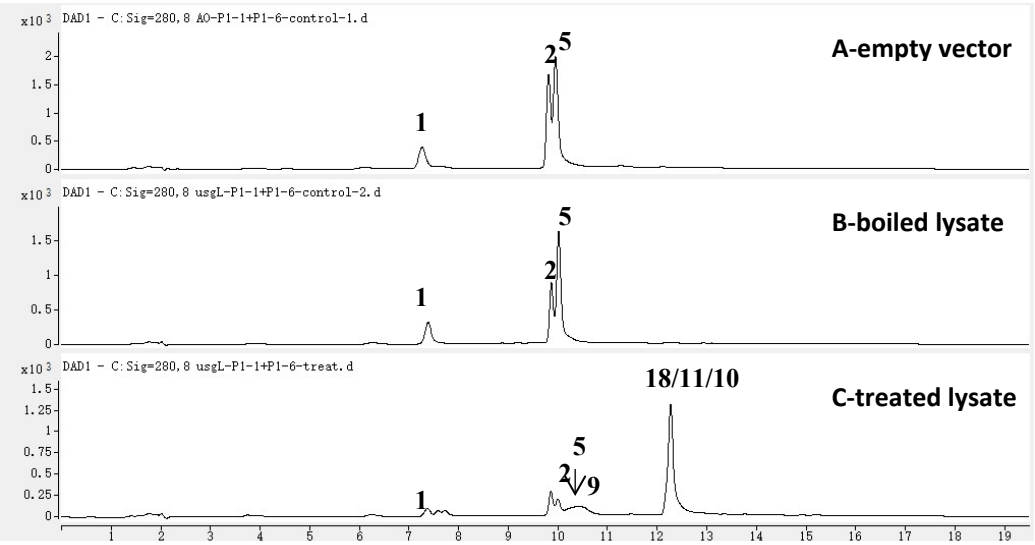
EXPL3: **1**+**3**+UsgL



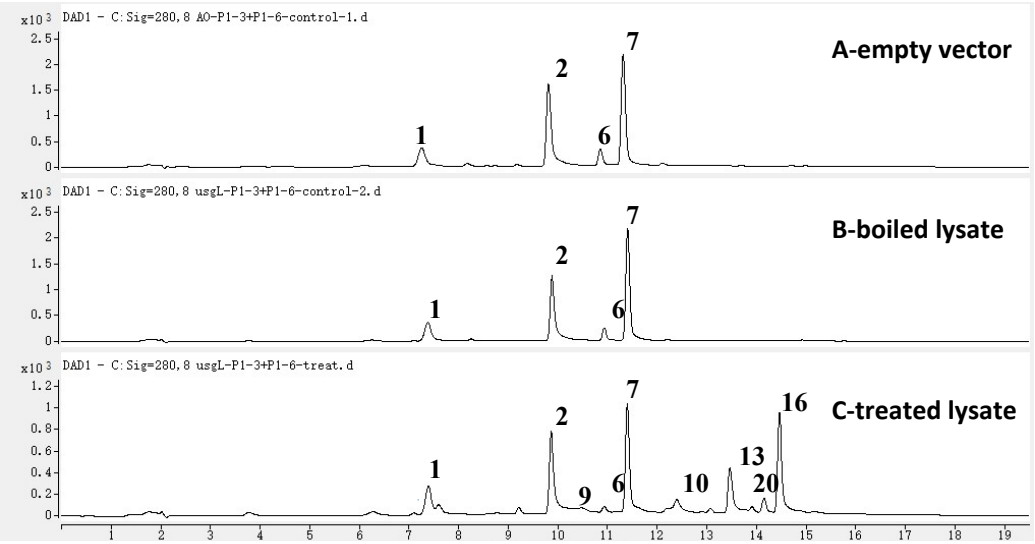
EXPL4: **1**+**4**+UsgL



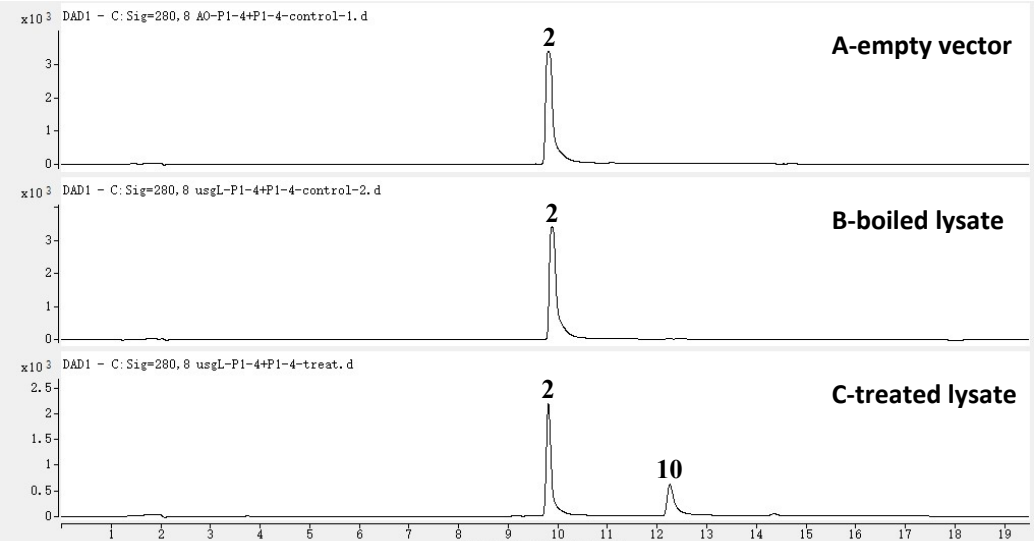
EXPL5: **1**+**5**+UsgL



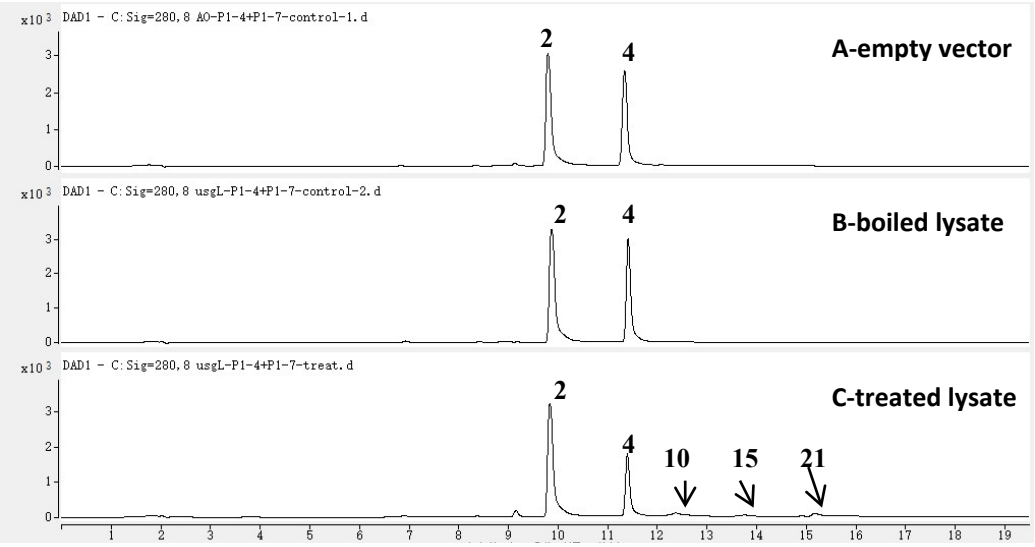
EXPL6: **1**+**7**+UsgL



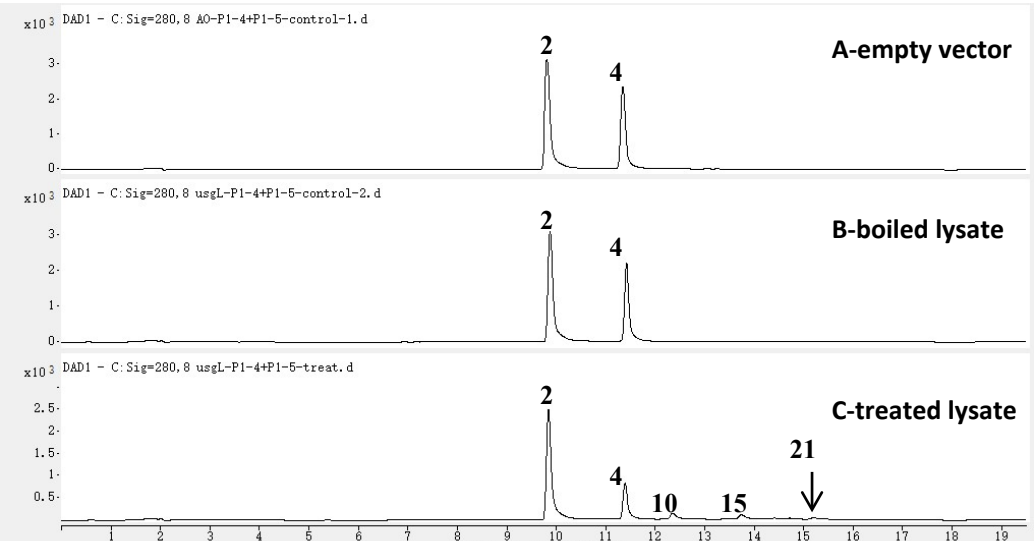
EXPL7: **2**+UsgL



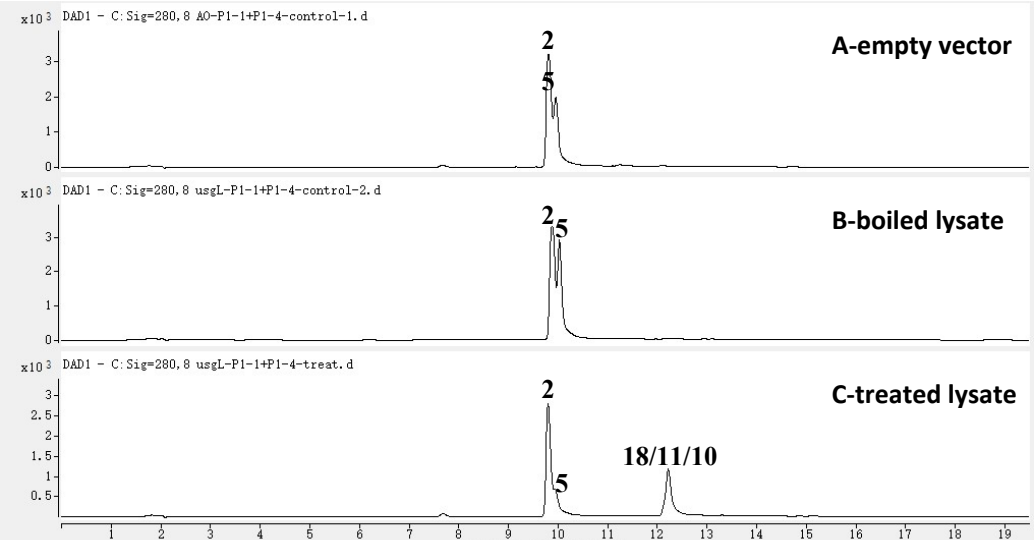
EXPL8: **2**+**3**+UsgL



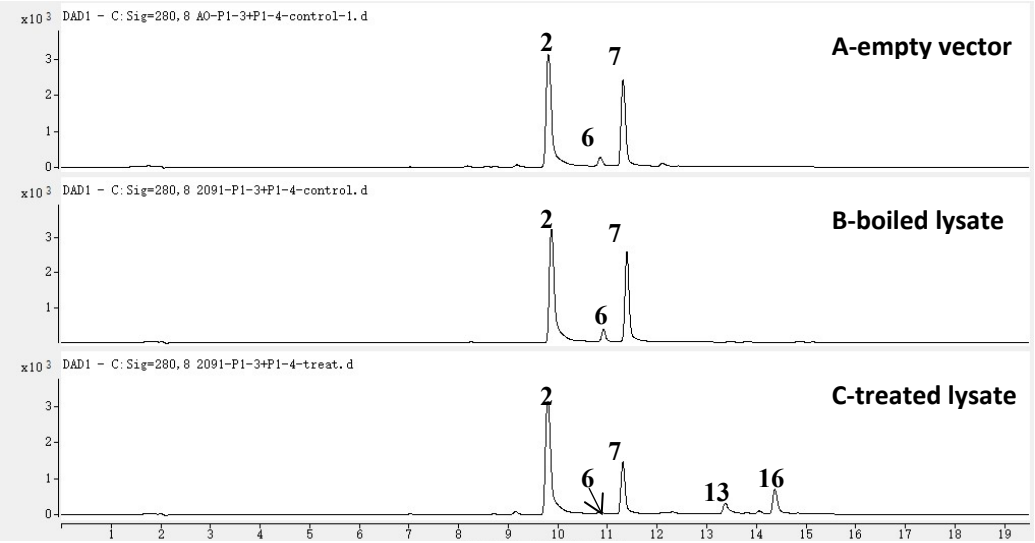
EXPL9: **2**+**4**+UsgL



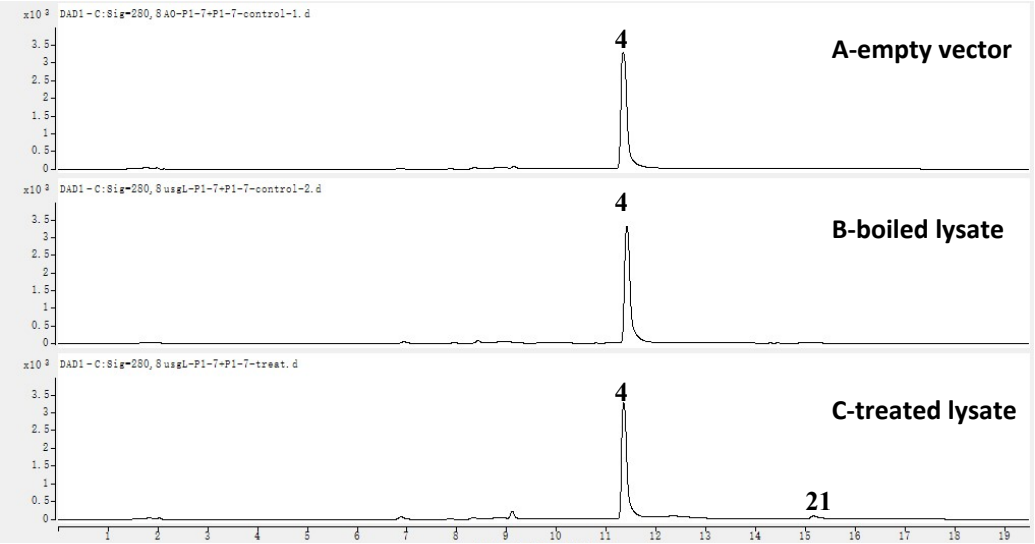
EXPL10: **2**+**5**+UsgL



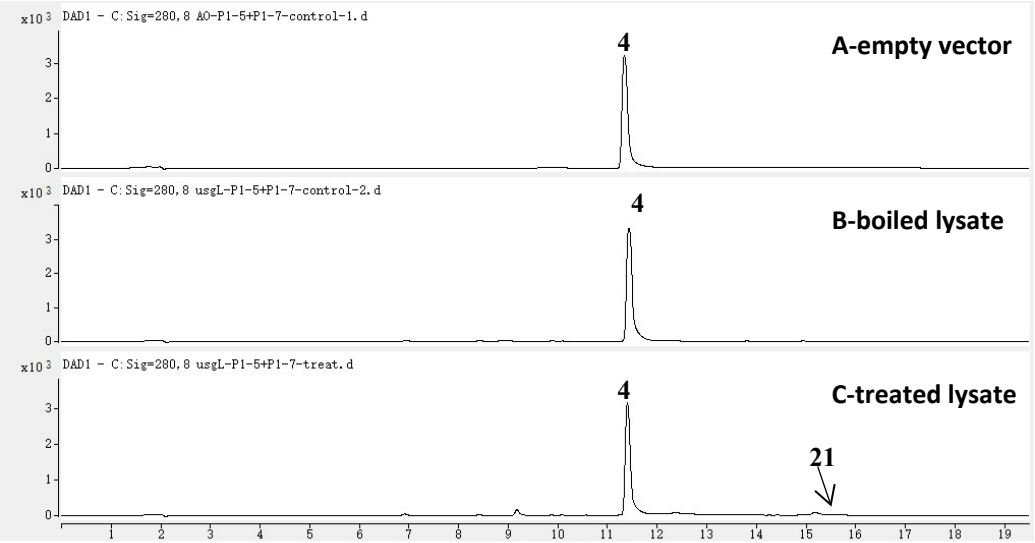
EXPL11: **2**+**7**+UsgL



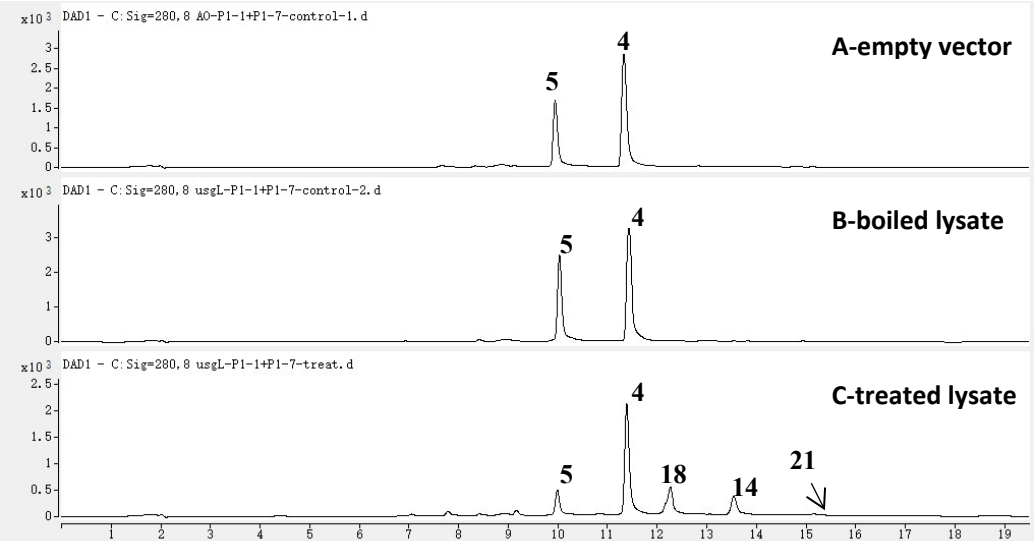
EXPL12: **3**+UsgL



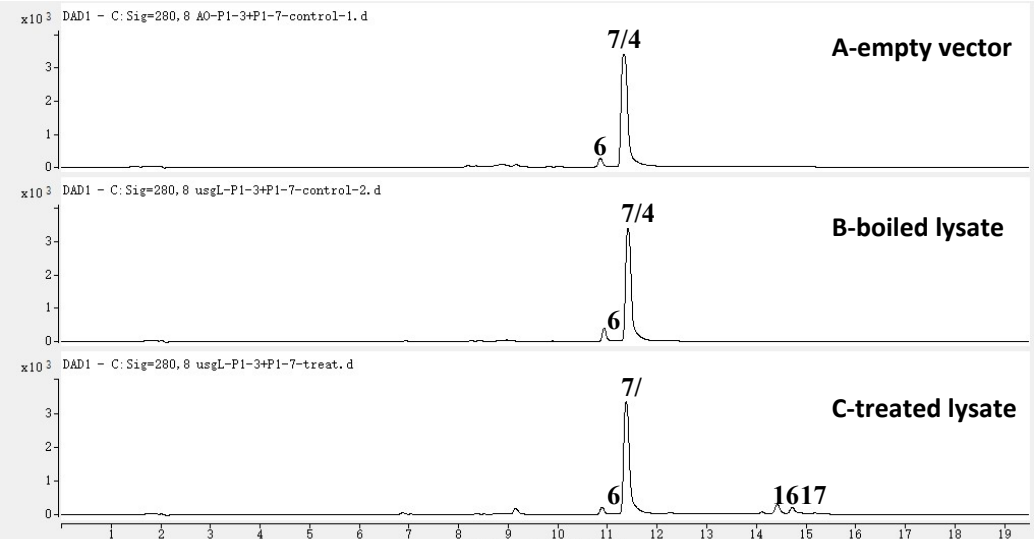
EXPL13: **3**+**4**+UsgL



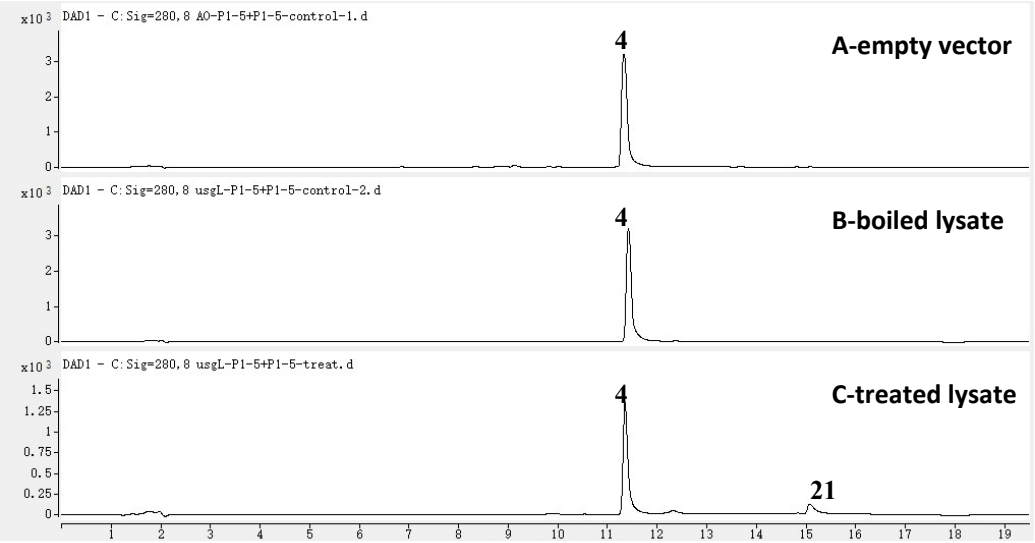
EXPL14: **3**+**5**+UsgL



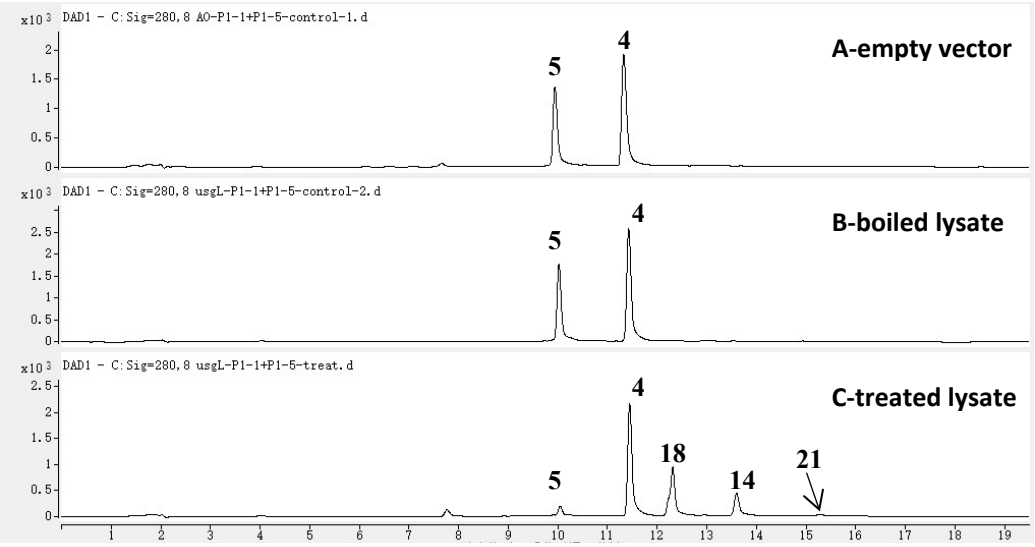
EXPL15:**3**+**7**+UsgL



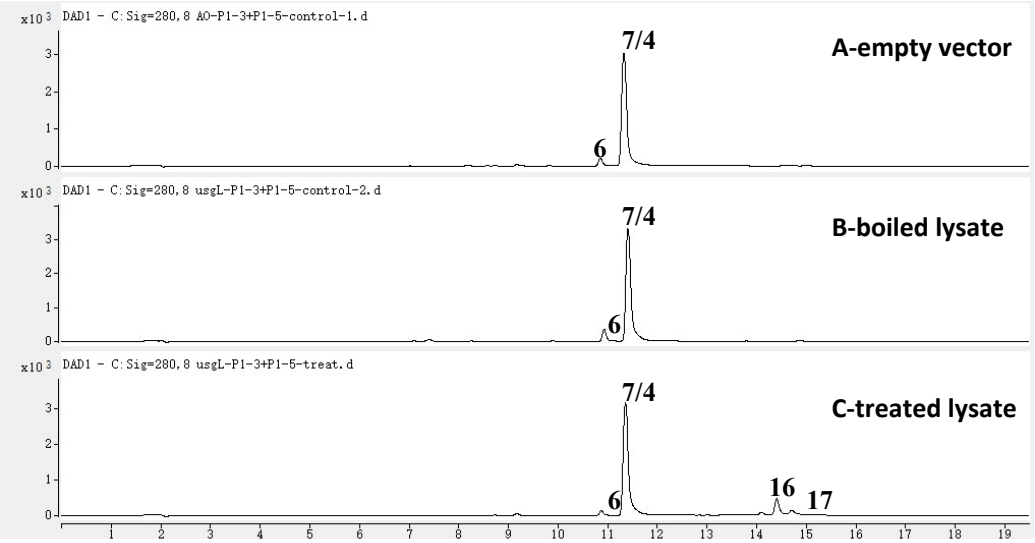
EXPL16: **4**+UsgL



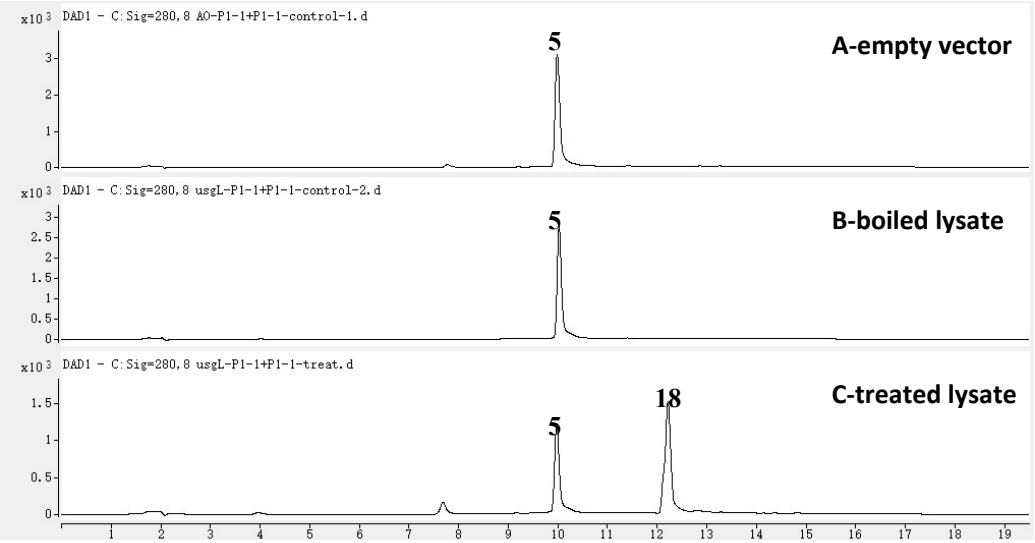
EXPL17: **4**+**5**+UsgL



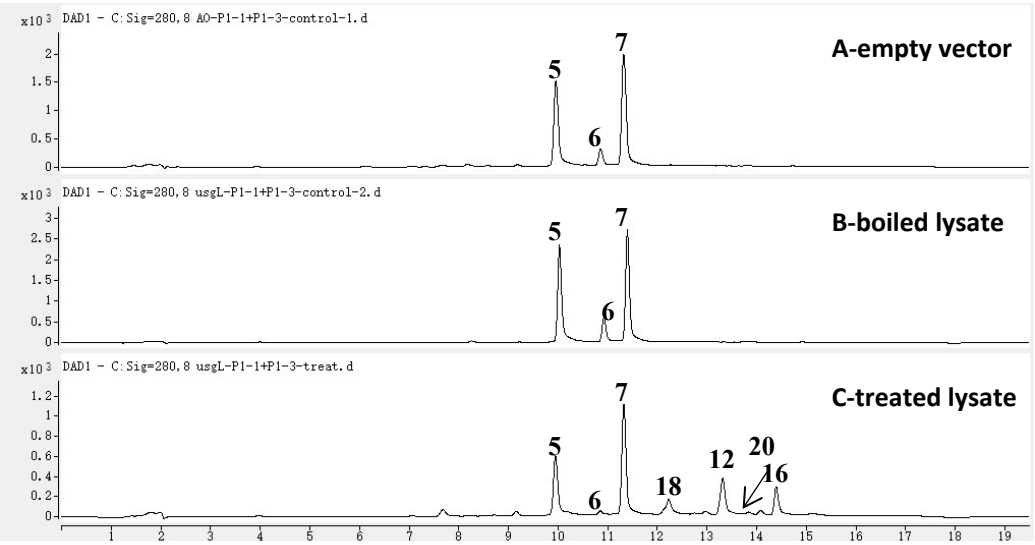
EXPL18: **4**+**7**+UsgL



EXPL19: **5**+UsgL



EXPL20: **5**+**7**+UsgL



EXPL21: **7**+UsgL

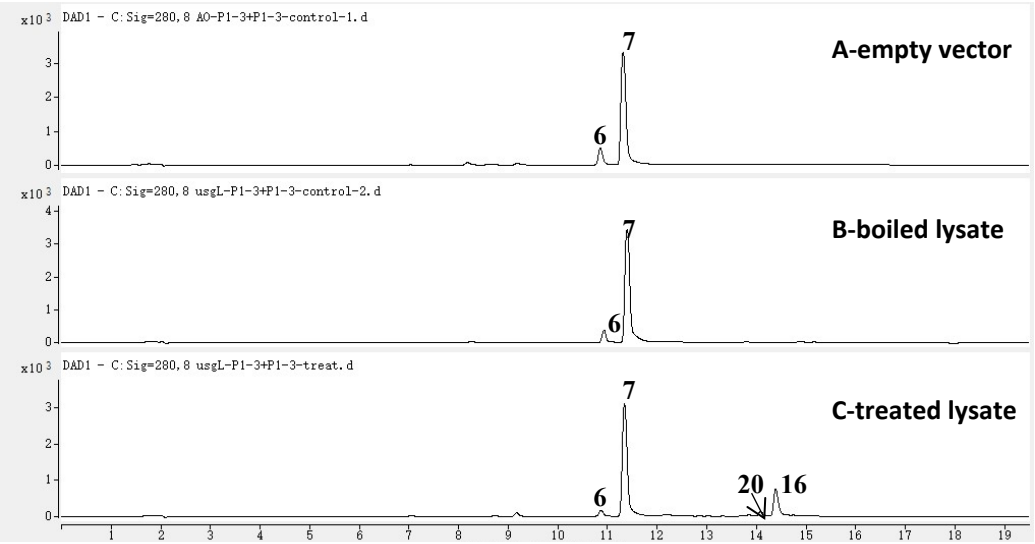
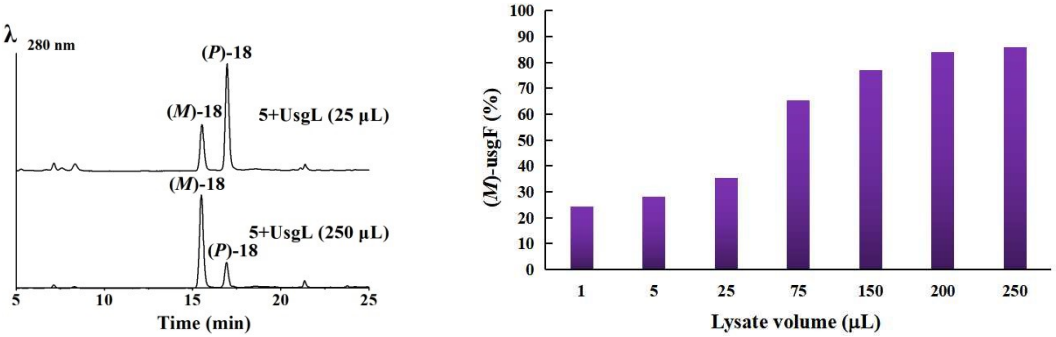


Fig. S9 LC-MS analysis of *in vitro* dimerization catalyzed by UsgL (24 h, λ = 280 nm).

(A) (B)



(C)

