

# Total and Engineered Biosynthesis of Cinatrin: Inhibitors of Phospholipase A<sub>2</sub> and Squalene Synthase

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# 1. Sequencing and Informatics

## 1.1 Genome Sequencing and Annotation

Sequencing of the *H. monticulosa* genome, its assembly and annotation, were described in Wibberg et al 2023.<sup>1</sup> Predictions of genes and their functions were made based on the funnannotate pipeline.<sup>2</sup>

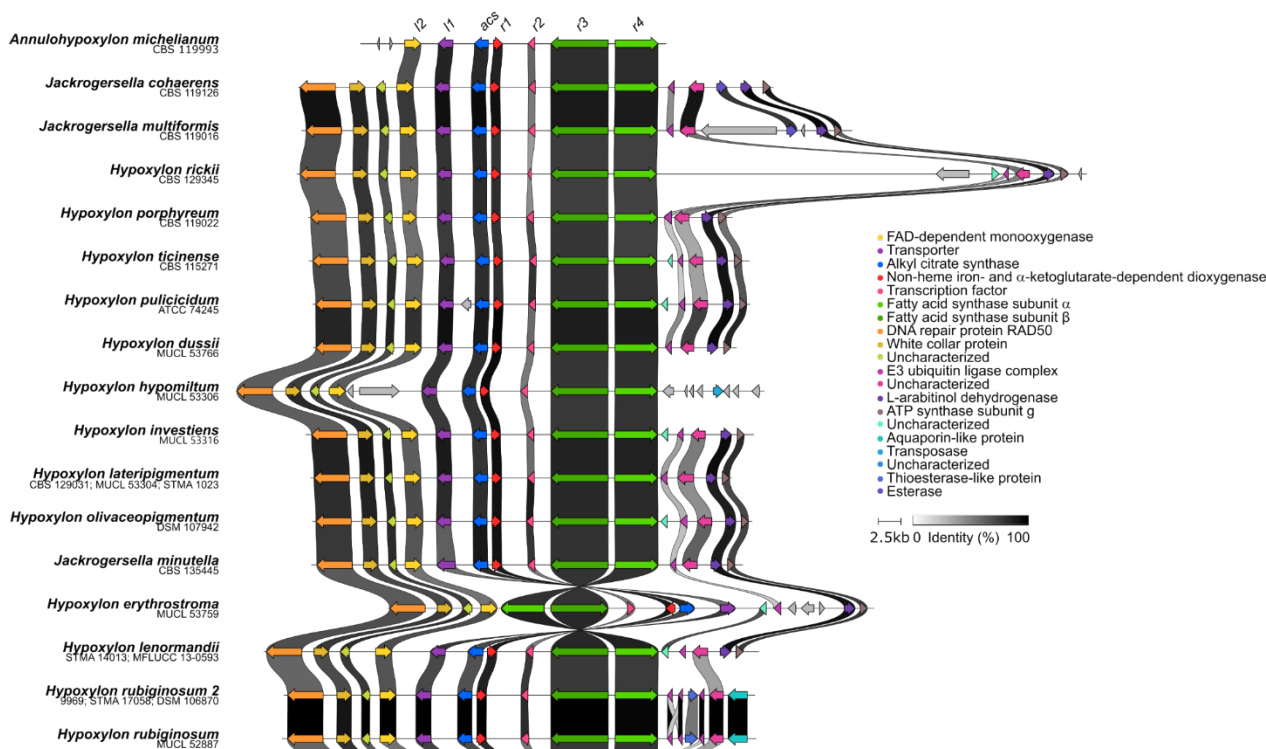
**Table S1** Sequencing results for *H. monticulosa*

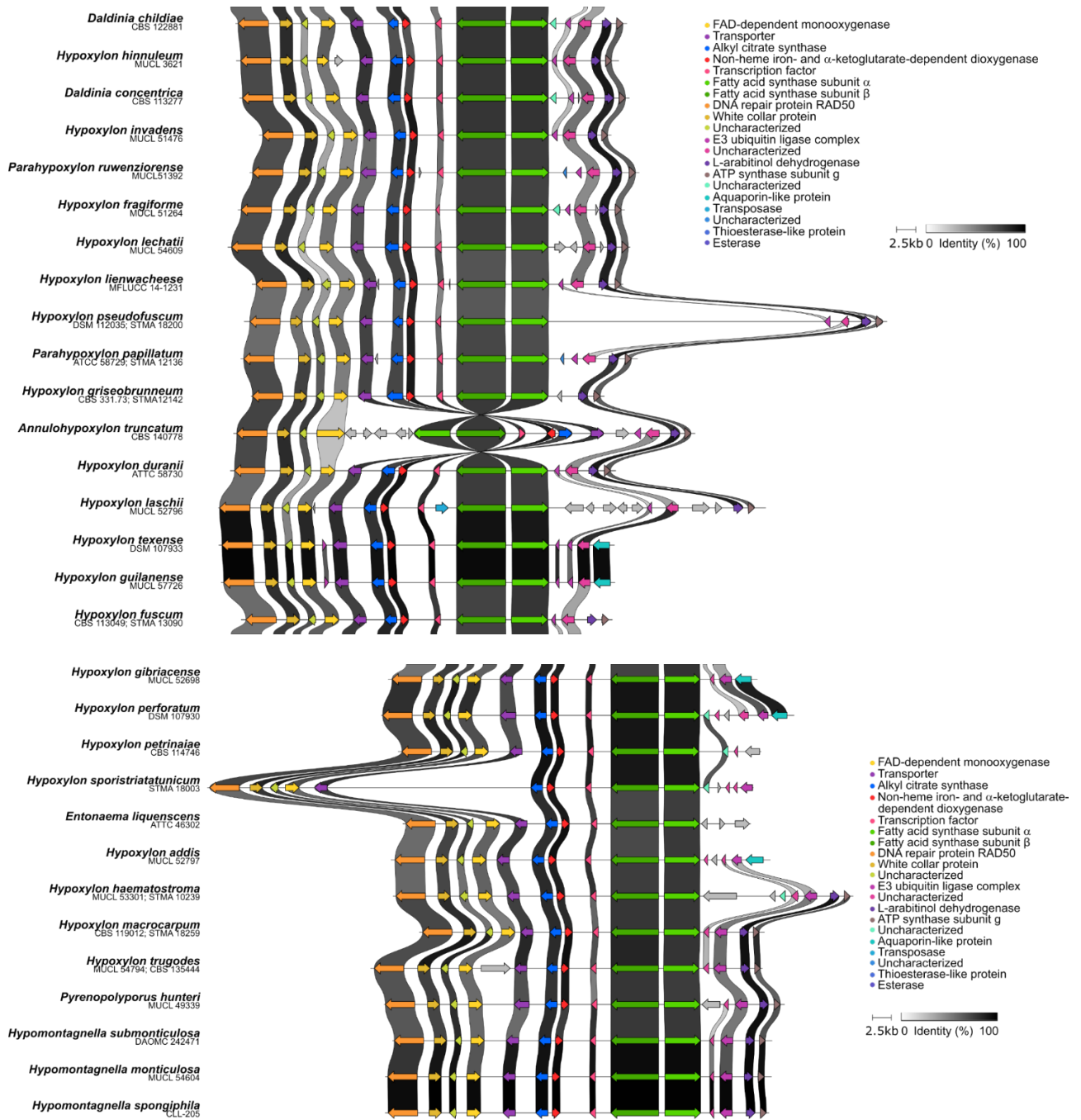
| Strain                           | Genome size [bp] | Sequencing Method          | Scaffolds/ contigs | N50 value              | Annotated genes |
|----------------------------------|------------------|----------------------------|--------------------|------------------------|-----------------|
| <i>H. monticulosa</i> MUCL 54604 | 42769229         | Illumina & Oxford Nanopore | 28                 | 3.44 x 10 <sup>6</sup> | 12254           |

Genome sequence data for *H. monticulosa* MUCL 54604 is available at genbank: **GCA\_902806465.1**  
The annotated *ctr* BGC is uploaded to genbank: **PX666452**

## 1.2 Synteny Analysis of the *ctr* Biosynthetic Gene Cluster

The search for the *ctr* BGC was performed via a manual search strategy using the Blast algorithm: the ACS from *H. monticulosa* that was already identified in our previous study,<sup>3</sup> was used against the translated nucleotide database of the targeted organism with the software package Geneious v. 2022.1.1 using standard parameters (Max E-value 0.05, BLOSUM62 matrix, gap cost 11 1).<sup>3,4</sup> The genomes contained at least three CS homologues with two of them being associated with primary metabolism. A fourth ACS was present in strains that are known producers of sporothriolide and part of the *spo* cluster.<sup>5</sup> Identified *ctr* BGCs were manually curated and the synteny analysis was conducted with clinker.<sup>6</sup>





**Figure S1** Synteny analysis of the identified *ctr* alkyl citrate biosynthetic gene cluster with homologous BGCs from other *Hypoxylon* species.

### 1.3 Sequence Analysis of CtrR1

The protein databank was searched for sequences similar to CtrR1. The best match was Nvfl, an endoperoxide-forming enzyme from the novofumigatonin biosynthetic pathway in *Aspergillus novofumigatus*.<sup>7,8</sup> CtrR1, Mfr1 and Mfr2 were aligned with this sequence and residues known to be involved in catalysis were identified.

|       |     |   |     |
|-------|-----|---|-----|
| CtrR1 | 1   | MAAADV-ATTTTGLIGMWGGTRDGDGTFIDWASGATNVHDPKQVKIEAEDMRTMNPVPSYMKDGYGIAKHKTSITPEQF   | 79  |
| Mfr1  | 1   | MATA-I-LPSTSGVIGLWDGTTDGG-EGFMDYANGDTNVKQPKYEIQVHDIRKLDQPPTLLKNGYELVDIPTVVTDEQF   | 77  |
| Mfr2  | 1   | MATATTTLHSTTGTVYVADGTTDGG-VGYNHTDDSTNVIR-KPIPIEVEDARTLSKSPPTKAEQYQLVNFHTKIPEEHF   | 78  |
| Nvfl  | 1   | MVGSRTWCESEMLFVQPDAGTKEELYRVRTPKPGQTQANFNWTPHKVRFHDARPQRDSFDLNTHGFTFVEDAISPQLIER  | 80  |
|       |     |   |     |
| CtrR1 | 80  | L-AGNDPEGKGFLEDVYYKEVEELVKQITGS-SIVVPYVFRIRQNSMKAGEFMAAKLS-----HSALPVAHVD         | 152 |
| Mfr1  | 78  | IeSGKSDEGNAYIKDVYFAECKRIIEEVSGGvDLIIPVSFRMREQKGEK-ESTTKKLGNIesrYAPRPVAHLDRDPTAI   | 156 |
| Mfr2  | 79  | L-NSKLPENKELIEEVYFDECRRLVQEVGTGA-AEAYPYVYRVRNQEQNAKESNKSNFHTDF-----VPIVHVDRDDVTAP | 151 |
| Nvfl  | 81  | I----RADDTAAVEGDYFASVAALVKRVTGA-DHVVCFSPYTRKENSEK-----GIFG--QPARTVHCDHTPAAAI      | 144 |
|       |     |   |     |
| CtrR1 | 153 | DGVREVLG-EQADELIKQKRYAQVNIWRSIDQPIKWKPLCFINHAGVEGWNYDShLARVYPTNDPRIAIRGEKKHDSVV   | 231 |
| Mfr1  | 157 | TVLEETVGGKEKAQELLSKHKRWAQVNVWRPIGNPATMWPCLFLNHDRIPWNYDTHVGHVWSLNDPRVSDRGQKTYDCVV  | 236 |
| Mfr2  | 152 | QRLRASLGAEKADMLLSKYKSYGSINVWRPVKNMVQKWPLMLVDHKSIEDWDYSTHMFTLHSSNDERVATRGAKEHETIL  | 231 |
| Nvfl  | 145 | ELTHKLCG-EDAVRLLQS--RFRAFSVWRPLVEPVLDWPLAVVDGRTIAPDDLHPVHWLRYEKKDTEPPFQ-----L     | 213 |
|       |     |   |     |
| CtrR1 | 232 | KYDPNKYHYVSNLIDIDEVLVFSFSDS--VSKVVPHGAFWDDASPDAPT                                 | 293 |
| Mfr1  | 237 | KHDDRyDYHYVSDLRPEECLVFCSPDSI--PKYAMPHSAFWDDNNVPADAPNRSIEVRSLVFF--                 | 297 |
| Mfr2  | 232 | THDKRYRYIYASDMTPEEAWLFFAFHSD--PALGIPIHGAFWDDSTKKEALTRCSIEVRIWVFFD-                | 293 |
| Nvfl  | 214 | SFSETQKWYLLSRQSRDEVSIVKNYDSEvvPSPRSAHCAFKHPFVVKDAPPESIDVFCVLFVGGr                 | 278 |

**Figure S2** Protein sequence alignment of NHI oxygenases CtrR1, Mfr1, Mfr2 and Nvfl using COBALT multiple sequence alignment tool.<sup>9</sup> Fe-binding sites are marked in yellow and  $\alpha$ -KG-binding residues in green based on the position in the Nvfl sequence. Endoperoxise Nvfl from *Aspergillus novofumigatus* was included in the alignment as the closest CtrR1 homolog contained in the Swiss-Prot database and a well characterized non-heme iron - and  $\alpha$ -ketoglutarate ( $\alpha$ -KG)-dependent dioxygenase.

## 2. Microbiology and Vector Construction

### 2.1 General Procedures

Molecular biology kits were used according to manufacturer's protocols. Analytical PCR was carried out using OneTaq<sup>®</sup> DNA Polymerase (New England Biolabs), PCR for cloning was performed with Q5<sup>®</sup> High-Fidelity DNA Polymerase (New England Biolabs). Restriction endonucleases were purchased from New England Biolabs.

### 2.2 Growth and Maintenance of Strains

*Escherichia coli* OneShot<sup>™</sup> Top10, *Escherichia coli* OneShot<sup>™</sup> ccdB Survival<sup>™</sup> 2 T1<sup>R</sup> were purchased from Thermo Fisher Scientific. *Saccharomyces cerevisiae* CEN.PK2 was purchased from Euroscarf. *Aspergillus oryzae* NSAR1 was provided by the Lazarus group.<sup>10</sup> *Hypomontagnella monticulosa* MUCL 54604 was obtained from the Stadler group in Braunschweig.<sup>11</sup>

**2.2.1 *Escherichia coli*** strains were cultivated on liquid or solid LB medium/ agar with appropriate antibiotics. Cultures were incubated at 37 °C for 12 to 18 h shaking at 200 rpm. For long term storage glycerol stocks (25 % - 50 % glycerol) were stored in cryo storage vials at -80 °C.

**2.2.2 *Saccharomyces cerevisiae*** strain CEN.PK2 was cultured on YPAD agar at 30 °C for 3-5 days. For liquid cultures a single colony was inoculated into YPAD medium and cultivated at 30 °C and 130 rpm for 18 h.

**2.2.3 *Aspergillus oryzae*** NSAR1 was grown on DPY agar at 28 °C for 5-7 days. Spores were suspended in H<sub>2</sub>O; the suspension was mixed with glycerol to a final concentration of 50 % (v/v) and stored in cryo storage vials at -80 °C. Secondary metabolite production was achieved in liquid cultures. 500 mL baffled flasks containing 100 mL DPY medium were inoculated with 2 mL of a mycelia and spore suspension in H<sub>2</sub>O from a 7 day old plate. The cultures were incubated at 28 °C and 110 rpm for 7 days.

**2.2.4 *Hypomontagnella monticulosa*** was grown on DPY-plates at 28 °C for 10-14 days. Stocks and genomic DNA was prepared from fresh mycelium, which was produced as follows: 100 mL DPY medium was inoculated with 1 mL of a spore suspension collected from a DPY plate and incubated at 28 °C for 5-7 days with shaking at 110 rpm. For storage, the cell suspension was mixed with glycerol to a final concentration of 50 % and stored in cryo storage vials at -80 °C.

### 2.3 Fungal gDNA and cDNA Preparation

A pea-sized portion of mycelia was dried with paper towels and incubated in 500 µL lysis buffer at 65 °C for 30 min. Next, it was incubated on ice for 5 min. 200 µL of 8 M KOAc were added, the mixture was mixed thoroughly and centrifuged for 15 min at 13,000 rpm. 300 µL of the liquid phase was added to 300 µL isopropanol in a new tube and the tube was inverted 8-10 times. After another centrifugation step for 3 min at 13,000 rpm the supernatant was removed. The pellet was washed with 1 mL 70 % high quality ethanol. After another centrifugation step for 3 min at 13,000 rpm the supernatant was removed and the pellet dried at 65 °C for ca. 10 min with an open lid. The genomic DNA was resuspended in 100 µL water and stored at 4 °C.

RNA extraction was performed with sterile consumables in a sterile clean bench. Fungal mycelia were collected by vacuum filtration. The cells were homogenized by mechanical disruption

using beads. RNA extraction from the homogenized mycelia was performed with Trizol™ (Thermo Fisher Scientific) following the manuals' instruction. RNA was optionally treated with DNase I (Zymo Research) according to the manufacturer's recommendations. Purified RNA was converted to cDNA immediately with RevertAid Premium Transcriptase kit (Thermo Fisher Scientific) and the Manufacturers' instructions were followed. For determination of gDNA contamination in the RNA, PCR amplifications of known intron-containing sequences were performed.

## 2.4 Cloning Procedures

**2.4.1 Yeast Homologous Recombination.** Transformation of *S. cerevisiae* CEN.PK2 was performed using a LiAc/SS-carrier DNA/PEG method.<sup>12</sup> 10 mL YPAD seed culture inoculated with a single colony was grown overnight for up to 18 h at 30 °C and 130 rpm. This seed culture was used to inoculate 40 mL YPAD medium (total volume was 50 mL after inoculation which was cultivated for 4-5 hours at the same conditions. Cells were harvested by centrifugation (3000 × g, 5 min, 4 °C) and the pellet was washed with 25 mL H<sub>2</sub>O and resuspended in 1 mL H<sub>2</sub>O in a 1.5 mL tube. The suspension was centrifuged again for 30 s at 11000 × g. The pellet was resuspended in 1 mL H<sub>2</sub>O, and the mixture was divided into 100 µL aliquots. After another centrifugation step for 30 s at 11000 × g the supernatant was discarded, and the pellet resuspended in 360 µL transformation mixture. The transformation mixture contained 240 µL PEG 3350 (50 % [w/v]), 36 µL LiOAc (1 M), 50 µL carrier DNA (2 mg/mL salmon testis DNA) and 34 µL DNA mix. The DNA mix comprised pTYGS-*argB/adeA/pE-YA* (up to 2000 ng) linearized and fragmented with *AscI* and PCR-derived genes of interest (in equimolar concentrations) that contained a 30 bp 3' and 5' overlap with the cut sites of the plasmid. Small DNA-'patches' were amplified from the intact pTYGS plasmid and used for recombination with integration sites which were not equipped with a gene of interest, so that the sequence at these sites was restored. The resulting mixture was incubated at 42 °C for 50 min, then centrifuged at 6000 × g for 1 min before the cell pellet was resuspended in 1 mL H<sub>2</sub>O. 200 µL were streaked on SM-URA agar plates and incubated at 30 °C until colonies appeared.

**2.4.2 Digest and Ligation.** Restriction enzyme digests of plasmids or PCR products were performed as indicated in the manufacturer's guidelines of the enzymes (New England Biolabs or Thermo Scientific). The reaction mixtures were incubated at the recommended temperature for up to 12 h. Heat inactivation of the restriction enzymes was done by heating the samples at 65 °C or 80 °C for 20 min. Purification of the digested DNA or plasmid was done using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) as described previously. Self-ligation of digested plasmids was prevented by dephosphorylation using shrimp alkaline phosphatase (SAP). SAP was added after the enzymatic digest, and the mixture was incubated for 1 h at 37 °C. Ligation of the plasmids and inserts (molar ratio of 1 : 5) was done with T4 DNA ligase (New England Biolabs) following the manufacturer's instructions. After overnight incubation of the ligation reaction at 4 °C heat inactivation was performed for 10 min at 65 °C and plasmids were transformed to *E. coli* Top10.

**2.4.3 Gateway® Technology.** For *in vitro* recombination of fungal vectors the Gateway LR Clonase enzyme mix II kit (Invitrogen) was used according to the manufacturer's instructions.

**Table S2** Fungal vectors constructed by yeast homologous recombination.

| Vector description                   | ONT for construction   | Template                                |
|--------------------------------------|--|---|
| pE-YA- <i>ctrR3</i>                  | HH54 + HH55 ( <i>ctrR3_1</i> ) <sup>a</sup><br>HH56 + HH57 ( <i>ctrR3_2</i> ) <sup>a</sup>   | <i>Hypomontagnella monticulosa</i> gDNA |
| pE-YA- <i>ctrL1</i>                  | HH168 + HH169 ( <i>ctrL1</i> ) <sup>b</sup>  | <i>Hypomontagnella monticulosa</i> gDNA |
| pTYGS- <i>argB-ctrR4</i>             | HH58 + HH59 ( <i>ctrR4_1</i> ) <sup>b</sup><br>HH60 + HH61 ( <i>ctrR4_2</i> ) <sup>b</sup>   | <i>Hypomontagnella monticulosa</i> gDNA |
| pTYGS- <i>ade-ctrACS-ctrR1-ctrL2</i> | HH62 + HH63 ( <i>ctrACS</i> ) <sup>b</sup><br>HH64 + HH65 ( <i>ctrR1</i> ) <sup>b</sup><br>HH66 + HH67 ( <i>ctrL2</i> ) <sup>b</sup> | <i>Hypomontagnella monticulosa</i> gDNA |
| pTYGS- <i>ade-ctrACS-ctrR1</i>       | HH62 + HH63 ( <i>ctrACS</i> ) <sup>b</sup><br>HH64 + HH65 ( <i>ctrR1</i> ) <sup>b</sup>  | <i>Hypomontagnella monticulosa</i> gDNA |
| pTYGS- <i>ade-ctrACS</i>             | HH62 + HH63 ( <i>ctrACS</i> ) <sup>b</sup>   | <i>Hypomontagnella monticulosa</i> gDNA |

<sup>a</sup> Annealing temperature was set to 55 °C in PCRs with OneTaq® and 57 °C for PCRs with Q5®.

<sup>b</sup> Annealing temperature was set to 58 °C in PCRs with OneTaq® and 60 °C for PCRs with Q5®.

**Table S3** Fungal vectors constructed by Gateway® cloning.

| Vector description             | Entry vector        | Destination vector       |
|--------------------------------|---------------------|--------------------------|
| pTYGS- <i>argB-ctrR3-ctrR4</i> | pE-YA- <i>ctrR3</i> | pTYGS- <i>argB-ctrR4</i> |

**Table S4** Fungal expression vectors obtained.

| Vector name | Vector description         | Supplier/ Reference |
|-------------|----------------------------|---------------------|
| KELI52A     | pTYGS- <i>sC-mfr1</i>      | ref 13              |
| KELI52B     | pTYGS- <i>sC-mfr2</i>      | ref 13              |
| KELI52C     | pTYGS- <i>sC-mfr1-mfr2</i> | ref 13              |

**Table S5** *E. coli* expression plasmids constructed in the project.

| Vector name       | Vector ID | ONT for construction | Template                  |
|-------------------|-----------|----------------------|---------------------------|
| pET28a(+)-CtrR1   | pHHII04   | HH128 + HH129        | synthetic intron-free DNA |
| pETM22-TrxA-CtrR1 | pHHII05   | HH10 + HH02          | synthetic intron-free DNA |
| pET28a(+)-Mfr1    | pHHII08   | HH170 + HH171        | KEL152A                   |
| pETM22-TrxA-Mfr1  | pHHII09   | HH172 + HH171        | KEL152A                   |
| pET28a(+)-Mfr2    | pHHII10   | HH173 + HH174        | KEL152B                   |
| pETM22-TrxA-Mfr2  | pHHII11   | HH175 + HH174        | KEL152B                   |
| pET28a(+)-SQSAo   | pHHII12   | pET28a_F + pET28a R  | synthetic intron-free DNA |

## 2.5 Transformation Protocols

**2.5.1 *Escherichia coli*.** Frozen chemically-competent *E. coli* cells were kept in the -80 °C freezer. Cells were thawed on ice and 1 µL of purified plasmid DNA or 10 µL of ligation mixture were added. The mixture was kept on ice for 30 min and subsequently the heat shock was performed for 45 seconds at 42 °C in the thermoblock. The cells were kept on ice for another 2 min before 100 µL of SOC medium was added. Afterwards, the cells were incubated at 37 °C for 45 min at 200 rpm. The cell suspension was spread on LB-agar plates treated with the correct antibiotic. Transformants were picked after overnight incubation at 37 °C.

**2.5.2 *Aspergillus oryzae* NSAR1.**<sup>14</sup> 50 mL GN-medium in a 250 mL baffled flask was inoculated with *A. oryzae* NSAR1 spores and mycelium from a DPY-plate and incubated at 28 °C and 110 rpm overnight for approx. 18 h. The mycelium was harvested by filtration with a miracloth filter, washed with approximately 50 mL 0.8 M NaCl and incubated in 10 mL sterilized protoplasting solution for 3-4 hours. The protoplasts were released from the hyphae by gentle pipetting with a wide bore pipette. The hyphae were removed by filtration through a miracloth filter. The filtrate containing the protoplasts was centrifuged at 3000 × g for 5 min at 4 °C and the visible pellet of protoplasts was resuspended in transformation solution 1 (100 µL per transformation). 500 - 4000 ng of prepared DNA (depending on the number of vectors used) was added to 100 µL protoplast suspension and incubated on ice for 2 min. Next, 1 mL of transformation solution 2 was added to the mixture and incubated at room temperature for 20 min. 5 mL of the respective selective soft-agar was added to the mixture and added to prepared plates with the respective selective agar. Plates were incubated at 28 °C for 4-6 days until colonies appeared. Colonies were picked and transferred to a fresh plate of selective agar. This was repeated two times before transformants were moved to DPY-plates.

## 2.6 Oligonucleotides

**Table S6** Oligonucleotides used in this study.

| ONT name    | Sequence                       |
|-------------|--------------------------------|
| T7 seq F    | TAATACGACTCACTATAGGG           |
| T7 seq R    | CTAGTTATTGCTCAGCGGT            |
| Peno plugF  | CTTCTTAAATATCGTTGTAAGTTCCTGA   |
| Peno plugR  | CGAAGTATATTGGGAGACTATAGCTACTAG |
| Padh plugF  | ATTCACCACTATTATCCACCCCTATAATA  |
| Padh plugR  | GAGACGAAACAGACTTTTTTCATCGTAAAA |
| PgdpA plugF | CTTTTCTTTTCTCTTTCTTTTCCCATCTTC |
| PgdpA plugR | TGACCTCCTAAAACCCAGTG           |
| M13 rev -29 | CAGGAAACAGCTATGACC             |
| M13 uni -21 | TGTAAAACGACGGCCAGT             |

**Table S7** Oligonucleotides designed in this study.

| ONT name | Sequence  |
|----------|---|
| HH54     | GCCAACTTTGTACAAAAAAGCAGGCTCCGCATGTTGGACATAGGAGACGG    |
| HH55     | GGAGTCCTTCTTGAACCATACTTCAAAGTCCTCATCC                 |
| HH56     | GGATGAGGACTTTGAAGTATGGTTCAAGAAGGACTCC                 |
| HH57     | TGCCAACTTTGTACAAGAAAGCTGGGTCGGTCACGACATCCCGATATTGC    |
| HH58     | TTCTTTCAACACAAGATCCCAAAGTCAAAGATGGCGATAGCACTTCAACG    |
| HH59     | CAGACATAAGTCTATTATGAGTCGGCCCATCTCC                    |
| HH60     | GGAGATGGGCCGACTCATAATAGGACTTATGTCTG                   |
| HH61     | TTTCATTCTATGCGTTATGAACATGTTCCCTAGAATCGCGCCGATGCGT     |
| HH62     | TTTCTTTCAACACAAGATCCCAAAGTCAAATGGAACAATACCTCGAAGTC    |
| HH63     | TTCACTTCTATGCGTTATGAACATGTTCCCTCTAGTTTCGCTAGCGGCTTAAC |
| HH64     | TAACAGCTACCCCGCTTGAGCAGACATCACATGGCTGCTGCTGATGTCGC    |
| HH65     | ACGACAATGTCCATATCATCAATCATGACCTTAGTCAAAGAAGACCCACGAGC |
| HH66     | TCGACTGACCAATTCCGCAGCTCGTCAAAGATGGAATACGTCGACGCTTTG   |
| HH67     | AGGTTGGCTGGTAGACGTCATATAATCATATTAAGCCGAGAGCTTCAAATC   |
| HH108    | CGGCAGCCATATGATGGAACAATACCTCGAAGTC                    |

|          |                                     |
|----------|-------------------------------------|
| HH109    | TGGTGGTGCTCGAGCTAGTTCGCTAGCGGCTTAAC |
| HH128    | GGCAGCCATATGATGGCTGCTGCTGATGTC      |
| HH129    | CTCGAGTGCGGCCGCTTAGTCAAAGAAGACCCA   |
| HH130    | AGGGGCCCATGGATATGGCTGCTGCTGATGTC    |
| HH170    | GGCAGCCATATGATGGCGACAGCTATCCTTC     |
| HH171    | TCGAGTGCGGCCGCTAAAGAACAACCAAGGAG    |
| HH172    | AGGGGCCCATGGATATGGCGACAGCTATCCTTC   |
| HH173    | GGCAGCCATATGATGGCGACTGCAACAACAAC    |
| HH174    | TCGAGTGCGGCCGCTAGTCAAAGAAAACCCAG    |
| HH175    | AGGGGCCCATGGATATGGCGACTGCAACAACAAC  |
| pET28a_F | CGGCAGCCATATGACCGAAACACAGAAAGCCTG   |
| pET28a_R | CTCGAGTGCGGCCGCTTAGGCTTCTTGACGTGC   |

## 2.7 Media, Agar and Solutions

**Table S8** Media and agar used in this study.

| Medium                              | Composition  |
|-------------------------------------|--|
| CZD/S1 agar                         | 3.50 % (w/v) Czapek Dox broth (Duchefa Biochemie)<br>18.22 % (w/v) D-sorbitol (= 1 M) (Roth)<br>0.10 % (w/v) ammonium sulfate (Roth)<br>0.15 % (w/v) L-methionine (Roth)<br>1.50 % (w/v) agar (Duchefa Biochemie)  |
| CZD/S1 soft agar                    | 3.50 % (w/v) Czapek Dox broth (Duchefa Biochemie)<br>18.22 % (w/v) D-sorbitol (= 1 M) (Roth)<br>0.10 % (w/v) ammonium sulfate (Roth)<br>0.15 % (w/v) L-methionine (Roth)<br>0.80 % (w/v) agar (Duchefa Biochemie)  |
| CZD/S1 agar without methionine      | 3.50 % (w/v) Czapek Dox broth (Duchefa Biochemie)<br>18.22 % (w/v) D-sorbitol (= 1 M) (Roth)<br>0.10 % (w/v) ammonium sulfate (Roth)<br>1.50 % (w/v) agar (Duchefa Biochemie)  |
| CZD/S1 soft agar without methionine | 3.50 % (w/v) Czapek Dox broth (Duchefa Biochemie)<br>18.22 % (w/v) D-sorbitol (= 1 M) (Roth)<br>0.10 % (w/v) ammonium sulfate (Roth)<br>0.80 % (w/v) agar (Duchefa Biochemie)  |
| DPY agar                            | 2.00 % (w/v) dextrin from potato starch (Sigma Aldrich)<br>1.00 % (w/v) polypeptone (Roth)<br>0.50 % (w/v) yeast extract (Duchefa Biochemie)<br>0.50 % (w/v) monopotassium phosphate (Roth)<br>0.05 % (w/v) magnesium sulfate hexahydrate (Sigma Aldrich)<br>2.50 % (w/v) agar (Duchefa Biochemie) |
| DPY medium                          | 2.00 % (w/v) dextrin from potato starch (Sigma Aldrich)<br>1.00 % (w/v) polypeptone (Roth)<br>0.50 % (w/v) yeast extract (Duchefa Biochemie)<br>0.50 % (w/v) monopotassium phosphate (Roth)<br>0.05 % (w/v) magnesium sulfate hexahydrate (Sigma Aldrich)  |
| GN medium                           | 2.00 % (w/v) D(+)-glucose monohydrate (Roth)<br>1.00 % (w/v) nutrient broth Nr. 2 from Oxoid (Fisher Scientific)   |
| LB agar                             | 0.5 % (w/v) yeast extract (Duchefa Biochemie)<br>1 % (w/v) tryptone (Duchefa Biochemie)<br>0.5 % (w/v) sodium chloride (Roth or VWR)<br>1.5 % (w/v) agar (Duchefa Biochemie)   |
| LB medium                           | 0.5 % (w/v) yeast extract (Duchefa Biochemie)<br>1 % (w/v) tryptone (Duchefa Biochemie)<br>0.5 % (w/v) sodium chloride (Roth or VWR)   |
| PDB medium                          | 2.40 % (w/v) potato dextrose broth (Formedium)   |
| SM-URA agar                         | 0.17 % (w/v) yeast nitrogen base (Sigma Aldrich)<br>0.50 % (w/v) ammonium sulfate (Roth)<br>2.00 % (w/v) D(+)-glucose monohydrate (Roth)<br>0.077 % (w/v) complete supplement mixture minus uracil (Sigma Aldrich)<br>2.50 % (w/v) agar (Duchefa Biochemie)  |
| SOC medium                          | 93.75 mL SOB Medium<br>1.25 mL magnesium chloride hexahydrate (Roth) (2 M)<br>5 mL D(+)-glucose monohydrate (Roth) (20 %)<br>For 100 mL SOC  |
| YPAD                                | 1.00 % (w/v) yeast extract (Duchefa Biochemie)<br>2.00 % (w/v) tryptone (Duchefa Biochemie)<br>2.00 % (w/v) D(+)-glucose monohydrate (Roth)<br>0.03 % (w/v) adenine (Roth)   |

YPAD Agar

1.00 % (w/v) yeast extract (Duchefa Biochemie)  
2.00 % (w/v) tryptone (Duchefa Biochemie)  
2.00 % (w/v) D(+)-glucose monohydrate (Roth)  
0.03 % (w/v) adenine (Roth)  
1.50 % (w/v) agar (Duchefa Biochemie)

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**Table S9** Solutions and buffers used in this study.

| Application                       | Buffer or solution     | Composition  |
|-----------------------------------|------------------------|--|
| <i>A.oryzae</i><br>transformation | Solution 1             | 0.8 M sodium chloride<br>10 mM calcium chloride<br>50 mM Tris-HCl, pH 7.5  |
|                                   | Solution 2             | 60 % (w/v) PEG 3350<br>0.8 M sodium chloride<br>10 mM calcium chloride<br>50 mM Tris-HCl, pH 7.5   |
|                                   | Protoplasting solution | 10 mg/ mL lysing enzymes from <i>Trichoderma harzianum</i> (Sigma Aldrich) in 0.8 M NaCl<br>or<br>VinoTaste® Pro (Lamothe-Abiet) in 0.8 M NaCl |
| Fungal genomic DNA extraction     | Lysis buffer           | Lysis Buffer (100 mL)<br>5 mL 1M Tris (pH 7.2)<br>10 mL 0.5 M EDTA<br>3 g SDS<br>1 mL mercaptoethanol  |
|                                   | KOAc solution          | 8 M potassium acetate  |
| Agarose gel electrophoresis       | 50x TAE buffer         | 2 M Tris acetate pH 8.3<br>0.05 M EDTA   |

## 3. *In Vitro* Methods

### 3.1 Protein Production and Purification

**3.1.1 *E. coli* Culture Conditions.** Seed cultures with 2-3 colonies of *E. coli* BL21 containing the required plasmid were grown overnight at 30 °C in 5-10 mL LB medium containing the appropriate antibiotics. 1 L LB medium containing the appropriate antibiotics were inoculated with 5 mL seed culture. Cultures were incubated at 37 °C. OD<sub>600</sub> was monitored, and temperature was reduced to 18 °C at an OD<sub>600</sub> of 0.25-0.30. Cells were induced with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD<sub>600</sub> of 0.6.

Cells were harvested after ~ 15-19 h of induction period. A 10 minute centrifugation step was performed at 4 °C and 6000 g. Cell pellets were transferred to fresh 50 mL tubes. At this point pellets could be stored in the -80 °C fridge. For further purification process 20 mL appropriate lysis buffer were added to tubes containing the cell pellet of 1 L cell culture.

Sonication was performed using SonoPlus Ultrasonic homogenizer from Bandelin with the following settings: Amplitude: 34 %. Sonication was done for 7 min. The cells were intermittently sonicated on ice for 10 s with 10 s allowed for cooling. The cell lysate was cleared by centrifugation at 20000 rpm for 40 min at 4 °C. The obtained supernatant was used in further purification steps. Samples of the supernatant and the pellet were used for SDS-PAGE.