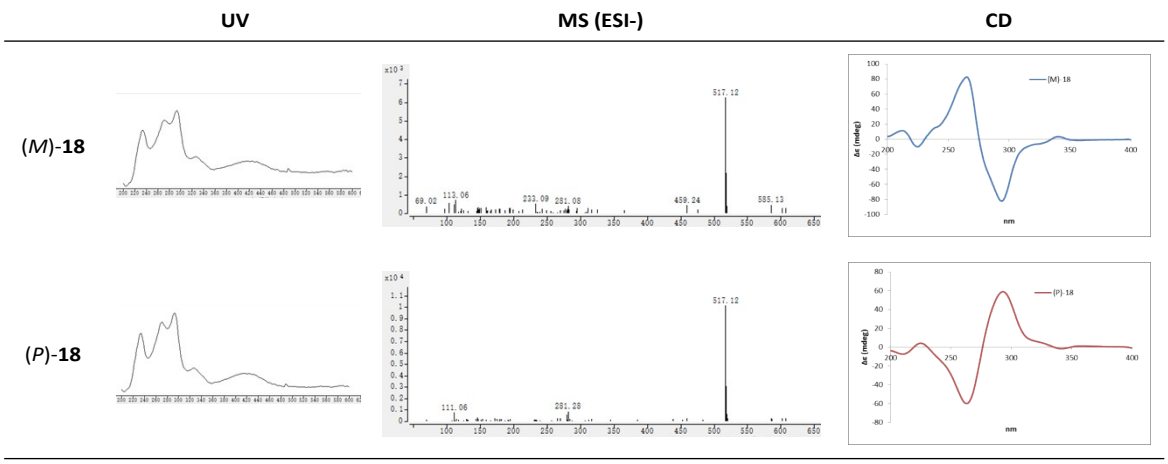
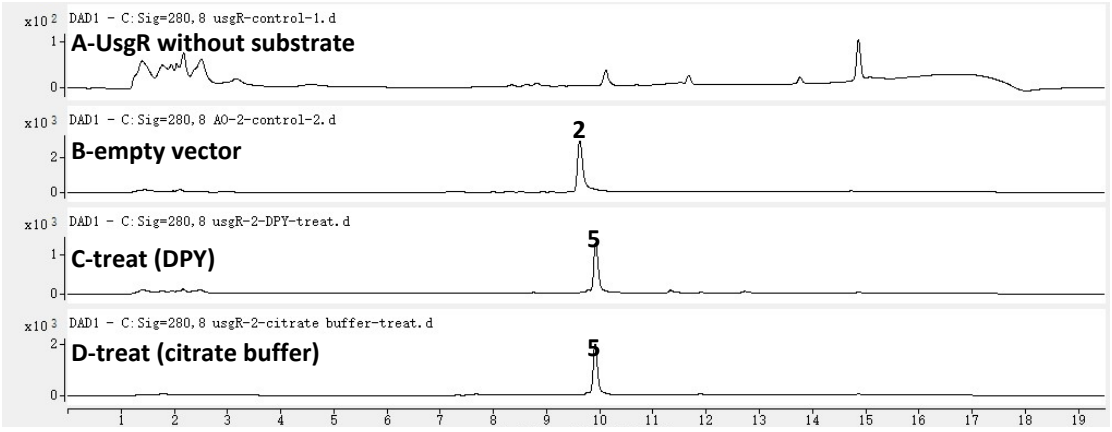


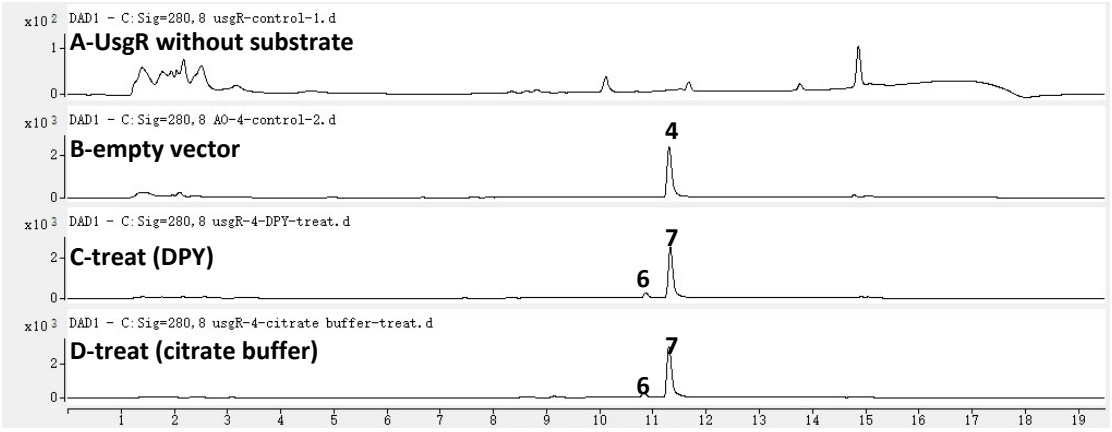
Fig. S10 The atropselectivity varied with different concentration of UsgL.
(A) HPLC analysis of the reaction products of **5** and UsgL. (B) percentage of (*M*)-usgF (**18**) varied as enzyme concentration changed. (C) UV, MS, and CD spectra of (*M*)/ (*P*)-**18**.

Note: (1) condition 1 was used for HPLC analysis; (2) percentage of (*M*)-usgF was calculated as follows: *M*/(*M*+*P*)*100%

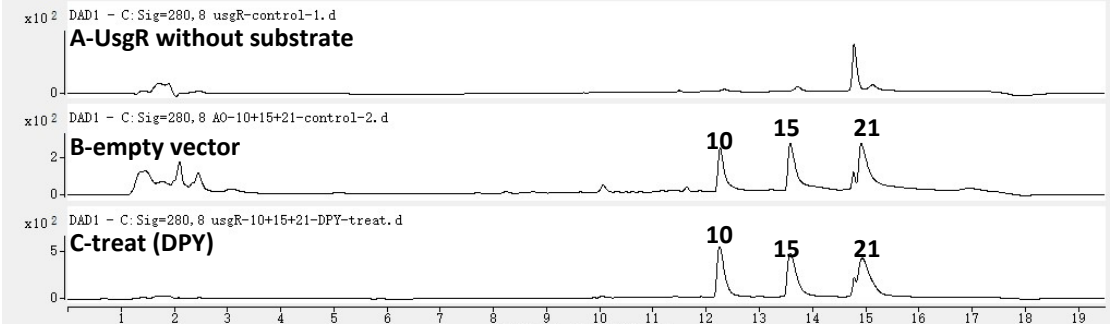
2+UsgR



4+UsgR



10+**15**+**21**+UsgR



29+UsgR

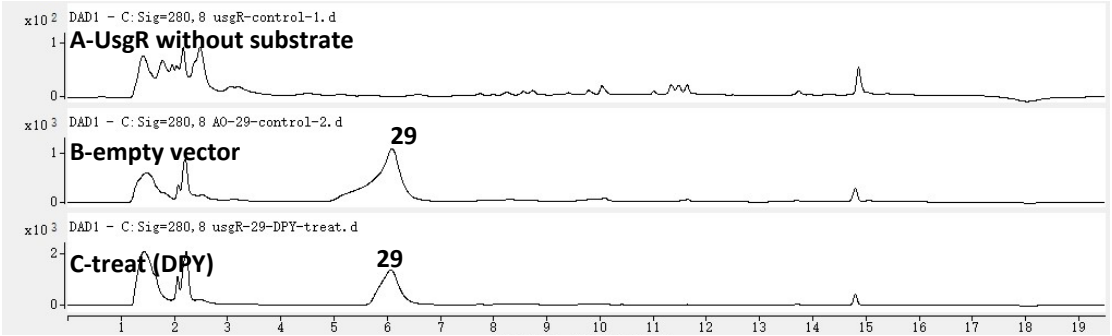
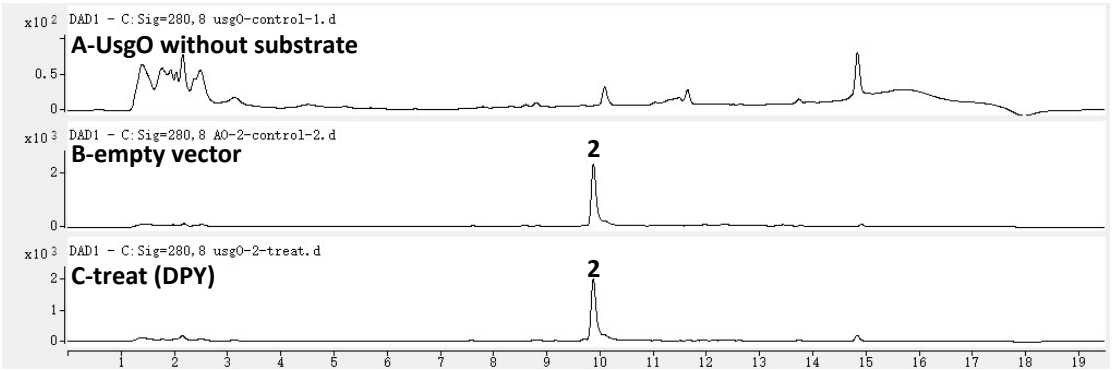
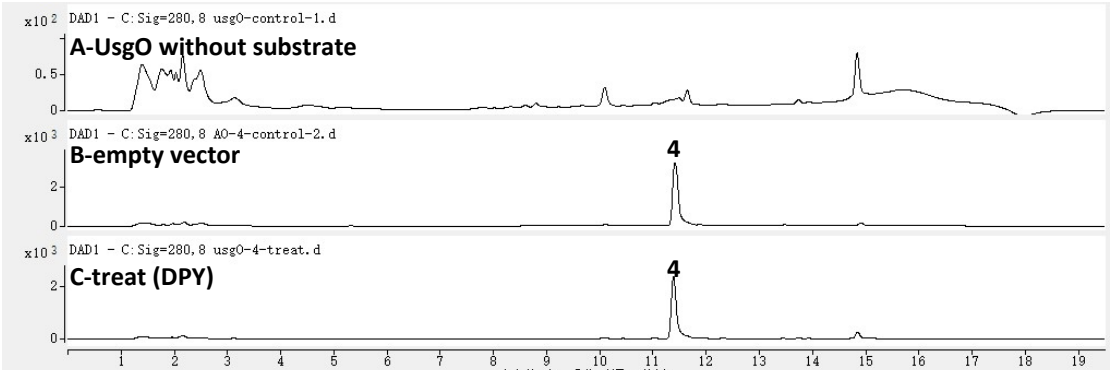


Fig. S11 LC-MS chromatograms of feeding experiments in AO-*usgR* (2 d, λ = 280 nm).

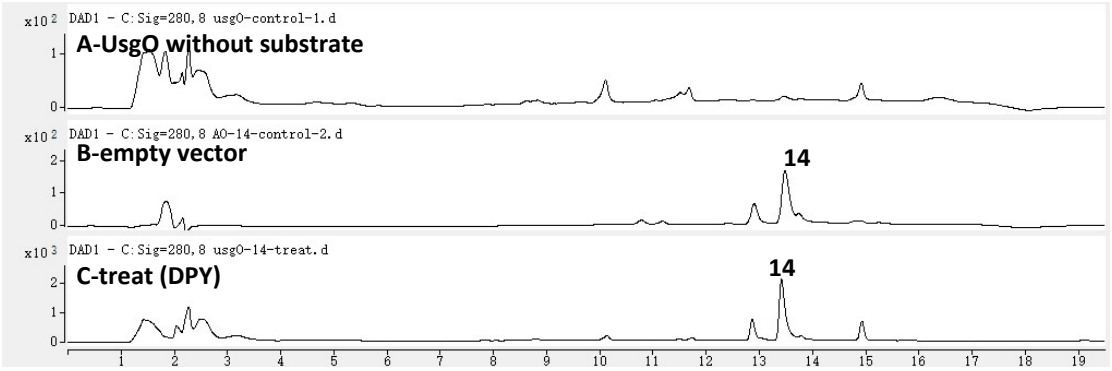
2+UsgO



4+UsgO



14+UsgO



17+UsgO

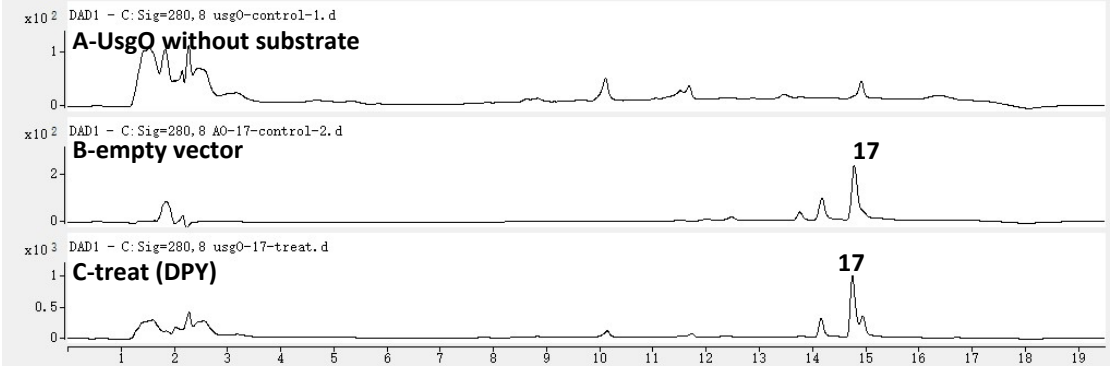


Fig. S12 LC-MS chromatograms of feeding experiments in AO-*usgO* (2 d, λ = 280 nm).

1+UsgM

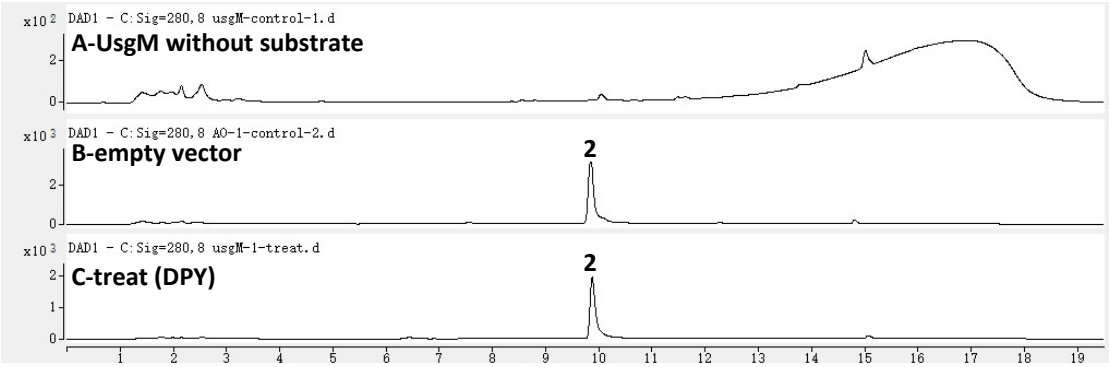


Fig. S13 LC-MS chromatograms of feeding experiments in AO-*usgM* (2 d, λ = 280 nm).

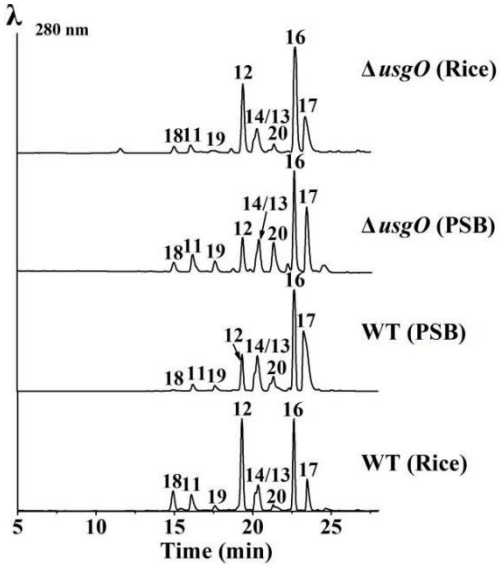


Fig. S14 HPLC-DAD analysis of Δ*usgO* and wild-type strain (WT) cultured on different media.
Note: condition 1 was used.