**3.1.2 Ni-NTA Affinity Chromatography.** For manual Ni-NTA affinity chromatography 2 mL resin in ethanol (Ni-NTA agarose (cube biotech)) per liter of culture were washed 3 times with 2 mL of the respective lysis buffer per liter of culture. After washing, 2 mL lysis buffer was added to the pure resin. The appropriate amount of cell lysate was added to tubes containing the resin and tubes were placed at a rocker at 4 °C for 1 h. A 10 mL syringe was prepared with a PE-filter (CHROMABOND®) and the suspension was added to the syringe after the incubation time. Once the lysate ran through (collected as 'FT' in a tube underneath the column), 10 mL of lysis buffer were placed on the column. Remaining resin in the used tubes was additionally added to the column by washing the tubes using the buffer. Once this liquid ran through (collected as 'LB'), 10-15 mL of the wash buffer were placed on the column. After this ran through (collected as 'WB'), 7-10 mL of elution buffer 1 were added to the column and the running through liquid was collected as 'E1). Next, 2 mL of elution buffer 2 were added and the flowthrough was collected as E2. Otherwise Ni-NTA affinity chromatography was performed with the ÄKTA™ pure system and a HisTrap™ column (GE Healthcare; column volumes (CV) = 5 mL) following the manufacturer's instructions enabling for a linear gradient. Filtered and degassed lysis and elution buffer 2 were used.

Elutions were either kept at 4 °C overnight or further processed on the same day. Before storage at -80 °C or -20 °C, proteins were concentrated to a concentration of approximately 15 mg/mL (quantified with a DeNovix® DS-11+ Spectrophotometer, calculation of protein molecular weight and extinction coefficients was done using Benchling) and protein concentration was done with Amicon Ultra centrifugal filters from Millipore.

**3.1.3 SDS-PAGE.** Samples for analysis in SDS-PAGE were taken immediately after the corresponding fraction was generated and 4 : 1 diluted in 4 × Lämmli buffer, except for lysate and flow through samples which were 1 : 10 diluted in water before being diluted 4 : 1 in 4 × Lämmli buffer. 15 μL of each sample were added to the pockets of the gel and 4 μL of the ladder (Color Prestained Protein Standard, Broad Range (10 - 250 kDa) or Color Prestained Protein Standard, Broad Range (11-245 kDa)) were used. Electrophoresis was done using BioRad gel electrophoresis systems and gels with 12 % polyacrylamide separating and 5 % stacking gel layers at 50 mA for 45 min and 1 × SDS running buffer was used as buffer.

Gels were incubated in 30 mL Coomassie staining solution for 30 min or overnight. Destaining was done using Coomassie bleach solution for 30 min or overnight. Gels were scanned using the Molecular Imager Gel doc XR+ (Bio-Rad) system and digital images of the gels were created with the associated software or with mobile phone cameras.

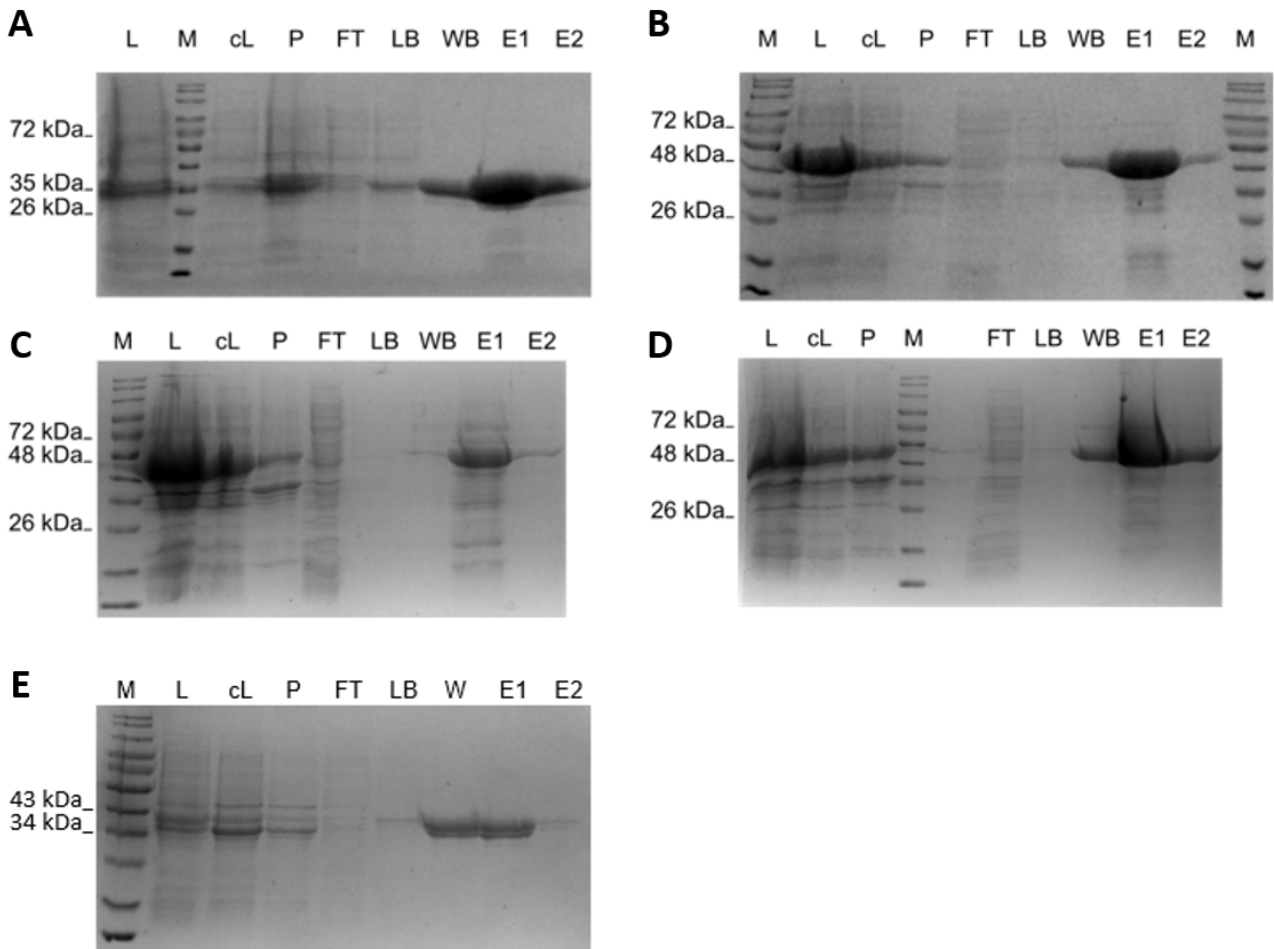
## 3.2 Expressed Gene Sequences

**Table S10** Gene sequences of expressed proteins. 6x-His Tag used for purification is labelled in bold letters.

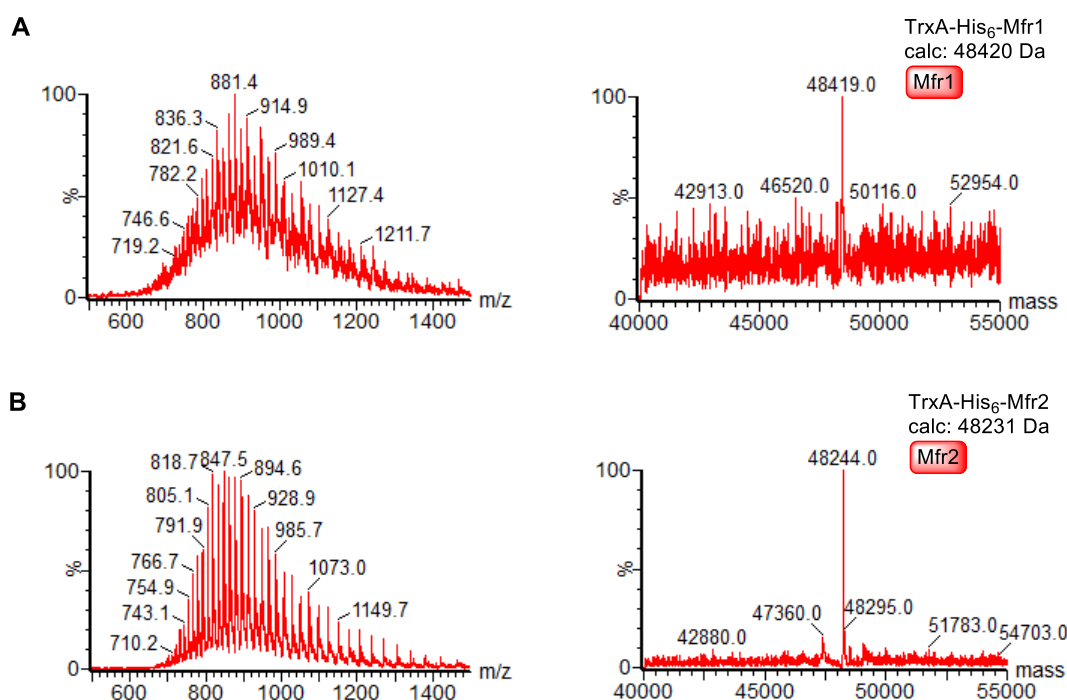
| Gene name                    | Sequence  |
|------------------------------|---|
| <i>SQSAo</i>                 | ATGGGCAGCAGCC <b>ATCATCATCATCAC</b> AGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGACCGA<br>AACACAGAAAGCCTGCTTTAAATTCCTGGATCTGACCAGCCGTAGCTTTAGCGCAGTTATTAAGAAGCTG<br>CATCCGGAAGTCTGCTGCCGGTTTGTGTTTTTATCTGGTTCTGCGTGGTCTGGATACCATTGAAGATG<br>ATACCAGCATTCCGCTGAAAACCAAAGAACCAGTCTGCGTGAATCAAAGATTATCTGGAACAGGATGG<br>TTGGACCTTTGATGGTAATCGTCCGGAAGAAAAAGATCGTGAAGTCTGGTTCAAGTTTCATAACGTGATTA<br>CCGAGTTTAAAAACATGAAACCTGCCTATCGCGAAATCGTGAAGATATACCCCGAAAATGGGTAATGG<br>CATGGCAGATTATTGTCGAAAGCAGAATTTGAAGATGCCAGCGTTAAAACCATCGAAGAGTATGATCTG<br>TATTGCTATTATGTTGCAGGTCTGGTTGGTGAAGGTCTGACCCGCTGTTTTGTTGAAGCCGAATTTGGTAA<br>TCCTGCACTGCTGAGCCGTCGCGCTCTGCATAAAAAGCATGGGTCTGTTTCTGAAAACCAACATTATT<br>CGTGATGTGCGCGAAGATCATGATGATGATCTATTTTTGGCCGAAAAGCTTTGGACAAATGTGTA<br>CCGAATTCGAGGACCTGTTAAACCGGAAAATCGCGAAACCGCACTGAATTGGTAGCGAAAATGGTTCT<br>GAATGCACTGGAACATGCAGAAGAATGTCTGTTCTATCTGGCAGGTCTGCGTGAACAGAGCGTTTTTAAC<br>TTTTGTGCAATTCGCGAGGCAATGGCAATTGCAACCCTGGAAGTGTTTTTCTGTAATCCGGATATGTTTGA<br>CCGCAACATCAAAATCACAAAGGTGAAGCATGTCAGCTGATGATGAAAAGCACCCAGAATCTGCATGTG<br>CTGTGTGATACCTTTTCGTGTTATGCACGTGATCCATAAAAAGAATACCCCGAAAATCCGAACTTCTCT<br>GAAAATTAGCATTGTGTGCGGCAAAATCGAGAAATTCATCGATACCATTTTTCCGCGAGCAGCCGAGCA<br>CAGGCAAAACTGAAAGTTCAGGGTGAAAAAGCGAAGCCGAAAAAGAAAAAGCACGTCAAGAAGCCTAA  |
| <i>ctrR1</i>                 | ATGGGCAGCAGCC <b>ATCATCATCATCAC</b> AGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGATGGC<br>TGCTGCTGATGTCGCTACTACCACCAGTGGCTCATTGGCATGTGGGCGGTACCCGGGACGGTGTCTAC<br>CGATGGATTCATTGACTGGGCCAGCGGTGCTACCAATGTCCACGACCCCAAGCAGGTTAAGATTGAGGC<br>CGAGGACATGCGAACCATGAACCCCGTCCCTTCTACATGAAGGATGGATACGGCATTGCCAAGCACAA<br>GACCTCTATACCCCGGAGCAGTTTCTGGCCGGCAACGACCCCGAAGGCAAGAAGTTCCTCGAGGATGT<br>CTACTACAAGGAGGTTGAGGAGCTGGTCAAGCAGATCACCGGCTCCAGCATCGTAGTCCCTACGTGTT<br>CCGCATCCCGCCAGAACAGCATGAAGGCGGGCGAGTTCATGGCCGCGAAGCTCTCTCACTCCGCTCTTCC<br>CGTCGCCACGTTGACCGTGACGCGATAACGGGCGAGGACGGTGTCCGCGAGGTGCTCGGCGAGCAG<br>GCCGACGAGCTCATCAAGCAGGGCAAGCGCTACGCCAGGTCAACATCTGGCGCAGCATCGACCAGCC<br>CATCAAGAAGTGGCCCTCTGCTTCAACACCAGCCGGCGTGGAGGGCTGGAAGTACGACTCCCACCT<br>CGCCCGCTTACCCACCAACGACCCCGCATTGCCATCCGAGGCGAGAAGAAGCACGACAGCGTCTG<br>TCAAGTACGACCCTAACTACAAGTACCCTACGTACGCAACCTCGACATCGACGAGGTCTCGTCTTCTC<br>GTGTTGACTCGGATGTGCTAAGGTGGTCCCCACGGTGTCTTCTGGGATGATGCCAGCCCTGACGA<br>TGCGCCACCCGACGATCCTCGAGTTGCTCGTGGGTCTCTTTGACTAA   |
| <i>ctrR1</i> (with TrxA-tag) | ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTAICTCAAAGCGGACGGGGCGA<br>TCCTCGTGATTTCTGGGCAGAGTGGTGCCTCGTGCAAAATGATCGCCCCGATTCTGGATGAAATCG<br>CTGACGAATATCAGGGCAAACCTGACCGTTGCAAAACTGAACATCGATCAAACCCCTGGCACTGCGCCGA<br>AATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGG<br>GTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCCGGTTCTGGTTCTGGCCATAT<br><b>GCACCATCATCATCAT</b> TTCTTCTGGTCTGGAAGTCTGTTCCAGGGGCCATGGATATGGTCTGCTGT<br>GATGTCGCTACTACCACCAGTGGCTCATTGGCATGTGGGCGGTACCCGGGACGGTGTACCCGATGG<br>ATTCATTGACTGGGCCAGCGGTGCTACCAATGTCCACGACCCCAAGCAGGTTAAGATTGAGGCCGAGGA<br>CATGCGAACCATGAACCCCGTCCCTTCTACATGAAGGATGGATACGGCATTGCCAAGCACAAAGACCTCT<br>ATCACCCCGAGCAGTTCCTGGCCGGCAACGACCCCGAAGGCAAGAAGTTCCTCGAGGATGTCTACTAC<br>AAGGAGGTTGAGGAGCTGGTCAAGCAGATCACCGCTCCAGCATCGTAGTCCCTACGTGTTCCCGCATC<br>CGCCAGAACAGCATGAAGGCGGGCGAGTTCATGGCCGCGAAGCTCTCTCACTCCGCTCTTCCCGTCTGC<br>CCAGTTGACCGTGACGCGATAACGGGCGAGGACGGTGTCCGCGAGGTGCTCGGCGAGCAGGCCGAC<br>GAGTCTCATCAAGCAGGGCAAGCGCTACGCCAGGTCAACATCTGGCGCAGCATCGACCAGCCCATCAA<br>GAAGTGGCCCTCTGCTTCACTCAACCACGCGCGTGGAGGGCTGGAAGTACGACTCCCACCTCGCC<br>GCGTCTACCCACCAACGACCCCGCATTGCCATCCGAGGCGAGAAGAAGCACGACAGCGTCTGCAAG<br>TAGGACCTAACTACAAGTACCCTACGTACGCAACCTCGACATCGACGAGGTCTCGTCTTCTCGTCTG<br>TCGACTCGGATGTGCTAAGGTGGTCCCCACGGTGTCTTCTGGGATGATGCCAGCCCTGACGATGCGC<br>CCACCCGACGATCCTCGAGTTGCTCGTGGGTCTCTTTGACTAA                |
| <i>mfr1</i> (with TrxA-tag)  | ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTAICTCAAAGCGGACGGGGCGA<br>TCCTCGTGATTTCTGGGCAGAGTGGTGCCTCGTGCAAAATGATCGCCCCGATTCTGGATGAAATCG<br>CTGACGAATATCAGGGCAAACCTGACCGTTGCAAAACTGAACATCGATCAAACCCCTGGCACTGCGCCGA<br>AATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGG<br>GTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCCGGTTCTGGTTCTGGCCATAT<br><b>GCACCATCATCATCAT</b> TTCTTCTGGTCTGGAAGTCTGTTCCAGGGGCCATGGATATGGCCAGC<br>TATCCTTCTTCTGACATCTGGCGTGATTGGGCTTTGGGATGGCACGACAGATGGTAAAGAGGGGTTTCAT<br>GGATTATGCAAAATGGTGATACGAACGTGAAACAGCCAAAGGAGTACGAGATTCAAGTCCACGACATTCTGA<br>AAGCTTGATCCTCAGCCTACTTTGCTTAAAGAACGGGTACGAGCTCGTGGATATTCCTACGGTAGTGACC<br>ATGAGCAATTTATTGAGTCTGGGAAGAGCGATGAAGGCAACGCGTACATCAAAGATGTCTACTCGCGGA<br>GTGCAAAAGGATCATAGAGGAGTCTCAGGGGGAGTTGATCTTATCTTCTGTTTCTTCCGATGCGT<br>GAACAGAAGGGCGAGAAAAGAGAGCACTACCAAGAAGCTCGGCAACATAGAGTCTAGATATGCTCCGCGT<br>CCCGTGGCGCATCTTGACCGAGACACCCCGACTGCCATCACGGTTCTAGAGGAGACTGTGGGCAAGGA<br>GAAGGCGCAGGAGCTGCTCTCGAAACCAAGCGCTGGGCCAGGTGAATGTATGGAGACCCATCGCGAA<br>ACCCCGCAACGATGTGGCCGCTTTGCTTCTGAATCATGATCGCATTCCACGTGGAAGTACGATACCCA<br>CGTTGGCCATGTCTGGTCTTTGAACGATCCAGAGTTTCGGACCCGCGGACAGAAGACCTATGACTGCGT<br>TGTCAAAACAGATGATCGATGACTACCACTACGTCAGTATCTTAGGCCAGAGGAATGCCTTGTCTTC<br>TGCTCTTTGACTCGATCCCGAAAATACGCTATGCCCATAGCGCTTTCTGGGACAACAATGTCCCGGCTG<br>ATGCCCGAATCGCCGTTCCATCGAGGTTGCTCCTTGGTGTCTTTAG |

*mfr2* (with TrxA-tag)

```
ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTAICTCAAAGCGGACGGGGCGA
TCCTCGTCTGATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCCCCGATTCTGGATGAAATCG
CTGACGAATATCAGGGCAAACCTGACCGTTGCAAACTGAACATCGATCAAAACCCCTGGCACTGCGCCGA
AATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGG
GTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCCGGTTCTGGTTCTGGCCATAT
GCACCATCATCATCATCATTCTTCTGGTCTGGAAGTTCTGTTCAGGGGGCCATGGATATGGCGACTGC
AACAACTACTTACACTCCACAACCTGGAACCGTCTACGTGCGTGTGGTACCACAGACGGCAAAGTAGGA
TATTACAACCACACGGATGATTCCACCAACGTGATAAGGAAGCCAATCCCGATTGAAGTGAAGATGCAC
GCACGCTAAGCAAGAGCCCAACGACAAAGGCAGAAGGCTATCAGCTAGTCAATTTCCACACTAAGATTCC
AGAGGAGCACTTTTTGAATTCGAACTTCCGGAGAACAAGGAACCTCATCGAAGAGGTGTACTTCCGACGAA
TGCCGTGCGCTGGTTCAAGAAGTCAACCGTGCAGCAGAAGCCTACCCGTACGTGTATCGCGTCAGGAAC
CAAGAGCAAAACGCCAAAGAATCCAATAAATCCAACCTCCACACCGACTTTGTCCCGATCGTGCACGTTG
ATCGCGACGATGTCACCTGCACCCAAAGACTGCGGGCCTCGCTTGGCGCGGAGAAAGCCGACATGCTA
TTGAGCAAATACAAGAGCTACGGATCGATCAATGTGTGGCGTCCAGTCAAGAACATGGTGCAGAAGTGG
CCTCTGATGCTCGTAGACCACAAGTCTATTGAGGATTGGGATTACAGCACGCACATGTTACCTTTGATT
CTAGCAACGACGAAAGAGTTGCAACTCGTGGGGCGAAAGAGCATGAAACGATCCTCACGCATGATAAGA
GATATCGATACATCTATGCGTCCGATATGACGCCGGAAGAAGCTTGGCTGTTCTTTGCTTTTCACTCGGA
CCCAGCGTTGGGGATACCACATGGGGCTTCTGGGATGATCCACGAAGGAAGAAGCTTTGACGAGATG
CTCTATTGAAGTGCAGGATCTGGGTTTTCTTTGACTAG
```



**Figure S3** Production of recombinant proteins from *E. coli* BL21. Bands in the SDS-PAGE were visualized by Coomassie staining. **A**, purification of His<sub>6</sub>-CtrR1 by Ni-NTA; **B**, purification of TrxA-His<sub>6</sub>-CtrR1 by Ni-NTA; **C**, purification of TrxA-His<sub>6</sub>-Mfr1 by Ni-NTA; **D**, purification of TrxA-His<sub>6</sub>-Mfr2 by Ni-NTA; **E**, purification of SQSAo by Ni-NTA. M: marker (Color Pres-tained Protein Standard, Broad Range, 10-250 kDa) L: protein sample prepared from lysate, cL: protein sample prepared from cleared lysate, P: protein sample prepared from insoluble pelleted fraction, FT: flow-through from Ni-NTA chromatog-raphy, LB: protein sample of wash with lysis buffer, WB: protein sample of wash with wash buffer (50 mM imidazole; 25 mM for TrxA-His<sub>6</sub>-Mfr1 and TrxA-His<sub>6</sub>-Mfr2), E1: protein sample of elution 1 (150 mM imidazol), E2: protein sample of elution 2 (500 mM imidazol).



**Figure S4** Mass spectrometry of TrxA-His<sub>6</sub>-Mfr1 and TrxA-His<sub>6</sub>-Mfr2 in H<sub>2</sub>O at a concentration of 1 mg/mL. **A**, mass spectrum of TrxA-His<sub>6</sub>-Mfr1 and zero-charge spectrum deconvoluted by MaxEnt; **B**, mass spectrum of TrxA-His<sub>6</sub>-Mfr2 and zero-charge spectrum deconvoluted by MaxEnt.

### 3.3 In Vitro Preparation of Alkyl Citrates

**3.3.1 Analytical *In Vitro* Preparation of 8, 9, 12, 13 and 14.** (TrxA-)His<sub>6</sub>-tagged NHI oxygenases were incubated at a concentration of 10 μM in a buffer containing 50 mM Tris, 0.1 mM FeSO<sub>4</sub>, 8 mM ascorbate, 5 mM α-ketoglutarate and 2.5 mM CJ-13,982 **7** (stock solution diluted in DMSO) for 2 h at 28 °C. Optionally, 75 % of the amount of the initially added NHI oxygenase solution were added sequentially every 20 min over the course of the reaction. After 2 h of reaction time, one volume of acetonitrile was added to the reaction, the mixture was vortexed, the proteins precipitated by centrifugation and clear supernatant was analyzed by LCMS.

**3.3.2 Preparative *In Vitro* Synthesis of 7-Hydroxy-CJ-13,982 **14**.** A volume of 5 mL reaction buffer containing 50 mM Tris, 0.1 mM FeSO<sub>4</sub>, 8 mM ascorbate, 5 mM α-ketoglutarate and 2.75 mM CJ-13,982 **7** (stock solution diluted in DMSO) and 10 μM TrxA-His<sub>6</sub>-Mfr2 was incubated for 36 hours. The reaction mixture was stirred at 28 °C for 36 h and 75 % of the amount of the initially added NHI oxygenase solution were added sequentially every hour during day-time (8am - 8pm) to the mixture. After the 36 h the mixture was kept at RT for 12 more days without shaking. 0.6 mg (12 %) of **12** were isolated from the reaction mixture by preparative LCMS using a linear gradient from 40 % to 90 % CH<sub>3</sub>CN.

## 3.4 Buffers

**Table S11** Buffers and solutions used in this study.

| Application  | Buffer or solution               | Composition   |
|--|----------------------------------|---|
| Protein purification<br>Tris buffer system<br>(TrxA-Mfr1, TrxA- Mfr2, CtrR1, TrxA-<br>CtrR1) | Lysis buffer                     | 50 mM Tris/HCl, pH 7.5<br>150 mM NaCl<br>10 mM imidazole<br>10 % (v/v) glycerol                               |
|  | Wash Buffer                      | 0.2 mL/ 0.5 mL/ 1 mL elution buffer 2<br>ad 10 mL lysis buffer  |
|  | Elution buffer 1                 | 3.5 mL lysis buffer<br>1.5 mL elution buffer 2  |
|  | Elution buffer 2                 | 50 mM Tris/HCl, pH 7.5<br>150 mM NaCl<br>500 mM imidazole<br>10 % (v/v) glycerol                              |
|  | Storage buffer                   | 50 mM Tris/HCl, pH 7.5<br>150 mM NaCl<br>10 % (v/v) glycerol  |
|  | SDS PAGE                         | 10x SDS buffer  |
| Coomassie dye  |                                  | 25 % (v/v) acetic acid<br>10 % (v/v) isopropanol<br>0.1 % (w/v) coomassie                                     |
| Coomassie bleach   |                                  | 25 % (v/v) acetic acid<br>10 % (v/v) isopropanol  |
| 4x Lämmli  |                                  | 10 % (v/v) $\beta$ -mercaptoethanol<br>0.25 % (w/v) bromophenol blue<br>30 % glycerol<br>0.25 % xylene cyanol |
| <i>In vitro</i> reactions  | Tris assay buffer                | 50 mM Tris/ HCl, pH 7.5   |
| <i>In vitro</i> reactions  | Sodium phosphate assay<br>buffer | 50 mM sodium phosphate buffer, pH 7.5   |

## 4. General Chemical Methods

### 4.1 Extraction of Secondary Metabolites

Liquid fungal cultures were homogenized with a blender and filtered by vacuum filtration. The filtrate was acidified with 2 M HCl to pH 2-4. The media was extracted twice with an equal amount of ethyl acetate, or three times if the extract was used for preparative LCMS. The organic layers were dried over anhydrous MgSO<sub>4</sub>. Solvent was removed with a rotary evaporator. The extract was then dissolved in an appropriate amount of solvent (methanol, acetonitrile, H<sub>2</sub>O) filtered with glass wool and analyzed by LCMS.

### 4.2 Protein Mass Spectrometry

MS data was obtained using a Quattro API mass spectrometer Waters system, which consists of a Waters 2767 autosampler and a Waters 2545 pump. Proteins were measured by direct injection (no column installed) of a protein solution (approx. 1 mg/mL) in a 50/50 mix of water/acetonitrile (0.05 % formic acid) at 0.2 mL/min. The mass spectrometer scanned over a range of 500 - 1500 *m/z*. Deconvolution of the protein peak area was done with MaxEnt<sup>®</sup> to give the molecular weight.<sup>15,16</sup>

### 4.3 High Resolution Mass Spectrometry

High Resolution Mass Spectrometry (HRMS) was performed with a Q-TOF Premier mass spectrometer (Waters) coupled to an Acquity UPLC system (Waters) by the MS department of the department of Organic Chemistry, Leibniz University Hannover.

### 4.4 Liquid Chromatography Mass Spectrometry

**4.4.1 Analytical Liquid Chromatography Mass Spectrometry.** Analytical liquid chromatography mass spectrometry (LCMS) data was obtained using a Waters LCMS system, which consists of a Waters 2767 autosampler, Waters 2545 pump, a Phenomenex Kinetex column (2.6 μm, C<sub>18</sub>, 100 Å, 4.6 × 100 mm) equipped with a Phenomenex Security Guard precolumn (Luna, C<sub>5</sub>, 300 Å). The system was used at a flow rate of 1 mL/min. Detection was carried out by a diode array detector (Waters 2998) in the range 210 to 600 nm and an ELSD detector (Waters 2424) together with a mass spectrometer, Waters SQD-2 mass detector, operating simultaneously in ES<sup>+</sup> and ES<sup>-</sup> modes between 150 and 1000 *m/z*. Typically, a solvent gradient was run over 15 min starting at 10 % acetonitrile/ 90 % HPLC grade water (0.05 % formic acid) and ramping to 90 % acetonitrile.

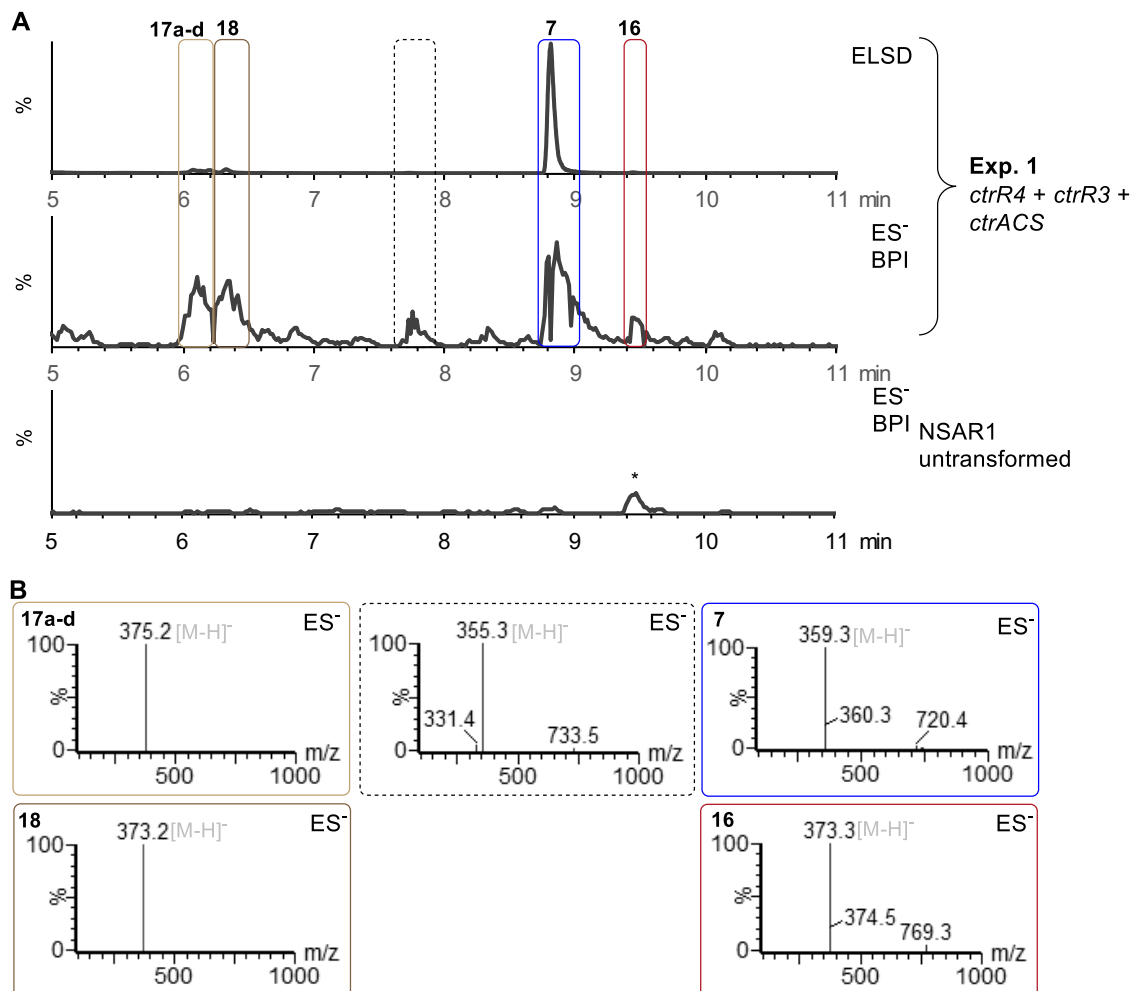
**4.4.2 Preparative Liquid Chromatography Mass Spectrometry.** Preparative LCMS was used to purify chemoenzymatically and chemically synthesized compounds or compounds from microbial extracts. The used system is a Waters mass-directed auto-purification system, which consists of a Waters 2767 autosampler, Waters 2545 pump, a Phenomenex Kinetex Axia column (5 μm, C<sub>18</sub>, 100 Å, 21.2 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna, C<sub>5</sub>, 300 Å). The system was used at a flow rate of 20 mL/min. Typically, a solvent gradient was run over 15 min starting at 10 % acetonitrile (solvent B, with 0.045 % formic acid) and 90 % HPLC grade water (solvent A; with 0.05 % formic acid) and ramping to 90 % solvent B. The post-column flow was split (100 : 1) and the minority flow was made up with HPLC-grade (1 : 1 solvent A/B) to 1.0 mL/ min for simultaneous analysis by diode array (Waters 2998) in the range 210 to 600 nm, an ELSD

detector (Waters 2424) together with a mass spectrometer, Waters SQD-2 mass detector, operating simultaneously in ES<sup>+</sup> and ES<sup>-</sup> modes between 150 and 1000 *m/z*. Purification was done mass-directed. Compounds were fractionated and collected into glass test tubes and combined fractions were lyophilized after residual solvent was removed *in vacuo*. Compounds were stored at - 20 °C. Mass data was analyzed with MassLynx™ (Waters).

## 4.5 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) measurements were performed by the NMR department of Organic Chemistry department, Leibniz University Hannover. NMR data were recorded using either a Bruker Ultrashield 500 (<sup>1</sup>H 500 MHz, <sup>13</sup>C 125 MHz), Bruker Ascend 600 (<sup>1</sup>H 600 MHz, <sup>13</sup>C 150 MHz), Bruker Avance III 600 (<sup>1</sup>H 600 MHz, <sup>13</sup>C 150 MHz) spectrometer. Chemical shifts are shown in parts per million (ppm) and referenced to solvents. The coupling constants *J* are given in Hz.

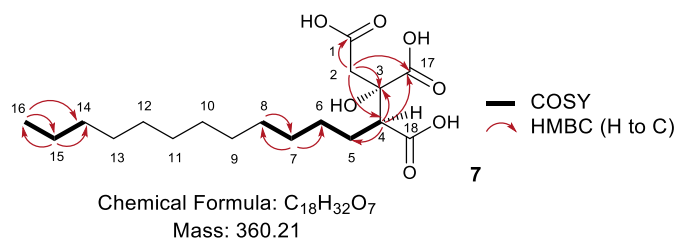
## 5. EXPERIMENT 1 (*ctrR4* + *ctrR3* + *ctrACS*) *in vivo*



**Figure S5** Heterologous expression of the structural genes of the *ctr* BGC: *ctrR4*, *ctrR3*, *ctrACS*. **A**, LCMS chromatograms of extracts of *A. oryzae* NSAR1 transformant and untransformed control strain (arbitrary units, ELSD smoothed in Excel, \* = unrelated compound); **B**, ES<sup>-</sup> spectra of compounds detected in *A. oryzae* NSAR1 transformants.