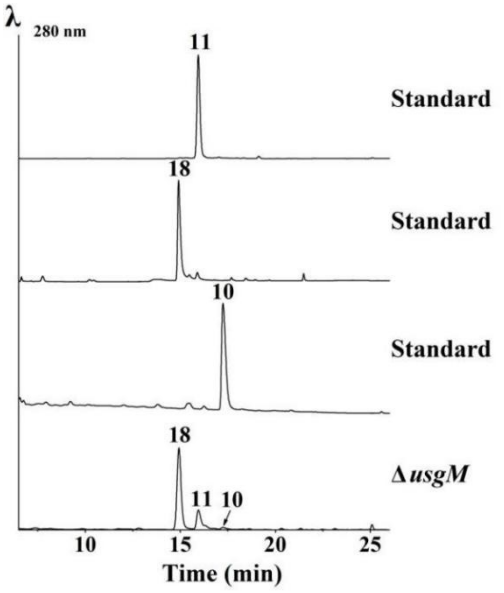


Fig. S15 HPLC-DAD analysis of Δ*usgM*.
Note: condition 1 was used.

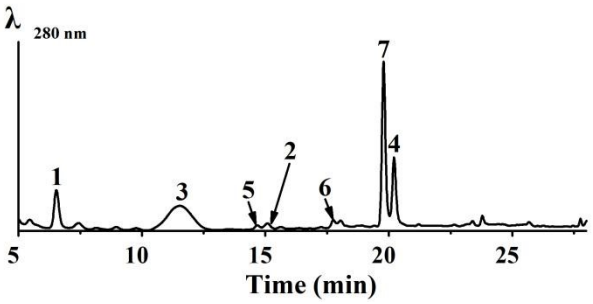


Fig. S16 HPLC-DAD analysis of Δ*usgD*/Δ*usgL*

Note: condition 2 was used.

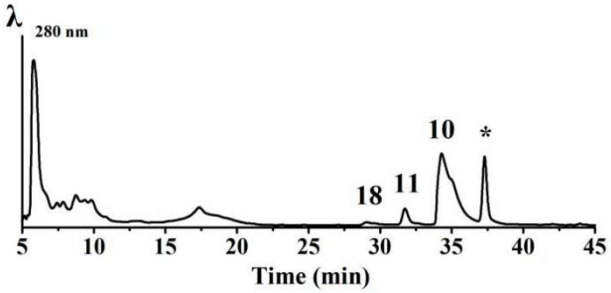


Fig. S17 HPLC-DAD analysis of Δ*usgM*/Δ*usgD*.

Note: condition 3 was used. *-unrelated peak.

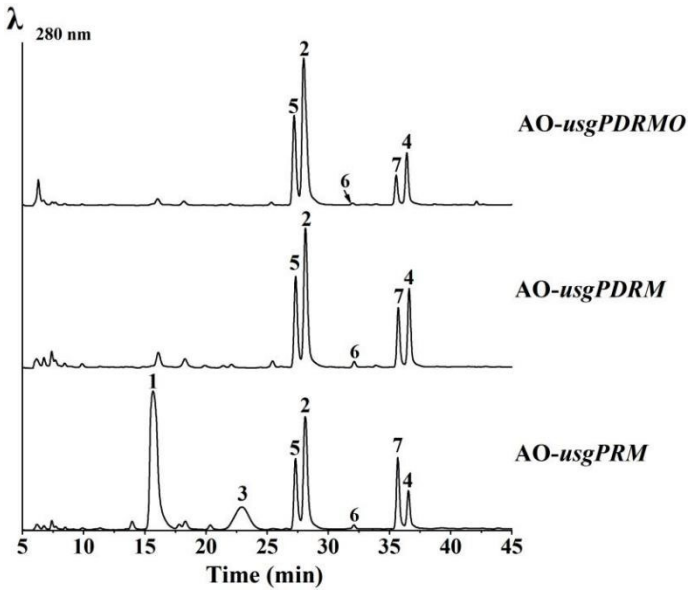


Fig. S18 HPLC-DAD analysis of AO-*usgPDRMO* (EXP15), AO-*usgPDRM* (EXP12) and AO-*usgPRM* (EXP10).

Note: condition 4 was used.

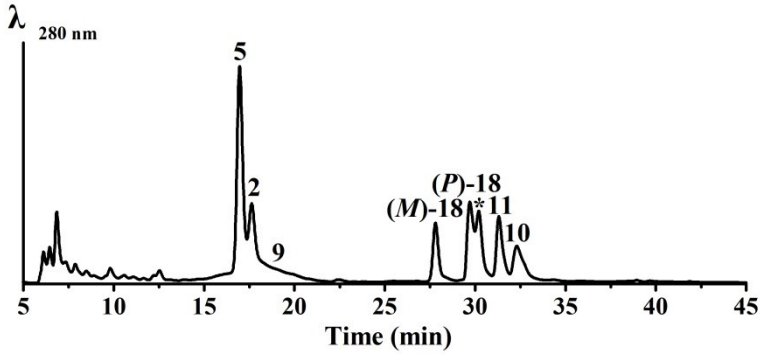


Fig. S19 HPLC-DAD analysis of AO-*usgPDRL* (EXP13 in Fig. 3).
Note: condition 5 was used.

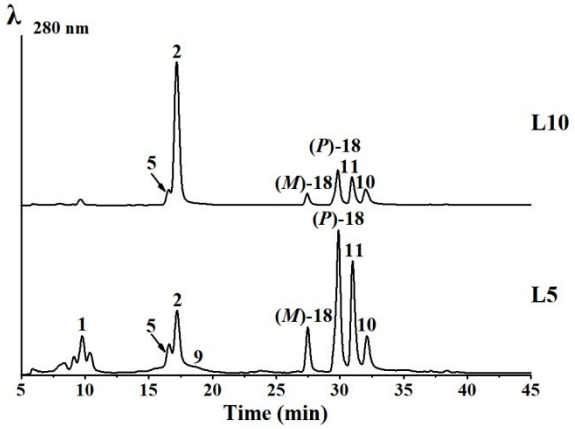
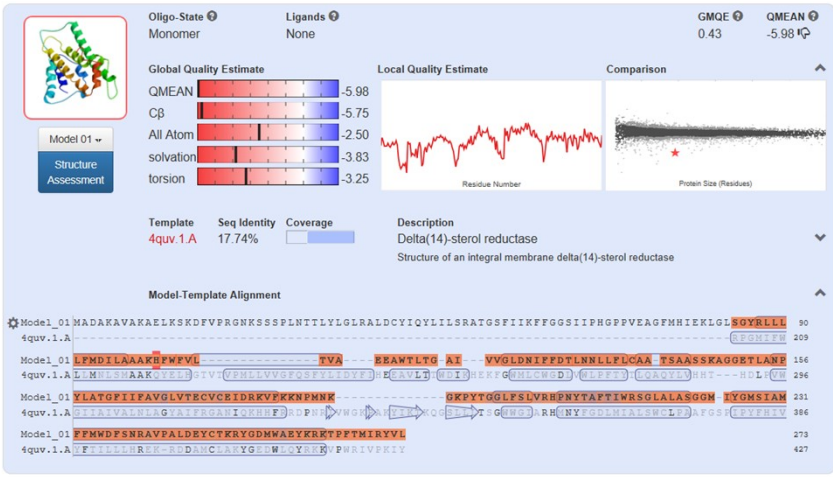


Fig. S20 HPLC-DAD analysis of *in vitro* reactions of AO-*usgL* cell-free lysate (EXP L10, L5).
Note: condition 5 was used. *-unrelated peak.

(A) Truncation 1: 76-273 AA



(B) Truncation 2: 160-264 AA

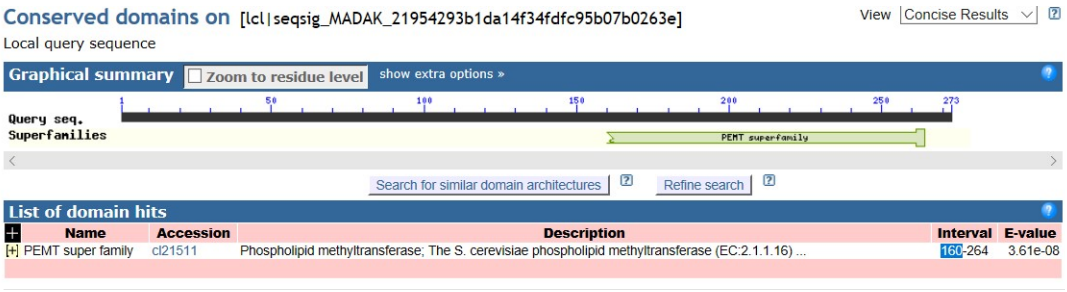


Fig. S21 Two truncated sequences of UsgR.

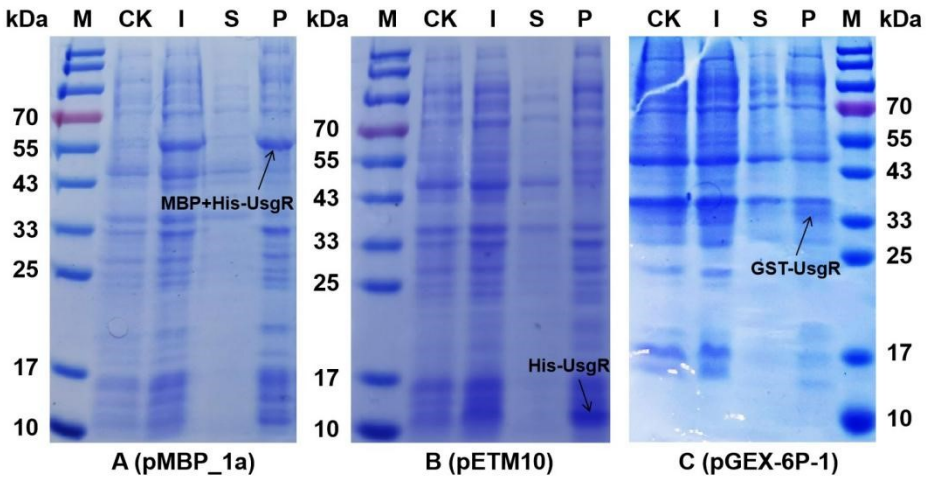


Fig. S22 Induced expression of UsgR$^{160\text{-}264}$ detected by SDS-PAGE electrophoresis.

The experiments were performed at 16°C overnight, with or without addition of IPTG. CK: Control, without IPTG; I: Induction with 0.1 mM IPTG; S: supernatant of I; P: Pellet of I; M: Protein molecular weight marker. A-C: different expression vectors indicated.

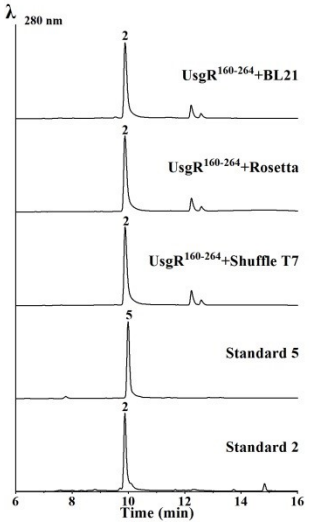


Fig. S23 LC-MS chromatograms of the feeding experiments in *E. coli* expressing UsgR$^{160\text{-}264}$

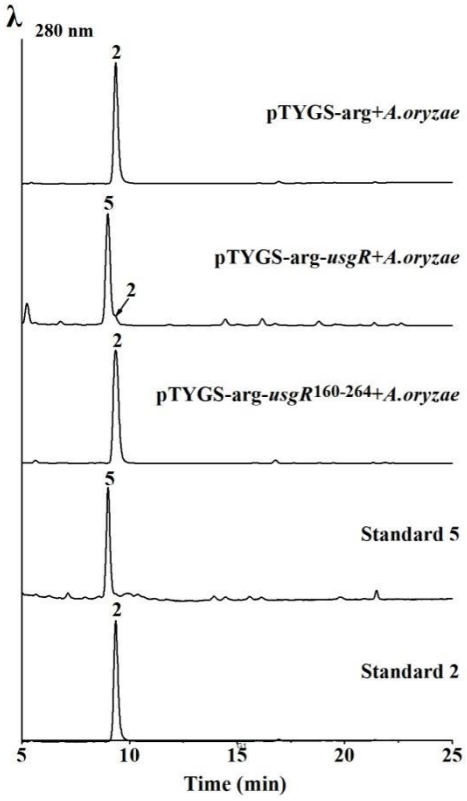


Fig. S24 HPLC analysis of the feeding experiments in *A. oryzae*-UsgR$^{160\text{-}264}$.
Note: condition 1 was used.

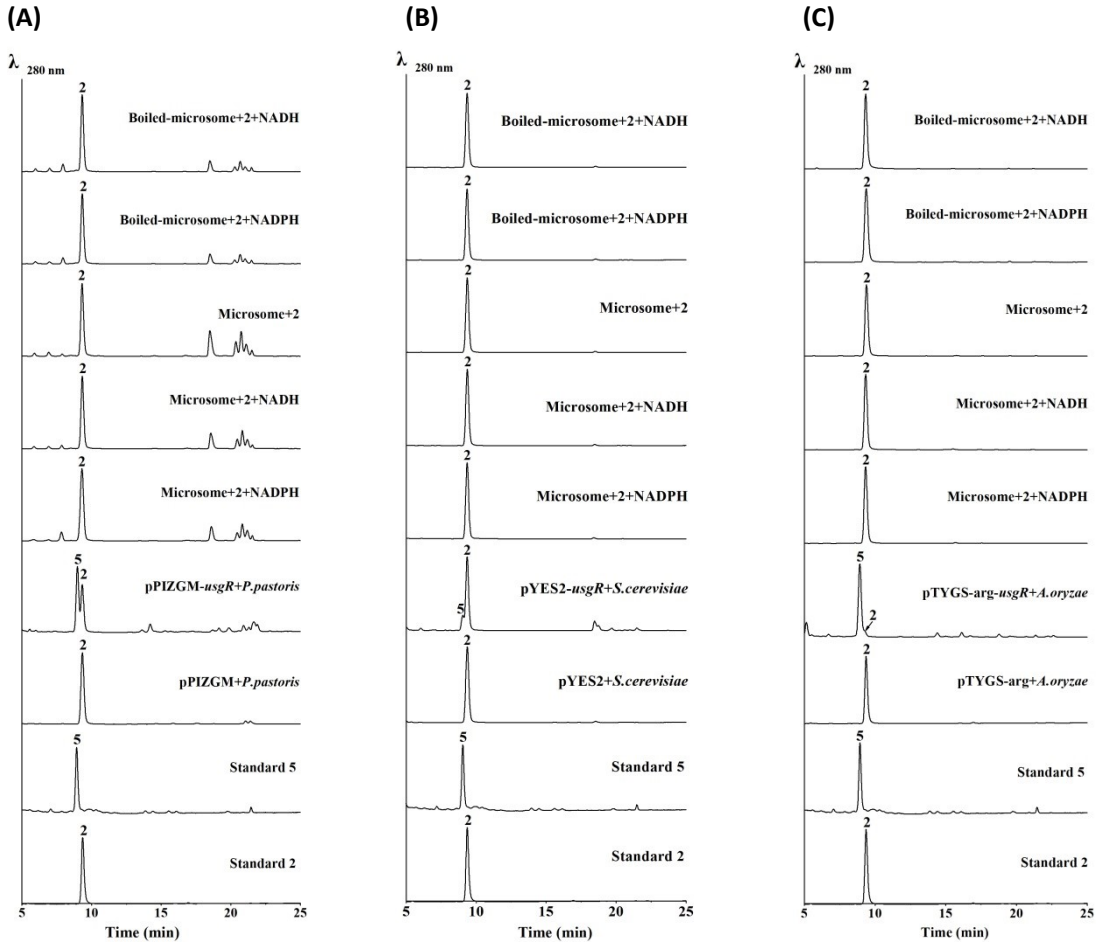
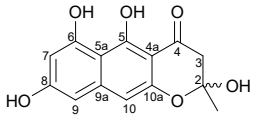


Fig. S25 *In vitro* reaction of microsomes with **2**. A) pPIZGM-*usgR P. pastoris* GS115, B) pYES2-*usgR S. cerevisiae* BY4741, C) pTYGS-*usgR A. oryzae*

Note: condition 1 was used.

Compound characterization data

YWA1 (1)



YWA1 (**1**)

Chemical Formula: $C_{14}H_{12}O_6$; Exact Mass: 276.0634

UV λ_{max}(MeOH): 229, 277, 323, 336, 408 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{14}H_{11}O_6$: 275.0561, found: 275.0567.

Compound was identified based on mass, UV-absorption, and NMR (Table S10, Fig. S28, S29), which was consistent with the literature. [11, 16]

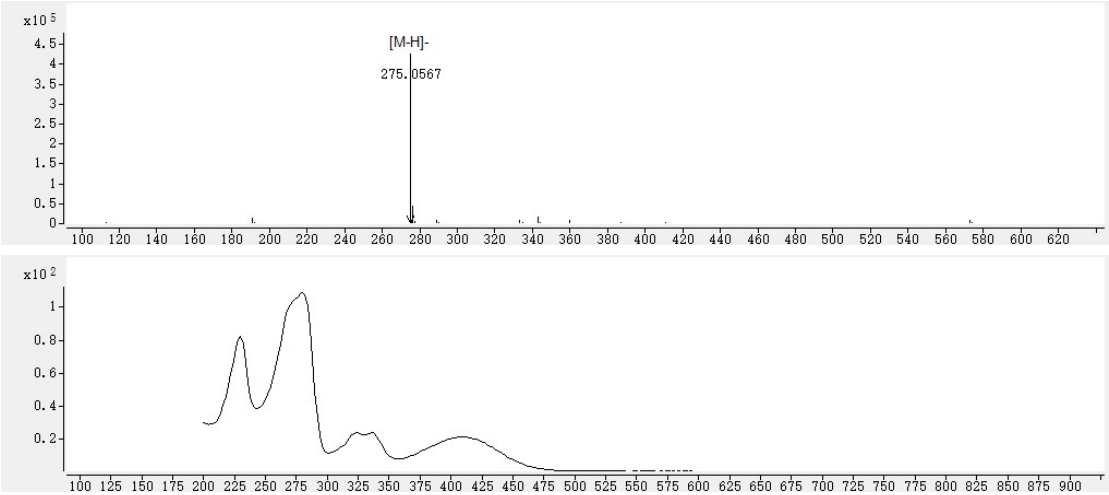


Fig. S26 HRESIMS (top) and UV (bottom) spectra of **1**.

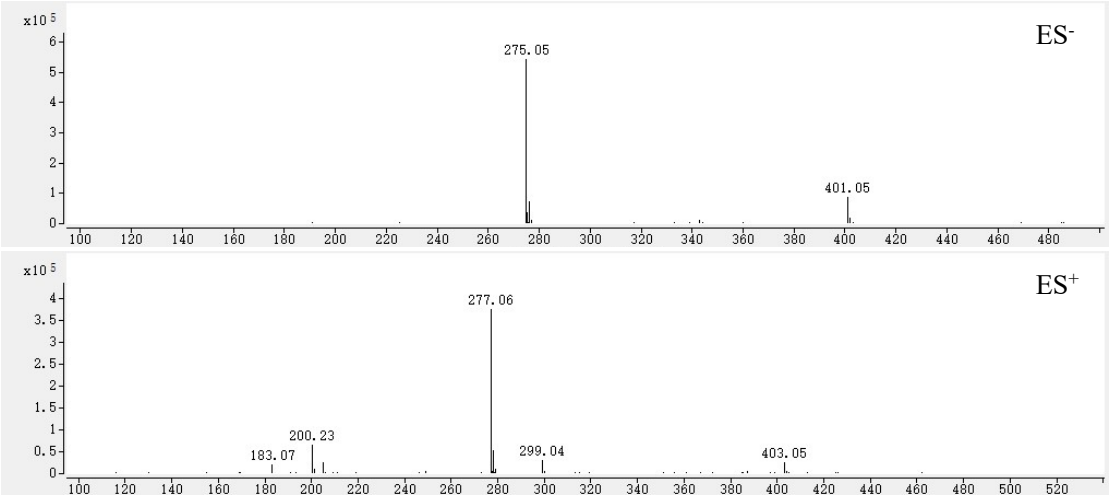


Fig. S27 MS spectra of **1**.

Table S10. ^{13}C and ^{1}H NMR data of **1** (CD_3COCD_3).

Position	δ_C, type	δ_C, type[16]	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)[11]
2	101.3 C	101.3 C		
3	47.7 CH_2	47.8 CH_2	3.17 d (17.1)	3.17 d (17.1)
			2.86 d (17.1)	2.86 d (17.1)
4	198.8 C	198.8 C		
4a	102.9 C	102.9 C		
5	164.8 C	165.0 C		
5a	104.9 C	105.0 C		
6	160.7 C	160.8 C		
7	100.7 CH	100.8 CH	6.28 d (2.2)	6.28 d (2.2)
8	162.7 C	162.8 C		
9	102.4 CH	102.4 CH	6.51 d (2.2)	6.51 d (2.2)
9a	143.5 C	143.6 C		
10	102.7 CH	102.8 CH	6.44 s	6.45 s
10a	154.1 C	154.1 C		
2-CH_3	28.3 CH_3	28.4 CH_3	1.72 s	1.72 s
5-OH			15.29 brs	15.4 brs
6-OH			9.43 brs	9.43 brs
8-OH			9.16 brs	n.d.

n.d.: not detected

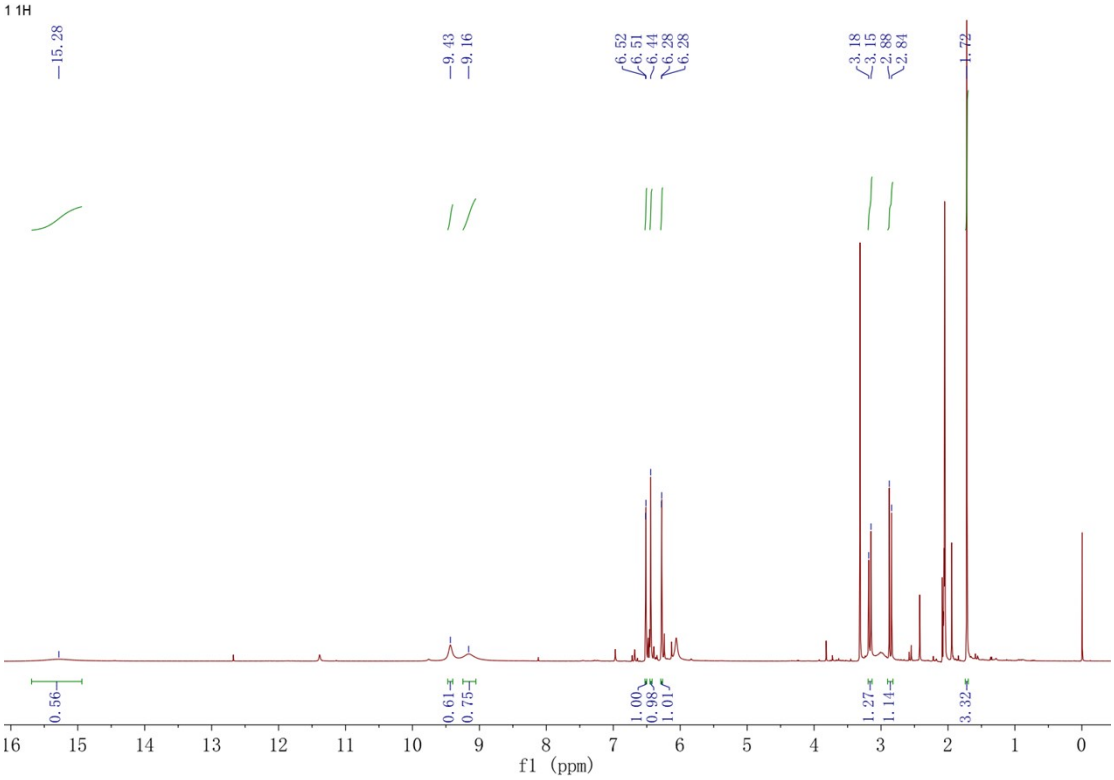


Fig. S28 ^{1}H NMR spectrum of **1** (500 MHz, CD_3COCD_3).

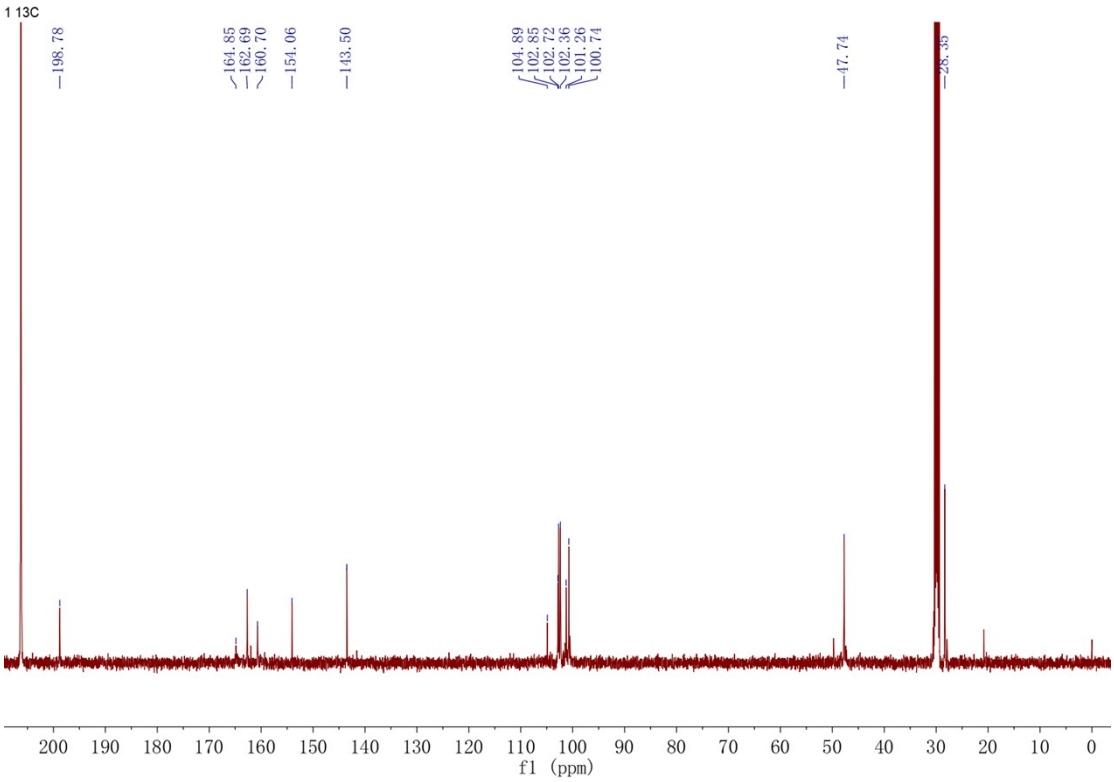
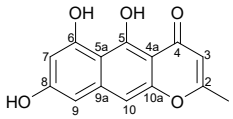


Fig. S29 ^{13}C NMR spectrum of **1** (125 MHz, CD_3COCD_3).

norrubrofusarin (2)



norrubrofusarin (**2**)

Chemical Formula: $C_{14}H_{10}O_5$, Exact Mass: 258.0528

UV λ_{max}(MeOH): 225, 277, 328, 413 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{14}H_9O_5$: 257.0455, found: 257.0454

Compound was identified based on mass and UV-absorption, and characterized by NMR (Table S11, Fig. S32,S33)[11, 17, 18].

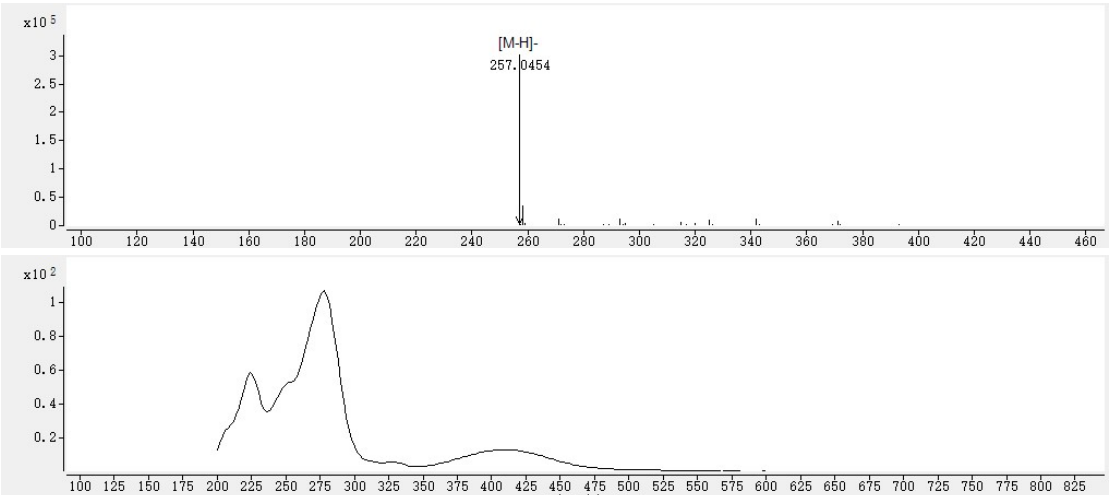


Fig. S30 HRESIMS (top) and UV (bottom) spectra of **2**.

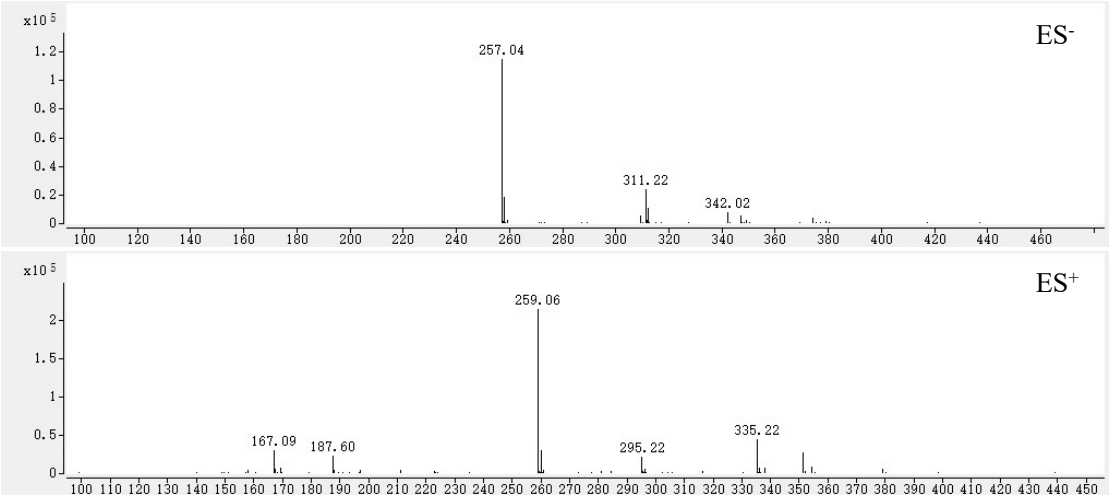


Fig. S31 MS spectra of **2**.

Table S11. ^{13}C and ^{1}H NMR data of **2** (DMSO-d_6).

position	δ_C, type	δ_C, type[18]	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)[18]
2	169.7 C	169.76 C		
3	105.8 CH	106.06 CH	6.19 s	6.17 s
4	183.4 C	183.48 C		
4a	101.8 C	101.68 C		
5	162.4 C	162.34 C		
5a	105.4 C	105.38 C		
6	158.5 C	158.45 C		
7	100.7 CH	100.66 CH	6.33 d (2.2)	6.32 s
8	160.8 C	161.90 C		
9	100.8 CH	100.74 CH	6.57 d (2.2)	6.57 s
9a	140.4 C	140.00 C		
10	99.8 CH	99.75 CH	7.00 s	6.97 s
10a	152.0 C	151.00 C		
2-CH_3	20.3 CH_3	20.3 CH_3	2.37 s	2.37 s
6-OH			9.85 s	9.84 s
8-OH			10.24 s	10.24 s
5-OH			15.77 brs	15.75 s

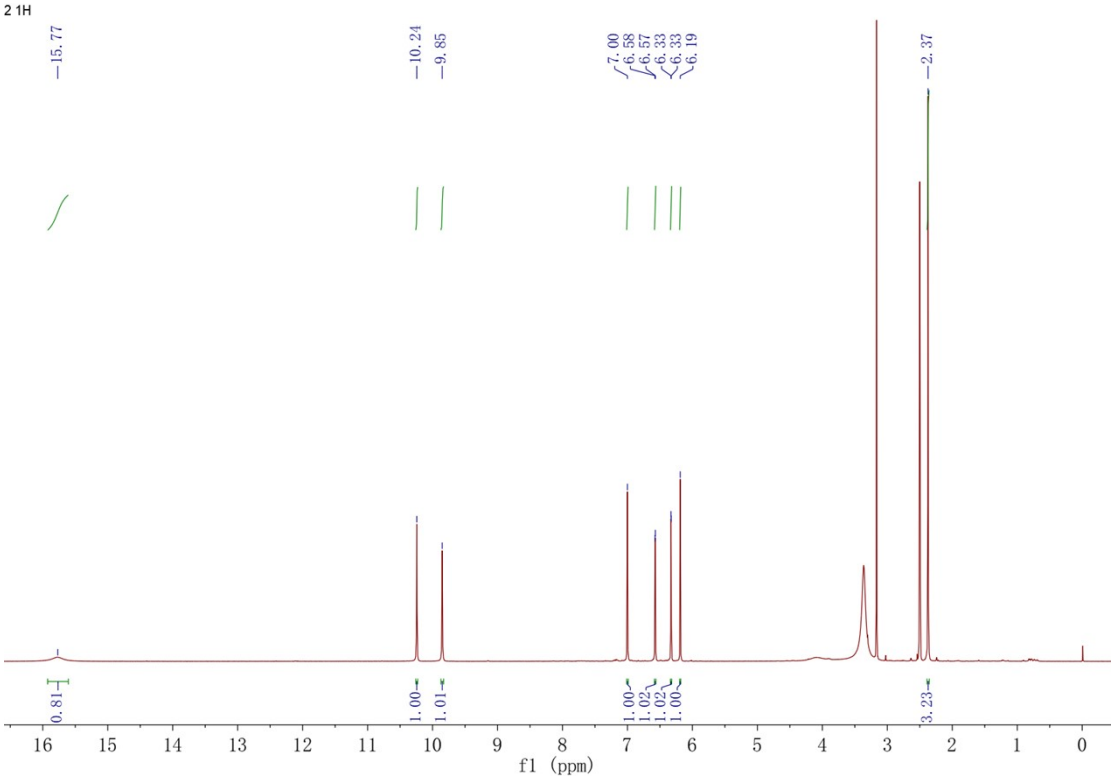


Fig. S32 ^{1}H NMR spectrum of **2** (500 MHz, DMSO-d_6).

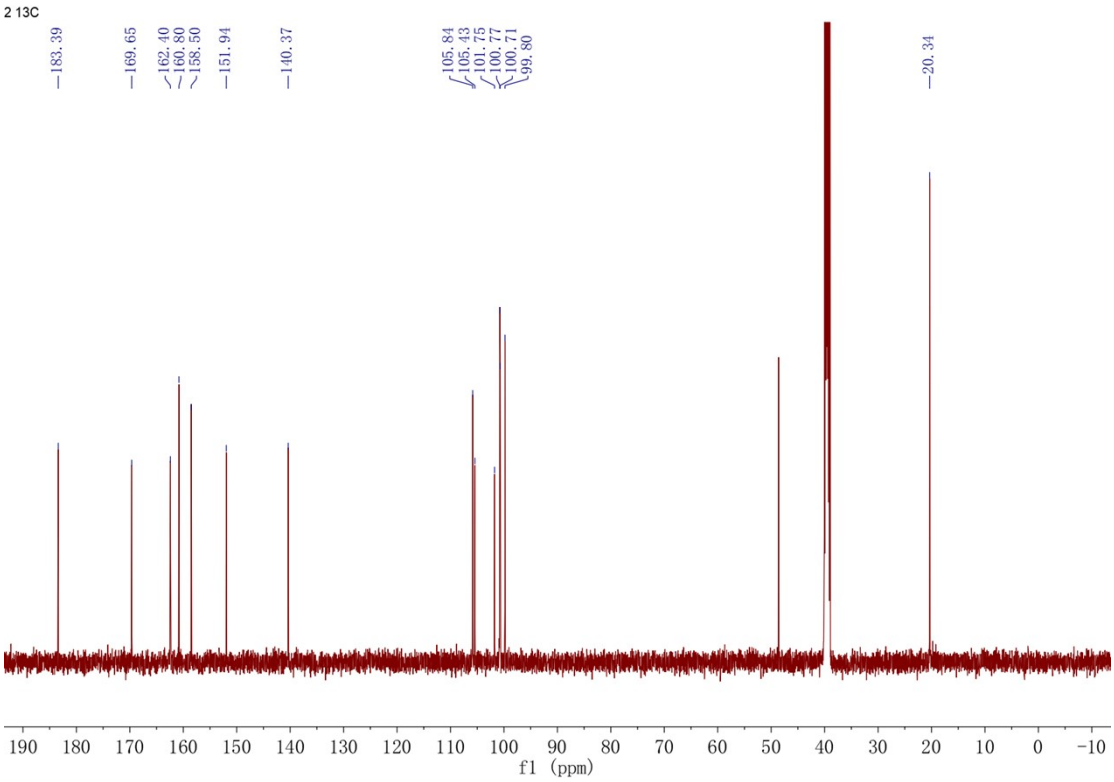
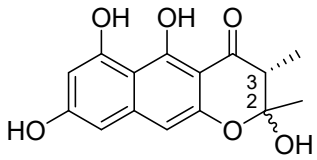


Fig. S33 ^{13}C NMR spectrum of **2** (125 MHz, DMSO-d_6).

3-methyl-YWA1 (3)



3-methyl-YWA1 (**3**)

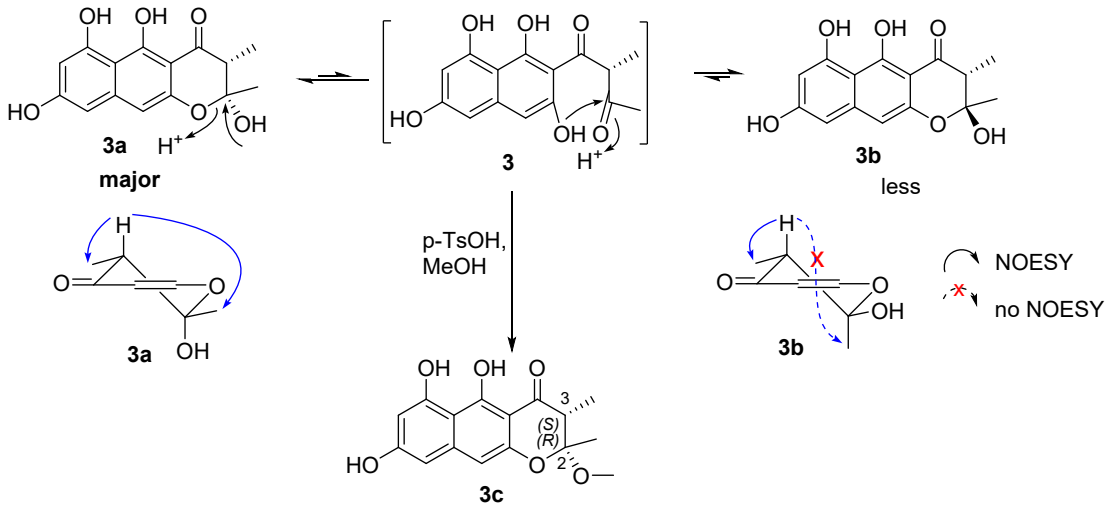
Chemical Formula: $C_{15}H_{14}O_6$, Exact Mass: 290.0790

UV λ_{max}(MeOH): 230, 279, 322, 334, 406 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{15}H_{13}O_6$: 289.0718, found: 289.0710

Compound was identified based on mass and UV-absorption, and characterized by NMR (Fig. S36, S37 and Table S12). This compound appeared as a mixture of 2,3-diastereomers (major/minor= ca. 1.73/1), as two sets of signals could be seen in the ^{1}H NMR spectrum (Fig. S36).

Compound **3** was revealed to be the 2-epimeric mixtures due to the presence of an unstable hemi-ketal group (scheme S1). The NOESY experiment (Fig. 32) indicated that the major one has 2,3-*trans*-dimethyl groups (**3a**), while the less one has a *cis*-dimethylated structure (**3b**). Upon treatment of **3** in MeOH with catalytic amount of *p*-TsOH, the methyl ketal were produced (scheme S1). After purification by semi-prepartive HPLC, the major ketal was isolated (**3c**), and characterized by ^{1}H NMR (Fig. S38, and Table S12), and MS (Fig. S39). The absolute configuration of **3c** was determined by ECD calculations. The calculated ECD spectrum of (2*R*,3*S*)-**3c** at B3LYP/6-31+G(d), PCM=MeOH// B3LYP/6-31G(d) level fitted well with the experimental data (Fig. S40), thus the absolute configuration was assigned as 2*R*, 3*S*. This then established the absolute configuration of **3** at C-3 (3*S*).



Scheme S1. Epimerism of **3** and its conversion to the methyl ketal (**3c**).

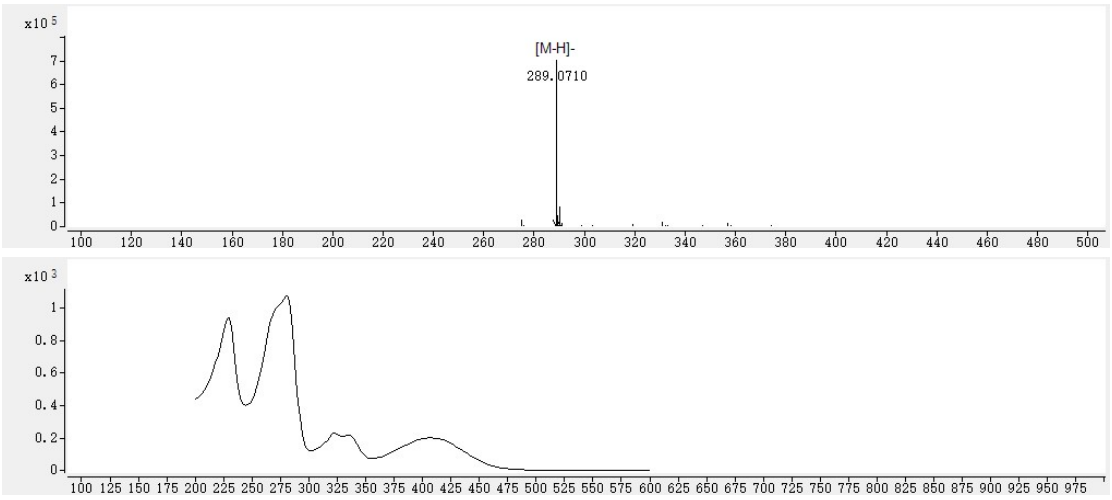


Fig. S34 HRESIMS (top) and UV (bottom) spectra of **3**.

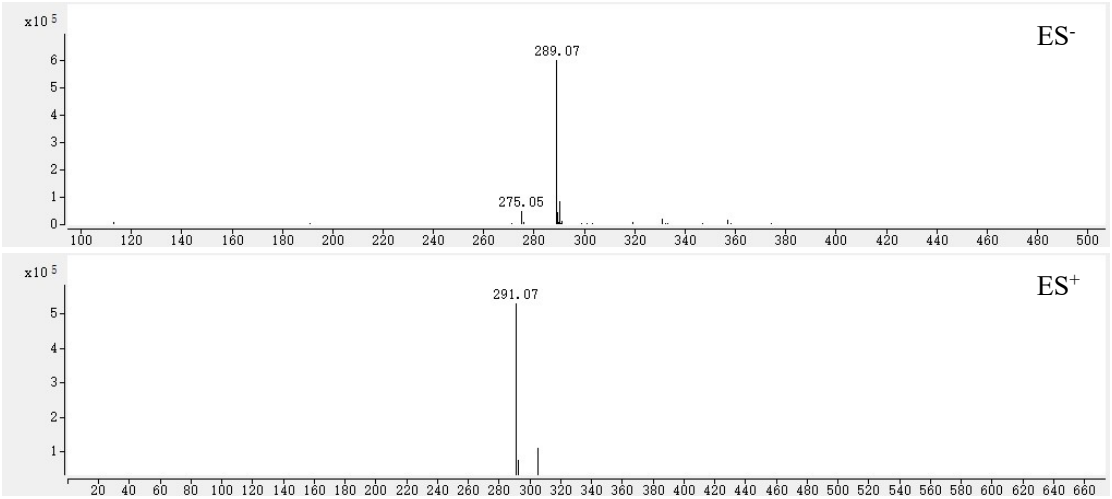


Fig. S35 MS spectra of **3**.

Table S12. ^{1}H NMR data of **3** (CD_3COCD_3).

Position	δ_H, mult. (*J* in Hz), **3a**	δ_H, mult. (*J* in Hz), **3b**	δ_H, mult. (*J* in Hz), **3c**
3	3.14 q (6.8)	2.79 q (7.4)	overlapped by water peak
7	6.23-6.32 overlapped	6.23-6.32 overlapped	6.31, s
9	6.56-6.36 overlapped	6.56-6.36 overlapped	6.56, s
10	6.56-6.36 overlapped	6.56-6.36 overlapped	6.54, s
2-CH_3	1.72 s	1.62 s	1.69, s
3-CH_3	1.33 d (6.8)	1.27 d (7.3)	1.28 d (7.3)
5-OH	15.28 brs	15.28 brs	15.17 brs
6-OH	9.37 brs	9.37 brs	9.38 brs
8-OH	9.13 brs	9.13 brs	-
2-OMe	-	-	3.24, s

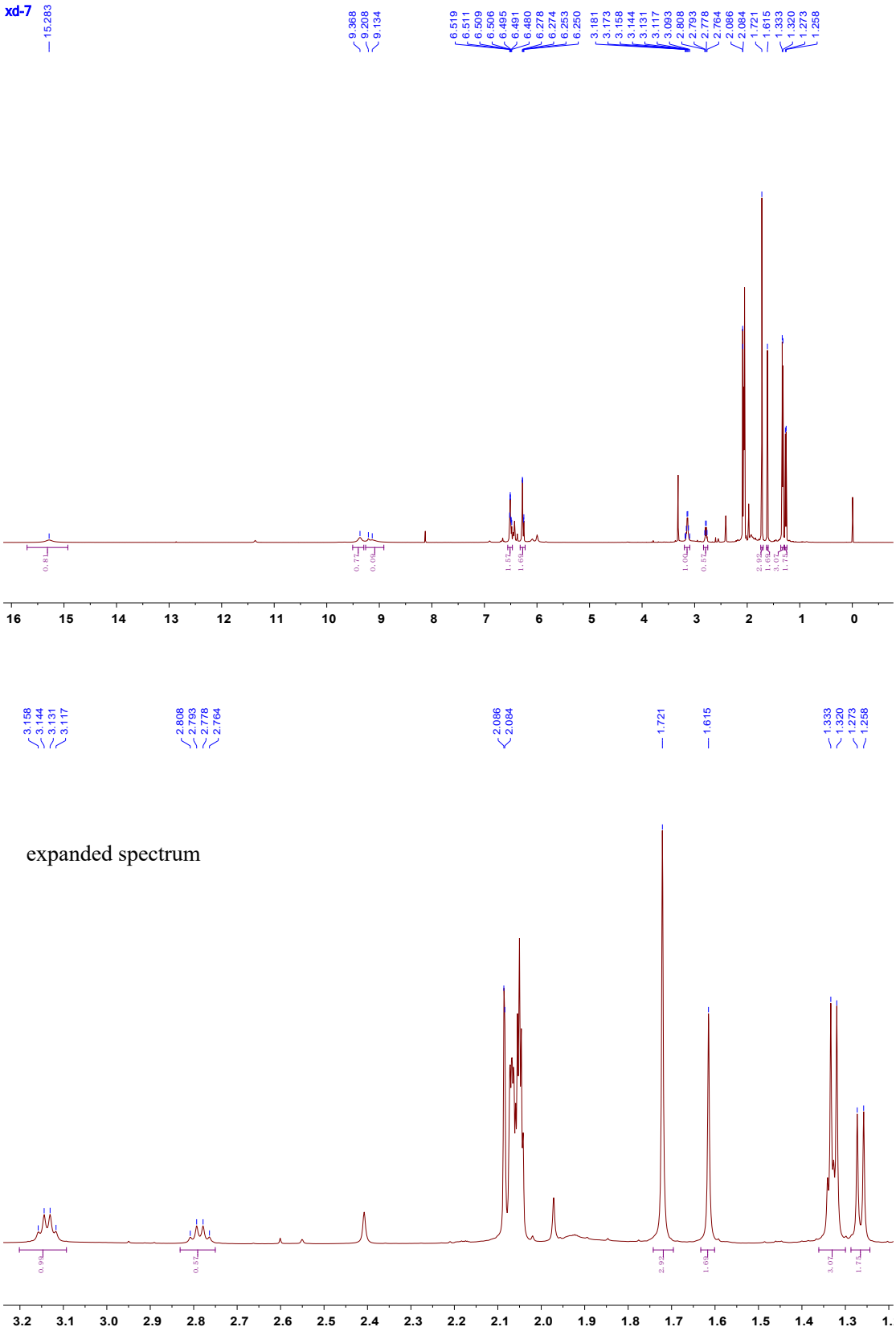


Fig. S36 ^{1}H NMR spectrum of **3** (500 MHz, CD_3COCD_3).