## 5.1 Characterisation of CJ-13,982, 7

**Table S12** Chemical shifts of CJ-13,982 7 in CD<sub>3</sub>OD (600 MHz).

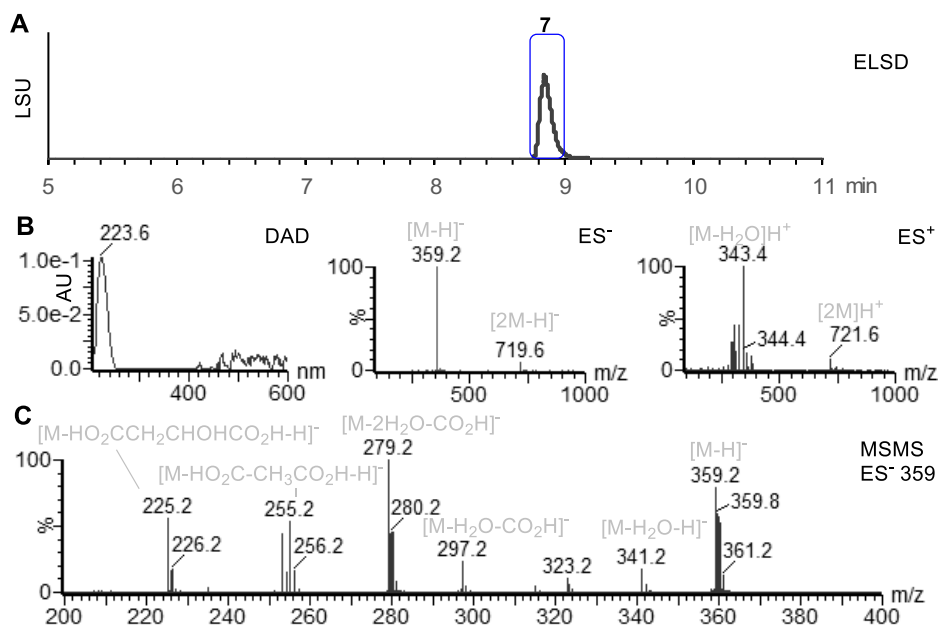


| Experimentally observed |                                  |                  |                 |             |          | Compound 7 literature in CD <sub>3</sub> OD <sup>17</sup> |                  |
|-------------------------|----------------------------------|------------------|-----------------|-------------|----------|---|------------------|
| Pos.                    | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY     | $\delta_H$ / ppm (mult, J in Hz)                          | $\delta_C$ / ppm |
| 1                       | -                                | 173.9            | CO              | -           | -        | -   | 174.5            |
| 2a                      | 3.07 (d, 16.4)                   | 42.3             | CH <sub>2</sub> | 1, 3, 17    | 2b       | 3.03 (d, 16.3)  | 43.1             |
| 2b                      | 2.69 (d, 16.4)                   | 42.3             | CH <sub>2</sub> | 1, 3, 4, 17 | 2a       | 2.69 (d, 16.3)  | 43.1             |
| 3                       | -                                | 76.8             | C               | -           | -        | -   | 77.0             |
| 4                       | 2.65 (dd, 11.8, 3)               | 54.8             | CH              | 2, 3, 5, 18 | 5, 6     | 2.65 (m)  | 54.5             |
| 5a                      | 1.81 (m)                         | 28.1             | CH <sub>2</sub> | 4, 6, 18    | 4, 5b, 6 | 1.81 (m)  | 28.3             |
| 5b                      | 1.49 (m)                         | 28.1             | CH <sub>2</sub> | 6, 18       | 4, 5a, 6 | 1.49 (m)  | 28.3             |
| 6                       | 1.3 (m)                          | 28.8             | CH <sub>2</sub> | 5, 7-14     | 5b, 7-14 | 1.17-1.39 (m)   | 28.8             |
| 7 <sup>†</sup>          | 1.3 (m)                          | 30.5             | CH <sub>2</sub> | 6, 8-14     | 6-14     | 1.17-1.39 (m)   | 30.4             |
| 8 <sup>†</sup>          | 1.3 (m)                          | 30.5             | CH <sub>2</sub> | 7-14        | 7-14     | 1.17-1.39 (m)   | 30.5             |
| 9 <sup>†</sup>          | 1.3 (m)                          | 30.5             | CH <sub>2</sub> | 7-14        | 7-14     | 1.17-1.39 (m)   | 30.6             |
| 10 <sup>†</sup>         | 1.3 (m)                          | 30.7             | CH <sub>2</sub> | 7-14        | 7-14     | 1.17-1.39 (m)   | 30.7             |
| 11 <sup>†</sup>         | 1.3 (m)                          | 30.7             | CH <sub>2</sub> | 7-14        | 7-14     | 1.17-1.39 (m)   | 30.7             |
| 12 <sup>†</sup>         | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14     | 1.17-1.39 (m)   | 30.7             |
| 13 <sup>†</sup>         | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14     | 1.17-1.39 (m)   | 30.8             |
| 14                      | 1.3 (m)                          | 33.1             | CH <sub>2</sub> | 7-14, 15    | 7-14     | 1.17-1.39 (m)   | 33.1             |
| 15                      | 1.3 (m)                          | 23.7             | CH <sub>2</sub> | 14, 16      | 14, 16   | 1.17-1.39 (m)   | 23.7             |
| 16                      | 0.90 (t, 7)                      | 14.4             | CH <sub>3</sub> | 14, 15      | 15       | 0.90 (t, 6.9)   | 14.4             |
| 17                      | -                                | 176.7            | CO              | -           | -        | -   | 177.5            |
| 18                      | -                                | 176.2            | CO              | -           | -        | -   | 177.0            |

<sup>†</sup> These signals are indistinguishable from each other

**Table S13** Optical rotation of CJ-13,982 7 in CD<sub>3</sub>OD.

| Experimentally observed                | Compound 7 literature in acetone <sup>18</sup> | Compound 7 literature in acetone <sup>19</sup> | Compound 7 literature in acetone <sup>17</sup> |
|--|--|--|--|
| $[\alpha]_D^{23}$ -12.7 (c 0.22, MeOH) | $[\alpha]_D^{23}$ -14.9 (c 0.52, acetone)      | $[\alpha]_D^{26}$ -18.3 (c 2.6, acetone)       | $[\alpha]_D^{25}$ -14.1 (c 0.25, acetone)      |



**D**

**Elemental Composition Report**

**Single Mass Analysis**

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction : Off

Number of isotopes peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

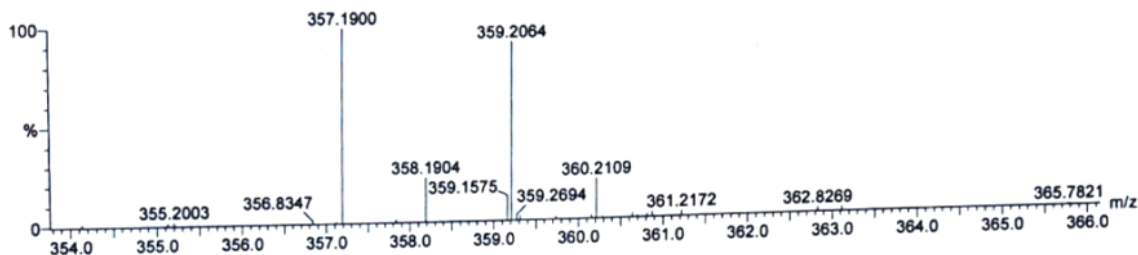
75 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-30 H: 0-50 O: 0-8 Na: 0-1

Heinemann QToF Premier HAB321  
HH II07 1357 (13.854) AM (Cen,4, 90.00, Ht,10000.0,554.26,0.70,LS 10)

1: TOF MS ES-  
1.27e+002



| Mass     | Calc. Mass | mDa  | PPM  | DBE  | i-FIT | i-FIT (Norm) | Formula       |
|----------|------------|------|------|------|-------|--------------|---------------|
| 359.2064 | 359.2070   | -0.6 | -1.7 | 3.5  | 24.7  | 0.6          | C18 H31 O7    |
|          | 359.2046   | 1.8  | 5.0  | 0.5  | 25.1  | 1.0          | C16 H32 O7 Na |
|          | 359.2011   | 5.3  | 14.8 | 12.5 | 27.0  | 2.9          | C25 H27 O2    |

**Figure S6** LCMS characterisation and structure elucidation of **7**. **A**, ELSD trace of **7** (arbitrary units; smoothed in Excel); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **7**; **C**, MSMS data obtained for **7**; **D**, HRMS analysis and calculated molecular formula of **7**.

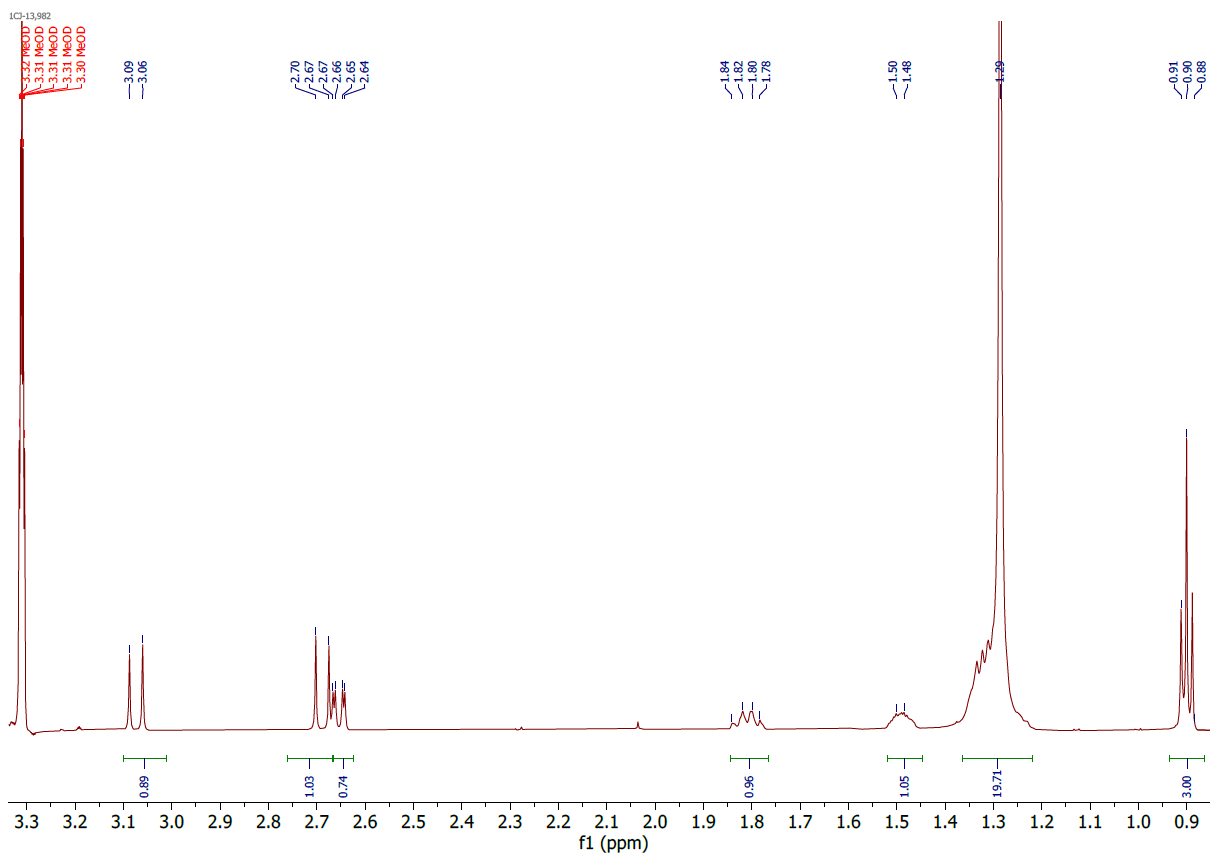


Figure S7 <sup>1</sup>H NMR of 7 in CD<sub>3</sub>OD (600 MHz). See Table S12 for assignment.

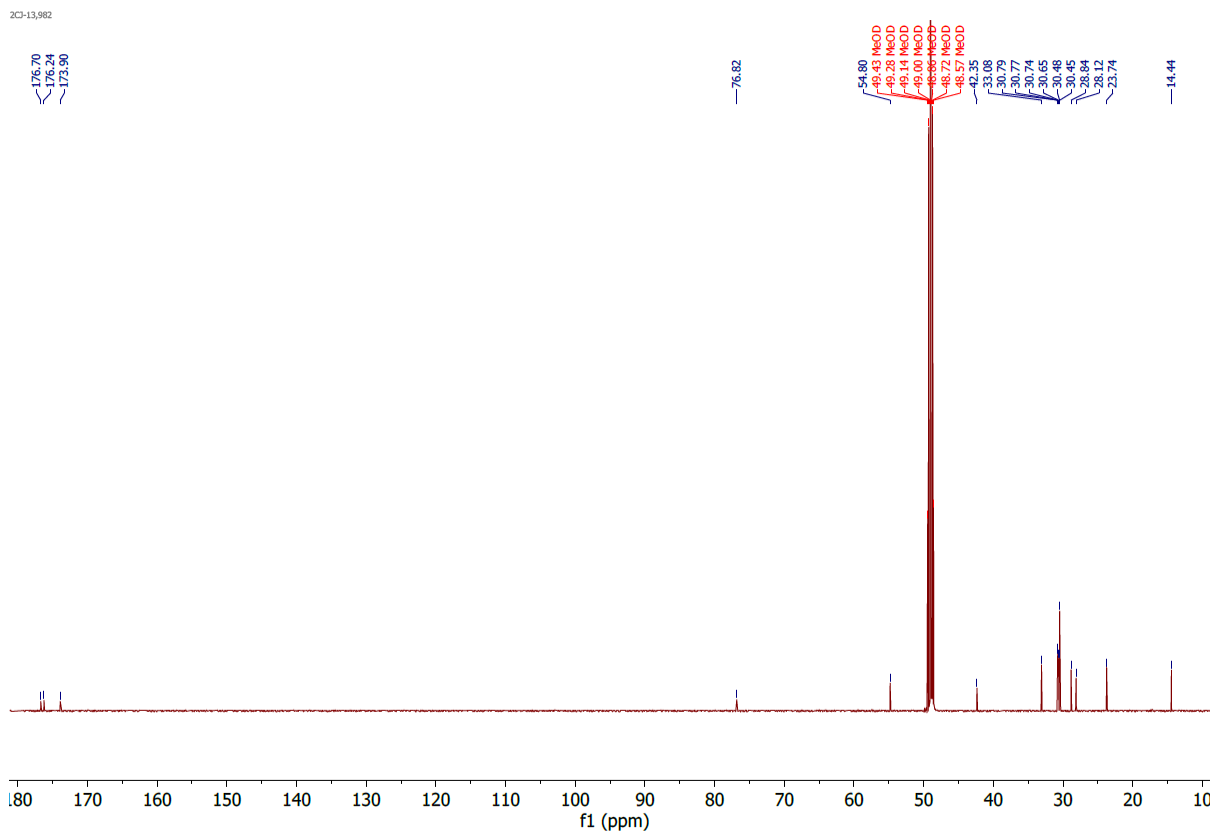


Figure S8 <sup>13</sup>C NMR of 7 in CD<sub>3</sub>OD (150 MHz). See Table S12 for assignment.

3C-13,982

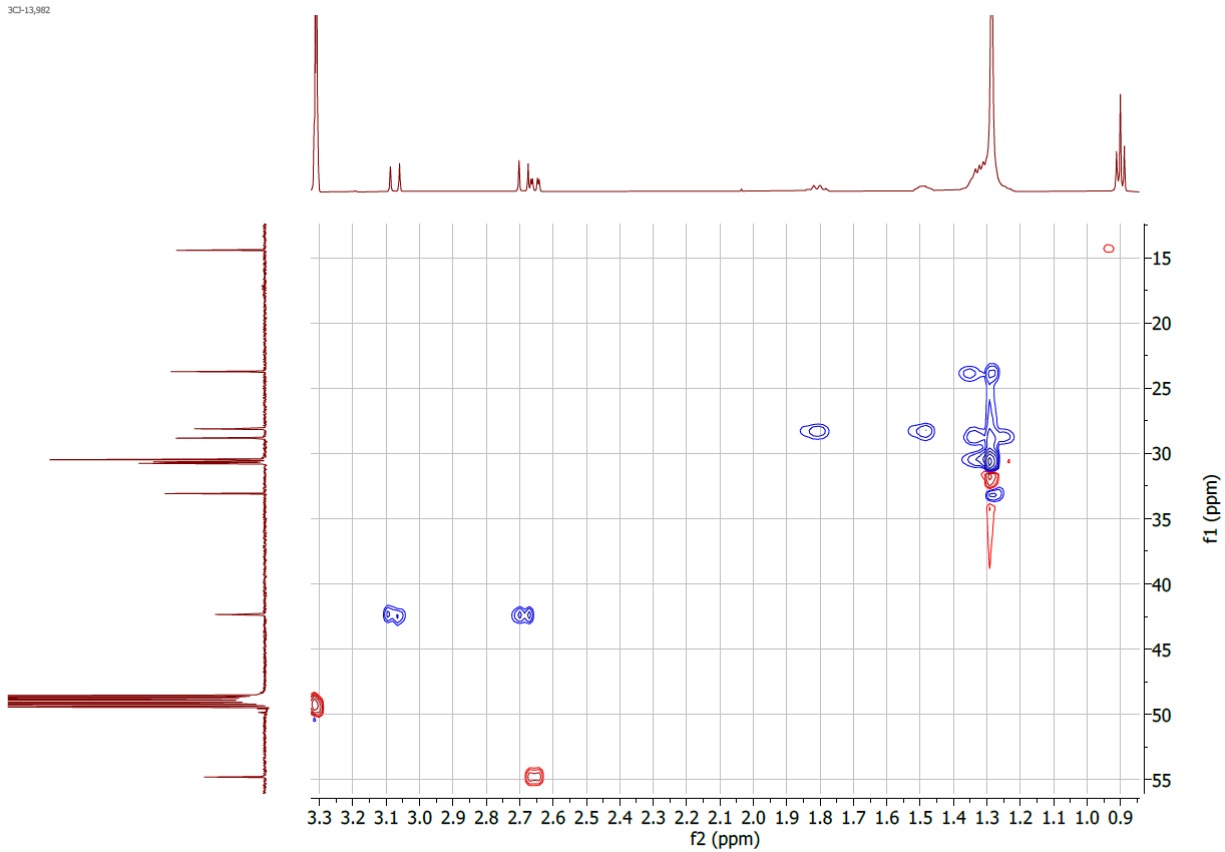


Figure S9 HSQC NMR of 7 in CD<sub>3</sub>OD.

4C-13,982

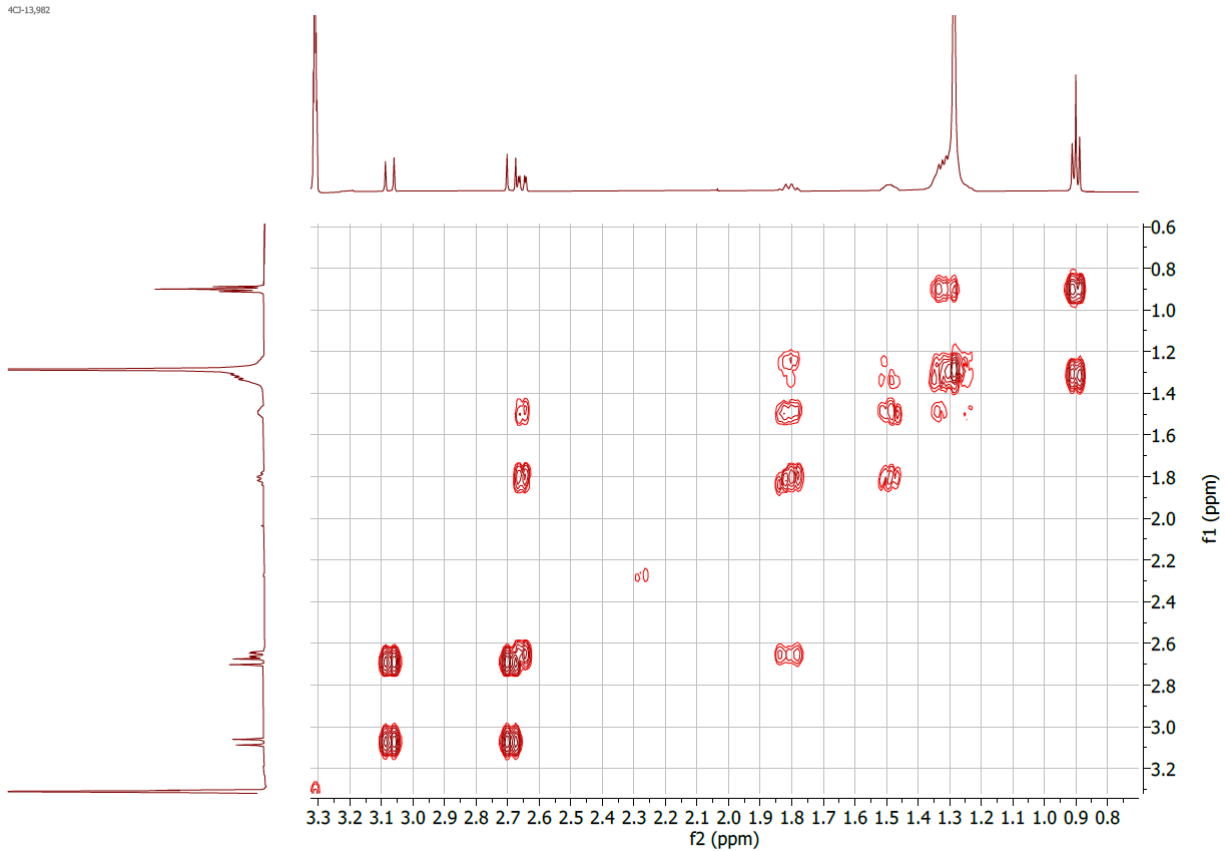


Figure S10 <sup>1</sup>H, <sup>1</sup>H COSY NMR of 7 in CD<sub>3</sub>OD.

5C-13,982

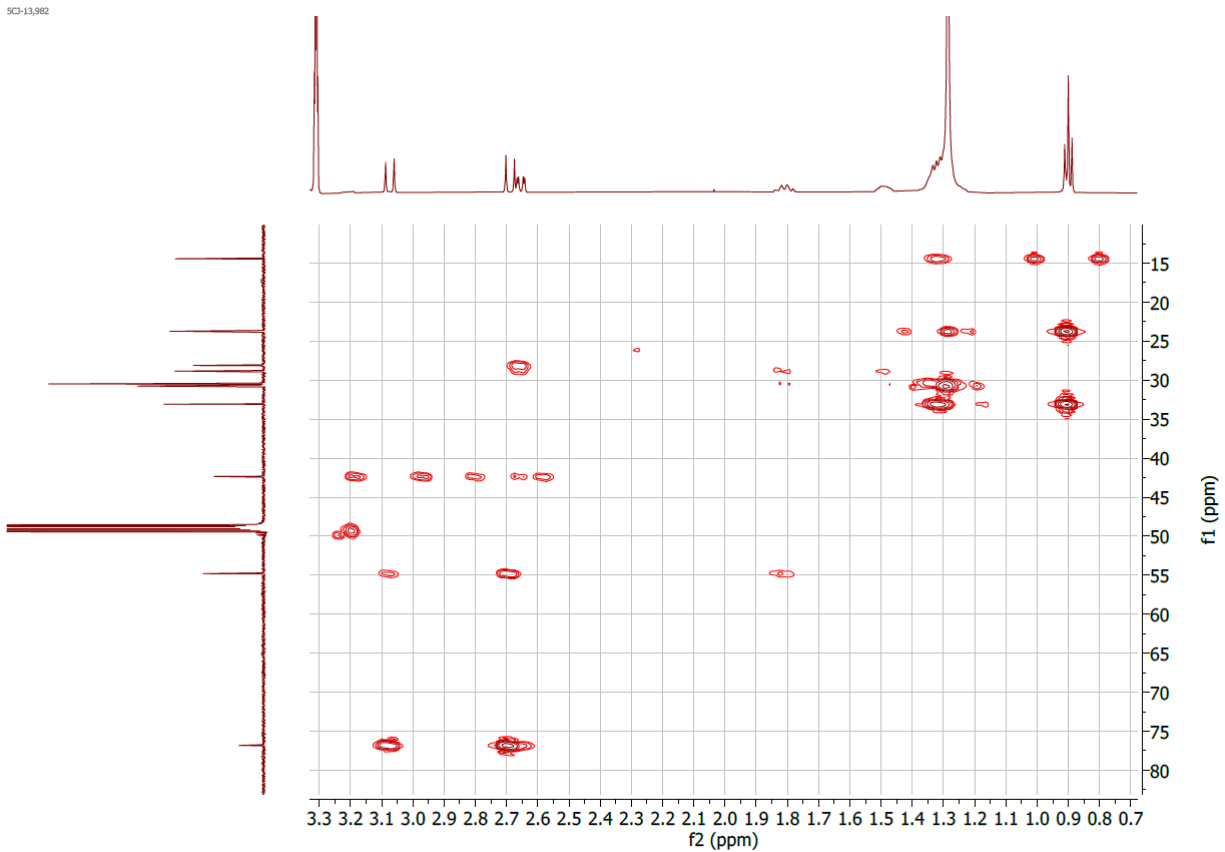


Figure S11 HMBC NMR of 7 in CD<sub>3</sub>OD.

6C-13,982

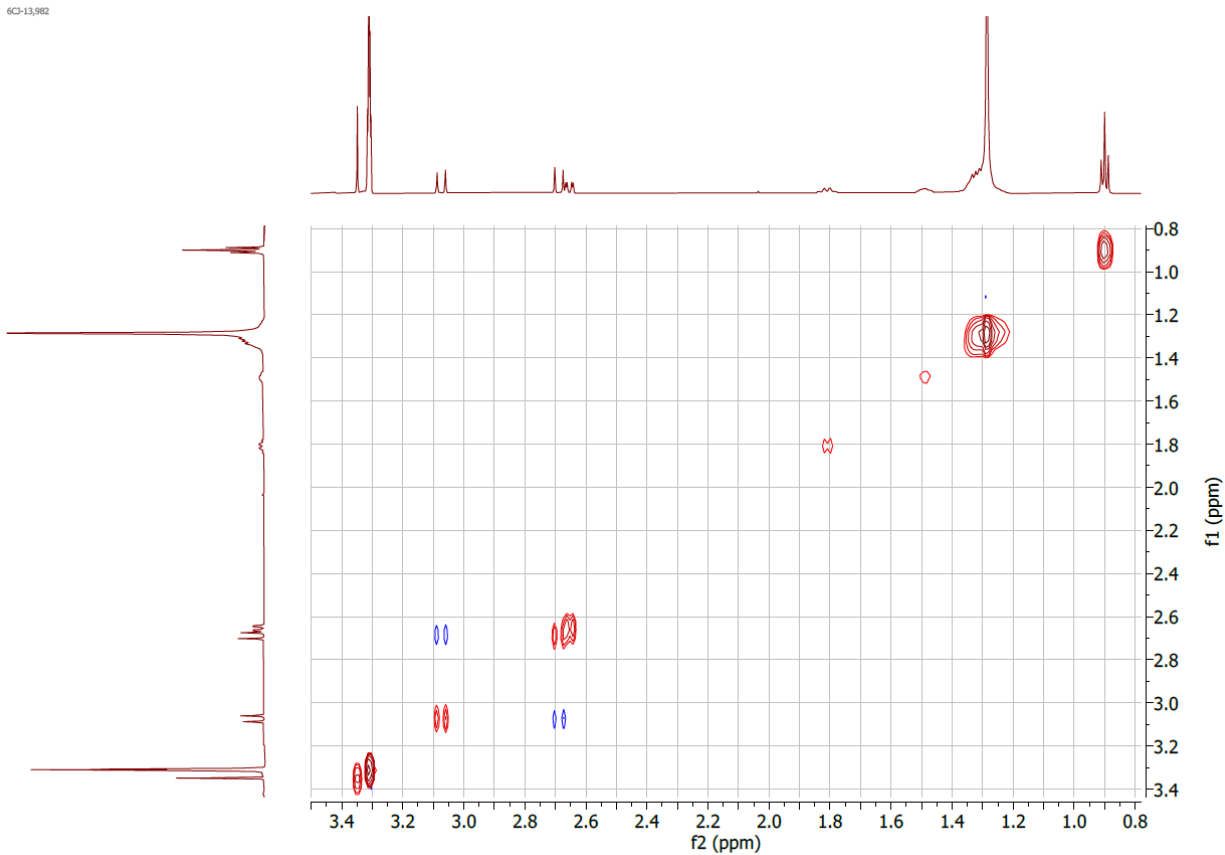
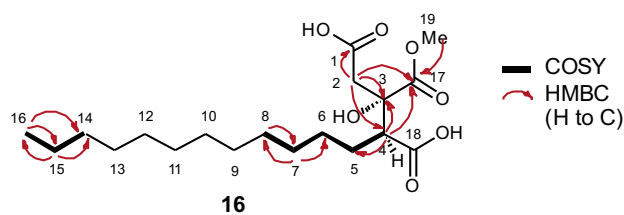


Figure S12 NOESY NMR of 7 in CD<sub>3</sub>OD.

## 5.2 Characterisation of CJ-13,982 17-methyl ester, 16

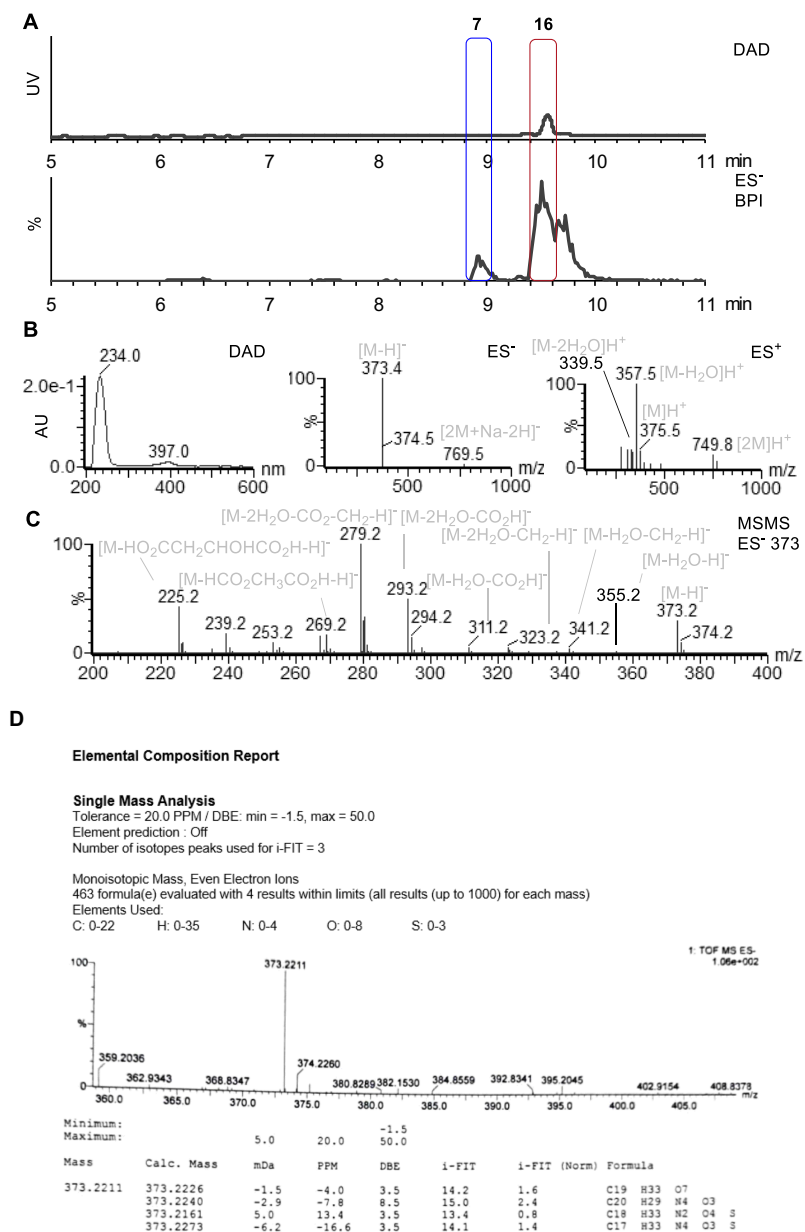
Table S14 Chemical shifts of 16 in CD<sub>3</sub>OD (600 MHz).



Chemical Formula: C<sub>19</sub>H<sub>34</sub>O<sub>7</sub>  
Exact Mass: 374.23

| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY     |
|-----------------------|----------------------------------|------------------|-----------------|-------------|----------|
| <b>1</b>              | -                                | 174.5            | CO              | -           | -        |
| <b>2a</b>             | 3.03 (d, 16.1)                   | 44.3             | CH <sub>2</sub> | 1, 3,       | 2b       |
| <b>2b</b>             | 2.64 (d, 16.1)                   | 44.3             | CH <sub>2</sub> | 1, 3, 4, 17 | 2a       |
| <b>3</b>              | -                                | 77.5             | C               | -           | -        |
| <b>4</b>              | 2.70 (dd, 11.7, 2.8)             | 54.4             | CH              | 3, 5, 18    | 5, 6     |
| <b>5a</b>             | 1.77 (m)                         | 28.6             | CH <sub>2</sub> | -           | 4, 5b, 6 |
| <b>5b</b>             | 1.40 (m)                         | 28.6             | CH <sub>2</sub> | -           | 5a, 6    |
| <b>6</b>              | 1.37 (m)                         | 28.9             | CH <sub>2</sub> | 5           | 5, 7     |
| <b>7<sup>†</sup></b>  | 1.3 (m)                          | 30.5             | CH <sub>2</sub> | 6, 8-14     | 6-14     |
| <b>8<sup>†</sup></b>  | 1.3 (m)                          | 30.6             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>9<sup>†</sup></b>  | 1.3 (m)                          | 30.6             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>10<sup>†</sup></b> | 1.3 (m)                          | 30.7             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>11<sup>†</sup></b> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>12<sup>†</sup></b> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>13<sup>†</sup></b> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>14</b>             | 1.27 (m)                         | 33.1             | CH <sub>2</sub> | 7-14        | 7-14     |
| <b>15</b>             | 1.3 (m)                          | 23.7             | CH <sub>2</sub> | 14, 16      | 14, 16   |
| <b>16</b>             | 0.90 (app. q, 7.1)               | 14.4             | CH <sub>3</sub> | 14, 15      | 15       |
| <b>17</b>             | -                                | 175.6            | CO              | -           | -        |
| <b>18</b>             | -                                | 177.4            | CO              | -           | -        |
| <b>19</b>             | 3.73 (s)                         | 52.8             | CH <sub>3</sub> | 17          | -        |

<sup>†</sup> These signals are indistinguishable from each other.



**Figure S13** LCMS characterisation and structure elucidation of **16**. **A**, DAD and ES<sup>-</sup> traces of **16** (arbitrary units); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **16**; **C**, MSMS analysis of **16**; **D**, HRMS analysis.

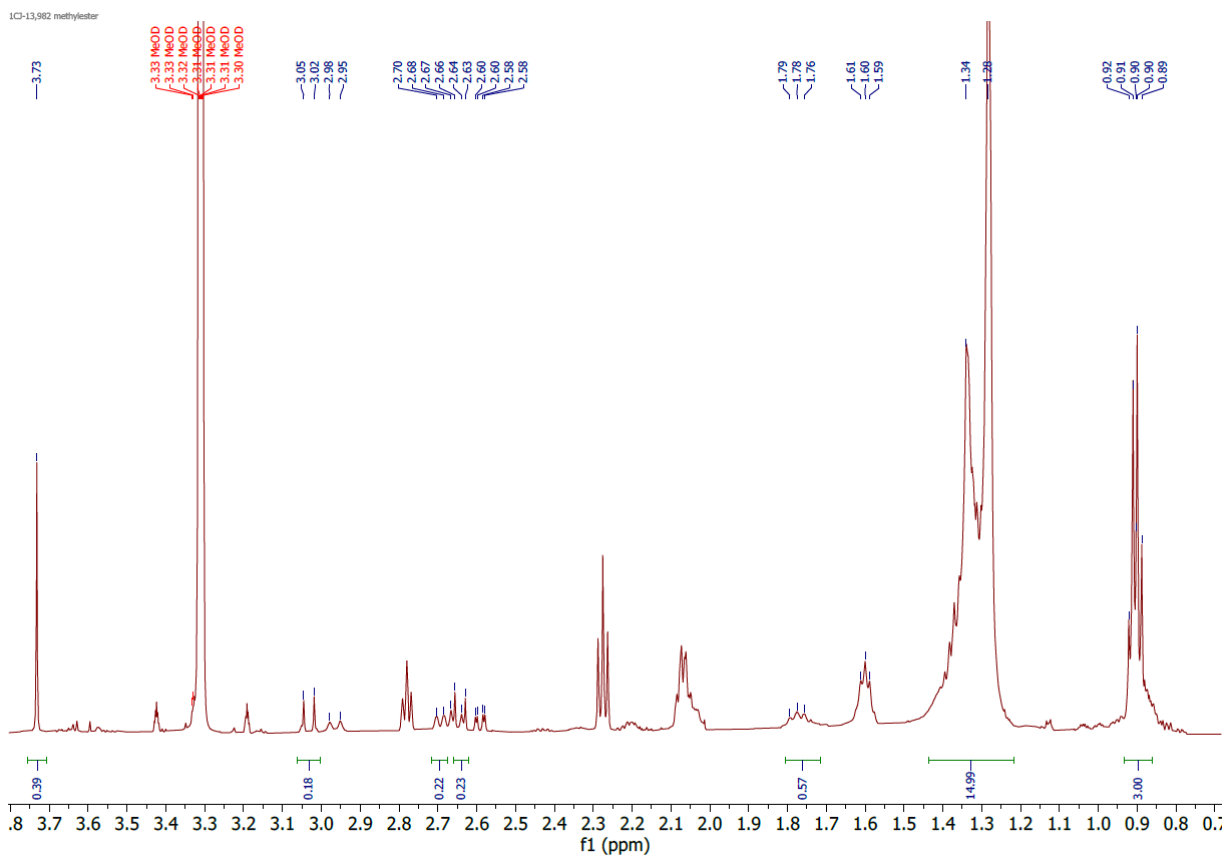


Figure S14  $^1\text{H}$  NMR of **16** in  $\text{CD}_3\text{OD}$  (600 MHz). See Table S14 for assignment.