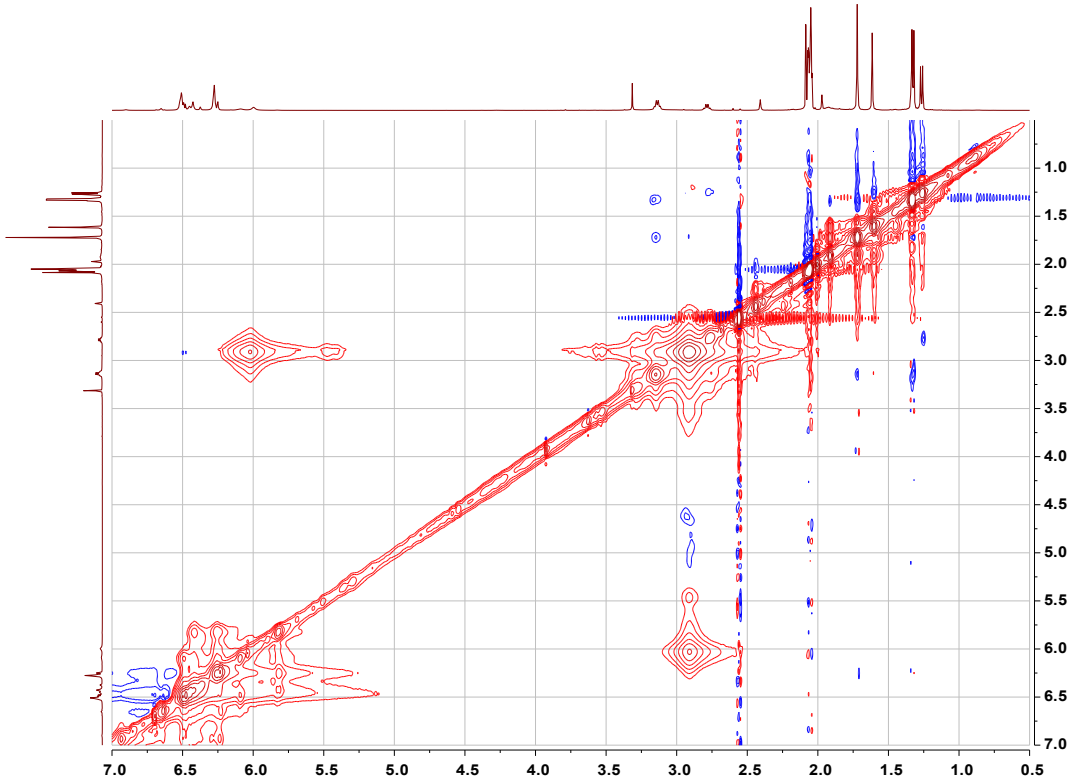


Fig. S37 NOESY spectrum of **3** (CD_3COCD_3).

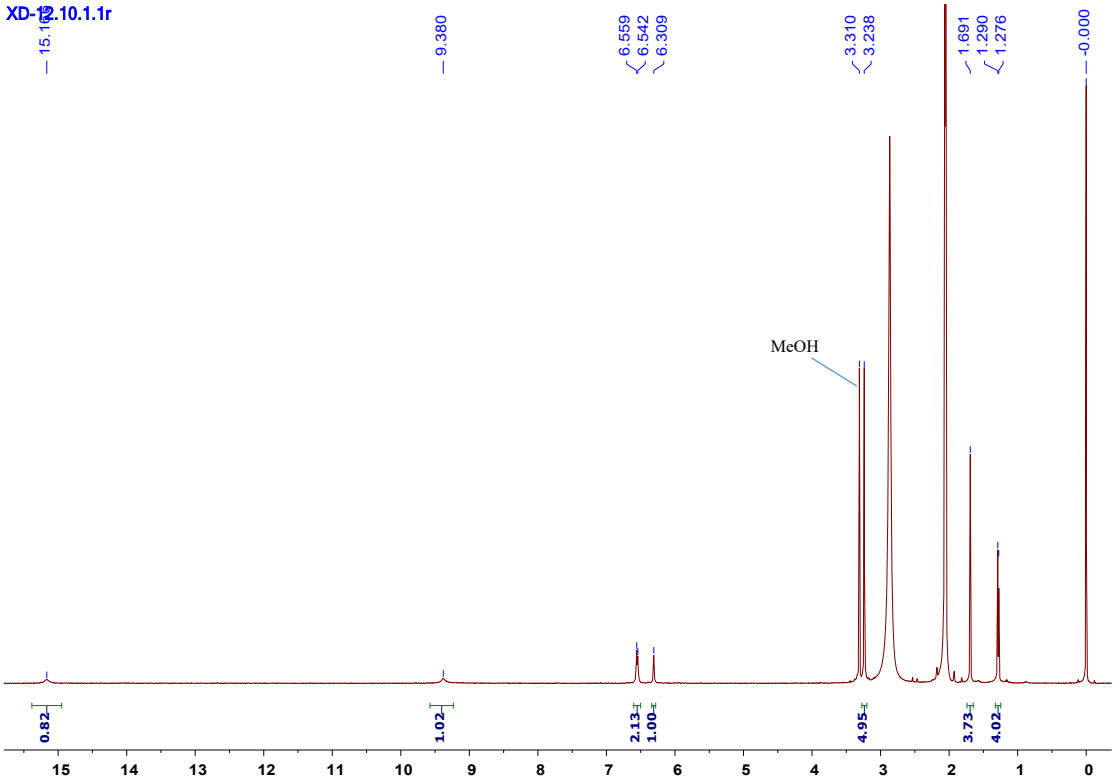


Fig. S38 ^1H NMR spectrum of **3c** (500 MHz, CD_3COCD_3)..

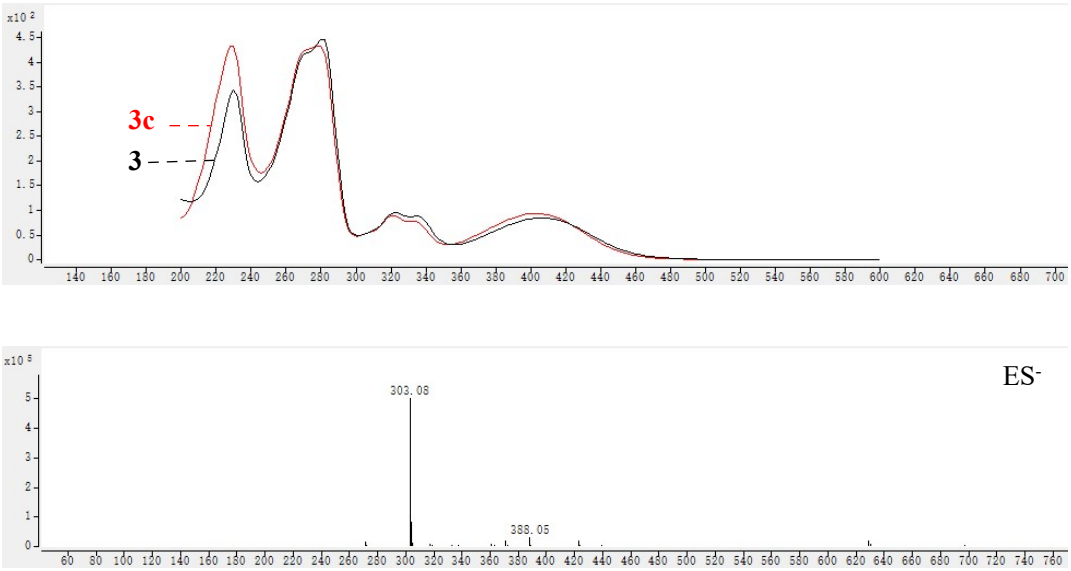


Fig. S39 UV and MS spectra of **3c**

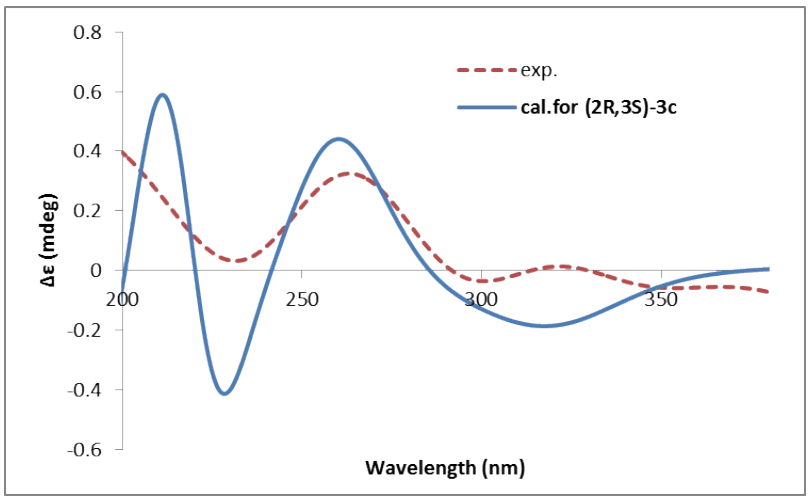
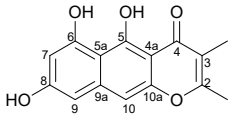


Fig. S40 Calculated ECD spectrum of (2*R*,3*S*)-**3c**, and the experimental data of **3c**.

3-methyl-norrubrofusarin (4)



3-methyl-norrubrofusarin (**4**)

Chemical Formula: $C_{15}H_{12}O_5$, Exact Mass: 272.0685

UV λ_{max}(MeOH): 227, 277, 327, 414 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{15}H_{11}O_5$: 271.0612, found: 271.0610

Compound was identified based on mass and UV-absorption, and characterized by NMR (Table S13, Fig. S43, S44)[19].

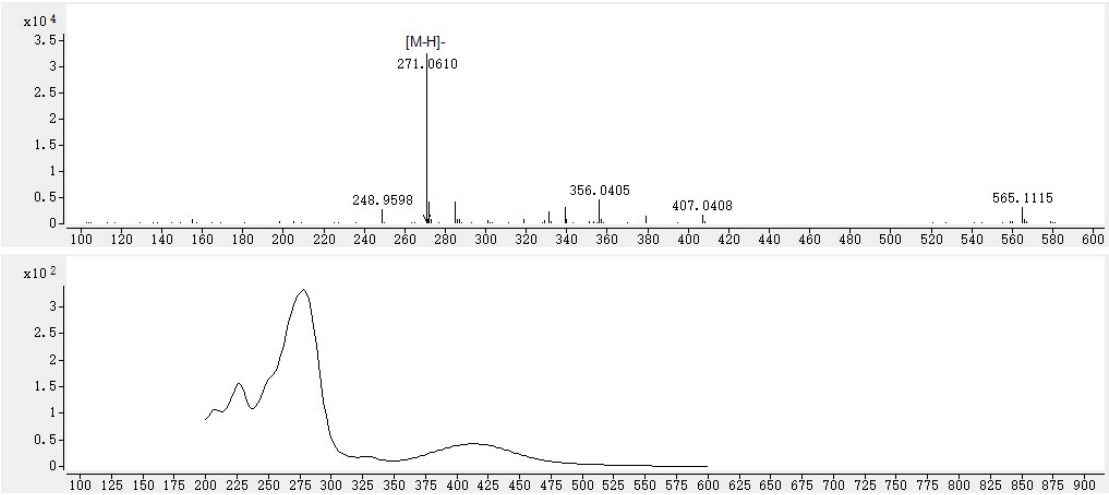


Fig. S41 HRESIMS (top) and UV (bottom) spectra of **4**.

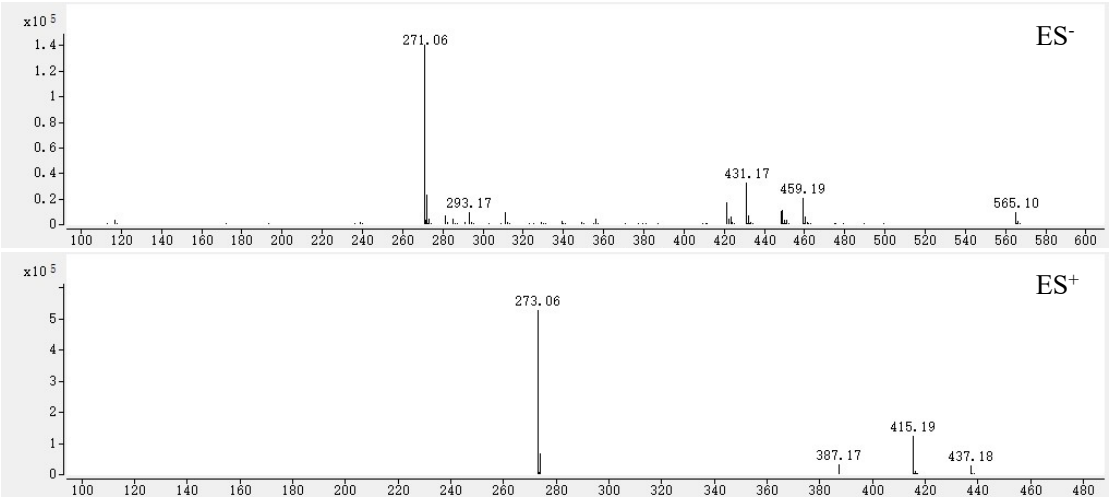


Fig. S42 MS spectra of **4**.

Table S13. ^{13}C and ^{1}H NMR data of **4** (DMSO-d_6).

Position	δ_C type	δ_H mult. (*J* in Hz)
2	165.2 C	
3	111.9 C	
4	182.7 C	
4a	101.3 C	
5	162.0 C	
5a	105.3 C	
6	158.4 C	
7	100.4 CH	6.31 d (2.0)
8	160.6 C	
9	100.5 CH	6.55 d (2.0)
9a	140.3 C	
10	99.2 CH	6.95 s
10a	151.4 C	
2-CH_3	18.6 CH_3	2.38 s
3-CH_3	8.7 CH_3	1.92 s
5-OH		16.04 brs
6-OH		10.20 s
8-OH		9.83 s

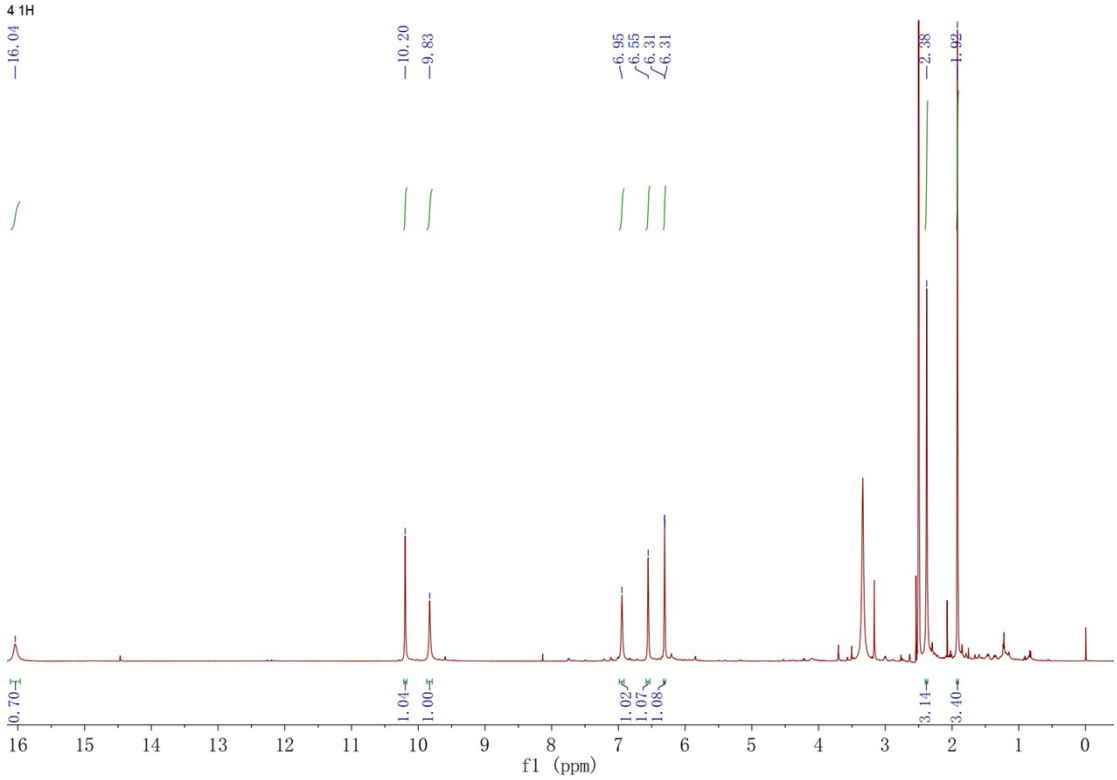


Fig. S43 ^{1}H NMR spectrum of **4** (500 MHz, DMSO-d_6).

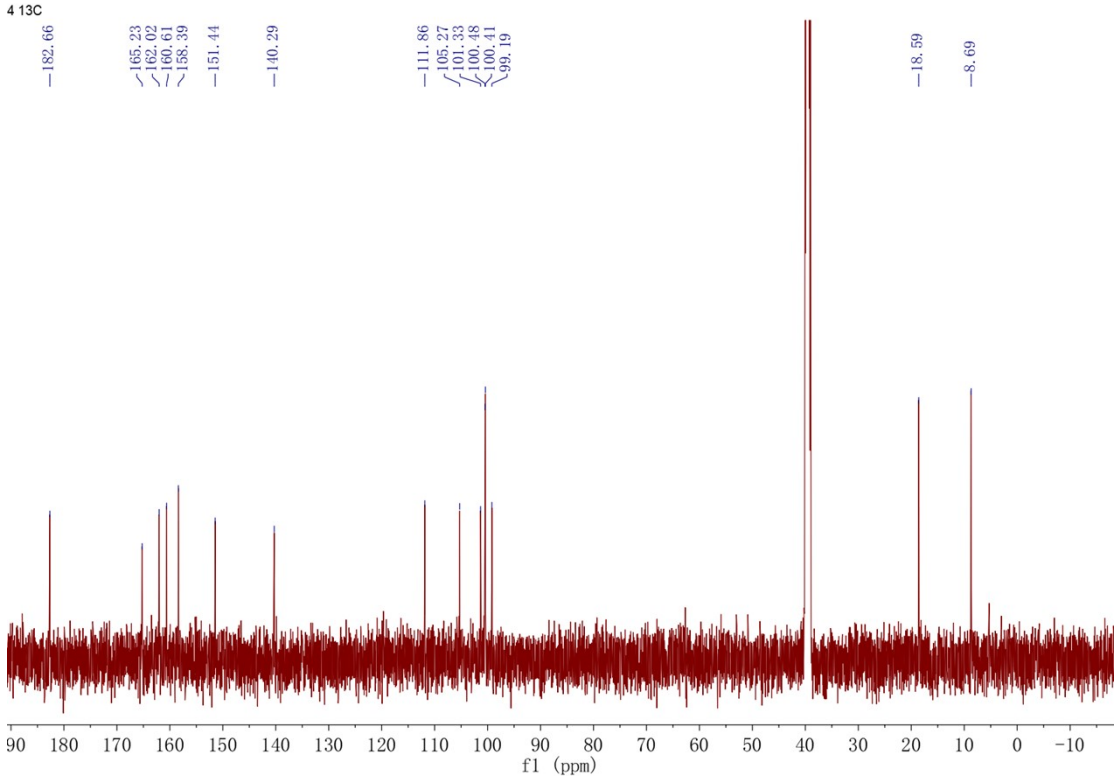
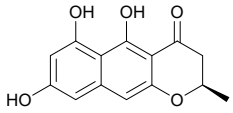


Fig. S44 ^{13}C NMR spectrum of **4** (125 MHz, DMSO-d_6).

hemiustilaginoidin F (5)



hemiustilaginoidin F (**5**)

Chemical Formula: $C_{14}H_{12}O_5$, Exact Mass: 260.0685

UV λ_{max}(MeOH): 231, 275, 325, 337, 413 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{14}H_{11}O_5$: 259.0612, found: 259.0614

Compound was identified based on mass, retention time and UV-absorption with the standard[13]

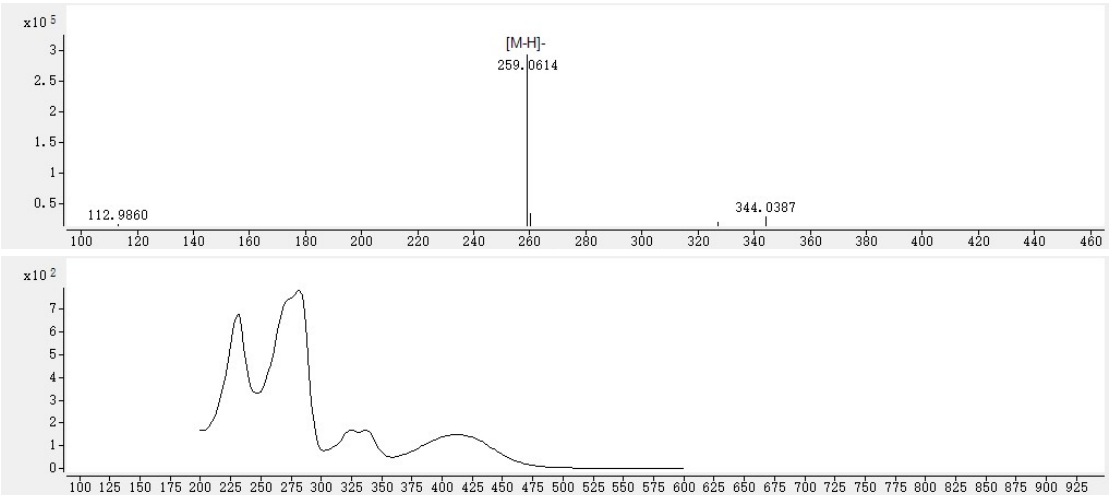


Fig. S45 HRESIMS (top) and UV (bottom) spectra of **5**.

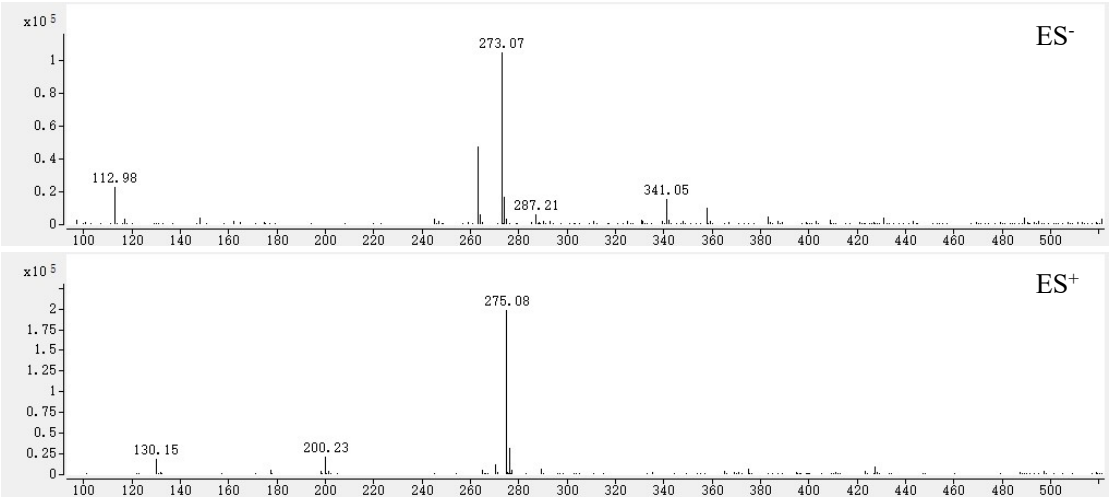
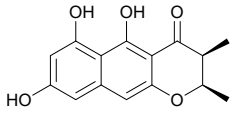


Fig. S46 MS spectra of **5**.

epihemiustilaginoidin D (6)



epihemiustilaginoidin D (**6**)

Chemical Formula: $C_{15}H_{14}O_5$, Exact Mass: 274.0841

UV λ_{max}(MeOH): 231, 280, 325, 337, 414 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{15}H_{13}O_5$: 273.0768, found: 273.0769

Compound was identified based on mass, retention time and UV-absorption with the standard[13]

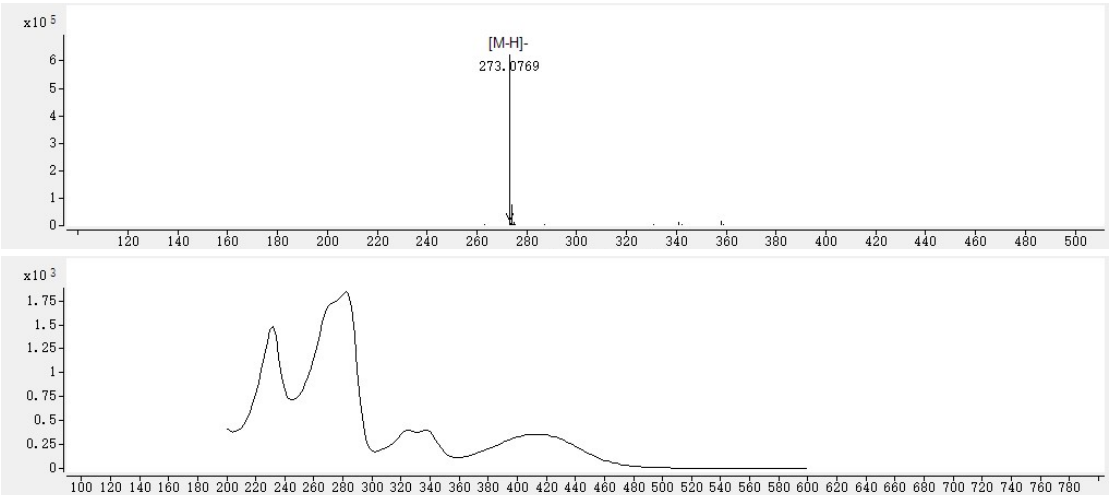


Fig. S47 HRESIMS (top) and UV (bottom) spectra of **6**.