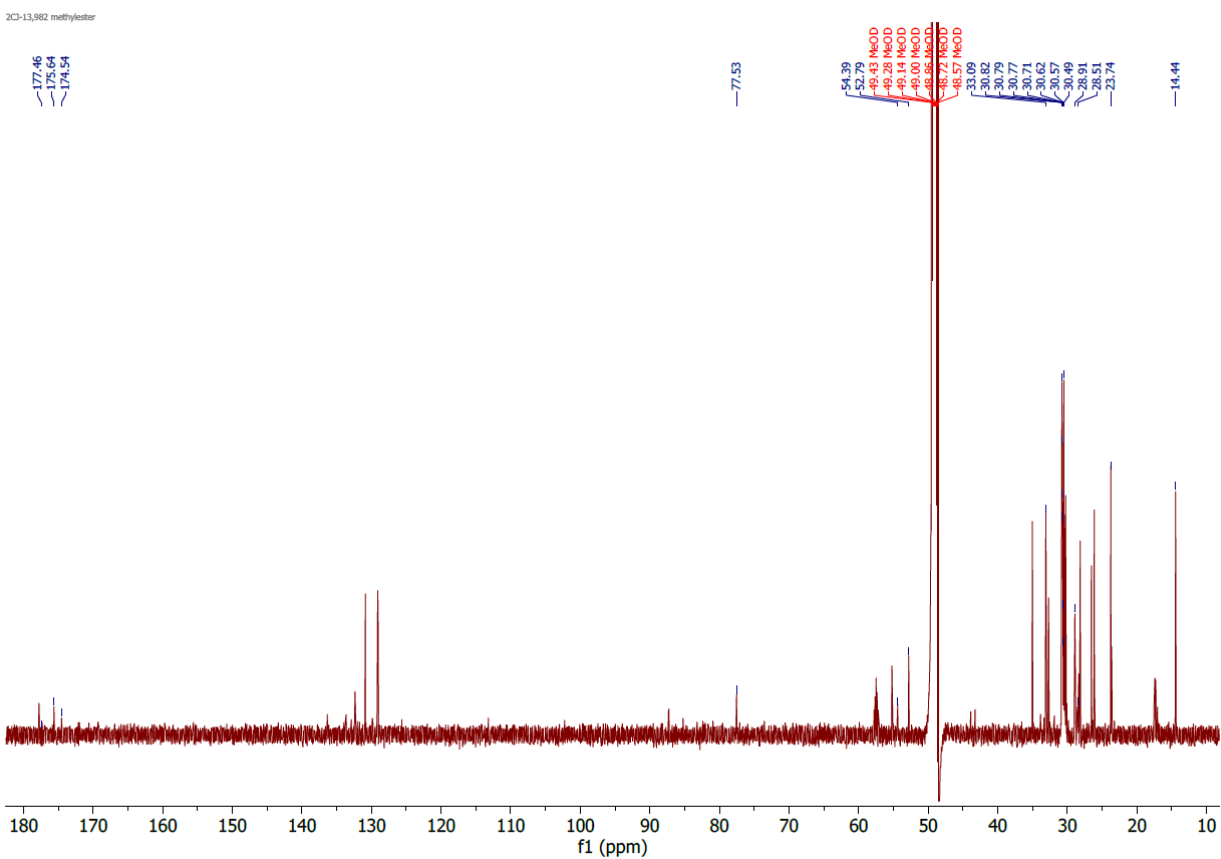


Figure S15  $^{13}\text{C}$  NMR of **16** in  $\text{CD}_3\text{OD}$  (150 MHz). See Table S14 for assignment.

3C-13,982 methylester

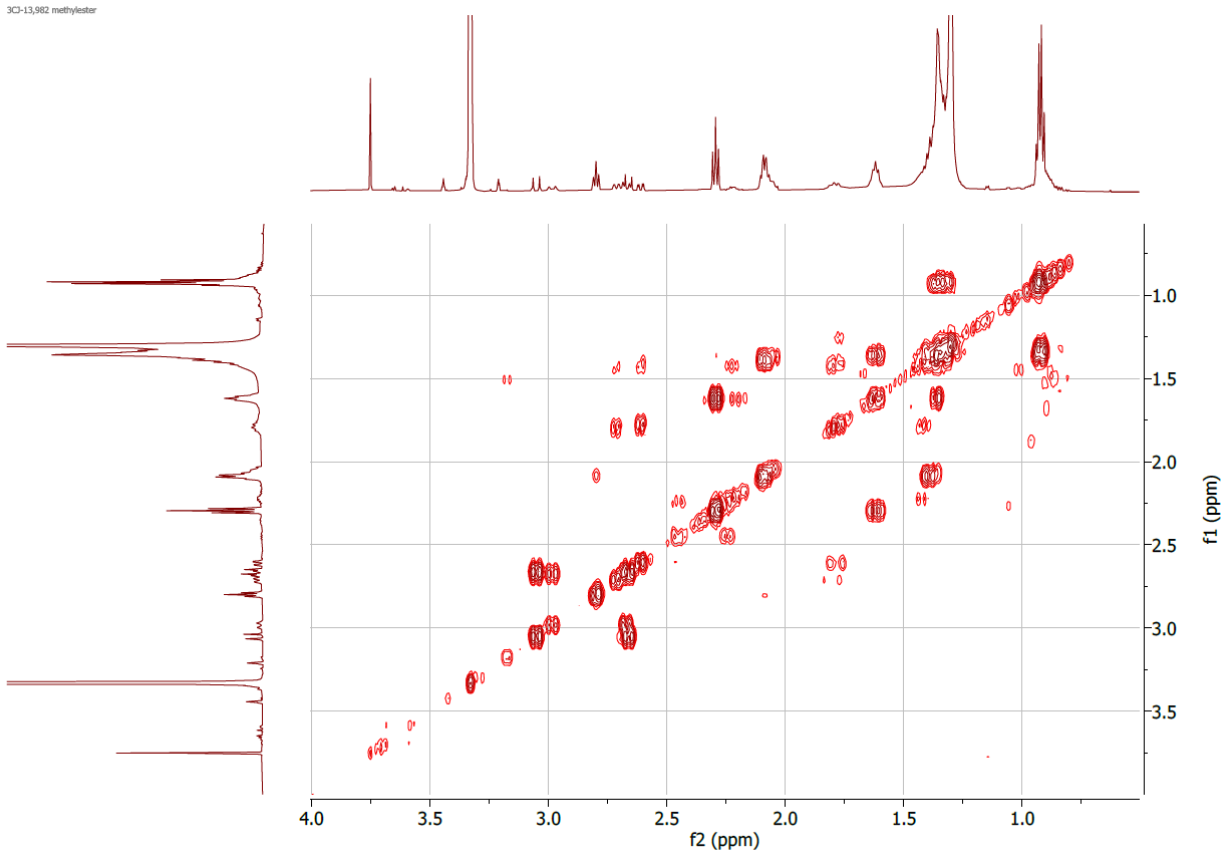


Figure S16  $^1\text{H}$ ,  $^1\text{H}$  COSY NMR of **16** in  $\text{CD}_3\text{OD}$ .

4C-13,982 methylester

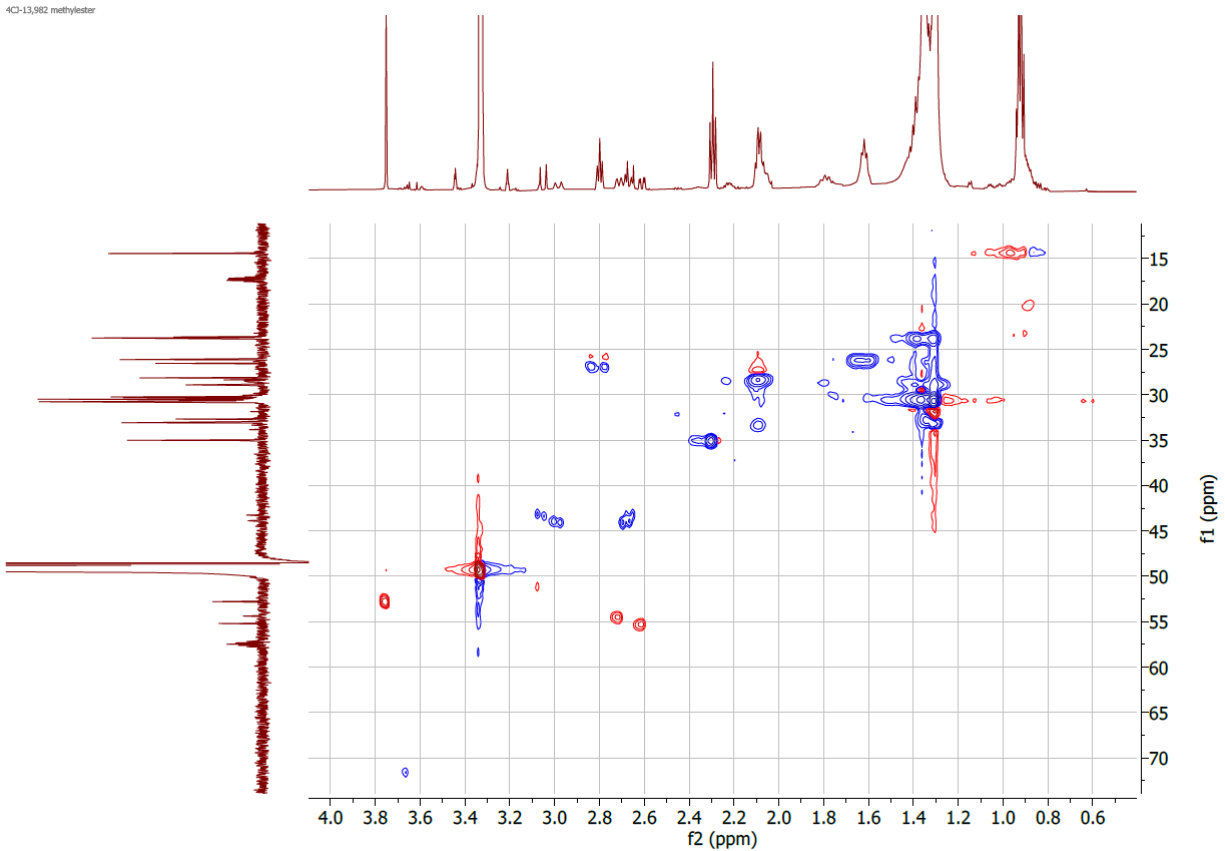


Figure S17 HSQC NMR of **16** in  $\text{CD}_3\text{OD}$ .

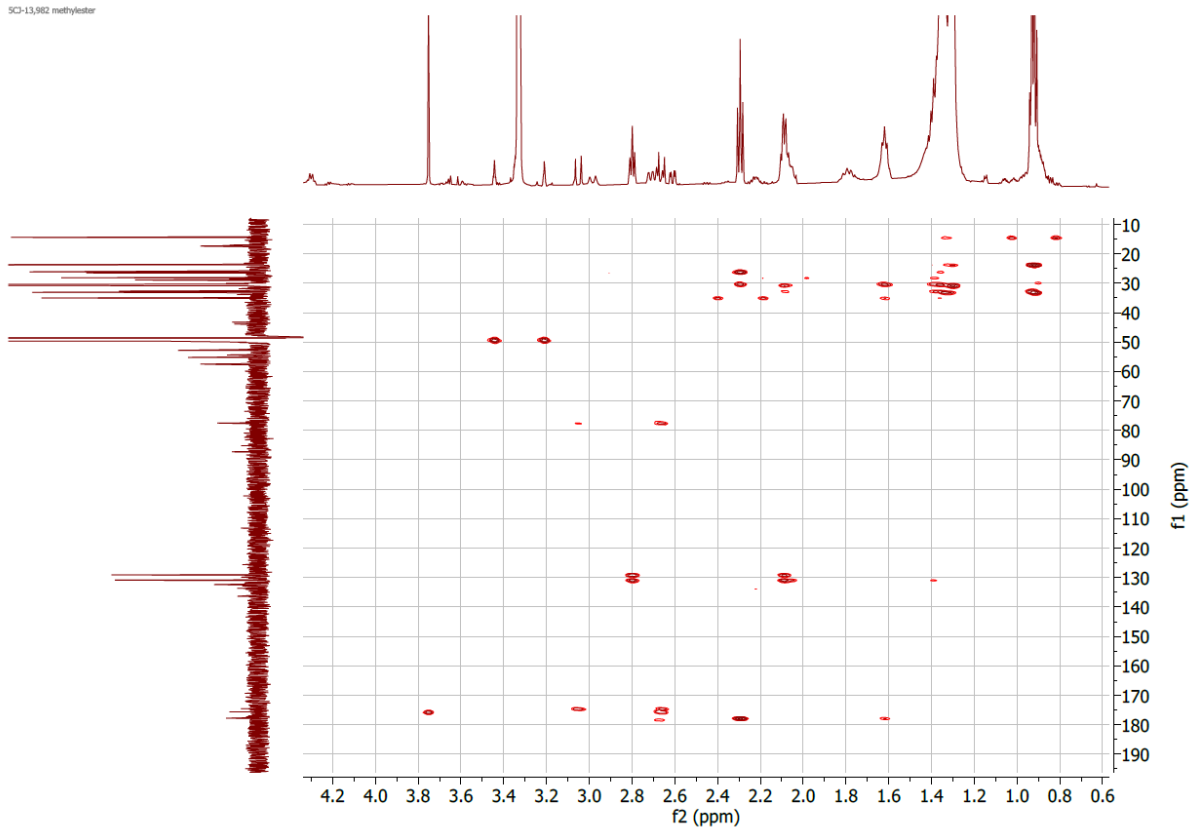
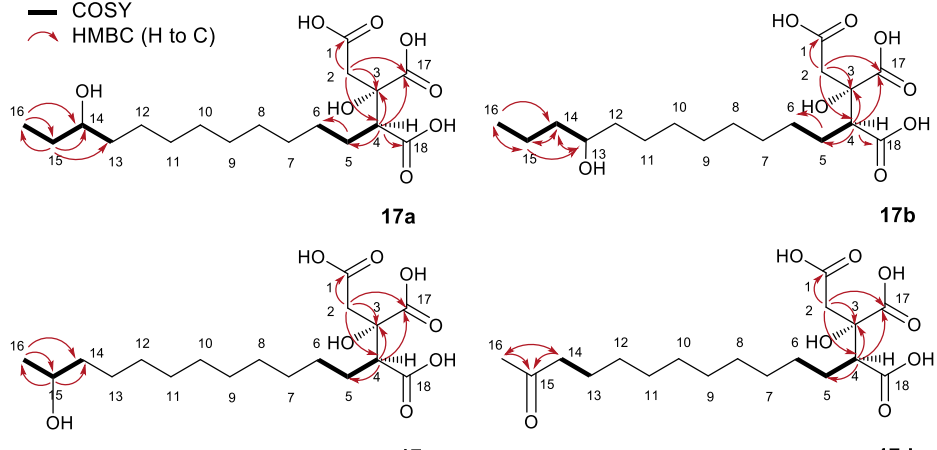


Figure S18 HMBC of 16 in CD<sub>3</sub>OD.

### 5.3 Characterisation of Inseparable Mixture of 13,14,15 hydroxy-CJ-13,982, 17a-c and 15-oxo- CJ-13,982 17d

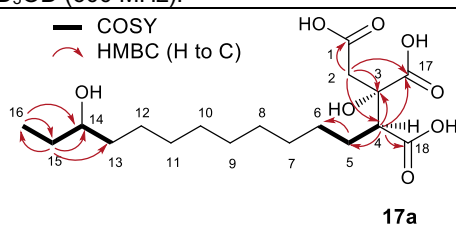
**Table S15** Chemical shifts of common NMR signals of **17a - d** in CD<sub>3</sub>OD (600 MHz).



| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C  | COSY |
|-----------------------|----------------------------------|------------------|-----------------|--------------|------|
| <b>1</b>              | -                                | 174.1            | CO              | -            | -    |
| <b>2a</b>             | 3.05 (d, 16.2)                   | 42.6             | CH <sub>2</sub> | 1, 3         | 2b   |
| <b>2b</b>             | 2.68 (d, 16.2)                   | 42.6             | CH <sub>2</sub> | 1, 3, 4, 17  | 2a   |
| <b>3</b>              | -                                | 76.9             | C               | -            | -    |
| <b>4</b>              | 2.65 (m)                         | 54.7             | CH              | 3, 5, 17, 18 | 5    |
| <b>5a</b>             | 1.81 (m)                         | 28.2             | CH <sub>2</sub> | 4, 6, 18     | 4, 6 |
| <b>5b</b>             | 1.47 (m)                         | 28.2             | CH <sub>2</sub> | 6            | 4, 6 |
| <b>6</b>              | 1.35 (m)                         | 28.8             | CH <sub>2</sub> | 7-14         | 5, 7 |
| <b>7<sup>†</sup></b>  | 1.3 (m)                          | 30.2             | CH <sub>2</sub> | 6, 7-14      | 6, 8 |
| <b>8<sup>†</sup></b>  | 1.3 (m)                          | 30.5             | CH <sub>2</sub> | 7-14         | 7-14 |
| <b>9<sup>†</sup></b>  | 1.3 (m)                          | 30.6             | CH <sub>2</sub> | 7-14         | 7-14 |
| <b>10<sup>†</sup></b> | 1.3 (m)                          | 30.6             | CH <sub>2</sub> | 7-14         | 7-14 |
| <b>11<sup>†</sup></b> | 1.3 (m)                          | 30.7             | CH <sub>2</sub> | 7-14         | 7-14 |
| <b>12<sup>†</sup></b> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14         | 7-14 |
| <b>17</b>             | -                                | 177.0            | CO              | -            | -    |
| <b>18</b>             | -                                | 176.5            | CO              | -            | -    |

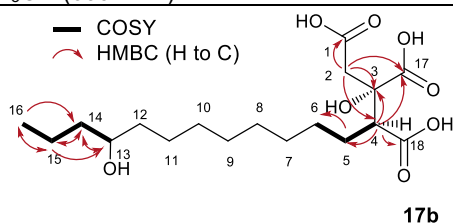
<sup>†</sup> These signals are indistinguishable from each other.

**Table S16** Chemical shifts of **17a** in CD<sub>3</sub>OD (600 MHz).



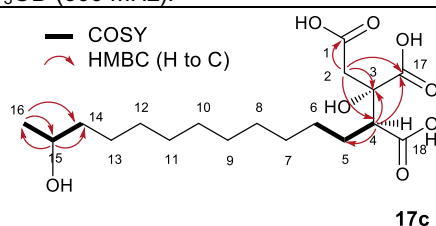
| Pos. | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY   |
|------|----------------------------------|------------------|-----------------|-------------|--------|
| 13   | 1.39 (m)                         | 40.7             | CH <sub>2</sub> | -           | 14, 12 |
| 14   | 3.43 (m)                         | 73.9             | CH              | -           | -      |
| 15   | 1.47 (m)                         | 31.0             | CH <sub>2</sub> | 13, 14, 16  | 14, 16 |
| 16   | 0.93 (t, 7.5)                    | 10.3             | CH <sub>3</sub> | 14, 15      | 15     |

**Table S17** Chemical shifts of **17b** in CD<sub>3</sub>OD (600 MHz).



| Pos.       | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY   |
|------------|----------------------------------|------------------|-----------------|-------------|--------|
| <b>13</b>  | 3.52 (m)                         | 72.2             | CH              | -           | 12, 14 |
| <b>14</b>  | 1.39 (m)                         | 40.7             | CH <sub>2</sub> | 13, 15      | 13, 15 |
| <b>15a</b> | 1.48 (m)                         | 19.7             | CH <sub>2</sub> | 13, 14, 16  | 14, 16 |
| <b>15b</b> | 1.36 (m)                         | 19.7             | CH <sub>2</sub> | 13, 14, 16  | 14, 16 |
| <b>16</b>  | 0.93 (t, 7.2)                    | 14.5             | CH <sub>3</sub> | 14, 15      | 15     |

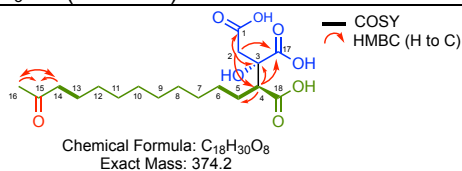
**Table S18** Chemical shifts of **17c** in CD<sub>3</sub>OD (600 MHz).



| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY   |
|-----------------------|----------------------------------|------------------|-----------------|-------------|--------|
| <b>13<sup>†</sup></b> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>14a</b>            | 1.46 (m)                         | 40.0             | CH <sub>2</sub> | -           | 15     |
| <b>14b</b>            | 1.39 (m)                         | 40.0             | CH <sub>2</sub> | -           | 15     |
| <b>15</b>             | 3.69 (m)                         | 68.6             | CH              | 14, 16      | 14, 16 |
| <b>16</b>             | 1.14 (d, 6.2)                    | 23.5             | CH <sub>3</sub> | 14, 15      | 15     |

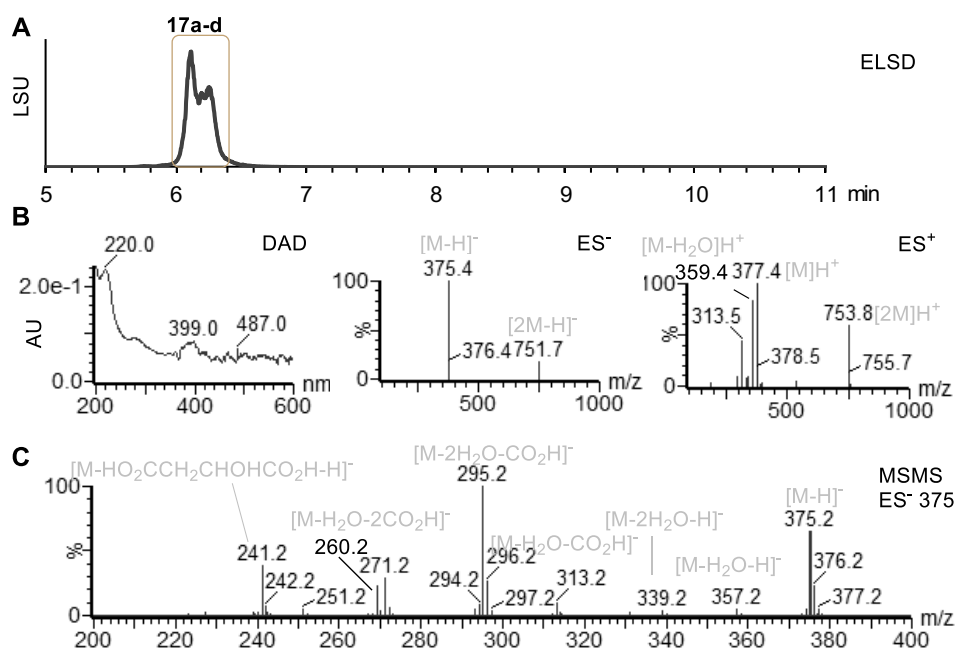
<sup>†</sup> This signal is not distinguishable from position 7 - 12 in Table S15.

**Table S19** Chemical shifts of **17d** in CD<sub>3</sub>OD (600 MHz).



| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY   |
|-----------------------|----------------------------------|------------------|-----------------|-------------|--------|
| <b>12<sup>†</sup></b> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>13</b>             | 1.53 (m)                         | 30.8             | CH <sub>2</sub> | 12 etc      | 12 etc |
| <b>14</b>             | 2.47 (t, 7.4 Hz)                 | 40.0             | CH <sub>2</sub> | 13, 15, 16  | 13     |
| <b>15</b>             | -                                | 212.3            | C               | -           | -      |
| <b>16</b>             | 2.12 (s)                         | 23.5             | CH <sub>3</sub> | 14, 15      | -      |

<sup>†</sup> This signal is not distinguishable from position 7 - 12 in Table S15.



**D**

#### Elemental Composition Report

##### Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction : Off

Number of isotopes peaks used for i-FIT = 3

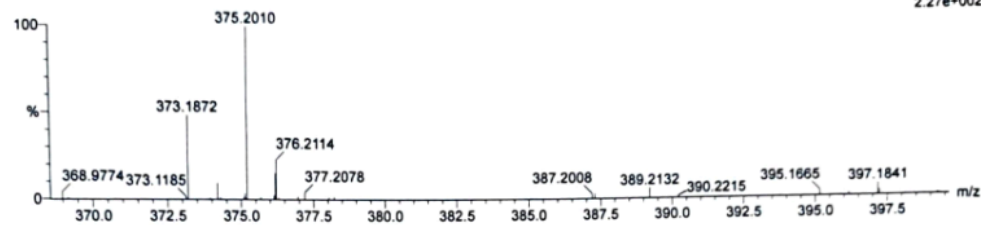
Monoisotopic Mass, Even Electron Ions

466 formula(e) evaluated with 9 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-22 H: 0-35 N: 0-4 O: 0-8 S: 0-3

1: TOF MS ES-  
2.27e+002



| Mass     | Calc. Mass | mDa  | PPM   | DBE  | i-FIT | i-FIT (Norm) | Formula          |
|----------|------------|------|-------|------|-------|--------------|------------------|
| 375.2010 | 375.2019   | -0.9 | -2.4  | 3.5  | 24.7  | 0.7          | C18 H31 O8       |
|          | 375.1994   | 1.6  | 4.3   | 7.5  | 27.8  | 3.8          | C22 H31 O3 S     |
|          | 375.2028   | -1.8 | -4.8  | 2.5  | 29.8  | 5.9          | C19 H35 O3 S2    |
|          | 375.2032   | -2.2 | -5.9  | 8.5  | 24.8  | 0.8          | C19 H27 N4 O4    |
|          | 375.1987   | 2.3  | 6.1   | -1.5 | 30.6  | 6.6          | C14 H35 N2 O5 S2 |
|          | 375.1987   | 2.3  | 6.1   | -1.5 | 30.6  | 3.9          | C15 H35 O8 S     |
|          | 375.2053   | -4.3 | -11.5 | -1.5 | 27.9  | 8.5          | C18 H35 N2 S3    |
|          | 375.1962   | 4.8  | 12.8  | 2.5  | 32.5  | 4.7          | C17 H31 N2 O5 S  |
|          | 375.1954   | 5.6  | 14.9  | 3.5  | 28.7  | 3.7          | C16 H31 N4 O4 S  |
|          | 375.2066   | -5.6 | -14.9 | 3.5  | 27.7  |              |                  |

**Figure S19** LCMS characterization and structure elucidation of **17a-d**. **A**, ELSD trace of **17a-d** (arbitrary units, smoothed in Excel); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **17a-d**; **C**, MSMS analysis of 375.4 ion; **D**, HRMS analysis and calculated molecular formula of **17a-c**.

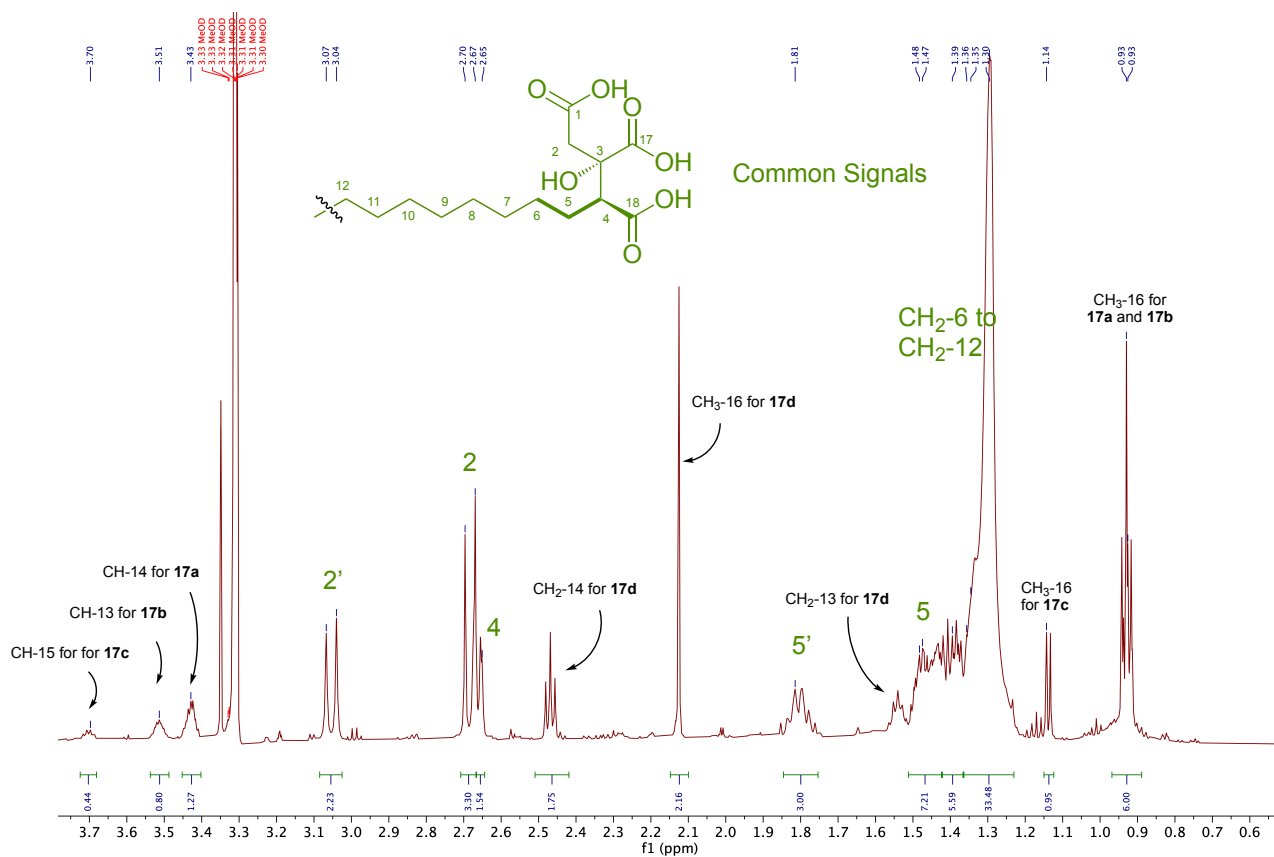


Figure S20 Annotated <sup>1</sup>H NMR spectrum of mixture of 17a-d in CD<sub>3</sub>OD.

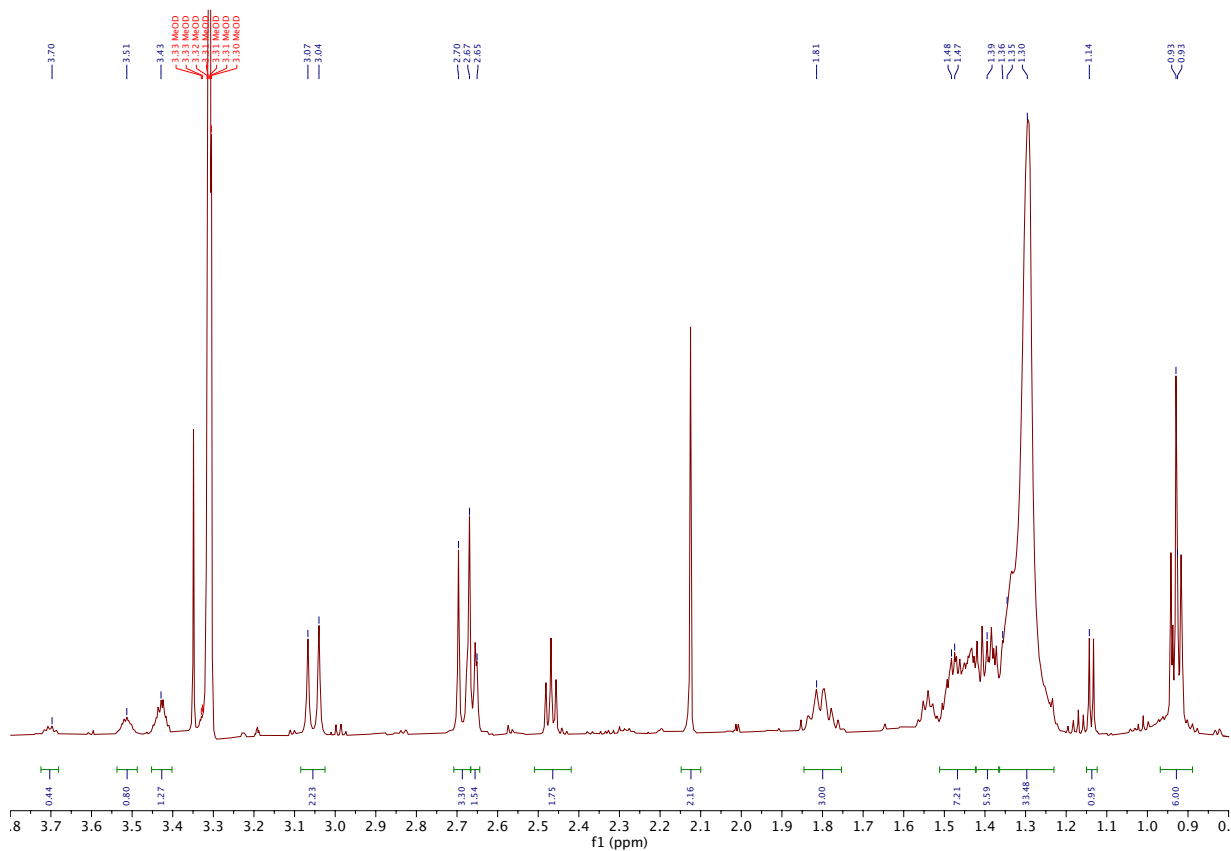


Figure S21 <sup>1</sup>H NMR of mixture of 17a-d in CD<sub>3</sub>OD.

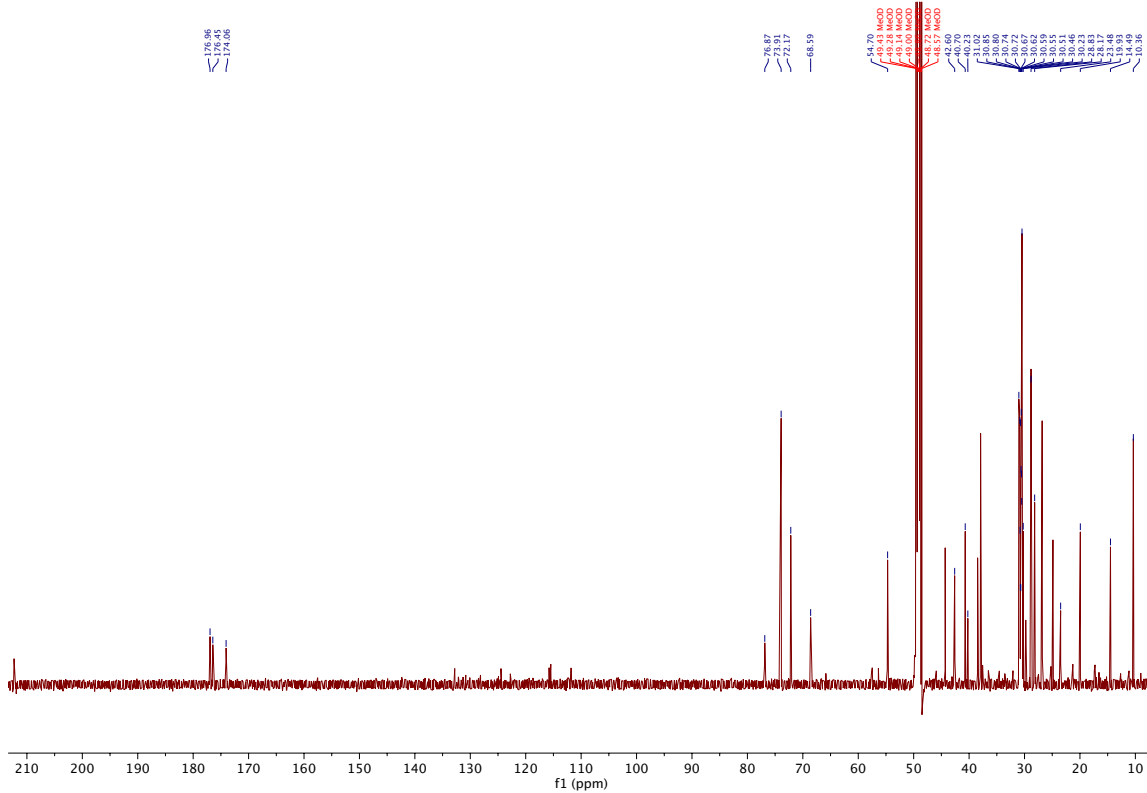


Figure S22  $^{13}\text{C}$  NMR of 17a-d in  $\text{CD}_3\text{OD}$ .

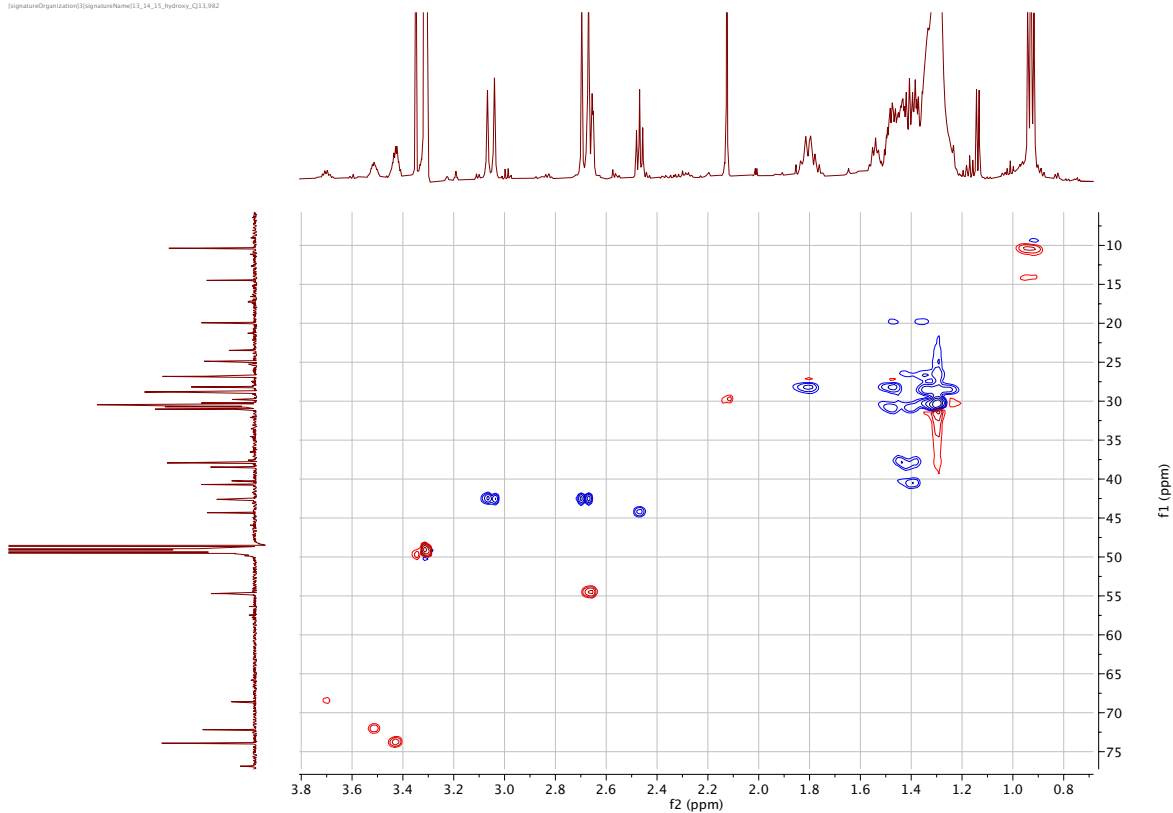


Figure S23 HSQC NMR of 17a-d in  $\text{CD}_3\text{OD}$ .