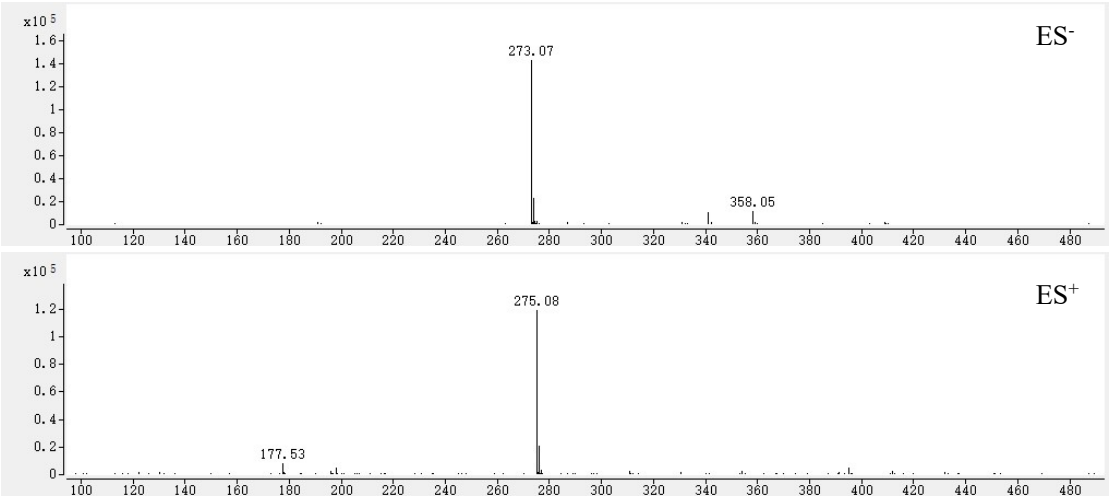
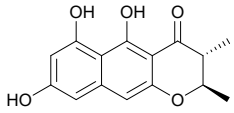


Fig. S48 MS spectra of **6**.

hemiustilaginoidin D (7)



hemiustilaginoidin D (**7**)

Chemical Formula: $C_{15}H_{14}O_5$, Exact Mass: 274.0841

UV λ_{max}(MeOH): 231, 280, 324, 337, 413 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{15}H_{13}O_5$: 273.0768, found: 273.0763

Compound was identified based on mass, retention time and UV-absorption with the standard[13]

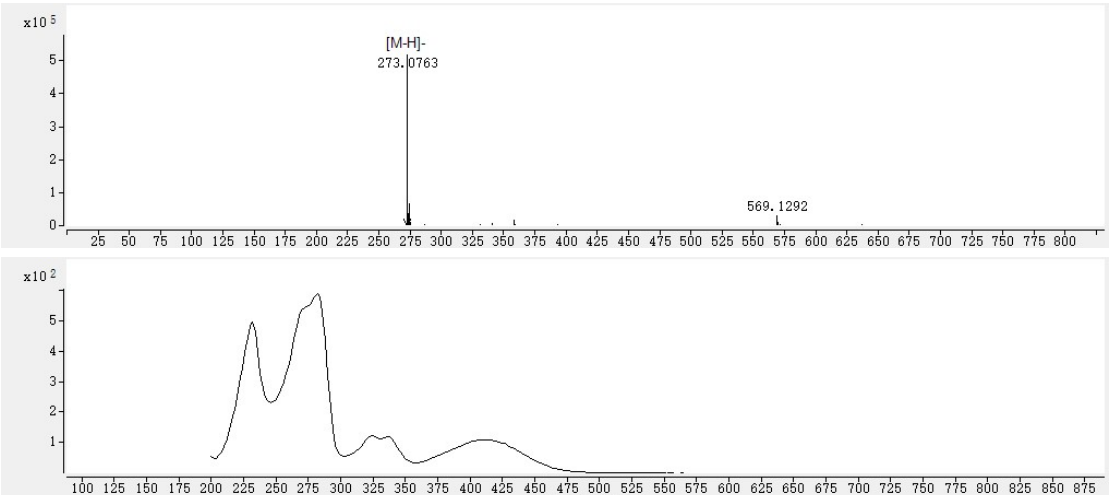


Fig. 49 HRESIMS (top) and UV (bottom) spectra of **7**.

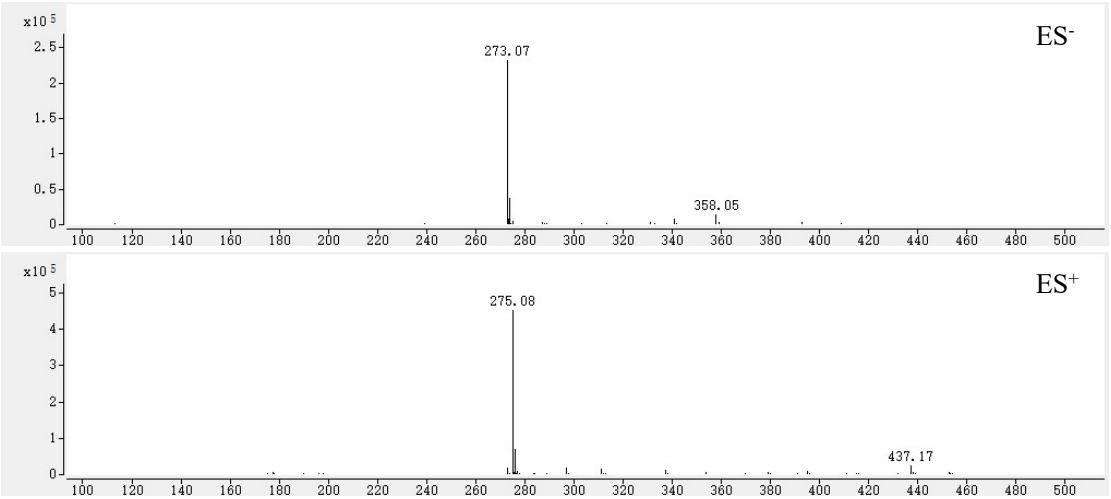
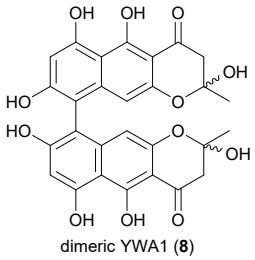


Fig. S50 MS spectra of **7**.

Dimeric YMA1 (8)



dimeric YWA1 (**8**)

Chemical Formula: $C_{28}H_{22}O_{12,}$ Exact Mass: 550.1111

UV λ_{max}(MeOH): 232, 270, 291, 326, 412 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{28}H_{21}O_{12}$: 549.1038, found: 549.1057

Compound **8** was identified based on mass, UV-absorption, and biosynthetic consideration, as well as the fact that it was quickly dehydrated to **9** and **10** upon isolation and purification.

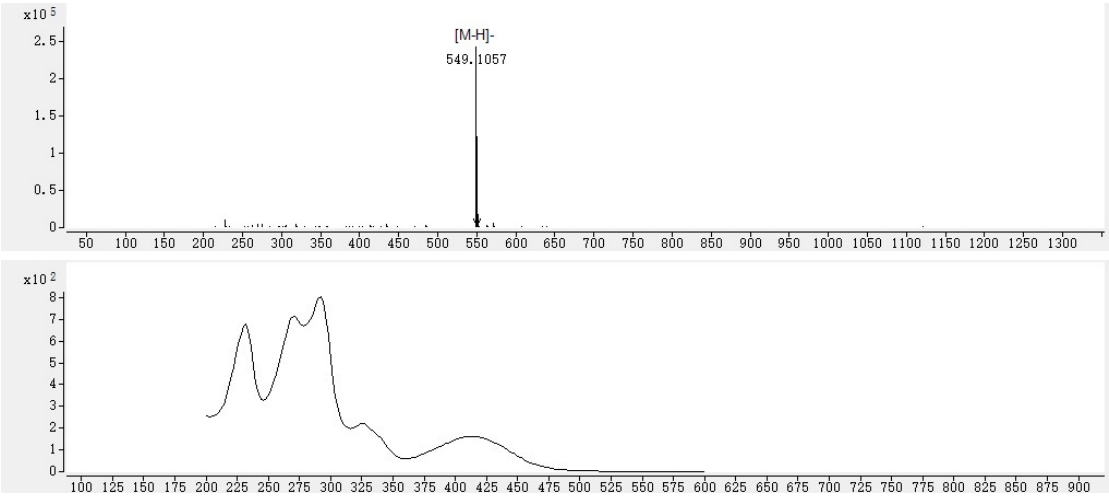


Fig. S51 HRESIMS (top) and UV (bottom) spectra of **8**.

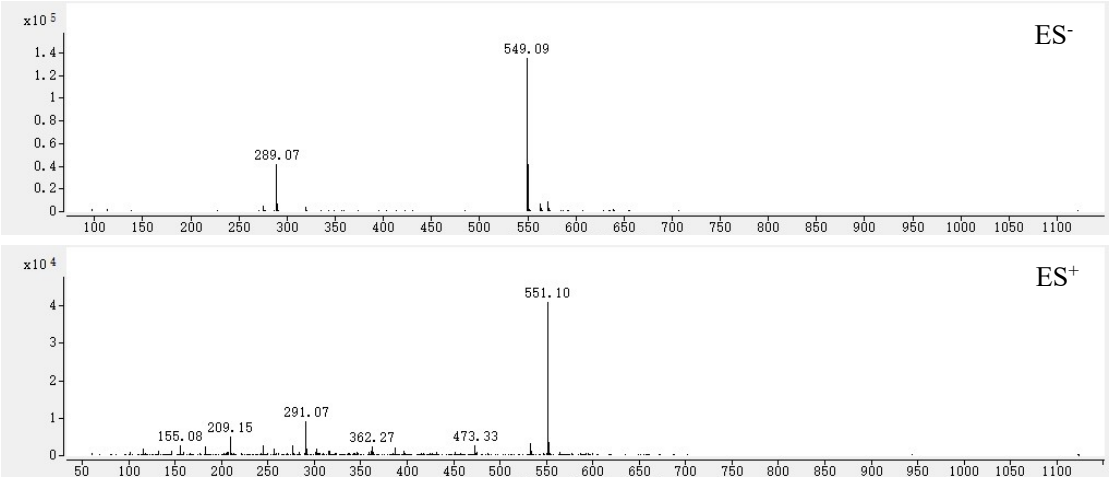
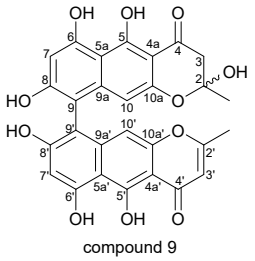


Fig. S52 MS spectra of **8**.

Compound 9



compound 9

Chemical Formula: $C_{28}H_{20}O_{11}$, Exact Mass: 532.1006

UV λ_{max}(MeOH): 229, 272, 290, 326, 416 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{28}H_{19}O_{11}$: 531.0933, found: 531.0918

Compound was identified based on mass and UV-absorption, and characterized by NMR (Table S14, Fig. S55). This compound appeared as a mixture of 2-epimers, as two sets of signals could be seen in the ^1H NMR spectrum (Fig. S55, and Table S14).

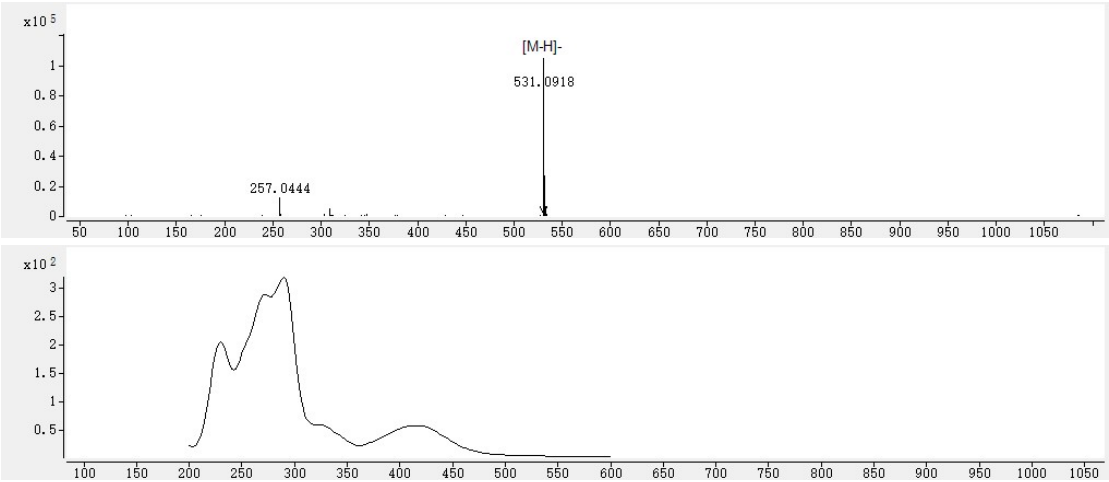


Fig. 53 HRESIMS (top) and UV (bottom) spectra of **9**.

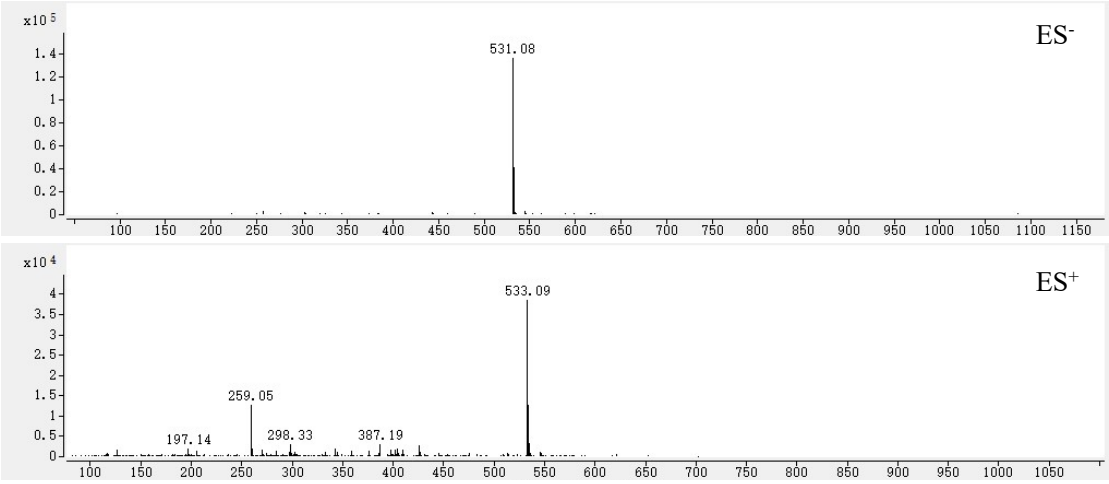


Fig. S54 MS spectra of **9**.

Table S14. ^{1}H NMR data of **9** (CD_3OD, 500 MHz).

Position	δ_H, mult. (*J* in Hz), **major**	δ_H, mult. (*J* in Hz), **minor**
3	~3.33, 3.14 overlapped [a]	~3.33, 3.14 overlapped [a]
3’	6.00 brs	6.00 brs
7/7’	6.34 brs	6.34 brs
10/10’	6.48/6.54 s [a]	6.48/6.54 s [a]
2-CH_3	1.52 s	1.49 s
2’-CH_3	2.18 s	2.25 s

[a] tentative assigned.

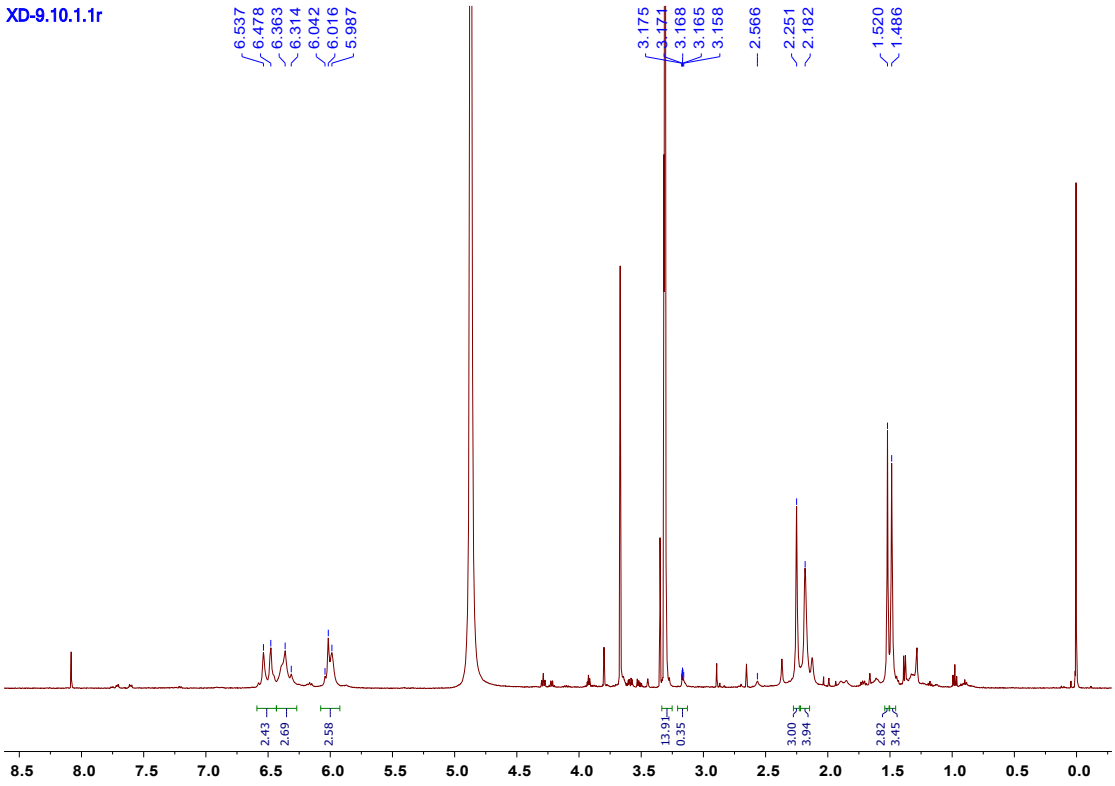
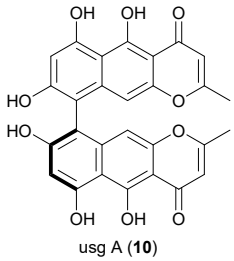


Fig. S55 ^{1}H NMR spectrum of **9** (500 MHz, CD_3OD)

usg A (10)



usg A (**10**)

Chemical Formula: $C_{28}H_{18}O_{10}$, Exact Mass: 514.0900

UV λ_{max}(MeOH): 227, 289, 416 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{28}H_{17}O_{10}$: 513.0827, found: 513.0810

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

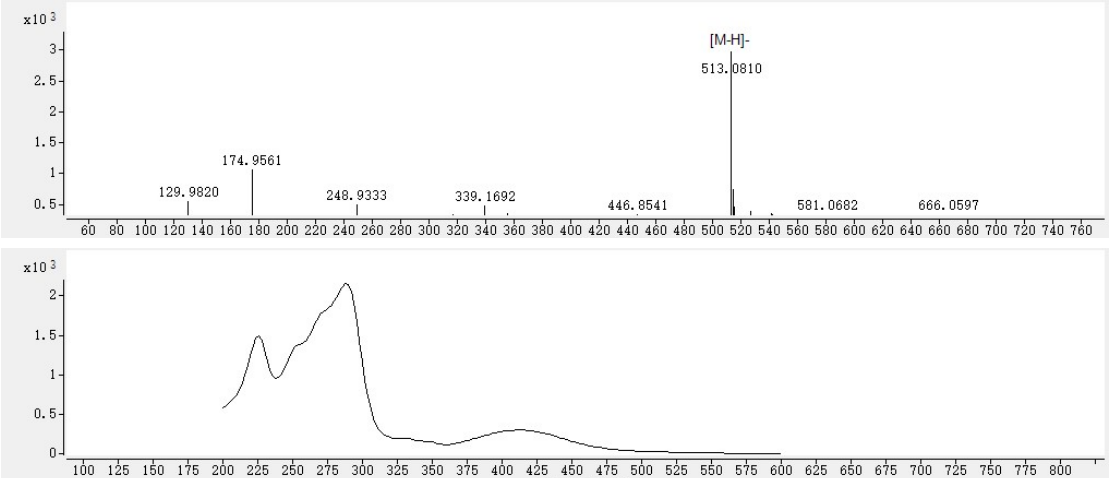


Fig. S56 HRESIMS (top) and UV (bottom) spectra of **10**.

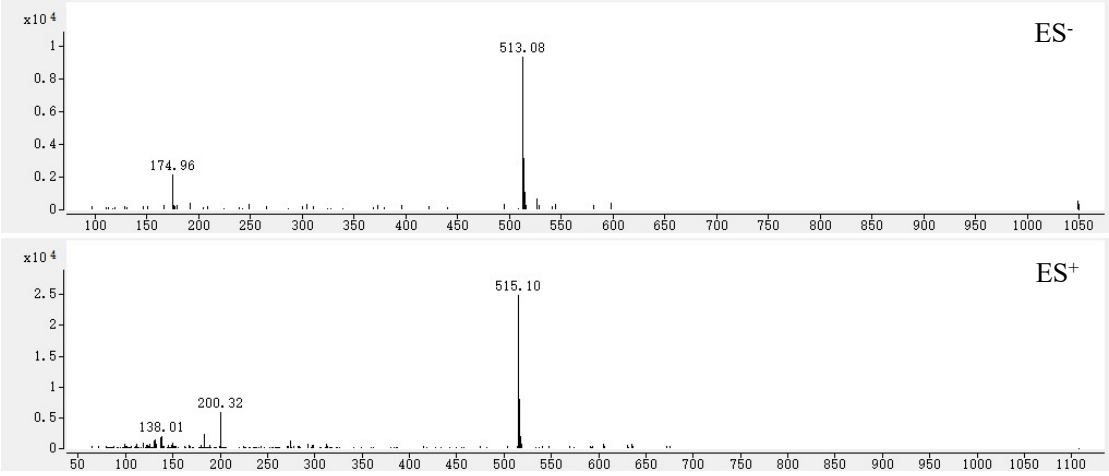
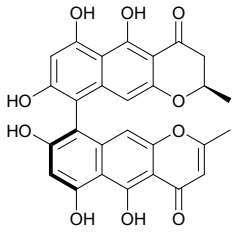


Fig. S57 MS spectra of **10**.

usg G (11)



usg G (**11**)

Chemical Formula: $C_{28}H_{20}O_{10}$, Exact Mass: 516.1057

UV λ_{max}(MeOH): 220, 272, 292, 327, 417 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{28}H_{19}O_{10}$: 515.0984, found: 515.1000

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

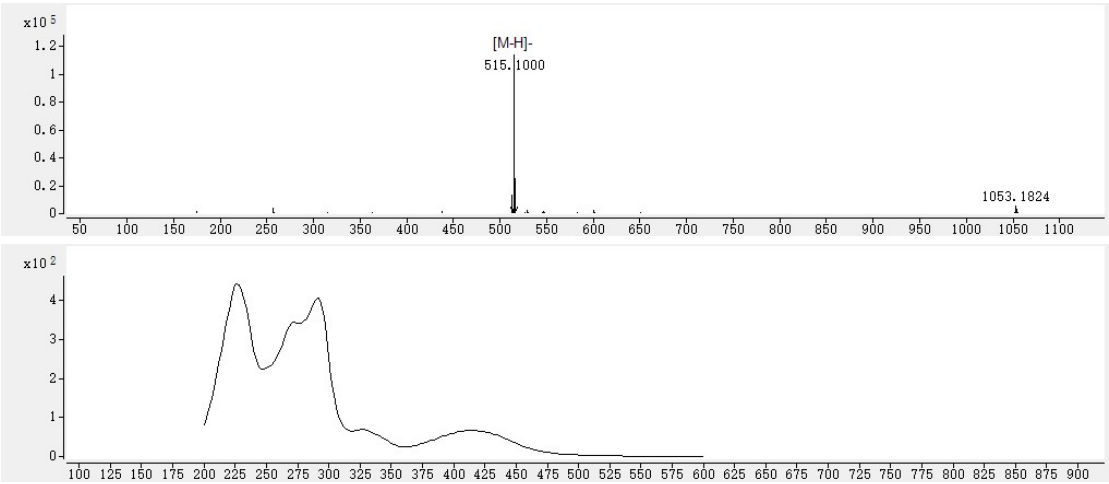


Fig. S58 HRESIMS (top) and UV (bottom) spectra of **11**.