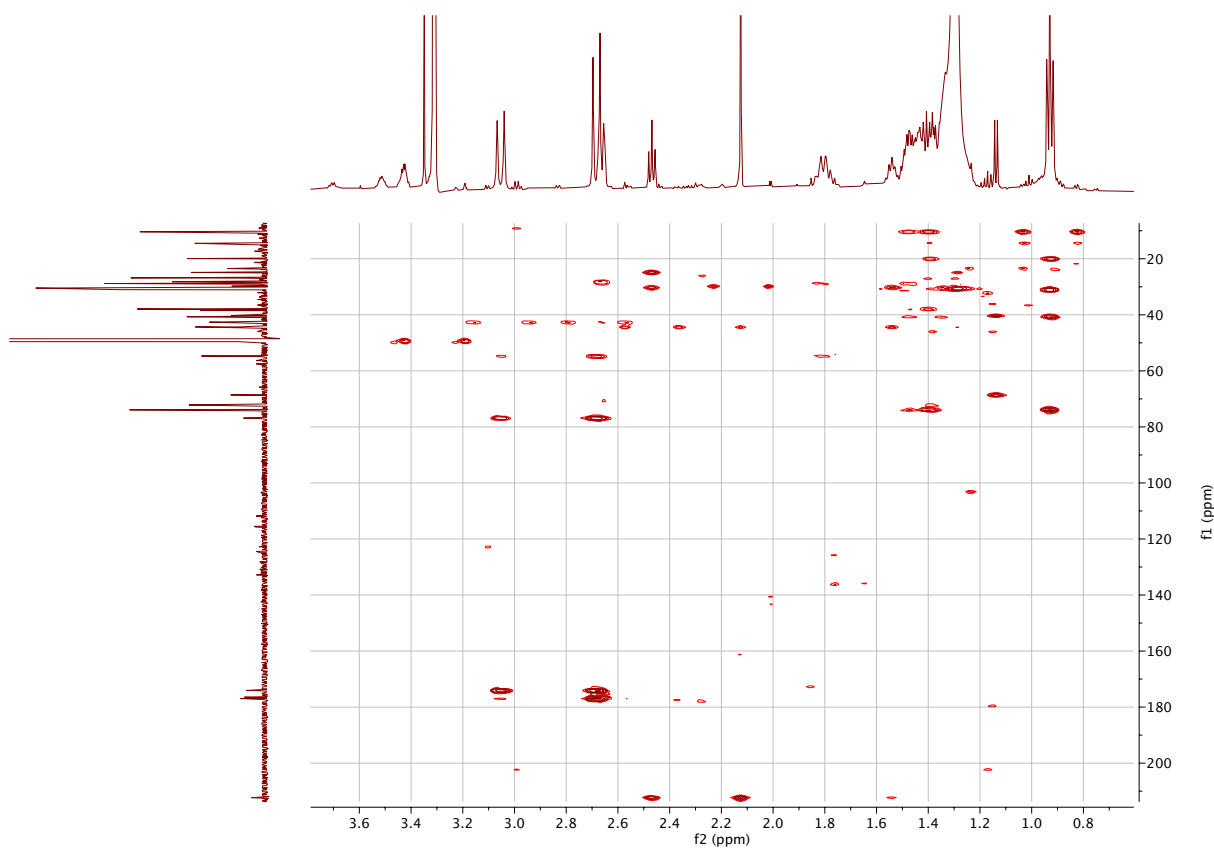


Figure S24 HMBC NMR of 17a-d in CD<sub>3</sub>OD.

Origin:Organization(S)SignatureName(13\_14\_15\_hydroxy\_Q13.982

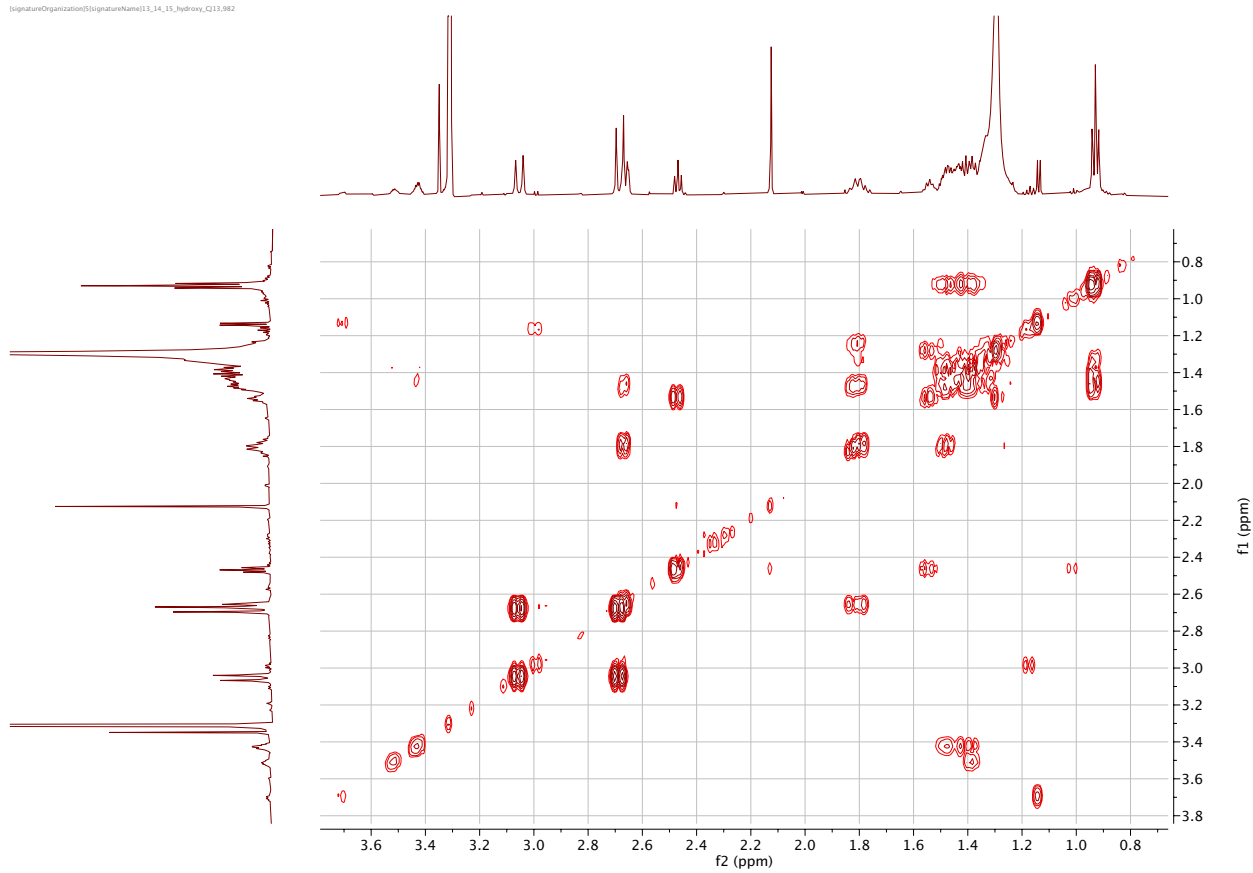
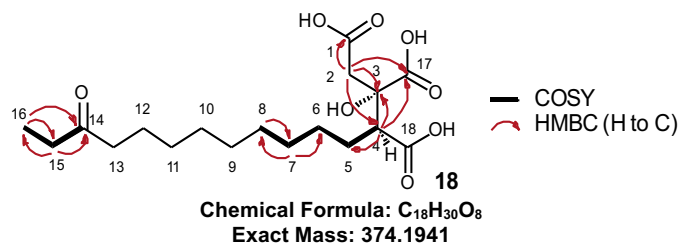


Figure S25 COSY NMR of 17a-d in CD<sub>3</sub>OD.

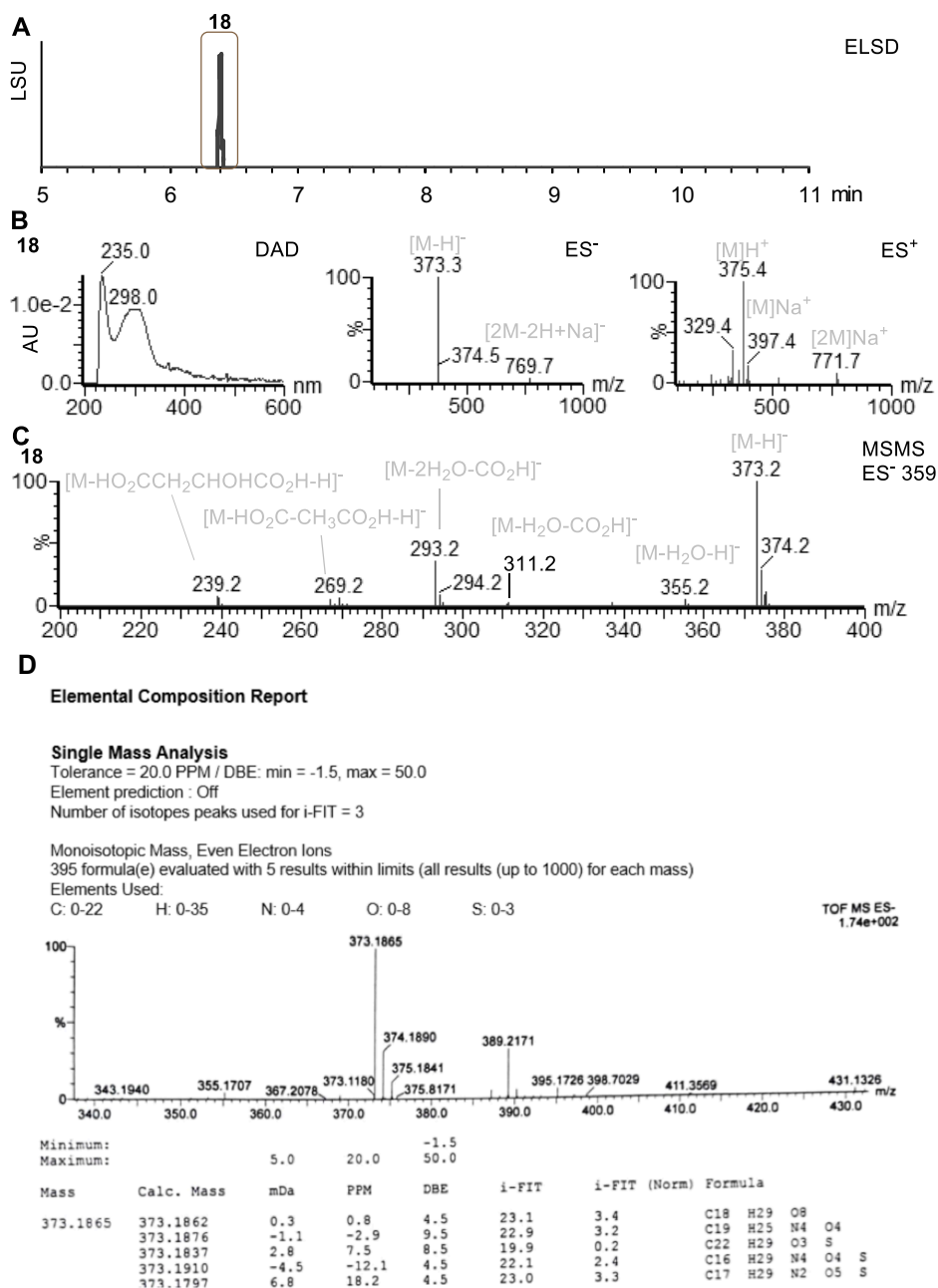
## 5.4 Characterisation of 14-oxo-CJ-13,982, 18

Table S20 Chemical shifts of 18 in CD<sub>3</sub>OD (600 MHz).

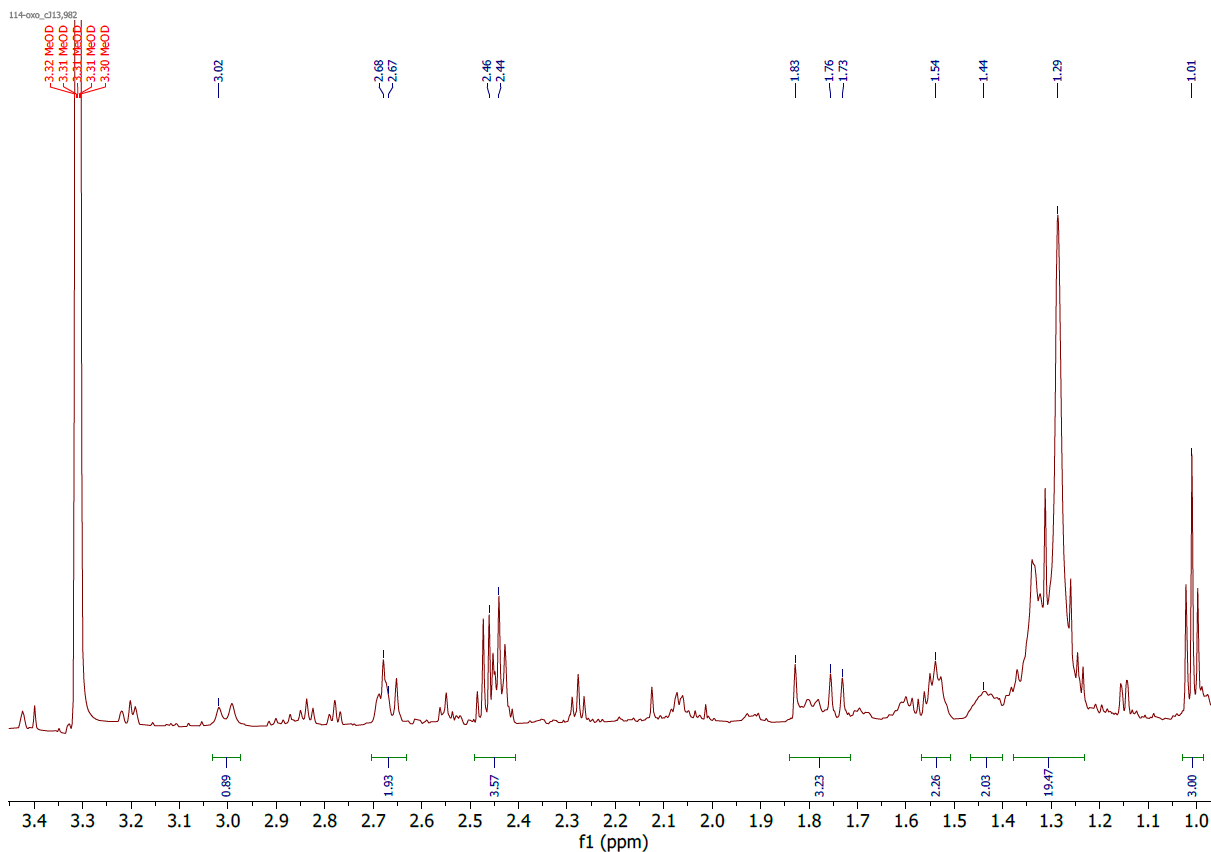


| Pos.            | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY     |
|-----------------|----------------------------------|------------------|-----------------|-------------|----------|
| 1               | -                                | 174.5            | CO              | -           | -        |
| 2a              | 3.01 (d, 16.2)                   | 43.1             | CH <sub>2</sub> | 1, 3        | 2b       |
| 2b              | 2.67 (d, 16.2)                   | 43.1             | CH <sub>2</sub> | 1, 3, 4, 17 | 2a       |
| 3               | -                                | 77.0             | C               | -           | -        |
| 4               | 2.68 (m)                         | 54.5             | CH              | 3, 5, 18    | 5a, b    |
| 5a              | 1.79 (m)                         | 28.8             | CH <sub>2</sub> | 4           | 4, 5b, 6 |
| 5b              | 1.43 (m)                         | 28.8             | CH <sub>2</sub> | 6           | 5a       |
| 6               | 1.3 (m)                          | 29.5             | CH <sub>2</sub> | 7-14        | 5b, 7-14 |
| 7 <sup>†</sup>  | 1.3 (m)                          | 30.5             | CH <sub>2</sub> | 7-14        | 7-14     |
| 8 <sup>†</sup>  | 1.3 (m)                          | 30.6             | CH <sub>2</sub> | 7-14        | 7-14     |
| 9 <sup>†</sup>  | 1.3 (m)                          | 30.6             | CH <sub>2</sub> | 7-14        | 7-14     |
| 10 <sup>†</sup> | 1.3 (m)                          | 30.7             | CH <sub>2</sub> | 7-14        | 7-14     |
| 11 <sup>†</sup> | 1.3 (m)                          | 30.8             | CH <sub>2</sub> | 7-14        | 7-14     |
| 12              | 1.54 (m)                         | 24.9             | CH <sub>2</sub> | 11, 13      | 7-14     |
| 13              | 2.44 (m)                         | 43.4             | CH <sub>2</sub> | 11, 12      | 14       |
| 14              | -                                | 214.7            | CO              | -           | -        |
| 15              | 2.46 (q, 7.3)                    | 36.5             | CH <sub>2</sub> | 16          | 14, 16   |
| 16              | 1.01 (t, 7.3)                    | 8.07             | CH <sub>3</sub> | 14, 15      | 15       |
| 17              | -                                | 177.0            | CO              | -           | -        |
| 18              | -                                | 177.5            | CO              | -           | -        |

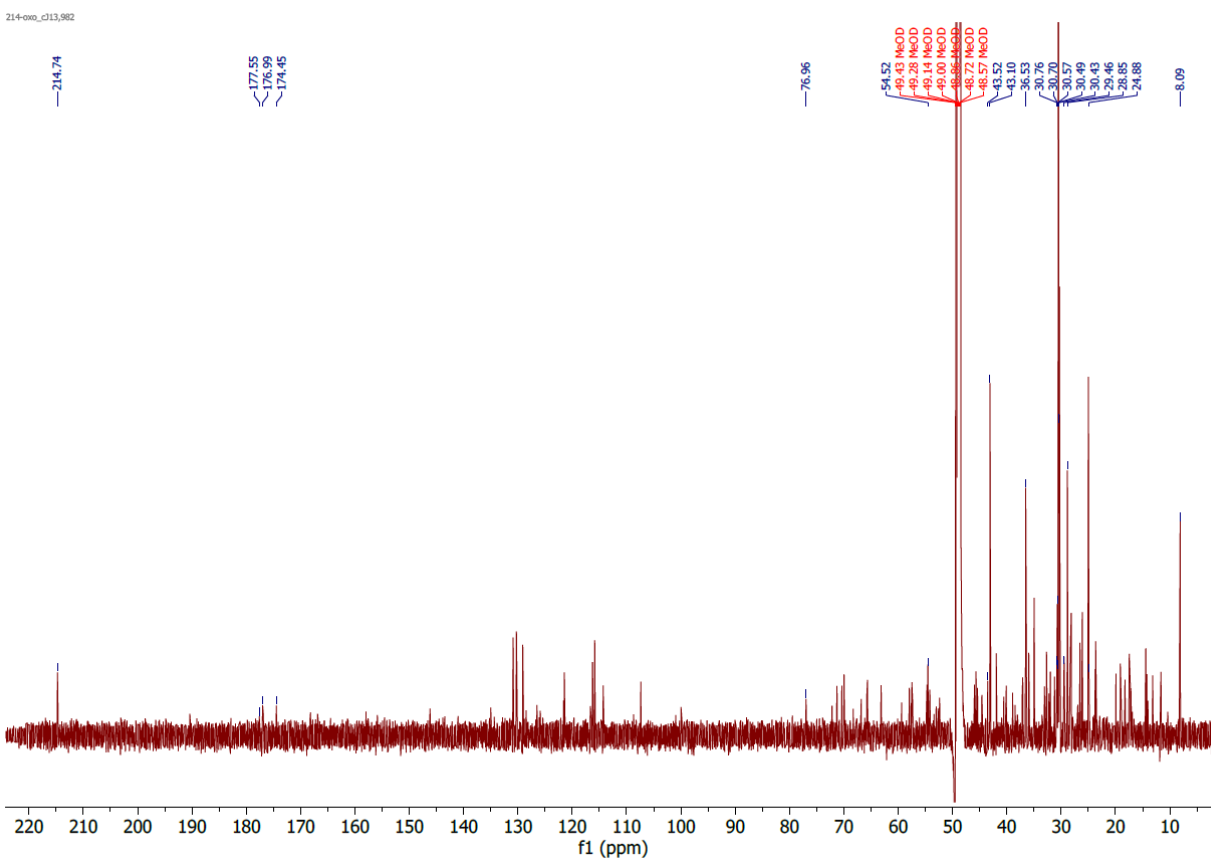
<sup>†</sup> These peaks are indistinguishable from each other.



**Figure S26** LCMS characterization and structure elucidation of **18**. **A**, ELSD trace of **18** (arbitrary units, smoothed in Excel); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **18**; **C**, MSMS analysis of **18**; **D**, HRMS analysis.



**Figure S27**  $^1\text{H}$  NMR of **18** in  $\text{CD}_3\text{OD}$  (600 MHz). See Table S20 for assignment.



**Figure S28**  $^{13}\text{C}$  NMR of **18** in  $\text{CD}_3\text{OD}$  (150 MHz). See Table S20 for assignment.

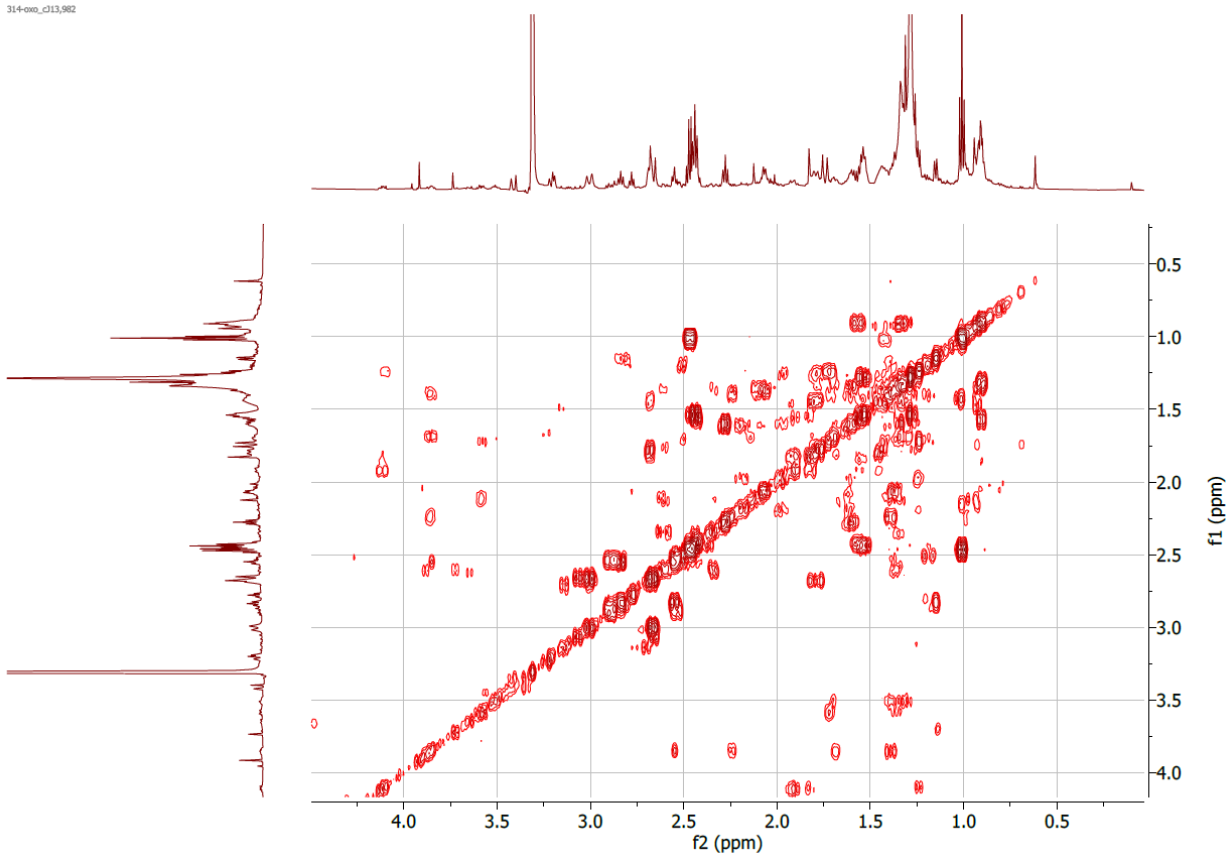


Figure S29  $^1\text{H}$ ,  $^1\text{H}$  COSY of **18** in  $\text{CD}_3\text{OD}$ .

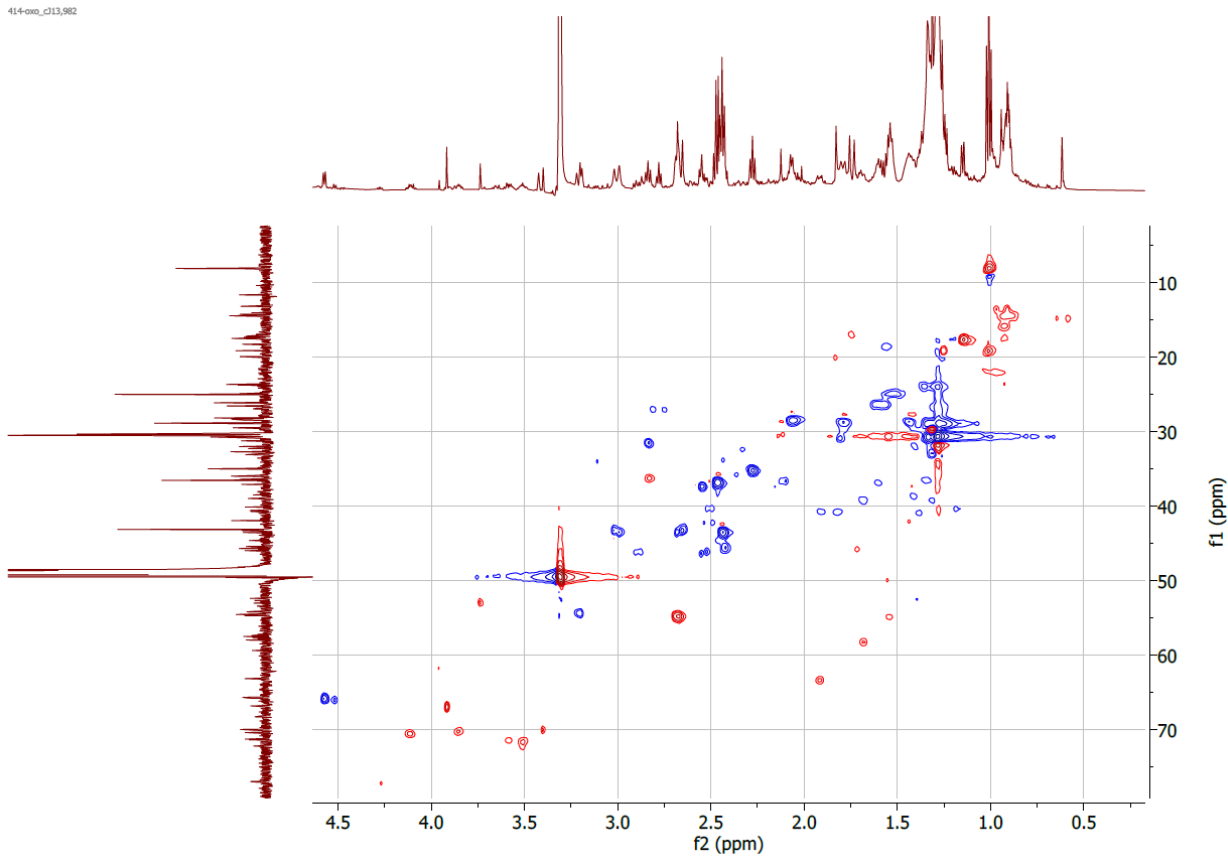


Figure S30 HSQC of **18** in  $\text{CD}_3\text{OD}$ .

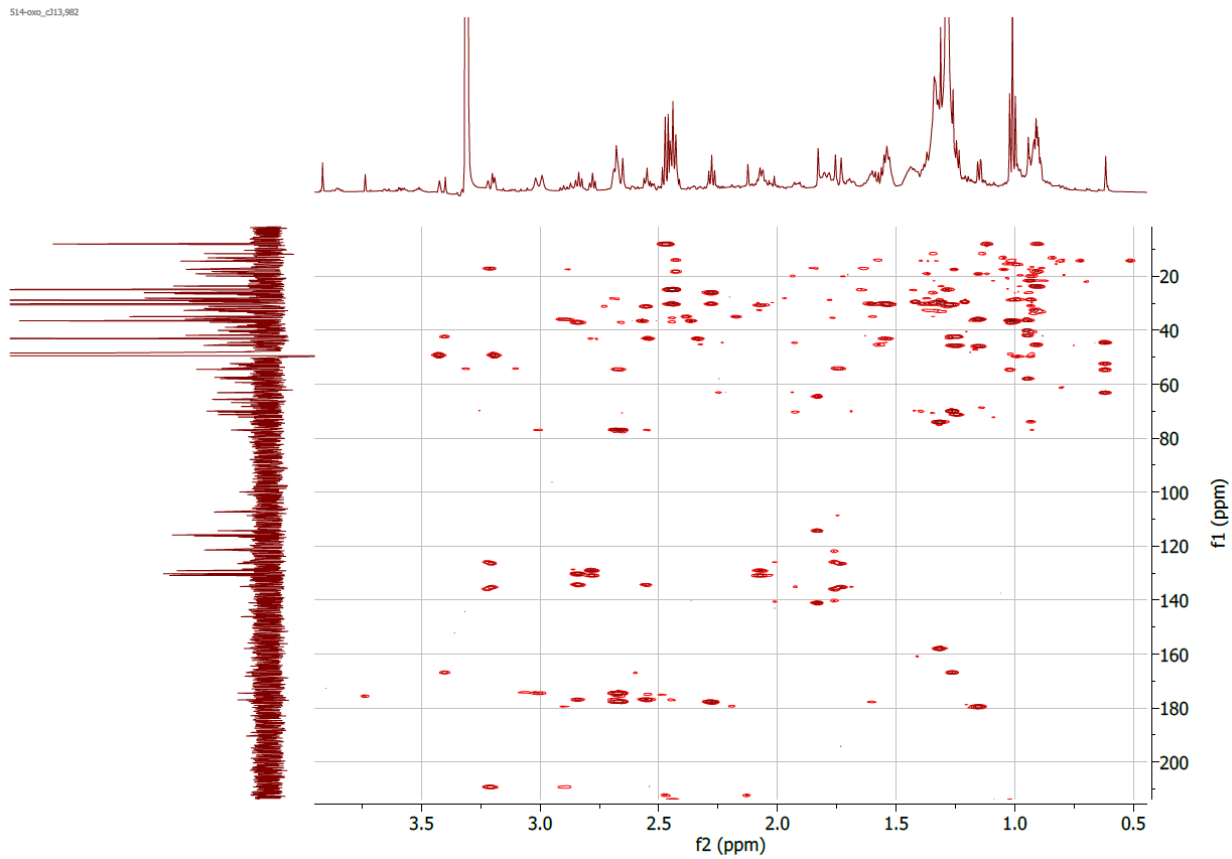
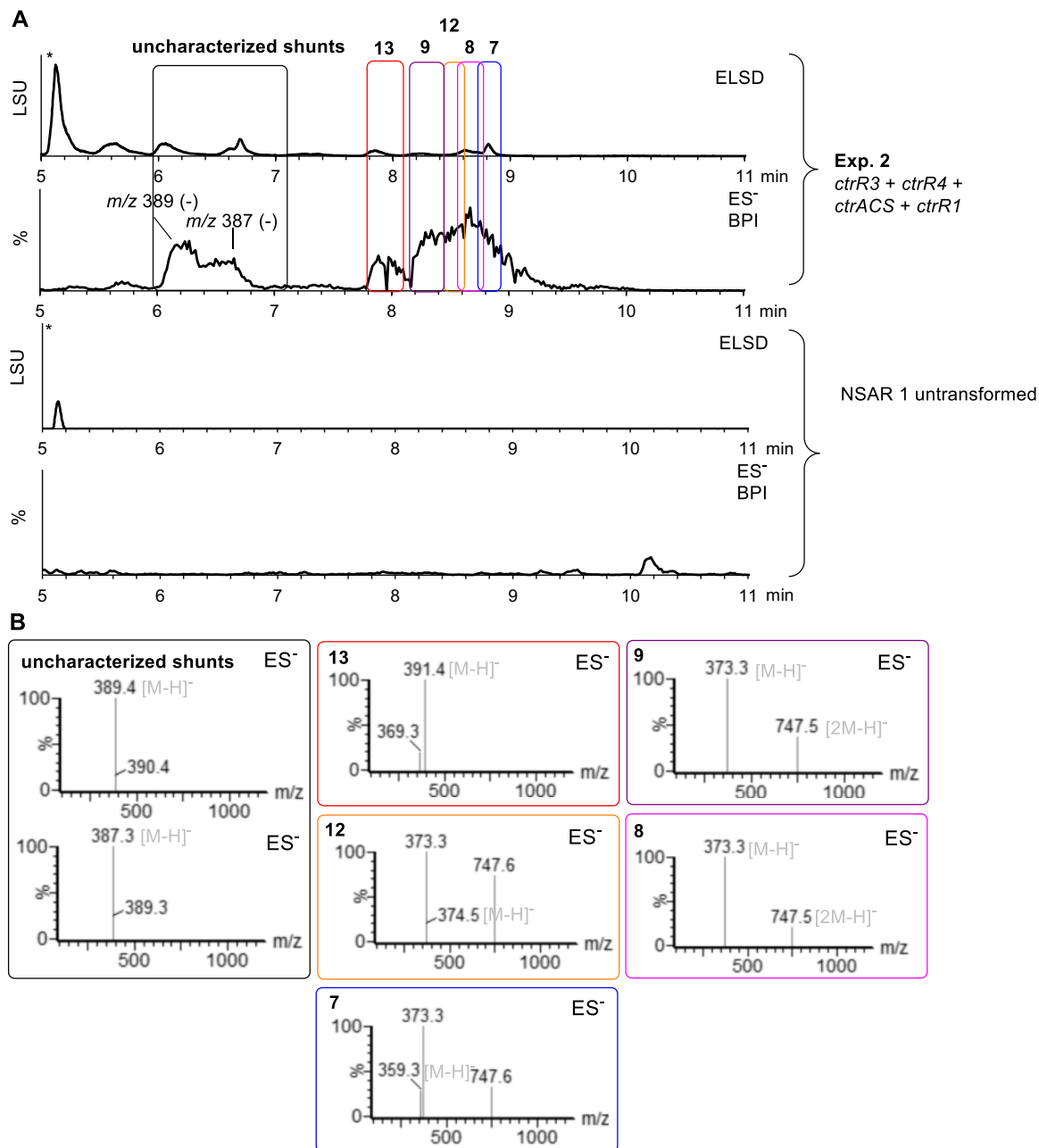


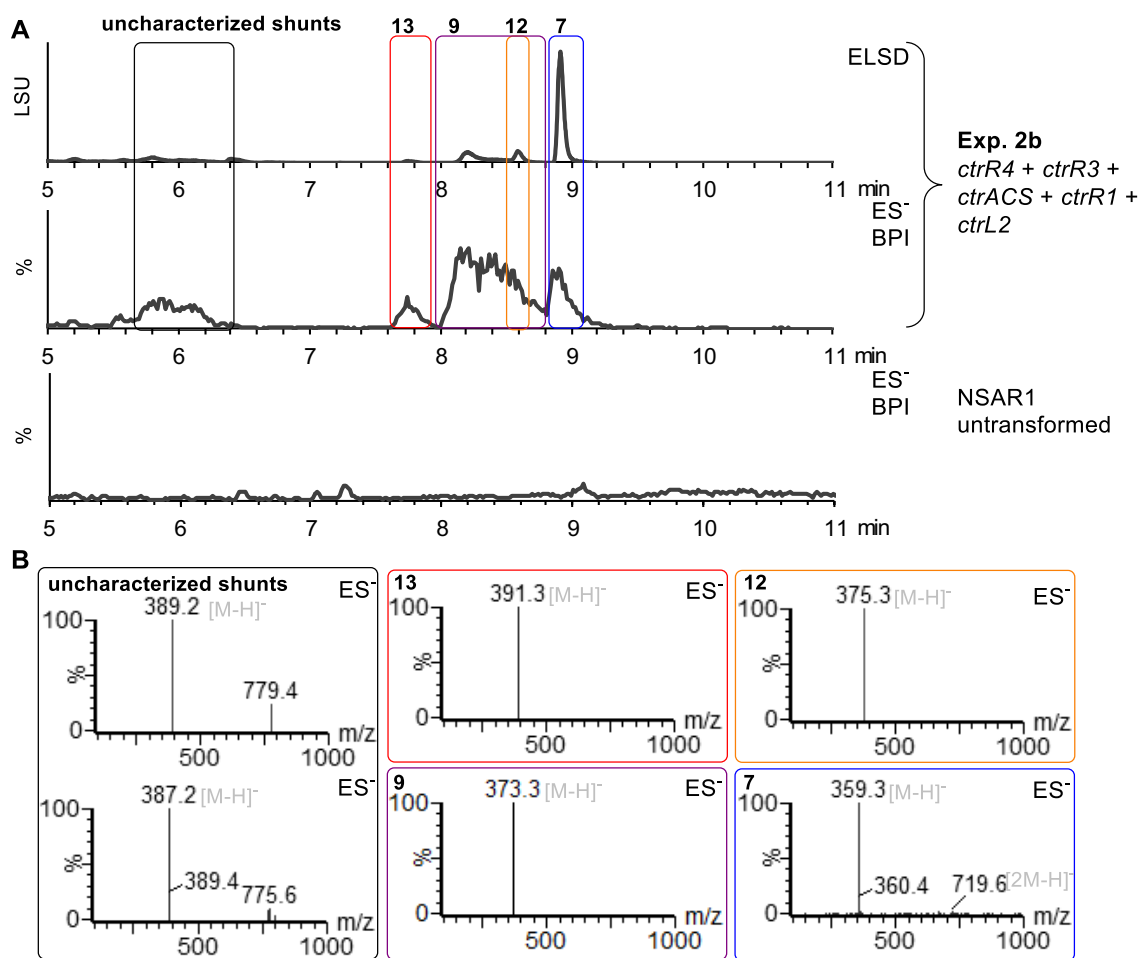
Figure S31 HMBC of 18 in CD<sub>3</sub>OD.

## 6. EXPERIMENT 2 (*ctrR4* + *ctrR3* + *ctrACS* + *ctrR1*) *in vivo*



**Figure S32** Heterologous expression of the structural genes of the *ctr* BGC: *ctrR4*, *ctrR3*, *ctrACS*, and *ctrR1*. **A**, LCMS chromatograms of extracts of *A. oryzae* NSAR1 transformant and untransformed control strain (arbitrary units, ELSD trace was smoothed in Excel); **B**, ES<sup>-</sup> spectra of compounds detected in *A. oryzae* NSAR1 transformants.

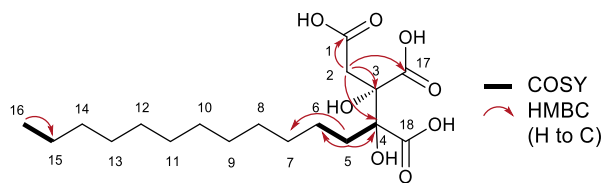
EXPERIMENT 2b (*ctrR4* + *ctrR3* + *ctrACS* + *ctrR1* + *ctrL2*) *in vivo*



**Figure S33** Heterologous expression of the structural genes of the *ctr* BGC: *ctrR4*, *ctrR3*, *ctrACS*, *ctrR1* and *ctrL2*. **A**, LCMS chromatograms of extracts of *A. oryzae* NSAR1 transformant and untransformed control strain (arbitrary units, ELSD trace was smoothed in Excel); **B**, ES<sup>-</sup> spectra of compounds detected in *A. oryzae* NSAR1 transformants.

## 6.1 Characterisation of 4-Hydroxy CJ-13,982, 12

Table S21 Chemical shifts of **12** in DMSO-d<sub>6</sub> (600 MHz).



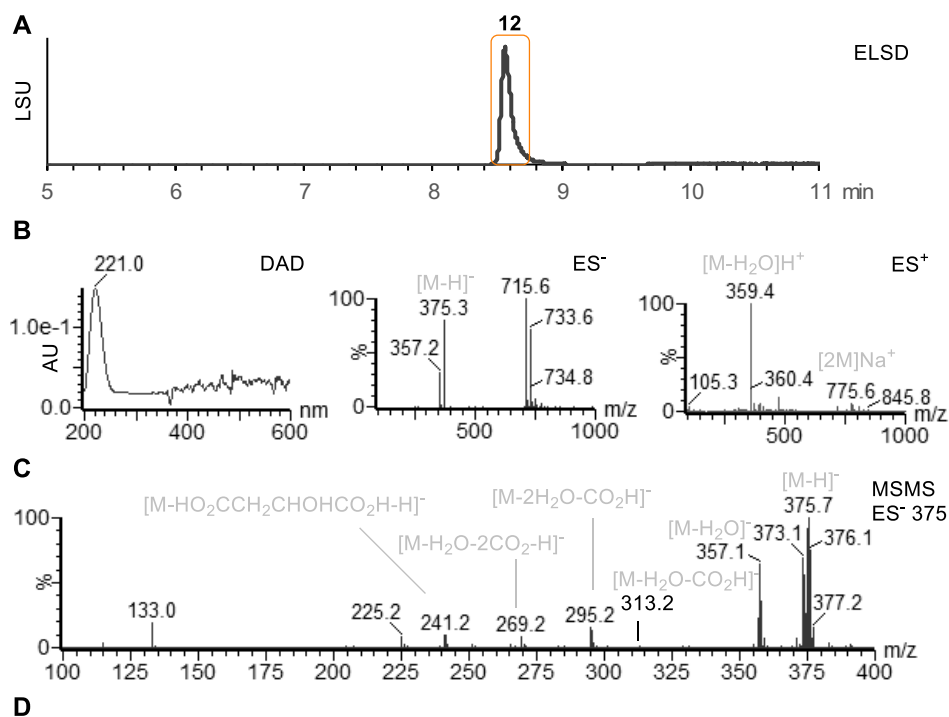
**12**

Chemical Formula: C<sub>18</sub>H<sub>32</sub>O<sub>8</sub>

Exact Mass: 376.21

| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY   |
|-----------------------|----------------------------------|------------------|-----------------|-------------|--------|
| <b>1</b>              | -                                | 174.8            | CO              | -           | -      |
| <b>2a</b>             | 3.13 (d, 17.7)                   | 43.4             | CH <sub>2</sub> | 1, 17       | 2b     |
| <b>2b</b>             | 2.41 (d, 17.7)                   | 43.4             | CH <sub>2</sub> | 1, 3, 4, 17 | 2a     |
| <b>3</b>              | -                                | 94.5             | C               | -           | -      |
| <b>4</b>              | -                                | 94.5             | C               | -           | -      |
| <b>5a</b>             | 1.81 (m)                         | 27.6             | CH <sub>2</sub> | 4           | 5b, 6  |
| <b>5b</b>             | 1.50 (m)                         | 27.6             | CH <sub>2</sub> | 4, 18       | 5a, 6  |
| <b>6</b>              | 1.3 (m)                          | 31.8             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>7<sup>†</sup></b>  | 1.3 (m)                          | 32.0             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>8<sup>†</sup></b>  | 1.3 (m)                          | 32.1             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>9<sup>†</sup></b>  | 1.3 (m)                          | 32.1             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>10<sup>†</sup></b> | 1.3 (m)                          | 32.2             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>11<sup>†</sup></b> | 1.3 (m)                          | 32.2             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>12<sup>†</sup></b> | 1.3 (m)                          | 32.2             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>13<sup>†</sup></b> | 1.3 (m)                          | 32.4             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>14</b>             | 1.3 (m)                          | 34.4             | CH <sub>2</sub> | 7-14        | 7-14   |
| <b>15</b>             | 1.3 (m)                          | 25.2             | CH <sub>2</sub> | 14          | 7-14   |
| <b>16</b>             | 0.86 (t, 6.8)                    | 17.1             | CH <sub>3</sub> | 14, 15      | 14, 15 |
| <b>17</b>             | -                                | 176.8            | CO              | -           | -      |
| <b>18</b>             | -                                | 172.4            | CO              | -           | -      |

<sup>†</sup> These signals are indistinguishable from each other.



### Elemental Composition Report

#### Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction : Off

Number of isotopes peaks used for i-FIT = 3

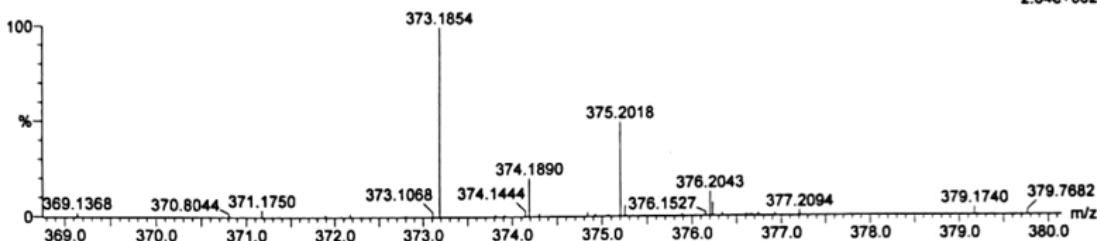
Monoisotopic Mass, Even Electron Ions

75 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-22 H: 0-50 O: 0-8 Na: 0-1

1: TOF MS ES-  
2.04e+002

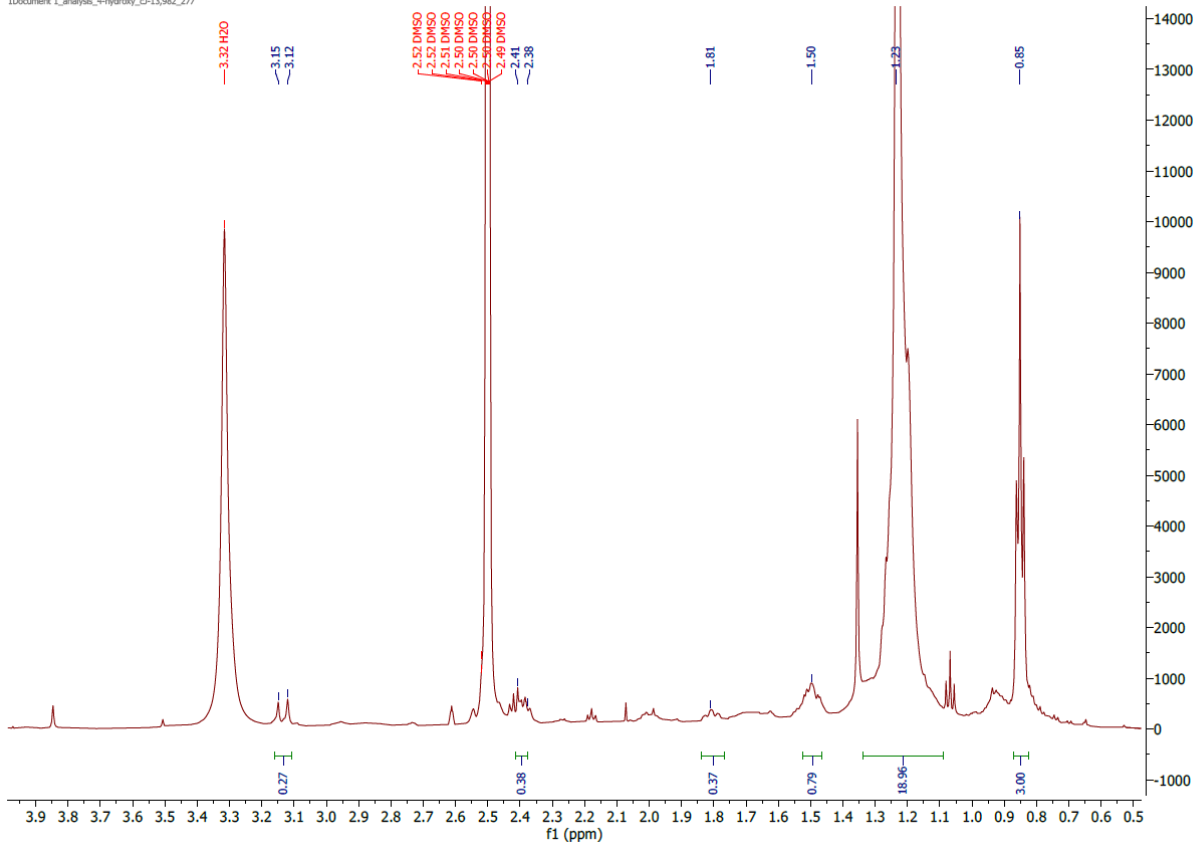


Minimum:  
Maximum:

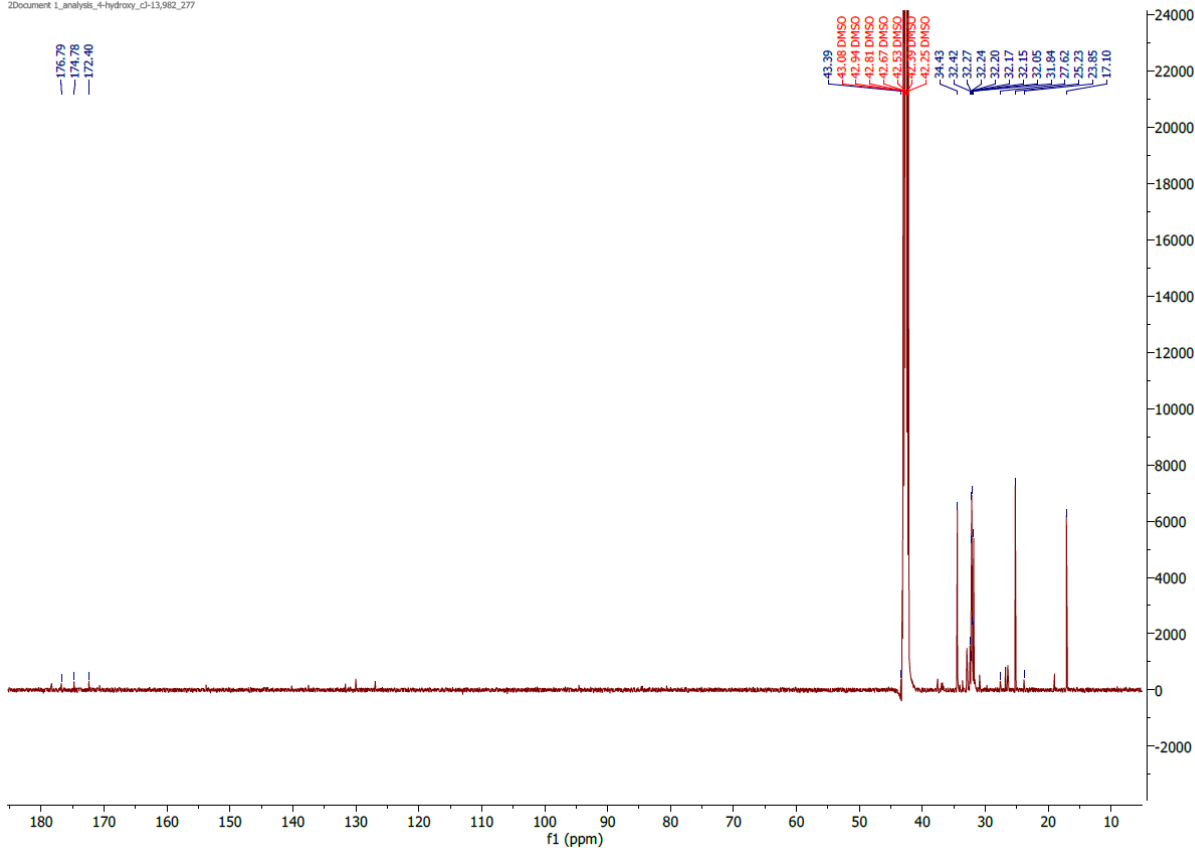
5.0 20.0 -1.5  
50.0

| Mass     | Calc. Mass | mDa  | PPM   | DBE  | i-FIT | i-FIT (Norm) | Formula       |
|----------|------------|------|-------|------|-------|--------------|---------------|
| 375.2018 | 375.2019   | -0.1 | -0.3  | 3.5  | 30.4  | 0.6          | C18 H31 O8    |
|          | 375.1995   | 2.3  | 6.1   | 0.5  | 31.0  | 1.3          | C16 H32 O8 Na |
|          | 375.1960   | 5.8  | 15.5  | 12.5 | 31.9  | 2.1          | C25 H27 O3    |
|          | 375.2089   | -7.1 | -18.9 | 13.5 | 32.8  | 3.0          | C27 H28 Na    |

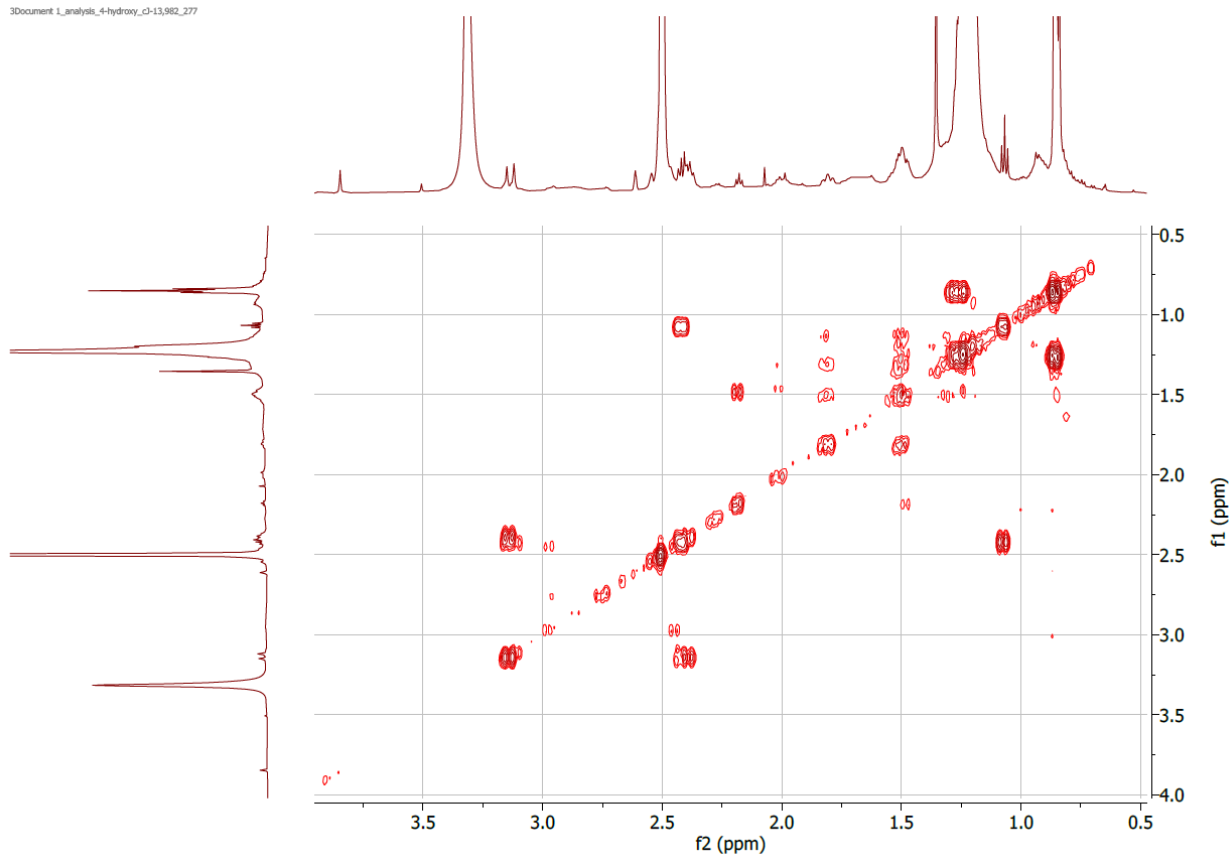
**Figure S34** LCMS characterization and structure elucidation of **12**. **A**, ELSD trace of **12** (arbitrary units, smoothed in Excel); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **12**; **C**, MSMS data obtained for **12**; **D**, HRMS analysis and calculated molecular formula of **12**.



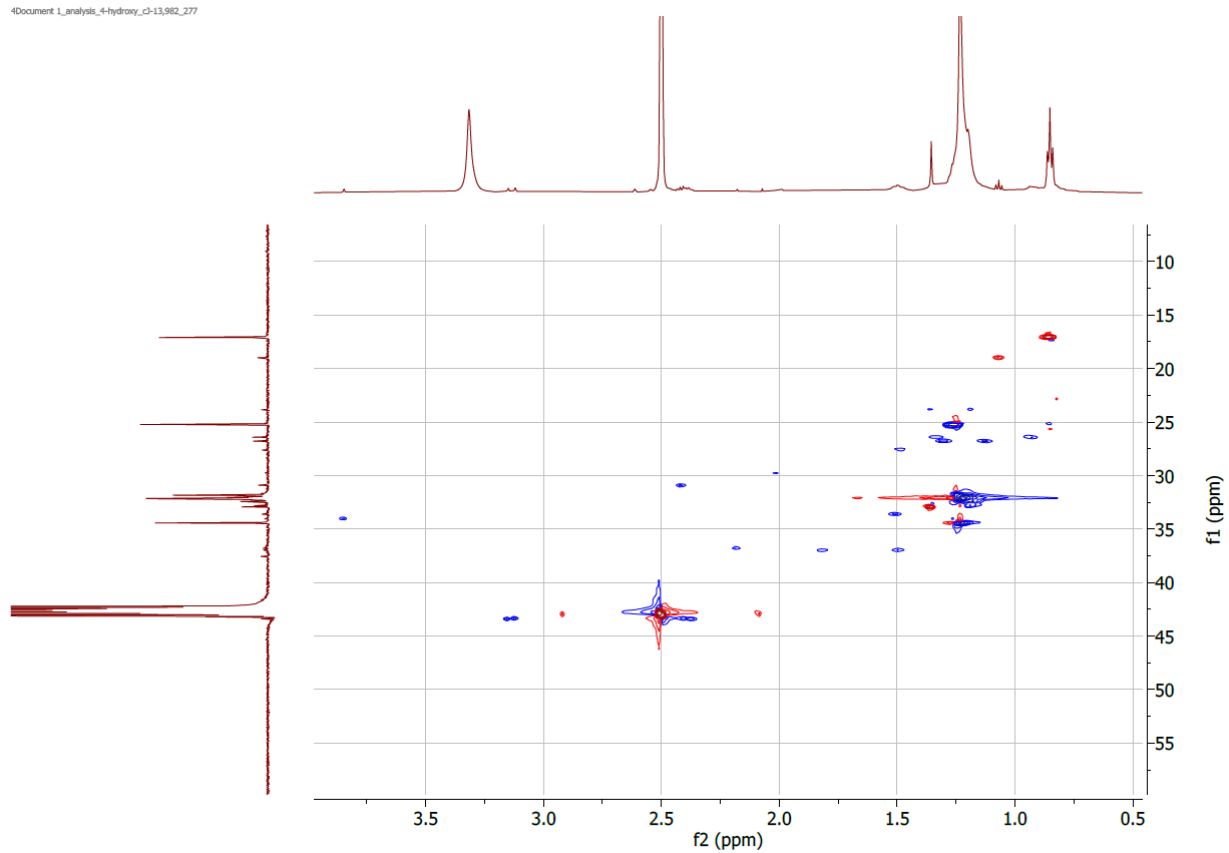
**Figure S35**  $^1\text{H}$  NMR of **12** in  $\text{DMSO-d}_6$  (600 MHz). See Table S21 for assignment.



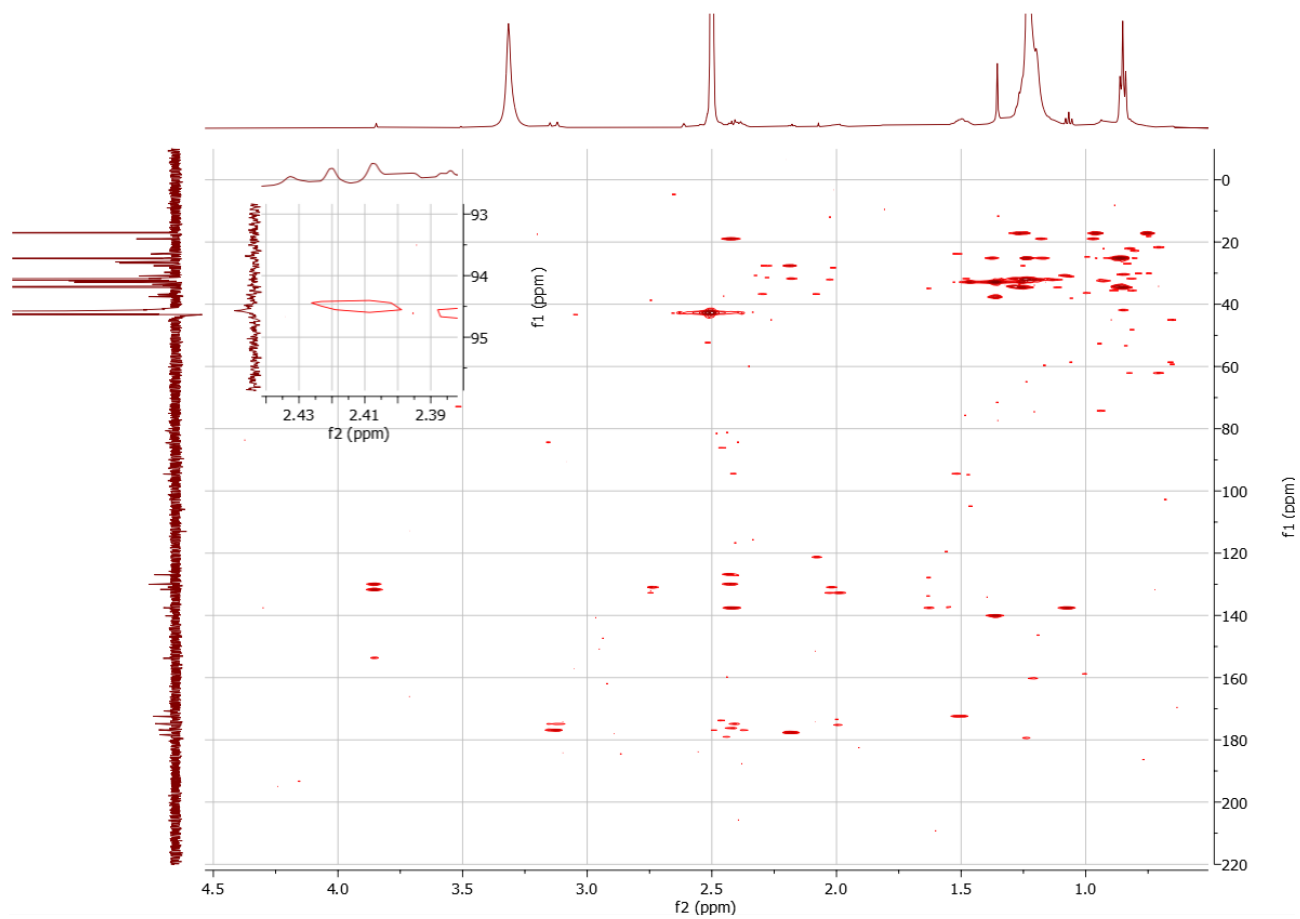
**Figure S36**  $^{13}\text{C}$  NMR of **12** in  $\text{DMSO-d}_6$  (150 MHz). See Table S21 for assignment.



**Figure S37** <sup>1</sup>H, <sup>1</sup>H COSY NMR of **12** in DMSO-d<sub>6</sub>.



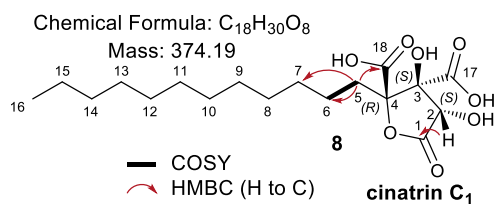
**Figure S38** HSQC NMR of **12** in DMSO-d<sub>6</sub>.



**Figure S39** HMBC NMR of **12** in DMSO-d<sub>6</sub>. Expansion window shows the HMBC correlation of H-2b to C-4.

## 6.2 Characterization of Cinatrin C<sub>1</sub> (8) and Cinatrin C<sub>3</sub> (9)

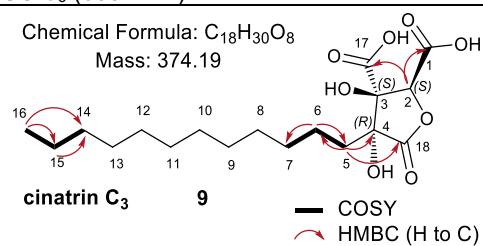
**Table S22** Chemical shifts of partial NMR of **8** in DMSO-d<sub>6</sub> (600 MHz). n.d. = not detected.



| Experimentally observed |   |                      |                 |             |      | Compound <b>8</b> literature in <sup>13</sup> C in DMSO-d <sub>6</sub> , <sup>1</sup> H in CD <sub>3</sub> OD <sup>20</sup> |                      |
|-------------------------|---|----------------------|-----------------|-------------|------|---|----------------------|
| Pos.                    | δ <sub>H</sub> / ppm<br>(mult, J in Hz) | δ <sub>C</sub> / ppm | C-type          | HMBC H to C | COSY | δ <sub>H</sub> / ppm<br>(mult, J in Hz)   | δ <sub>C</sub> / ppm |
| <b>1</b>                | -                                       | 172.9                | CO              | -           | -    | -   | 173.6                |
| <b>2</b>                | 4.72 (bs)                               | 72.5                 | CH              | 1           | -    | 4.69 (s)  | 73.1                 |
| <b>3</b>                | -                                       | n.d.                 | -               | -           | -    | -   | 84.0                 |
| <b>4</b>                | -                                       | n.d.                 | -               | -           | -    | -   | 86.5                 |
| <b>5a</b>               | 2.19 (app t, 7.4)                       | 33.5                 | CH <sub>2</sub> | 6, 7, 18    | 6    | 2.14 (app.t, 8.4)   | 30.8                 |
| <b>5b</b>               | n.d.                                    | -                    | -               | -           | -    | 1.7 (m)   | 30.8                 |
| <b>6a</b>               | 1.5 (m)                                 | 24.5                 | CH <sub>2</sub> | -           | 5    | 1.5 (m)   | 23.5                 |
| <b>6b</b>               | n.d.                                    | -                    | -               | -           | -    | 1.39 (m)  | 23.5                 |
| <b>7</b>                | 1.26 (m)                                | 28.5                 | CH <sub>2</sub> | -           | -    | 1.28 (m)  | 28.7                 |
| <b>8<sup>†</sup></b>    | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 28.8                 |
| <b>9<sup>†</sup></b>    | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 29.2                 |
| <b>10<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 29.0                 |
| <b>11<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 29.0                 |
| <b>12<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 29.0                 |
| <b>13<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 28.7                 |
| <b>14<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 31.3                 |
| <b>15<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 1.28 (m)  | 22.1                 |
| <b>16<sup>†</sup></b>   | n.d.                                    | -                    | -               | -           | -    | 0.89 (t, 6.9)   | 13.9                 |
| <b>17</b>               | -                                       | n.d.                 | CO              | -           | -    | -   | 170.4                |
| <b>18</b>               | -                                       | 174.3                | CO              | -           | -    | -   | 170.6                |

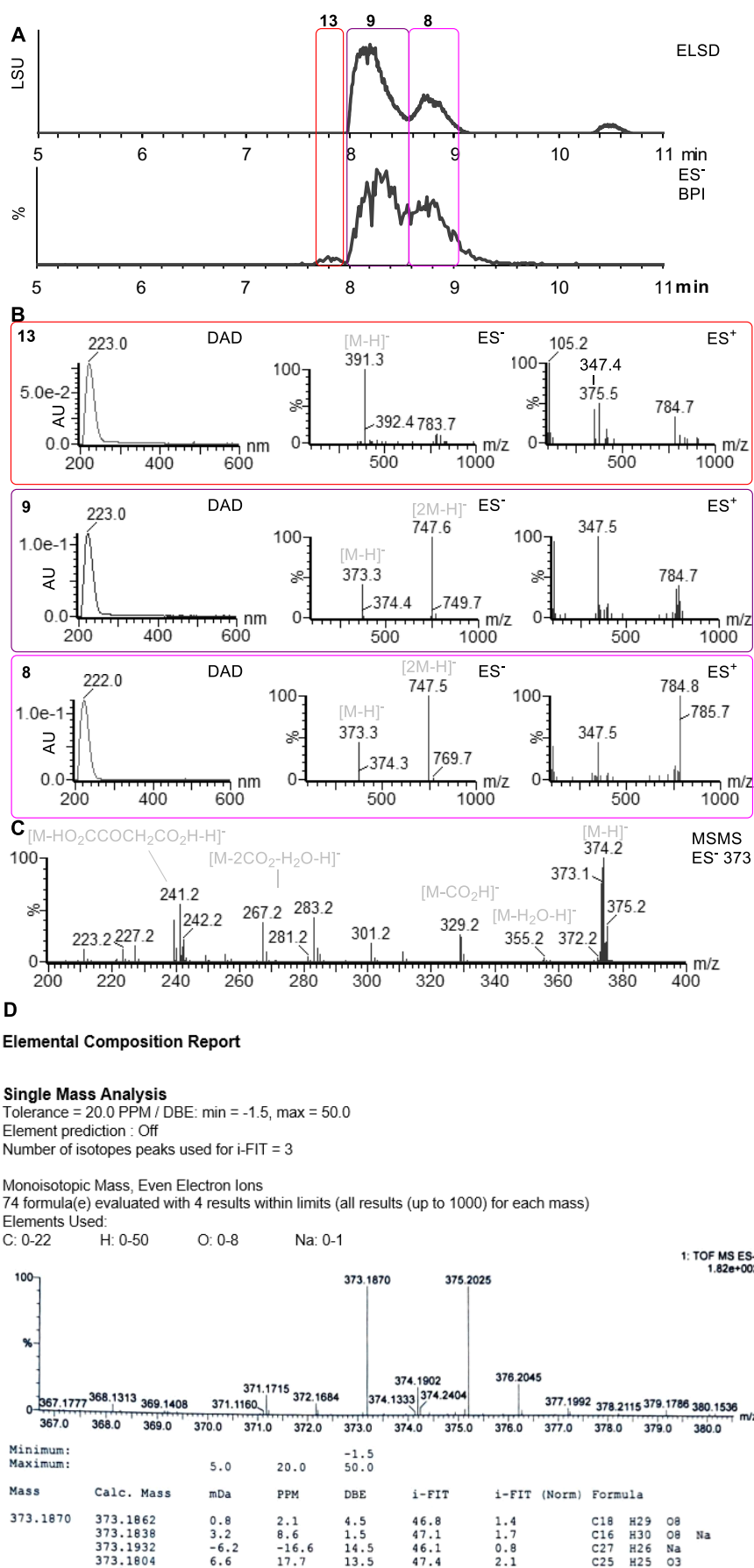
<sup>†</sup> These signals are indistinguishable from cinatrin C<sub>3</sub> signals.

**Table S23** Chemical shifts of **9** in DMSO-d<sub>6</sub> (600 MHz).



| Experimentally observed |                                  |                  |                 |             |      | Compound <b>9</b> literature in DMSO-d <sub>6</sub> <sup>21</sup> |                  |
|-------------------------|----------------------------------|------------------|-----------------|-------------|------|---|------------------|
| Pos.                    | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY | $\delta_H$ / ppm (mult, J in Hz)                                  | $\delta_C$ / ppm |
| <b>1</b>                | -                                | 167.6            | CO              | -           | -    | -   | 167.5            |
| <b>2</b>                | 5.21 (s)                         | 80.4             | CH              | 1, 17       | -    | 5.32 (s)  | 79.5             |
| <b>3</b>                | -                                | 78.2             | C               | -           | -    | 6.25 (br, 3-OH)   | 81.3             |
| <b>4</b>                | -                                | 78.2             | C               | -           | -    | -   | 78.7             |
| <b>5</b>                | 1.61 (m)                         | 30.5             | CH <sub>2</sub> | 4, 6, 7, 18 | 6    | 1.69 (m)  | 30.5             |
| <b>6</b>                | 1.32 - 1.38 (m)                  | 20.9             | CH <sub>2</sub> | 5, 7        | 7, 5 | 1.41 (m)  | 21.0             |
| <b>7</b>                | 1.25 (m)                         | 29.7             | CH <sub>2</sub> | 8-13        | 8-14 | 1.24 (m)  | 29.6             |
| <b>8<sup>†</sup></b>    | 1.25 (m)                         | 28.7             | CH <sub>2</sub> | 7-14        | 7-14 | 1.24 (m)  | 28.9             |
| <b>9<sup>†</sup></b>    | 1.25 (m)                         | 29.0             | CH <sub>2</sub> | 7-14        | 7-14 | 1.24 (m)  | 29.0             |
| <b>10<sup>†</sup></b>   | 1.25 (m)                         | 29.0             | CH <sub>2</sub> | 7-14        | 7-14 | 1.24 (m)  | 29.0             |
| <b>11<sup>†</sup></b>   | 1.25 (m)                         | 29.0             | CH <sub>2</sub> | 7-14        | 7-14 | 1.24 (m)  | 29.0             |
| <b>12<sup>†</sup></b>   | 1.25 (m)                         | 29.1             | CH <sub>2</sub> | 7-14        | 7-14 | 1.24 (m)  | 29.0             |
| <b>13<sup>†</sup></b>   | 1.25 (m)                         | 29.1             | CH <sub>2</sub> | 7-14        | 7-14 | 1.24 (m)  | 28.7             |
| <b>14</b>               | 1.25 (m)                         | 31.3             | CH <sub>2</sub> | 15          | 7-14 | 1.24 (m)  | 31.2             |
| <b>15</b>               | 1.26 (m)                         | 22.1             | CH <sub>2</sub> | 14, 16      | 14   | 1.24 (m)  | 22.0             |
| <b>16</b>               | 0.85 (t, 6.9)                    | 14.0             | CH <sub>3</sub> | 14, 15      | 15   | 0.86 (t)  | 13.9             |
| <b>17</b>               | -                                | 170.9            | CO              | -           | -    | -   | 170.4            |
| <b>18</b>               | -                                | 174.9            | CO              | -           | -    | -   | 174.6            |

<sup>†</sup> These signals are indistinguishable from each other.