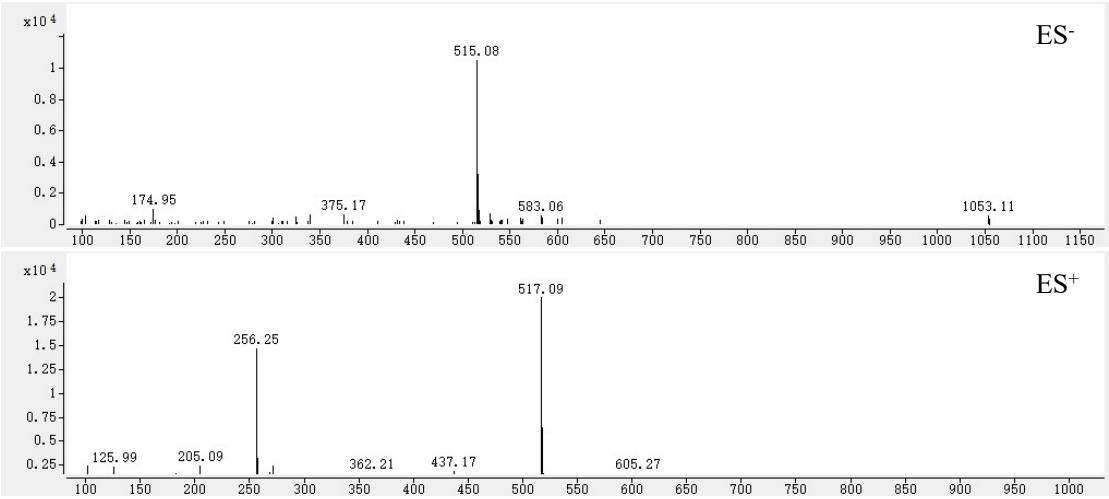
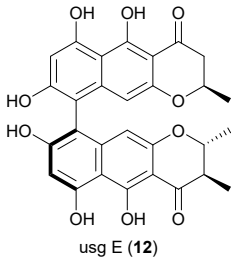


Fig. S59 MS spectra of **11**.

usg E (12)



usg E (**12**)

Chemical Formula: $C_{29}H_{24}O_{10}$, Exact Mass: 532.1370

UV λ_{max}(MeOH): 234, 271, 294, 327, 417 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{29}H_{23}O_{10}$: 531.1297, found: 531.1302

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

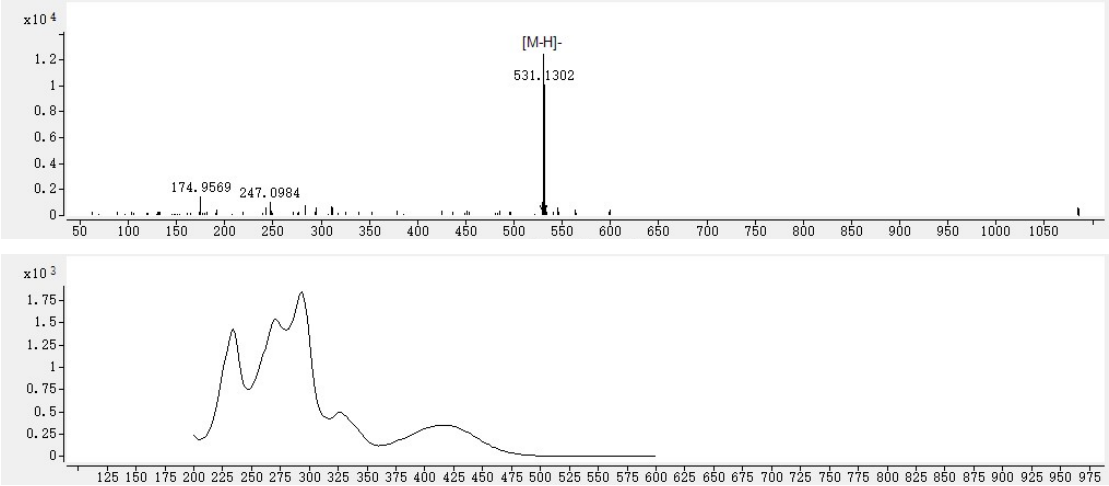


Fig. S60 HRESIMS (top) and UV (bottom) spectra of **12**.

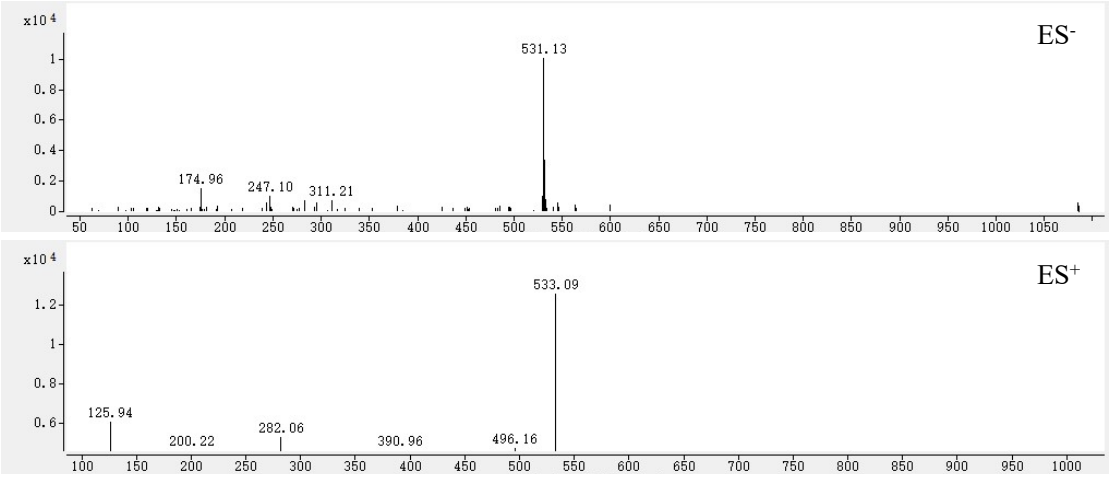
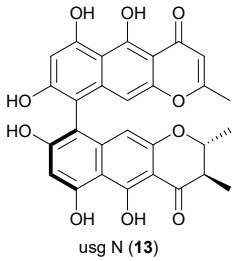


Fig. S61 MS spectra of **12**.

usg N (13)



usg N (**13**)

Chemical Formula: $C_{29}H_{22}O_{10}$, Exact Mass: 530.1213

UV λ_{max}(MeOH): 229, 273, 292, 327, 417 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{29}H_{21}O_{10}$: 529.1140, found: 529.1148

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

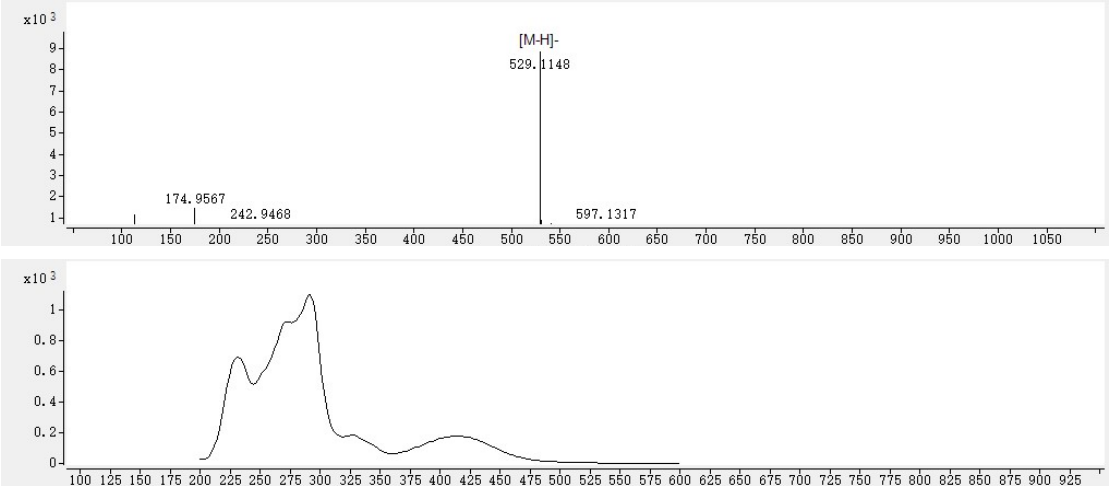


Fig. S62 HRESIMS (top) and UV (bottom) spectra of **13**.

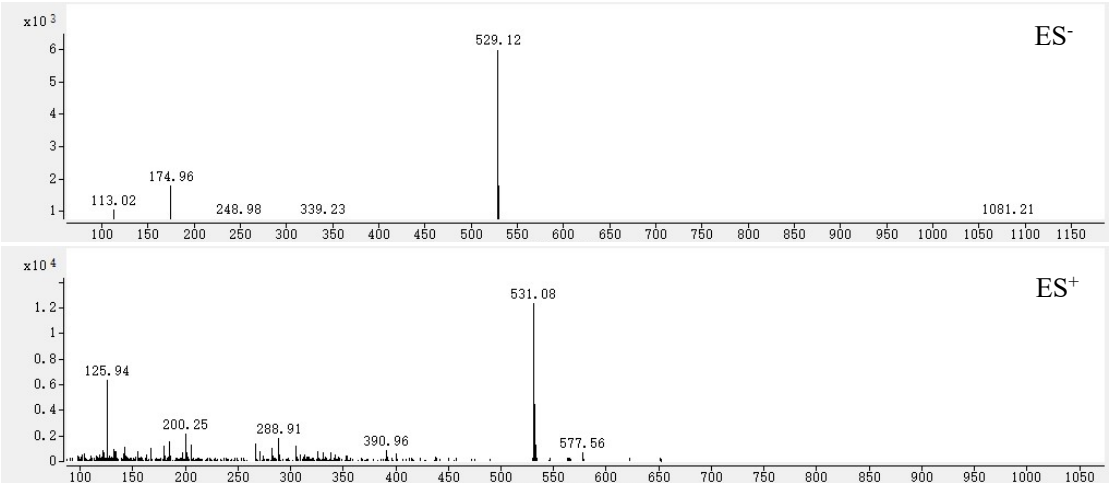
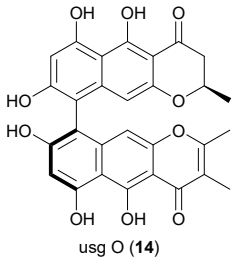


Fig. S63 MS spectra of **13**.

usg O (14)



usg O (**14**)

Chemical Formula: $C_{29}H_{22}O_{10}$, Exact Mass: 530.1213

UV λ_{max}(MeOH): 230, 272, 292, 327, 415 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{29}H_{21}O_{10}$: 529.1140, found: 529.1141

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

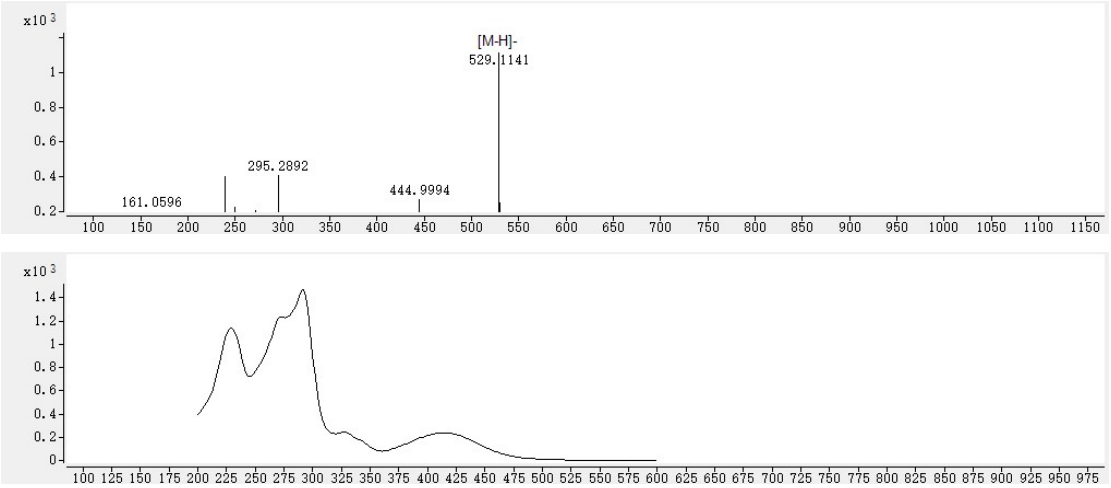


Fig. S64 HRESIMS (top) and UV (bottom) spectra of **14**.

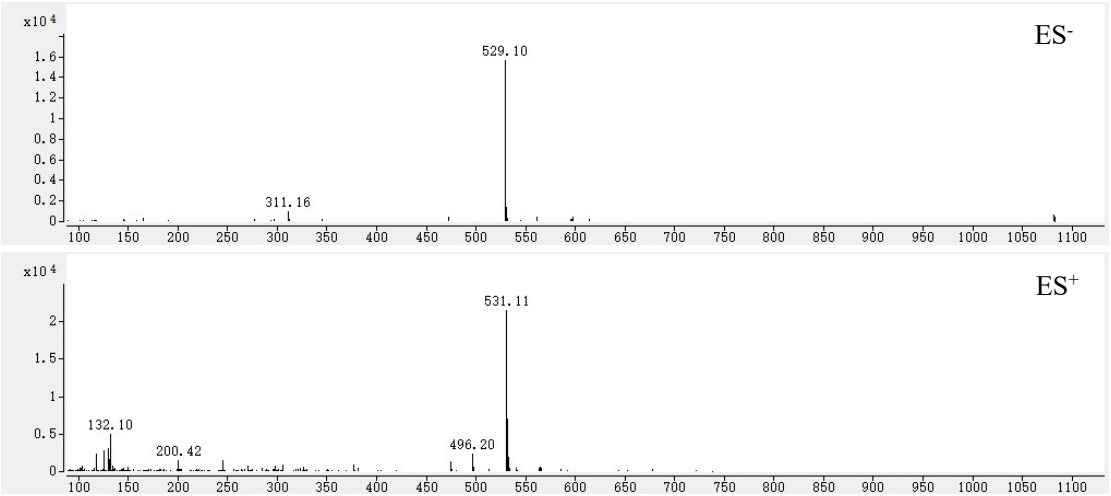
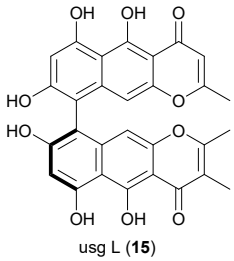


Fig. S65 MS spectra of **14**.

usg L (15)



usg L (**15**)

Chemical Formula: $C_{29}H_{20}O_{10}$, Exact Mass: 528.1056

UV λ_{max}(MeOH): 227, 290, 331, 416 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{29}H_{19}O_{10}$: 527.0984, found: 527.0959

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

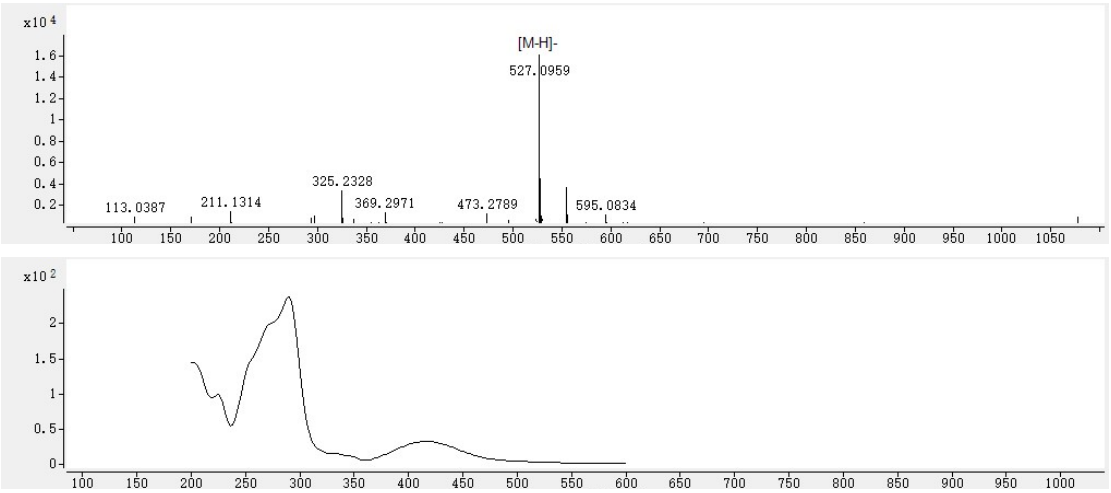


Fig. S66 HRESIMS (top) and UV (bottom) spectra of **15**.

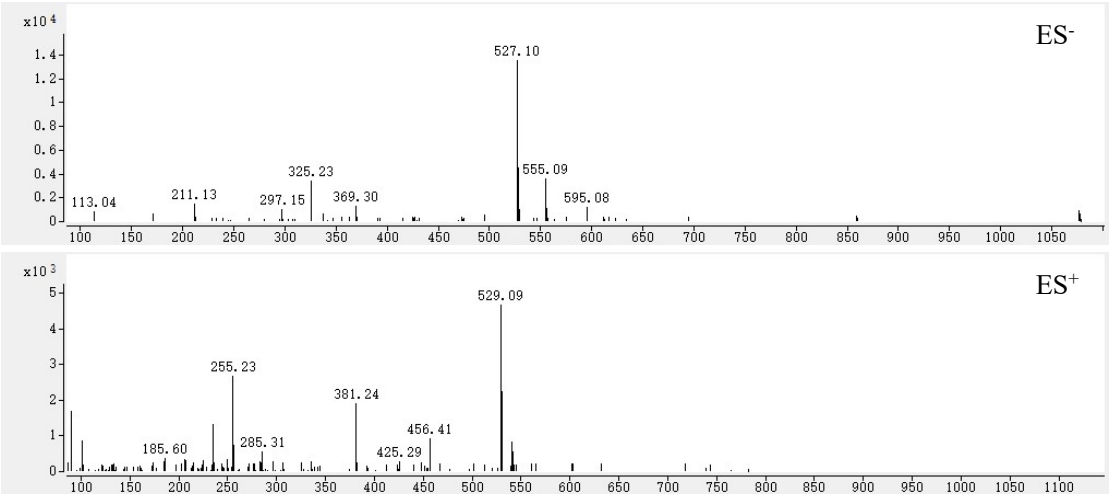
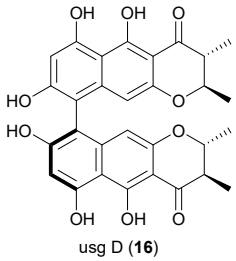


Fig. S67 MS spectra of **15**.

usg D (16)



usg D (**16**)

Chemical Formula: $C_{30}H_{26}O_{10}$, Exact Mass: 546.1526

UV λ_{max}(MeOH): 234, 271, 294, 327, 416 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{30}H_{25}O_{10}$: 545.1453, found: 545.1452

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

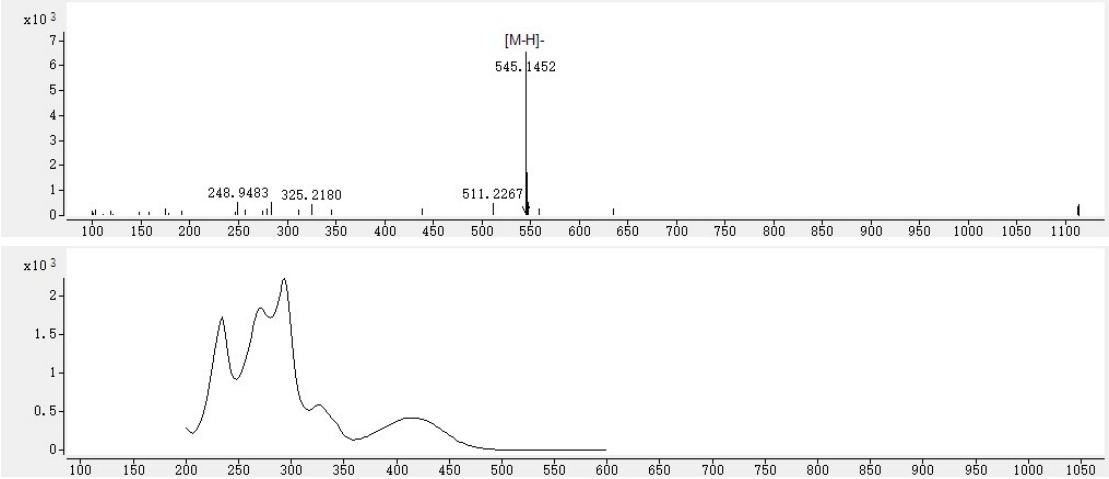


Fig. S68 HRESIMS (top) and UV (bottom) spectra of **16**.

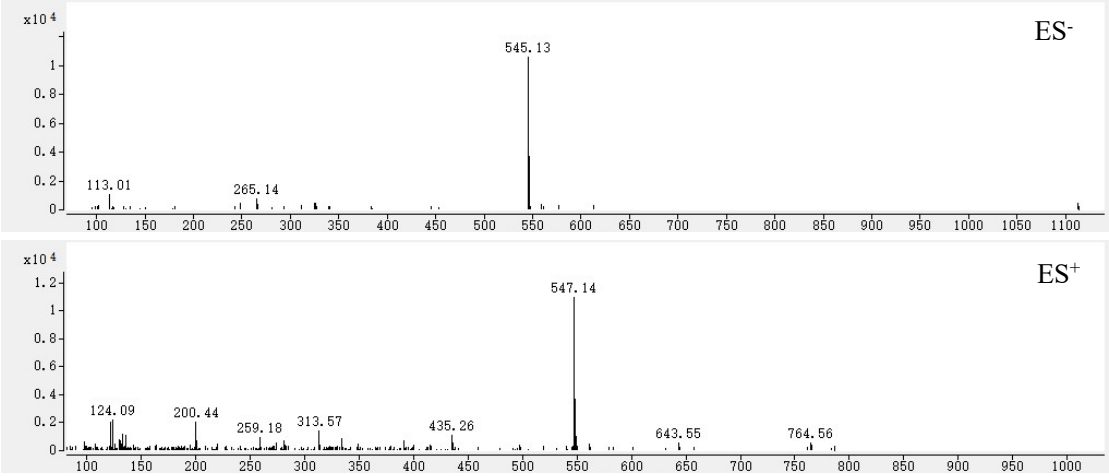
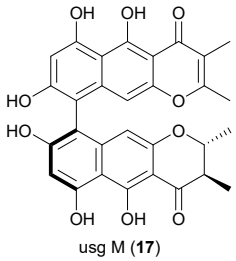


Fig. S69 MS spectra of **16**.

usg M (17)



usg M (**17**)

Chemical Formula: $C_{30}H_{24}O_{10}$, Exact Mass: 544.1370

UV λ_{max}(MeOH): 232, 272, 292, 327, 416 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{30}H_{23}O_{10}$: 543.1297, found: 543.1274

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

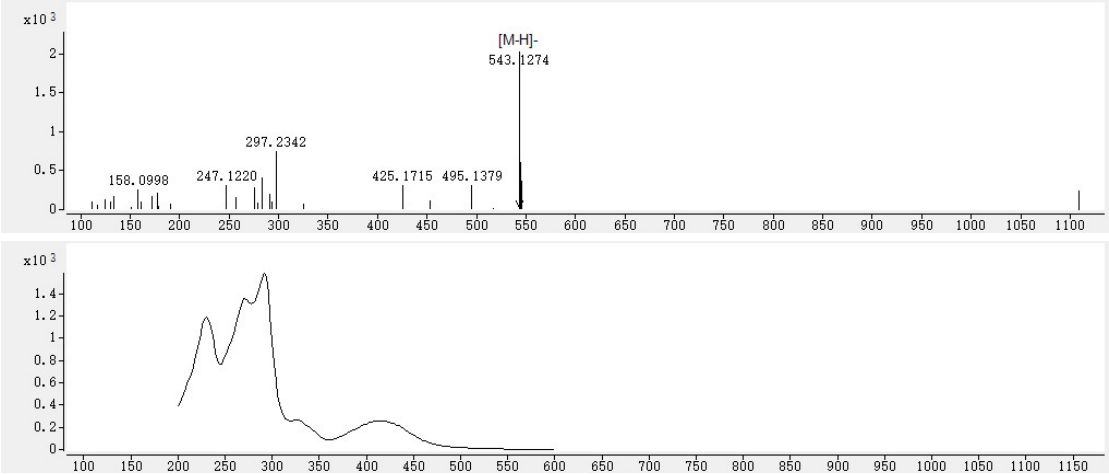


Fig. S70 HRESIMS (top) and UV (bottom) spectra of **17**.