**Figure S40** LCMS characterization of **8** and **9**. **A**, ELSD and ES<sup>-</sup> traces of **13**, **8** and **9** (arbitrary units, ELSD smoothed in Excel); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **13**, **8** and **9**; **C**, MSMS data obtained for **8** and **9**; **D**, HRMS analysis and calculated molecular formula of **8** and **9**.

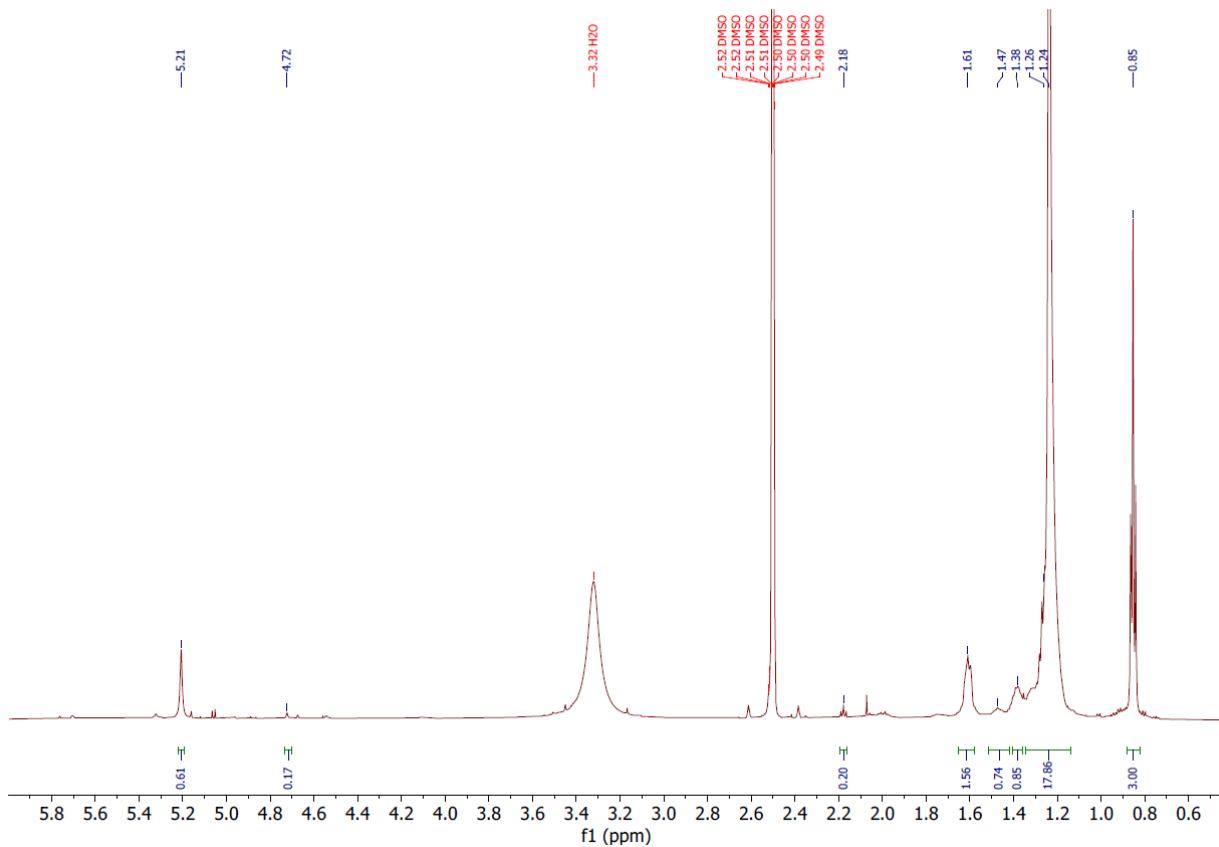


Figure S41  $^1\text{H}$  NMR of **8** and **9** in  $\text{DMSO-d}_6$  (600 MHz). See Table S22 and Table S23 for assignments.

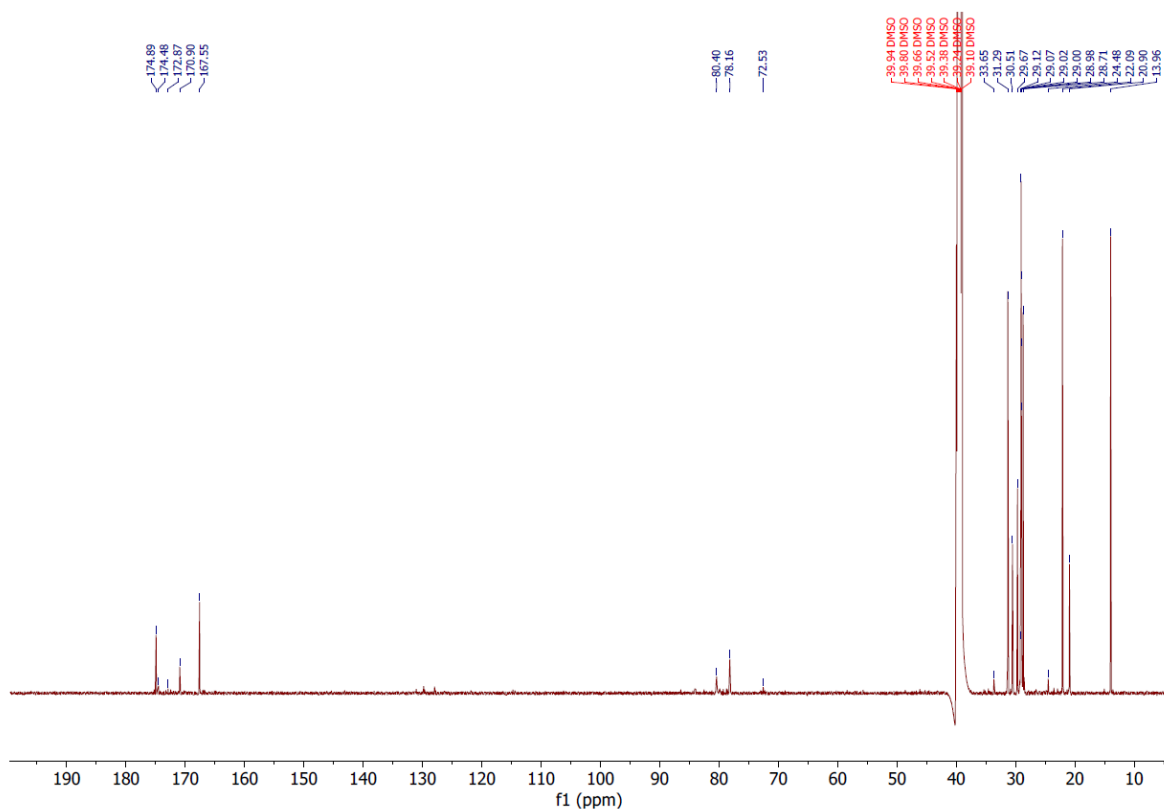
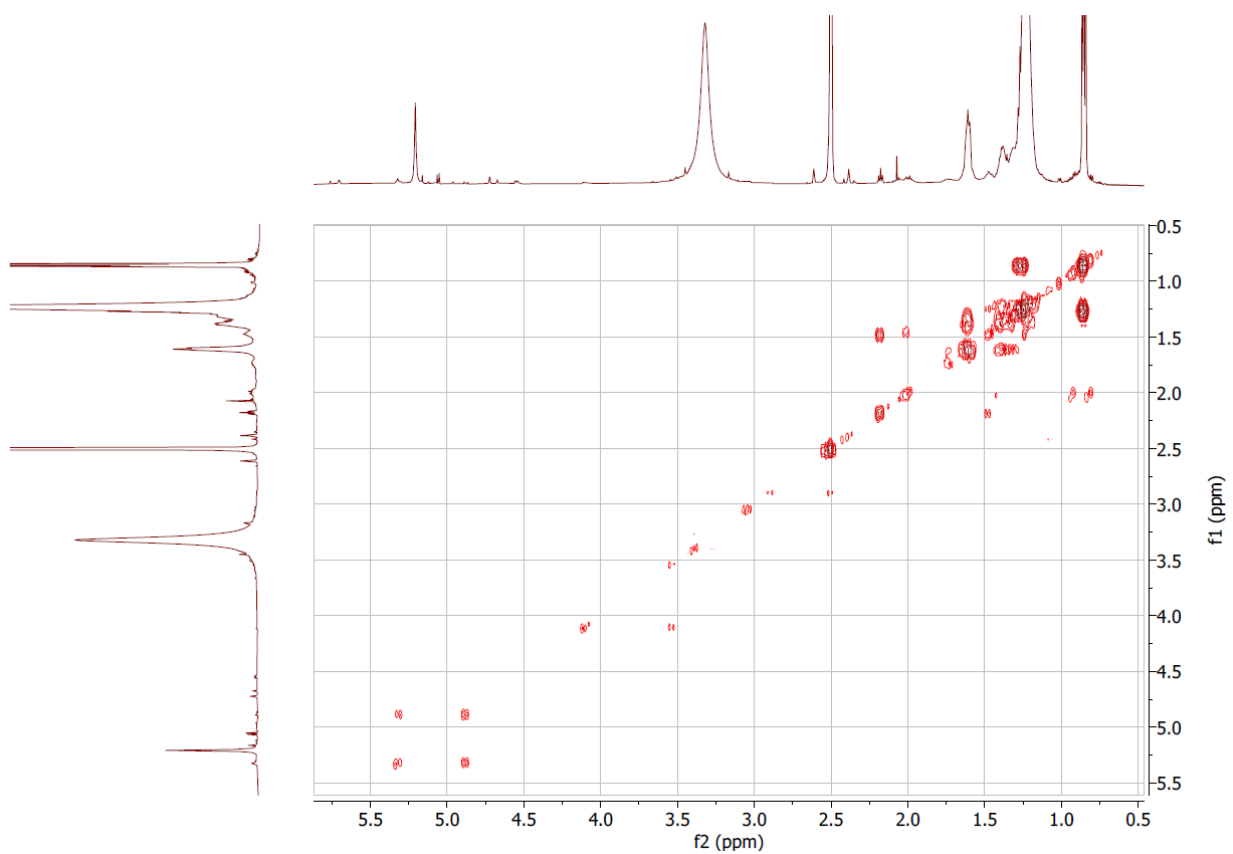
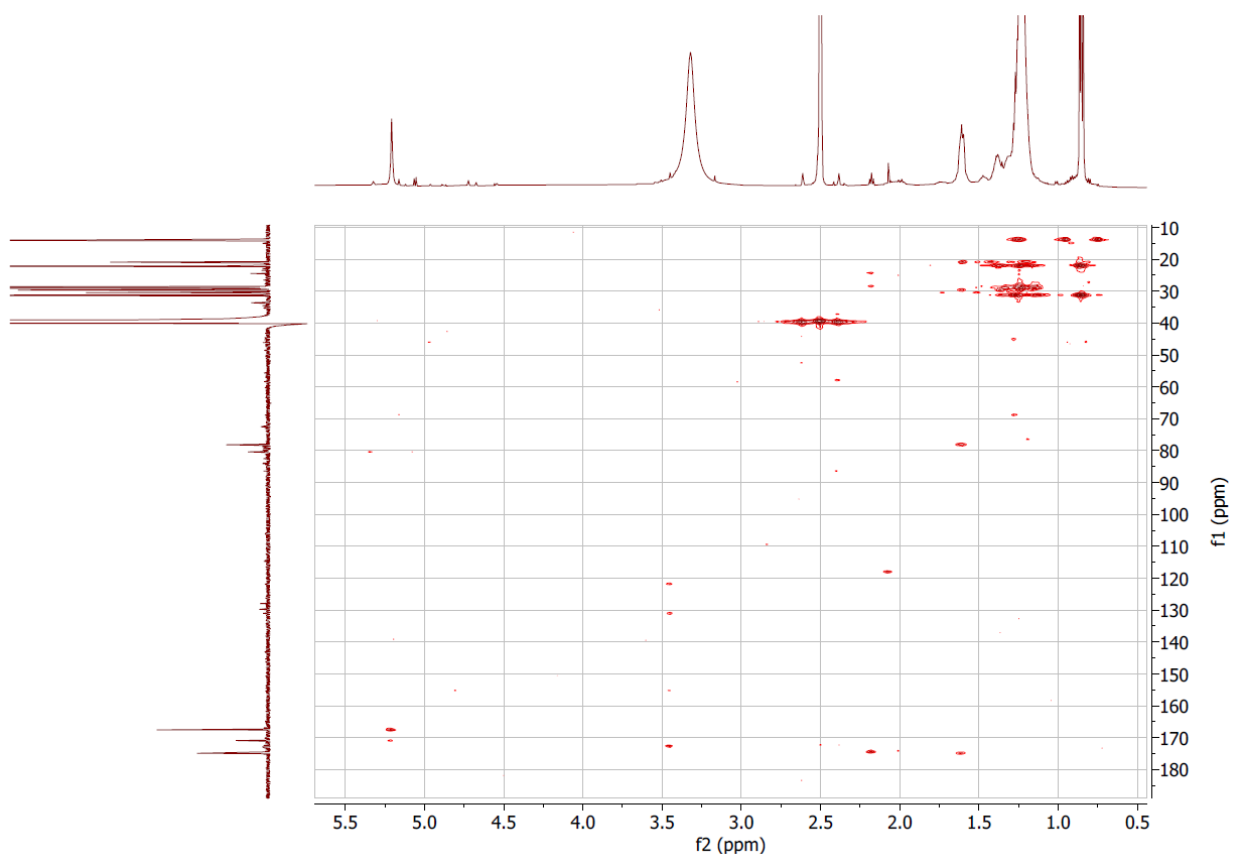


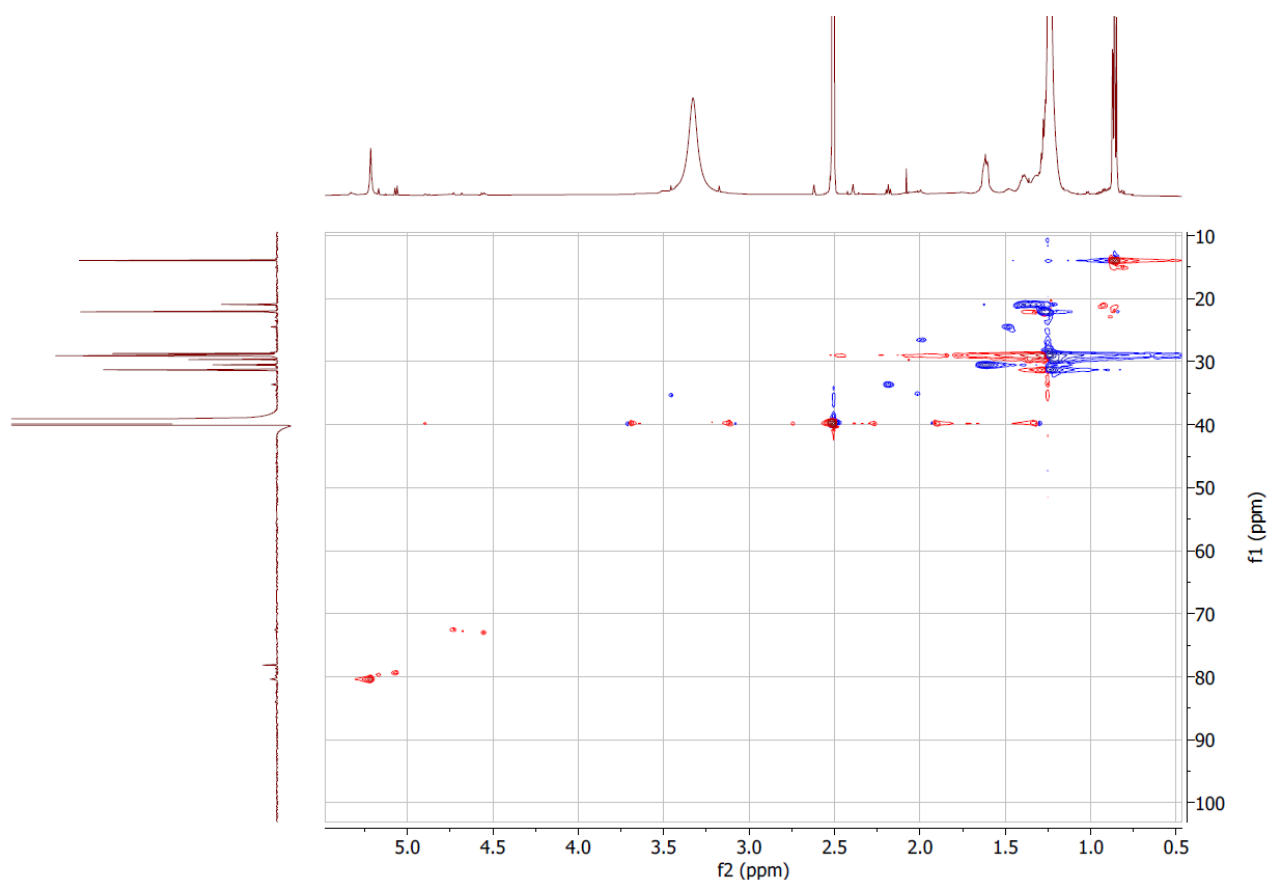
Figure S42  $^{13}\text{C}$  NMR of **8** and **9** in  $\text{DMSO-d}_6$  (150 MHz). See Table S22 and Table S23 for assignments.



**Figure S43**  $^1\text{H}$ ,  $^1\text{H}$  COSY NMR of **8** and **9** in  $\text{DMSO-d}_6$ .



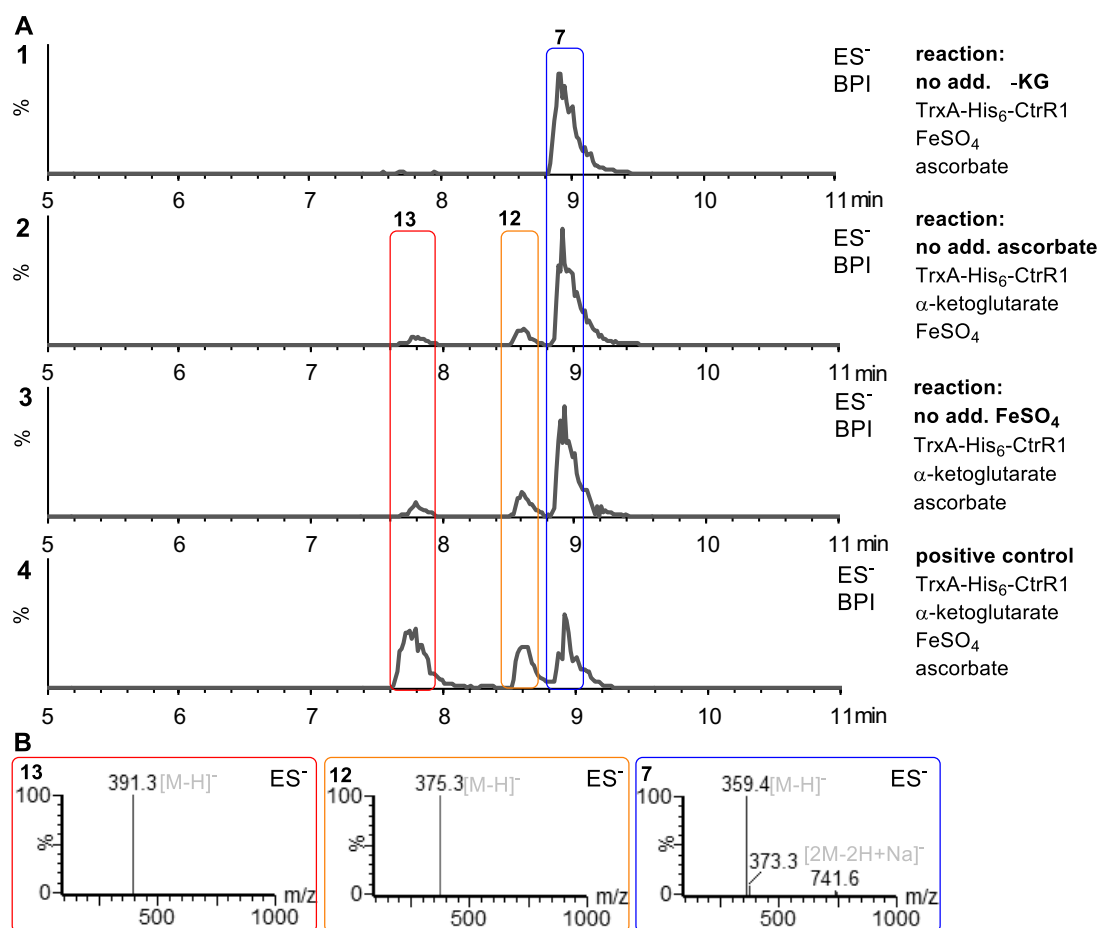
**Figure S44** HMBC NMR of **8** and **9** in  $\text{DMSO-d}_6$ .



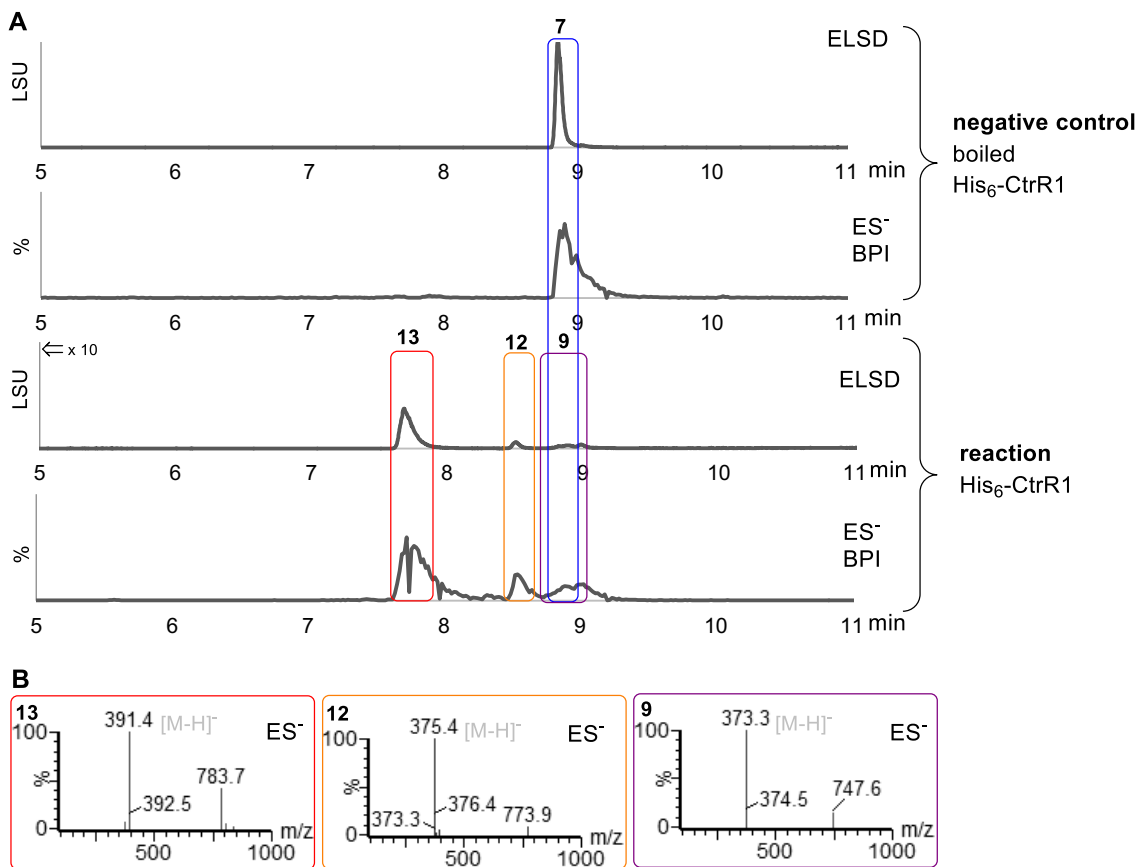
**Figure S45** HSQC NMR of **8** and **9** in  $\text{DMSO-d}_6$ .

## 7. EXPERIMENT 3 (7 + CtrR1) *in vitro*

Incubation of purified Trx-His<sub>6</sub>-CtrR1 with CJ-13,982 **7**, FeSO<sub>4</sub>,  $\alpha$ -ketoglutarate, and ascorbate at 28 °C for 2 h resulted in formation of **12** and **13** (Fig. S46A4), while omission of  $\alpha$ -ketoglutarate abolished the reaction (Fig. S46A1). In reactions with purified His<sub>6</sub>-CtrR1 sequential addition of 75 % of the amount of the initially added NHI oxygenase solution every 20 min over the course of the reaction, resulted in complete consumption of substrate CJ-13,982 **7** (Fig. S47).



**Figure S46** *In vitro* analysis of cofactor-dependency of TrxA-His<sub>6</sub>-CtrR1. **A**, LCMS analysis of reactions containing all or a subset of additives (arbitrary units); **B**, ES<sup>-</sup> spectra of substrate and products.

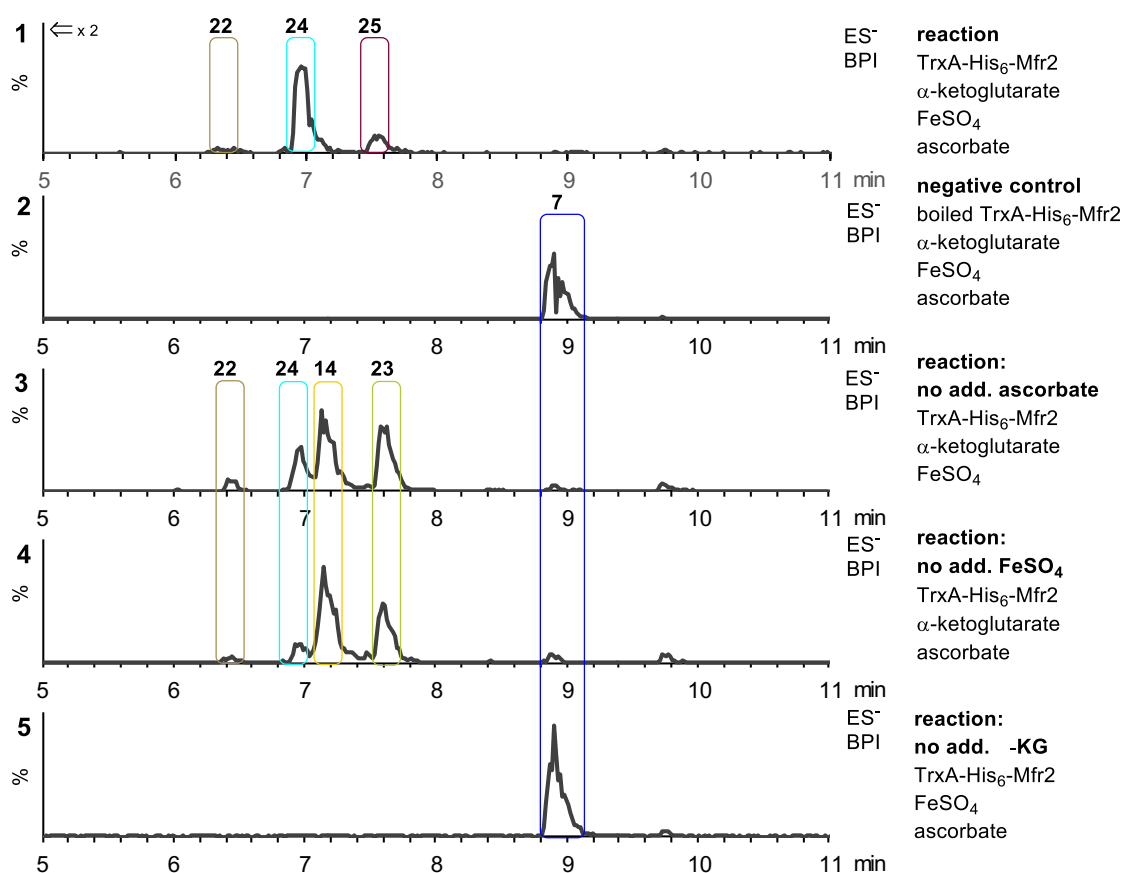


**Figure S47** *In vitro* studies with recombinant His<sub>6</sub>-CtrR1. **A**, LCMS analysis of *in vitro* assays with recombinant His<sub>6</sub>-CtrR1 and boiled His<sub>6</sub>-CtrR1 as control (arbitrary units, zoom is indicated by (←x) where applied, ELSD trace smoothed in Excel); **B**, ES<sup>-</sup> spectra of substrate and products.

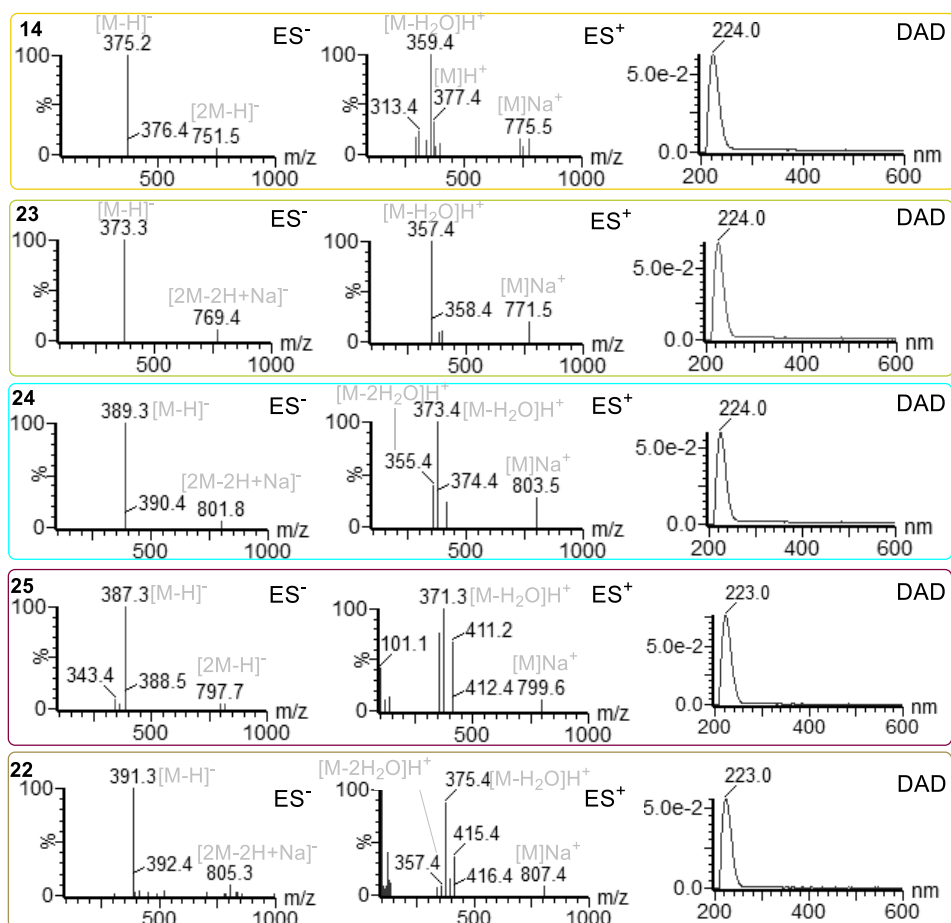


## 9. EXPERIMENT 5 (7 + Mfr2) *in vitro*

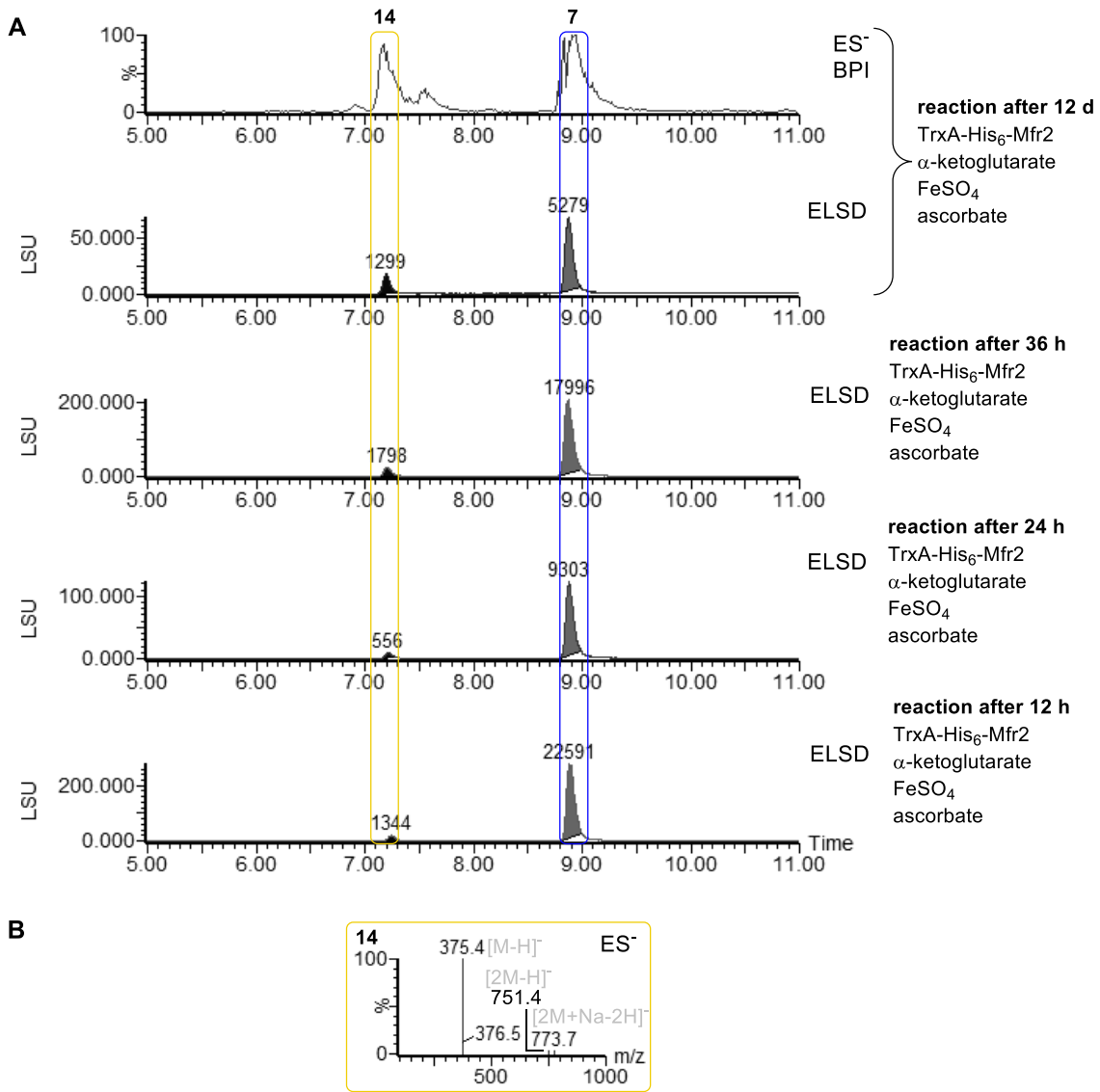
In the presence of TrxA-His<sub>6</sub>-Mfr2 with FeSO<sub>4</sub>, α-ketoglutarate, and ascorbate at 28 °C for 2 h CJ-13,982 **7** was fully converted (Fig. S49, 1) and three new peaks appeared at t<sub>R</sub> = 7.5 min with m/z ES<sup>-</sup> = 387 Da (Fig. S50, corresponds to 5,6-epoxy-7-oxo-CJ-13,982 **25**); t<sub>R</sub> = 6.9 min with m/z ES<sup>-</sup> = 389 (Fig. S50, corresponds to 5-hydroxy-7-oxo-CJ-13,982 **24**); t<sub>R</sub> = 6.5 min with m/z ES<sup>-</sup> = 391 Da (Fig. S50, corresponds to 5,7-dihydroxy-CJ-13,982, **22**). In reactions that lacked additional ascorbate or FeSO<sub>4</sub> (Fig. S49, 3-4) the peak at t<sub>R</sub> = 7.5 min with m/z ES<sup>-</sup> = 387 Da was absent, but two other peaks were detected: t<sub>R</sub> = 7.2 min with m/z ES<sup>-</sup> = 375 Da (Fig. S50, identical to **14**) and t<sub>R</sub> = 7.6 min with m/z ES<sup>-</sup> = 373 Da (Fig. S50, corresponds to 7-oxo-CJ-13,982 **23**). See Fig. S50 for MS spectra of corresponding peaks. Attempts to purify the produced compounds for characterization were not successful, except in the case of **14**. In a large scale *in vitro* reaction (Fig. S51) using TrxA-His<sub>6</sub>-Mfr2 and approximately 5 mg CJ-13,982 **7**, compound **14** (0.6 mg) was isolated and identified as 7-hydroxy-CJ-13,982 by NMR and HRMS analysis (see section 9.1).



**Figure S49** LCMS analysis of *in vitro* reactions with TrxA-His<sub>6</sub>-Mfr2 and boiled control (arbitrary units). See Fig. S75 for predicted structures.



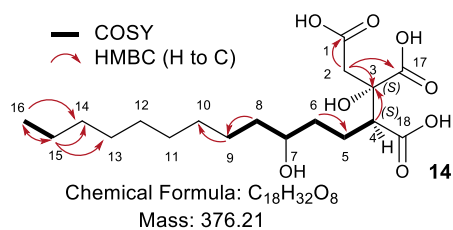
**Figure S50** MS spectra (ES<sup>-</sup>, ES<sup>+</sup>, DAD) of products generated by Mfr2 *in vitro*. See Fig. S75 for predicted structures.