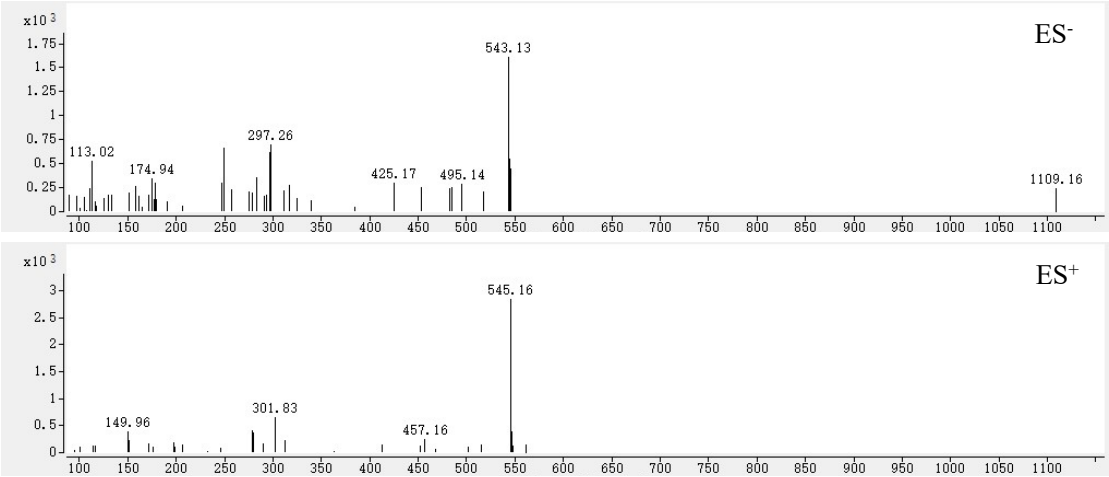
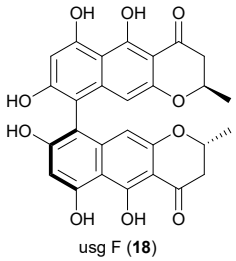


Fig. S71 MS spectra of **17**.

usg F (18)



usg F (**18**)

Chemical Formula: $C_{28}H_{22}O_{10}$, Exact Mass: 518.1213

UV λ_{max}(MeOH): 234, 271, 294, 328, 420 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{28}H_{21}O_{10}$: 517.1140, found: 517.1147

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

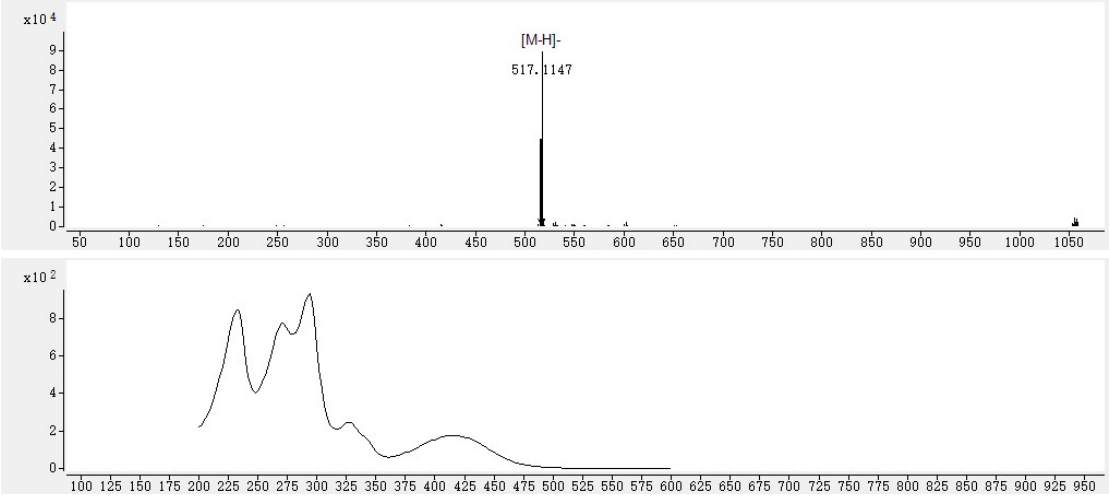


Fig. S72 HRESIMS (top) and UV (bottom) spectra of **18**.

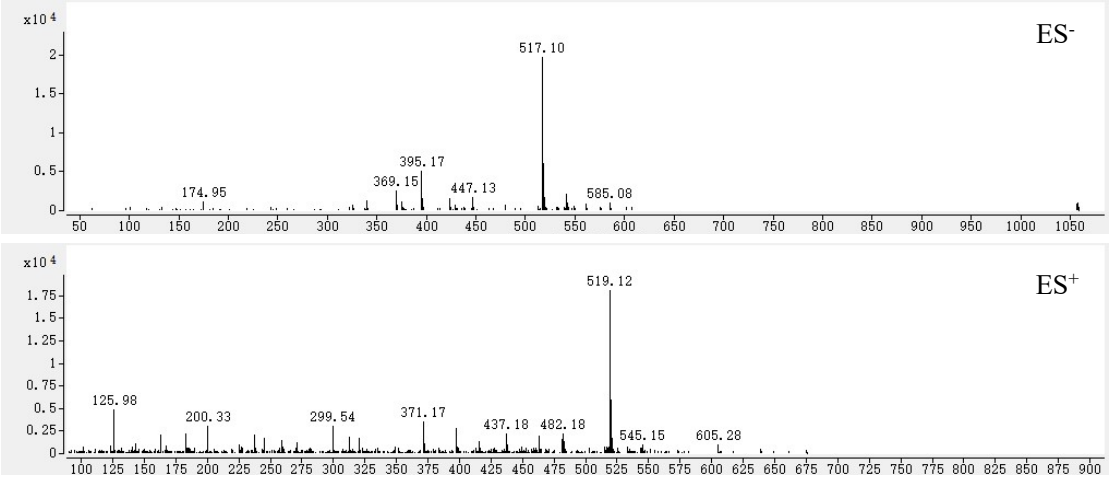
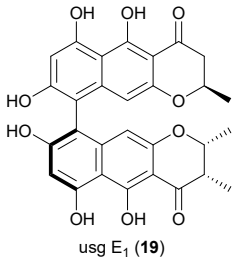


Fig. S73 MS spectra of **18**.

usg E_1 (19)



usg E_1 (**19**)

Chemical Formula: $C_{29}H_{24}O_{10}$, Exact Mass: 532.1370

UV λ_{max}(MeOH): 231, 273, 292, 327, 416 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{29}H_{23}O_{10}$: 531.1297, found: 531. 1319

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

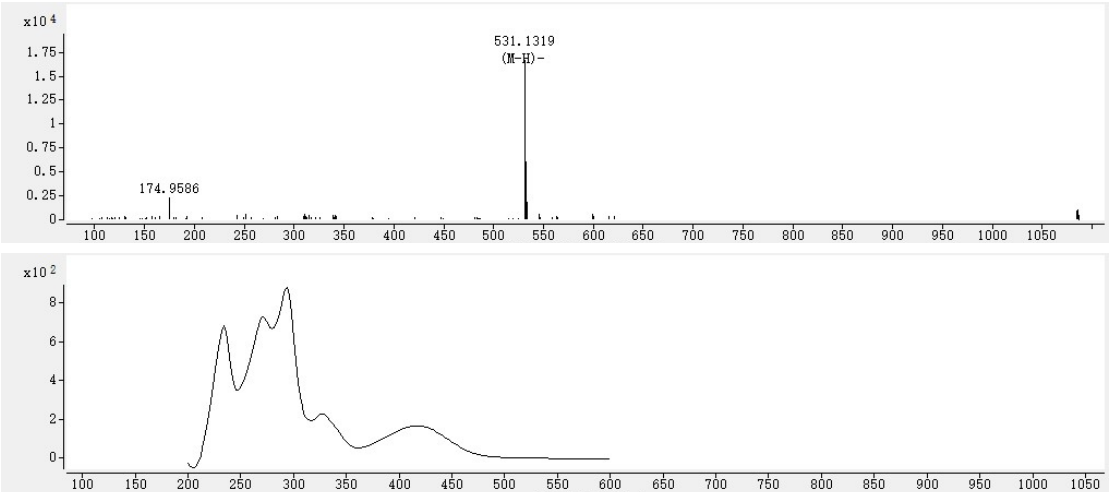


Fig. S74 HRESIMS (top) and UV (bottom) spectra of **19**.

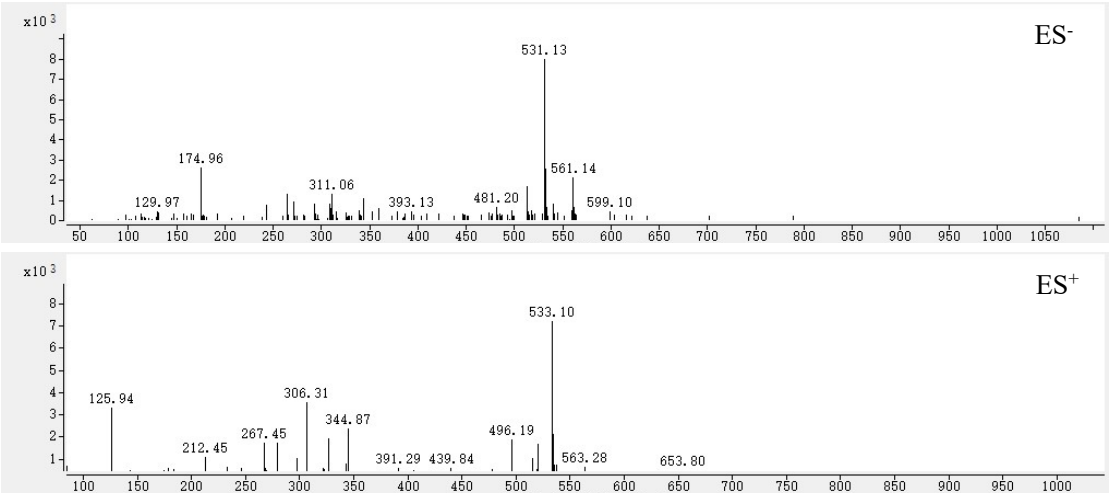
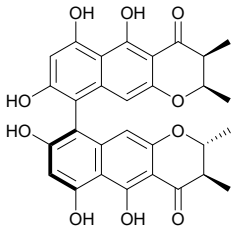


Fig. S75 MS spectra of **19**.

Isochaetochromin B_2 (20)



isochaetochromin B_2 (**20**)

Chemical Formula: $C_{30}H_{26}O_{10}$, Exact Mass: 546.1526

UV λ_{max}(MeOH): 234, 271, 294, 327, 420 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{30}H_{25}O_{10}$: 545.1453, found: 545.1433

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

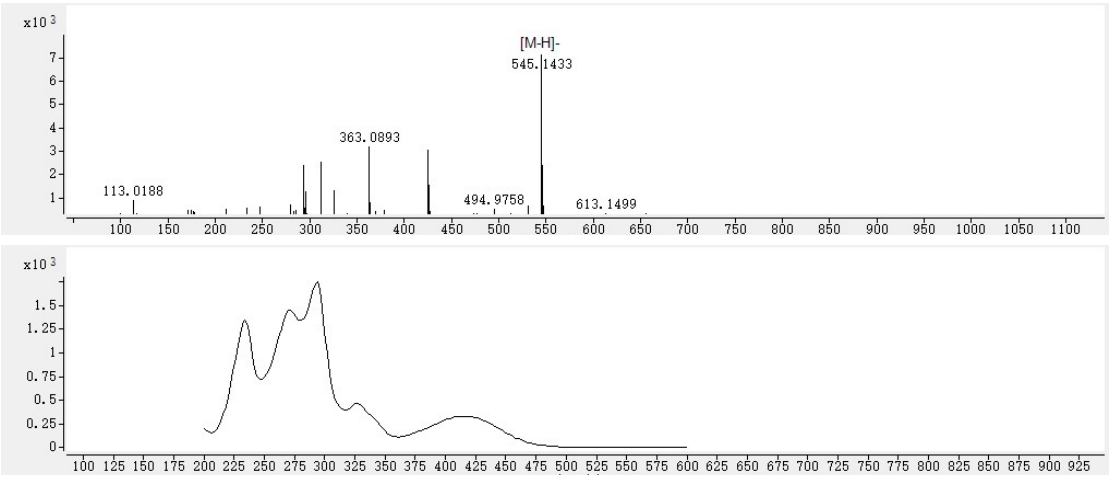


Fig. S76 HRESIMS (top) and UV (bottom) spectra of **20**.

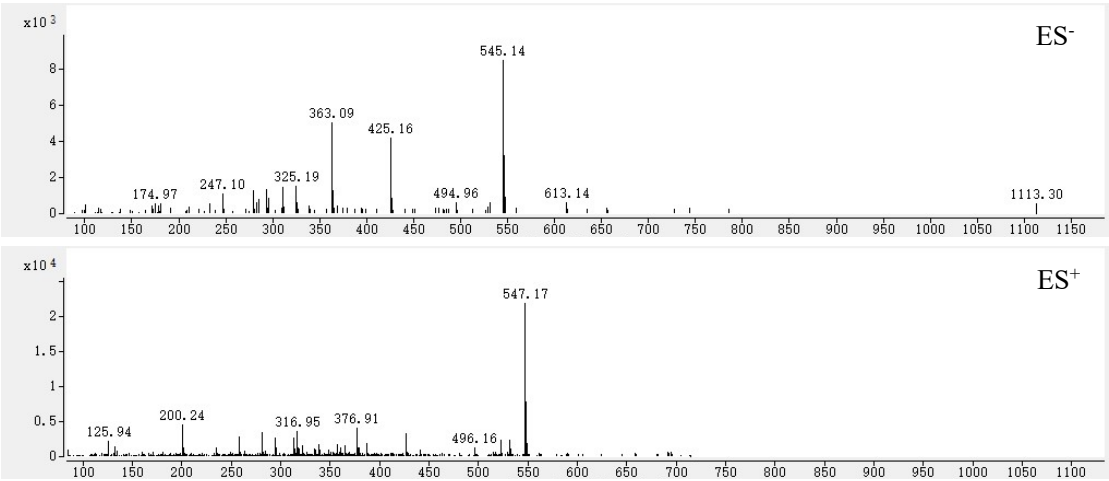
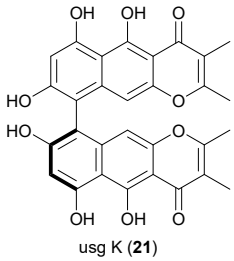


Fig. S77 MS spectra of **20**.

usg K (21)



usg K (**21**)

Chemical Formula: $C_{30}H_{22}O_{10}$, Exact Mass: 542.1213

UV λ_{max}(MeOH): 228, 291, 327, 420 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{30}H_{21}O_{10}$: 541.1140, found: 541.1145

Compound was identified based on mass, retention time and UV-absorption with the standard[12]

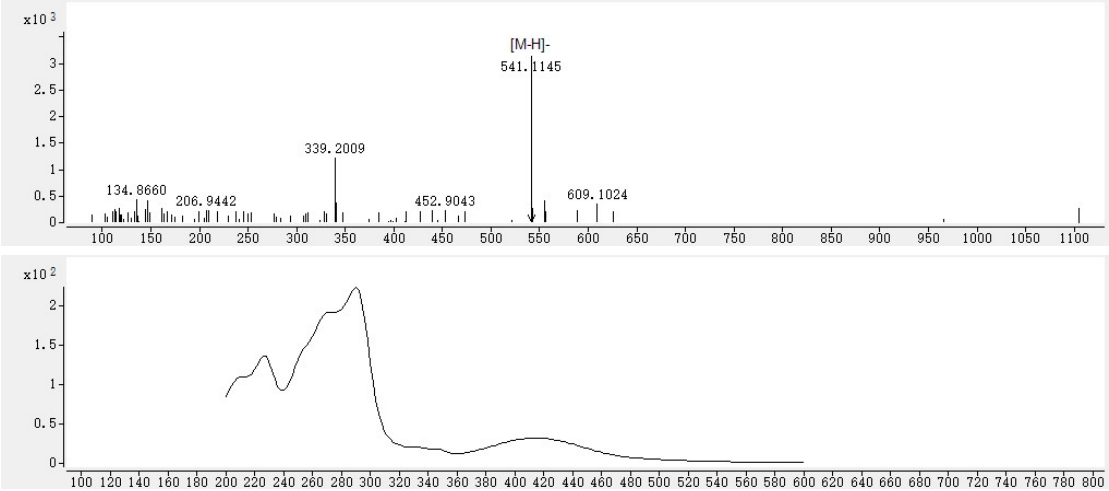


Fig. S78 HRESIMS (top) and UV (bottom) spectra of **21**.

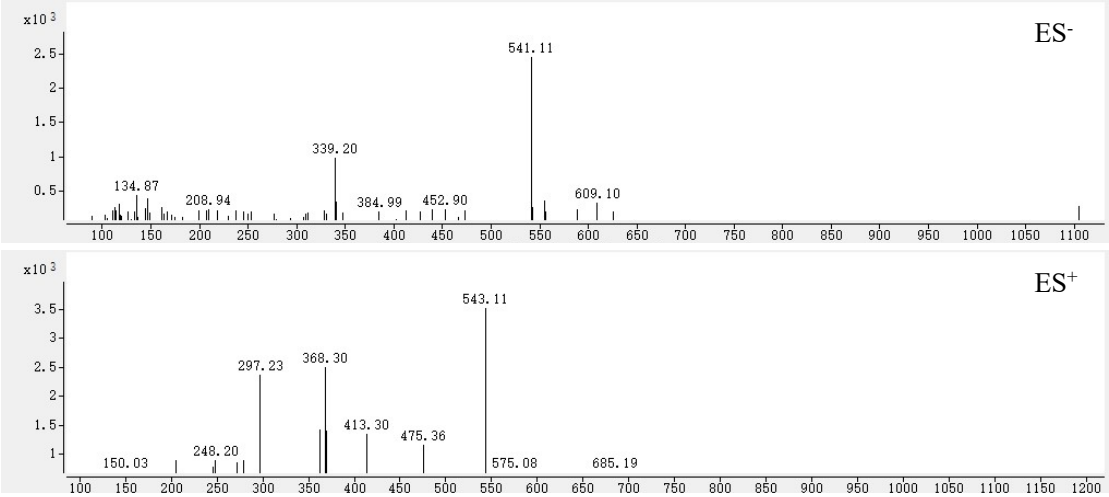
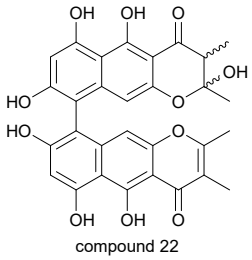


Fig. S79 MS spectra of **21**.

Compound 22



compound 22

Chemical Formula: $C_{30}H_{24}O_{11}$, Exact Mass: 560.1319

UV λ_{max}(MeOH): 228, 291, 327, 420 nm

HRESIMS: *m/z* $[M-H]^-$ calcd for $C_{30}H_{23}O_{11}$: 559.1246, found: 559.1262

Compound **22** was identified based on mass, UV-absorption, and biosynthetic consideration, as well as the fact that it was quickly dehydrated to **21** upon isolation and purification.

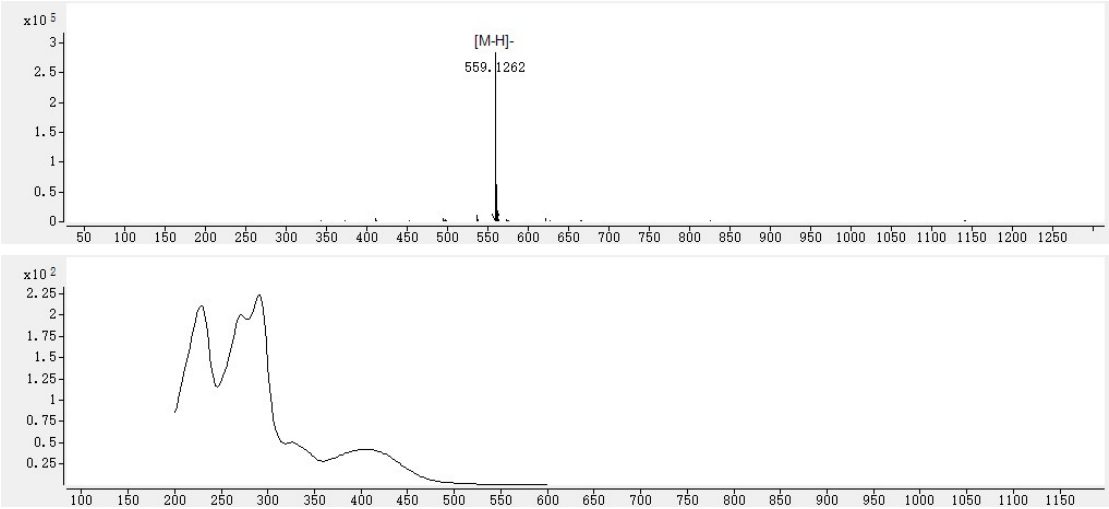


Fig. S80 HRESIMS (top) and UV (bottom) spectra of **22**.

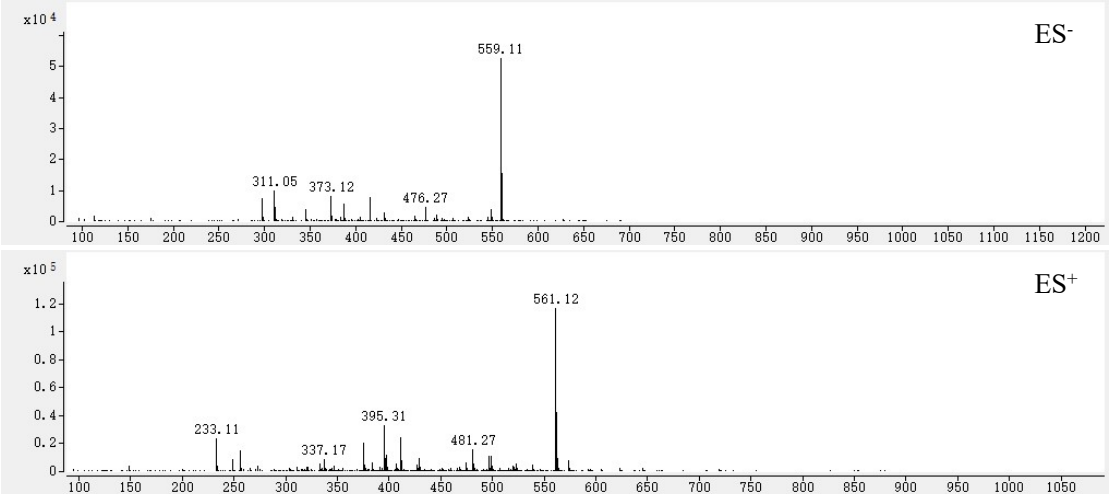


Fig. S81 MS spectra of **22**.

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