**Figure S51** LCMS analysis of large-scale TrxA-His<sub>6</sub>-Mfr2 *in vitro* assay. **A**, LCMS chromatograms; **B**, MS spectrum of product.

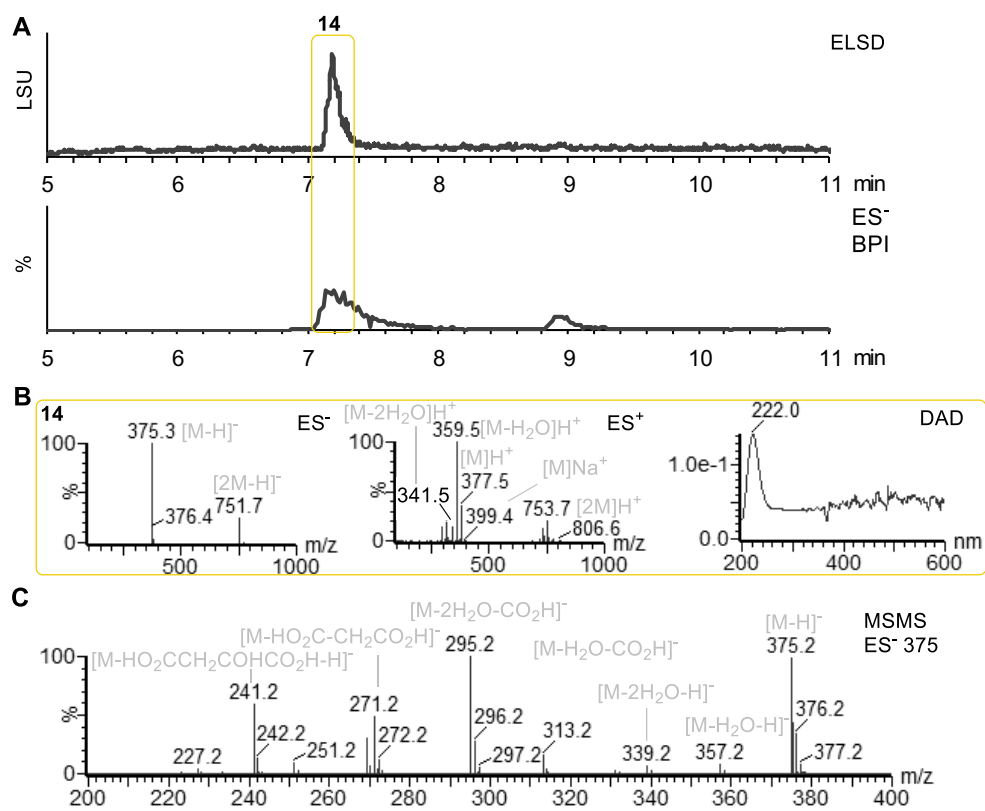
## 9.1 Characterization of 7-Hydroxy-CJ-13,982, 14

Table S24 Chemical shifts of **14** in CD<sub>3</sub>OD (600 MHz).



| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C | COSY      |
|-----------------------|----------------------------------|------------------|-----------------|-------------|-----------|
| <b>1</b>              | -                                | 174.8            | CO              | -           | -         |
| <b>2a</b>             | 3.0 (d, 16.5)                    | 43.7             | CH <sub>2</sub> | 1           | 2b        |
| <b>2b</b>             | 2.67 (d, 16.5)                   | 43.7             | CH <sub>2</sub> | 1, 3, 17    | 2a        |
| <b>3</b>              | -                                | 77.1             | C               | -           | -         |
| <b>4</b>              | 2.70 (m)                         | 54.4             | CH              | 3           | 5a, 5b    |
| <b>5a</b>             | 1.92 (m)                         | 24.6             | CH <sub>2</sub> | -           | 4, 5b, 6  |
| <b>5b</b>             | 1.50 (m)                         | 24.6             | CH <sub>2</sub> | -           | 4, 5a, 6  |
| <b>6</b>              | 1.42 (m)                         | 36.5             | CH <sub>2</sub> | 5           | 5a, 5b, 7 |
| <b>7</b>              | 3.52 (bs)                        | 72.0             | CH              | -           | 6, 8      |
| <b>8</b>              | 1.41 (m)                         | 38.3             | CH <sub>2</sub> | 9           | 7         |
| <b>9</b>              | 1.39 (m)                         | 26.8             | CH <sub>2</sub> | 10-13       | 10-14     |
| <b>10<sup>†</sup></b> | 1.29 (m)                         | 30.9             | CH <sub>2</sub> | 9, 11-13    | 9, 11-13  |
| <b>11<sup>†</sup></b> | 1.29 (m)                         | 30.8             | CH <sub>2</sub> | 10-13       | 10-13     |
| <b>12<sup>†</sup></b> | 1.29 (m)                         | 30.8             | CH <sub>2</sub> | 10-13       | 10-13     |
| <b>13<sup>†</sup></b> | 1.29 (m)                         | 30.5             | CH <sub>2</sub> | 10-13       | 10-13     |
| <b>14</b>             | 1.29 (m)                         | 33.1             | CH <sub>2</sub> | 10-13       | 10-13     |
| <b>15</b>             | 1.29 (m)                         | 23.8             | CH <sub>2</sub> | 13, 14, 16  | 16        |
| <b>16</b>             | 0.90 (t, 6.9)                    | 14.4             | CH <sub>3</sub> | 15, 14      | 15        |
| <b>17</b>             | -                                | 178.0            | CO              | -           | -         |
| <b>18</b>             | -                                | 177.3            | CO              | -           | -         |

† These signals are indistinguishable from each other.



**D**  
Elemental Composition Report

Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction : Off

Number of isotopes peaks used for i-FIT = 3

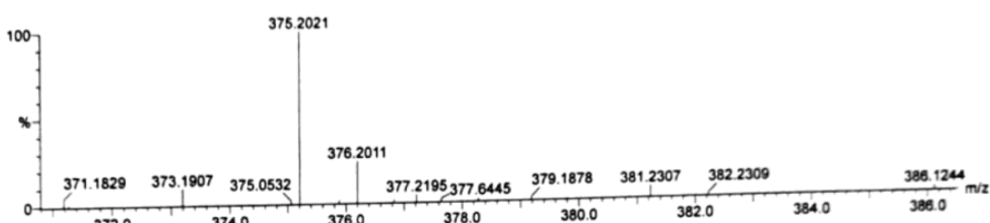
Monoisotopic Mass, Even Electron Ions

76 formula(e) evaluated with 8 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-40 H: 0-50 O: 0-8 Na: 0-1

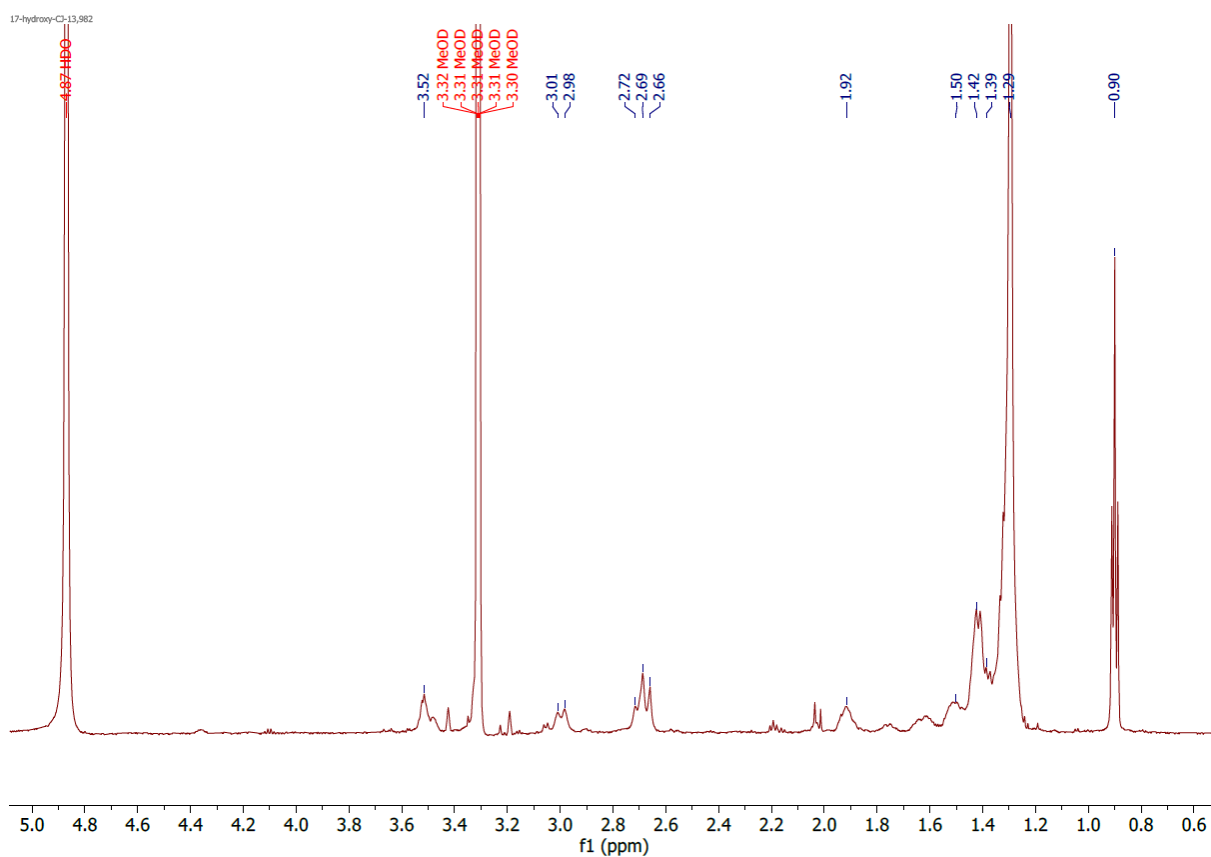
1: TOF MS ES-  
1.14e+002



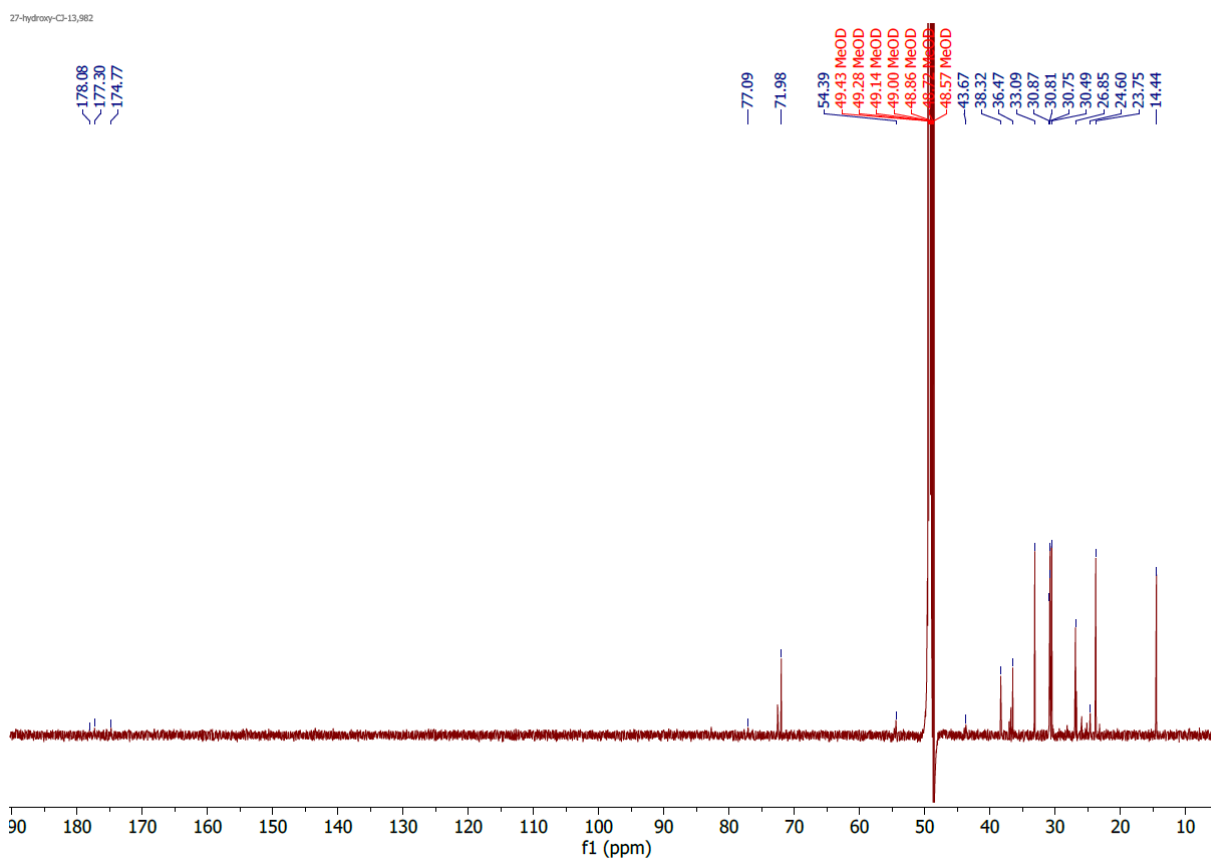
Minimum: 5.0 50.0 -1.5  
Maximum: 5.0 50.0 50.0

| Mass     | Calc. Mass | mDa   | PPM   | DBE  | i-FIT | i-FIT (Norm) | Formula       |
|----------|------------|-------|-------|------|-------|--------------|---------------|
| 375.2021 | 375.2019   | 0.2   | 0.5   | 3.5  | 12.8  | 1.5          | C18 H31 O8    |
|          | 375.1995   | 2.6   | 6.9   | 0.5  | 13.2  | 1.8          | C16 H32 O8 Na |
|          | 375.1960   | 6.1   | 16.3  | 12.5 | 13.0  | 1.7          | C25 H27 O3    |
|          | 375.2089   | -6.8  | -18.1 | 13.5 | 13.4  | 2.1          | C27 H28 Na    |
|          | 375.1936   | 8.5   | 22.7  | 9.5  | 13.2  | 1.9          | C23 H28 O3 Na |
|          | 375.2113   | -9.2  | -24.5 | 16.5 | 14.1  | 2.8          | C29 H27       |
|          | 375.2147   | -12.6 | -33.6 | 4.5  | 14.1  | 2.8          | C20 H32 O5 Na |
|          | 375.2171   | -15.0 | -40.0 | 7.5  | 14.5  | 3.2          | C22 H31 O5    |

**Figure S52** LCMS characterization and structure elucidation of **14**. **A**, ELSD and ES<sup>-</sup> traces of purified material (arbitrary units); **B**, DAD, ES<sup>-</sup> and ES<sup>+</sup> spectra of **14**; **C**, MSMS analysis of **14**; **D**, HRMS analysis.



**Figure S53** <sup>1</sup>H NMR of **14** in CD<sub>3</sub>OD (600 MHz). See Table S24 for assignment.



**Figure S54** <sup>13</sup>C NMR of **14** in CD<sub>3</sub>OD (150 MHz). Table S24 for assignment.

37-hydroxy-C-13,982

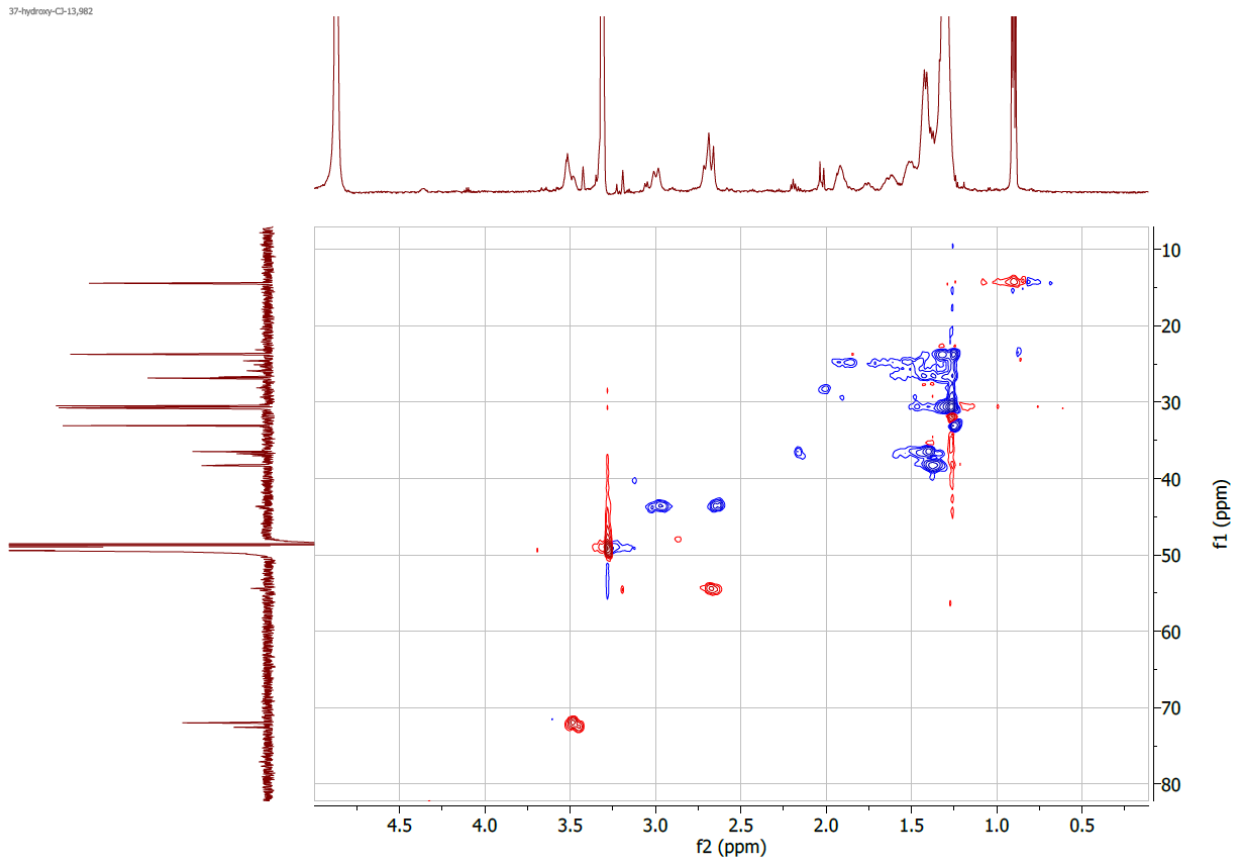


Figure S55 HSQC NMR of 14 in CD<sub>3</sub>OD.

47-hydroxy-C-13,982

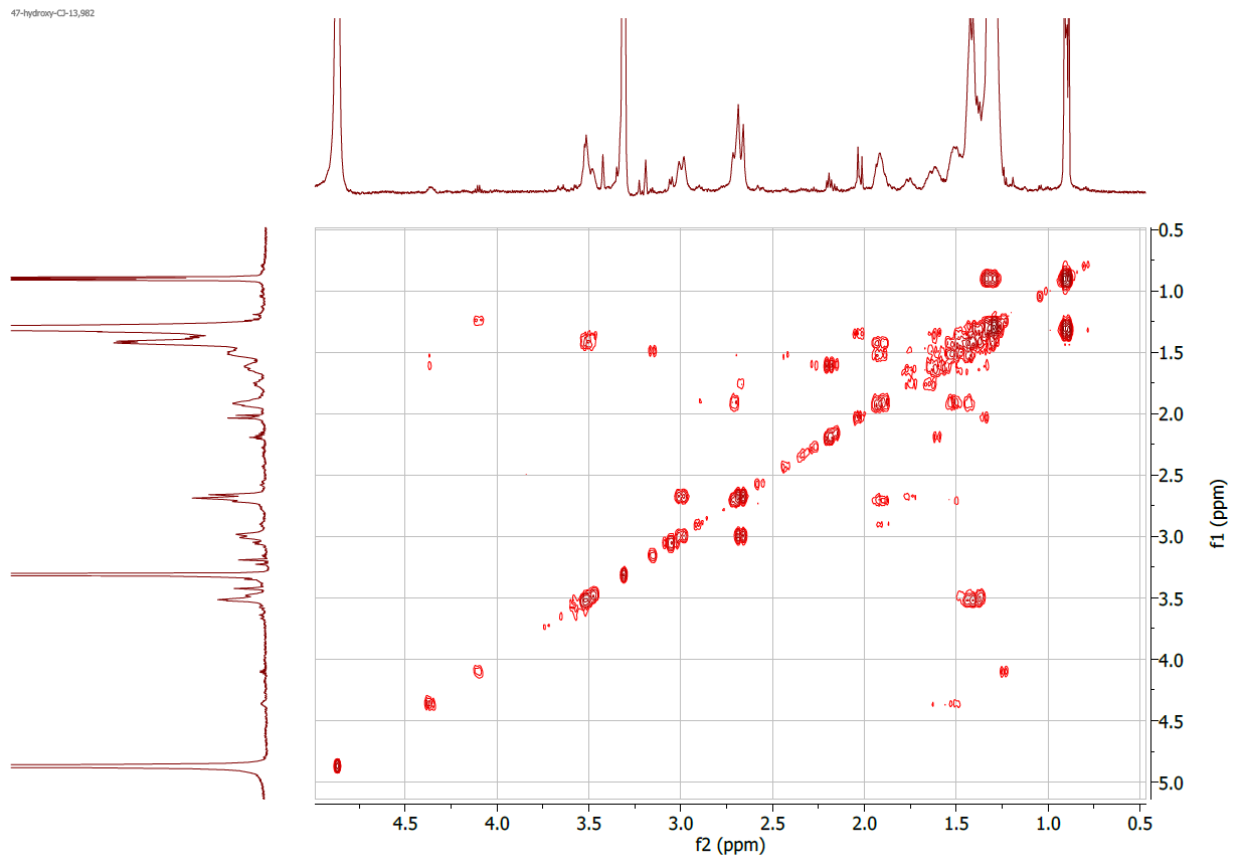


Figure S56 <sup>1</sup>H, <sup>1</sup>H COSY of 14 in CD<sub>3</sub>OD.

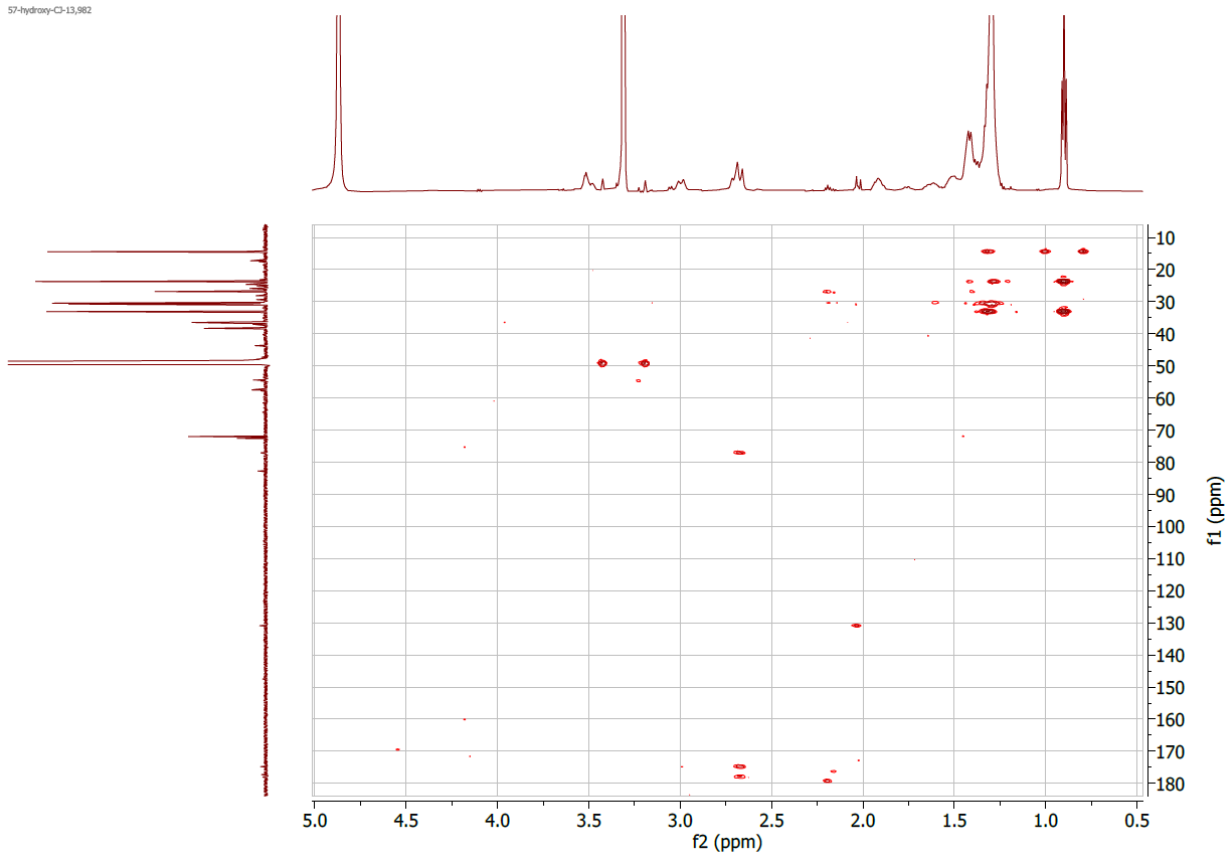
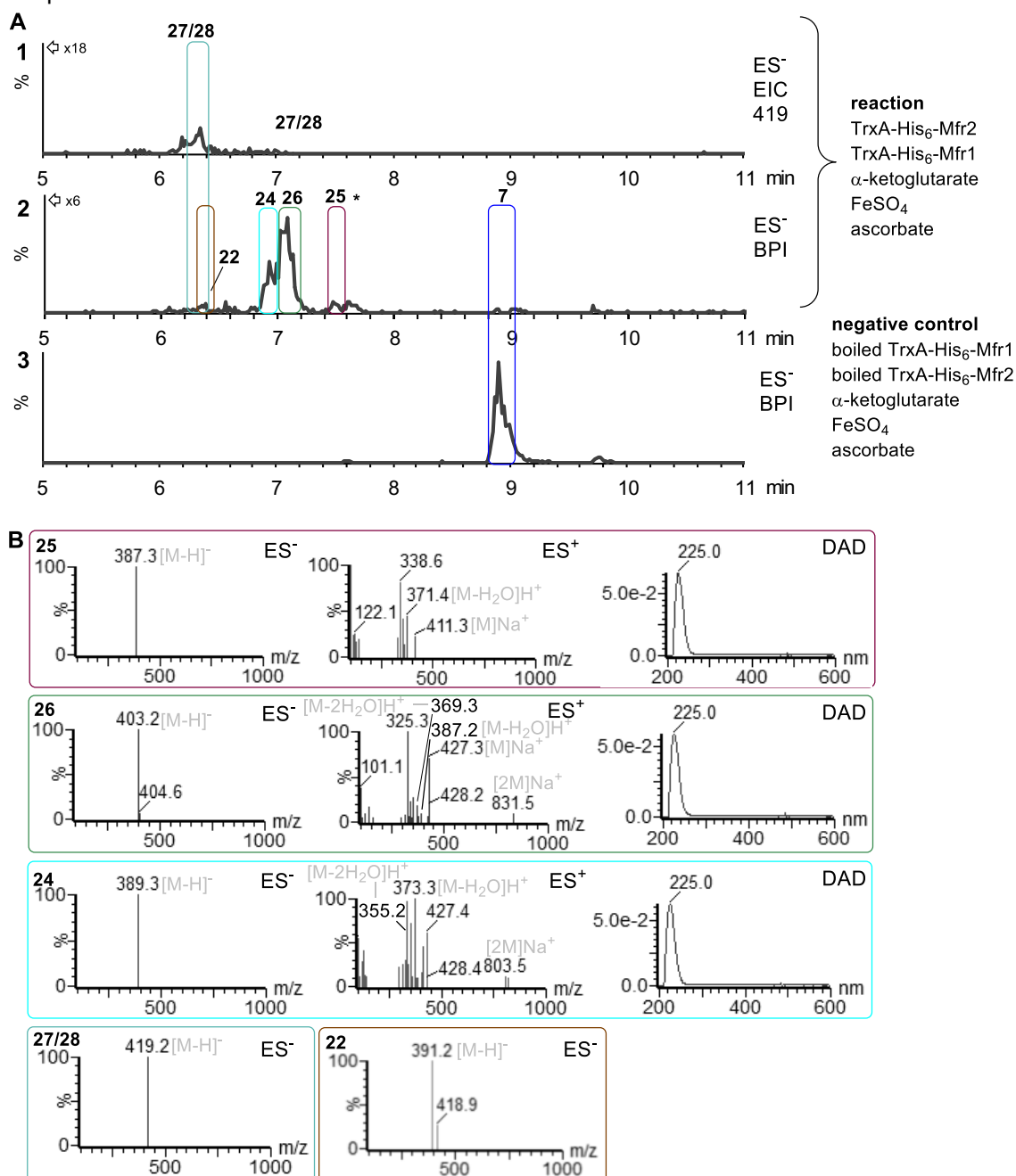


Figure S57 HMBC NMR of **14** in CD<sub>3</sub>OD.

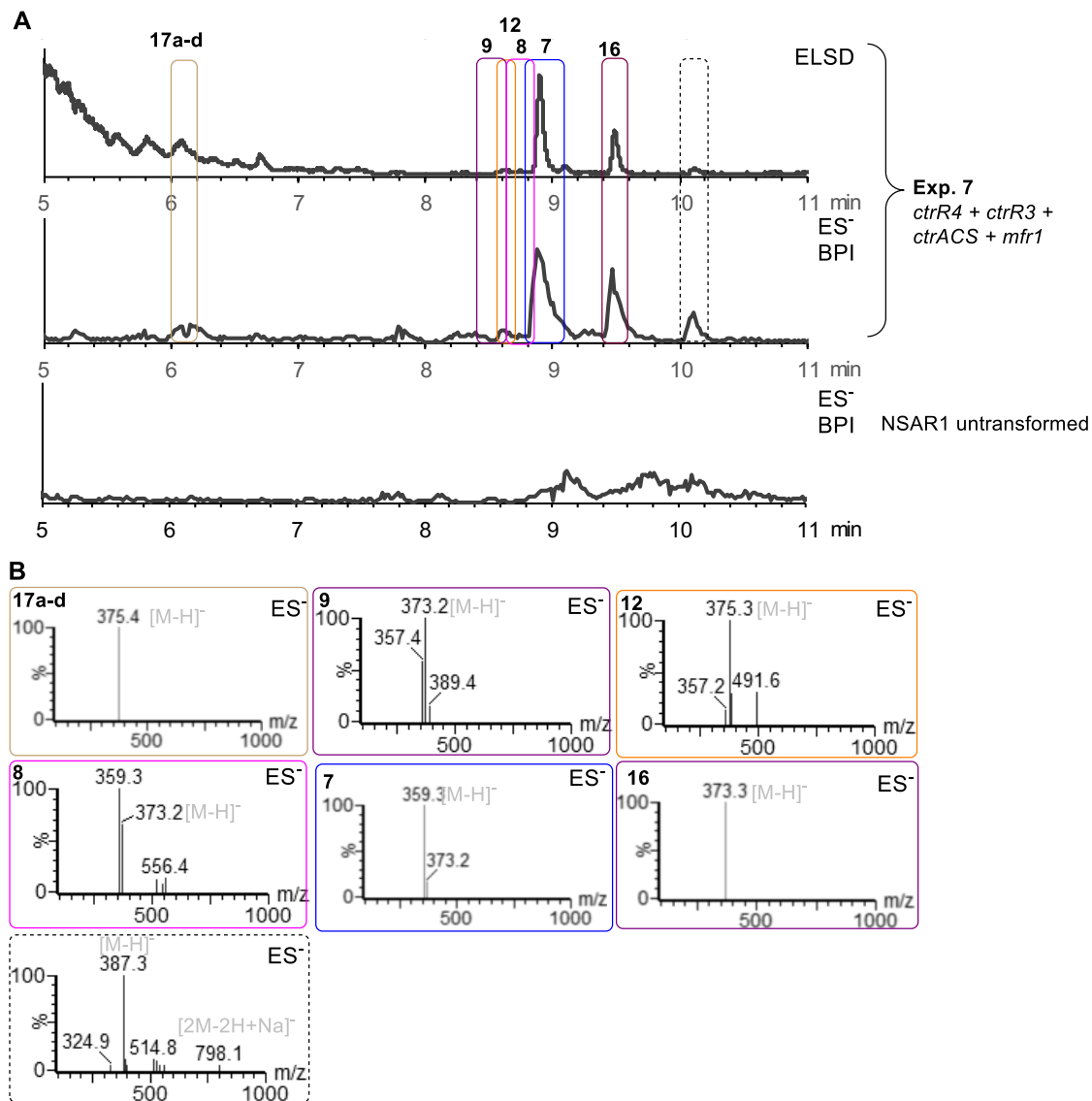
## 10. EXPERIMENT 6 (7 + Mfr1 + Mfr2) *in vitro*

In reactions that contained both, TrxA-His<sub>6</sub>-Mfr1 and TrxA-His<sub>6</sub>-Mfr2 (Fig. S58A2) the three compounds eluting at  $t_R = 7.5$  min with  $m/z$  ES<sup>-</sup> = 387 Da (**25**, Fig. S58B);  $t_R = 6.9$  min with  $m/z$  ES<sup>-</sup> = 389 Da (**24**, Fig. S58B);  $t_R = 6.5$  min with  $m/z$  ES<sup>-</sup> = 391 Da (**22**, Fig. S58B) were detected. Additionally another compound eluted at  $t_R = 7.1$  min with  $m/z$  ES<sup>-</sup> = 403 Da (corresponds to 5,6-epoxy-4-hydroxy-7-oxo-CJ-13,982 **26**, Fig. S58B)) that was not detected in reactions containing only one of the oxygenases. A barely detectable peak with  $m/z$  419.2 Da (Fig. S58A1, Fig. S58B) at 6.8 min may correspond to either **27** or **28**. Attempts to purify the produced compounds for characterization were not successful.



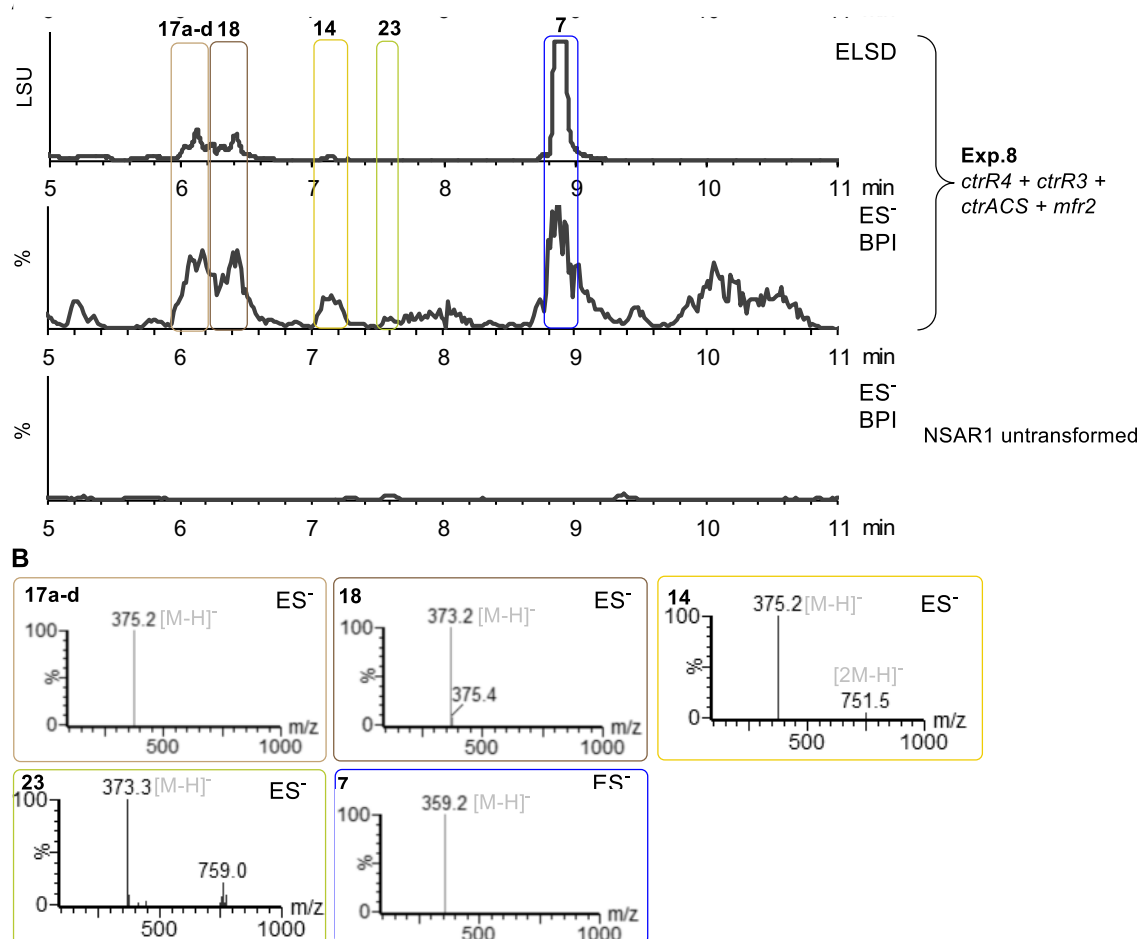
**Figure S58** *In vitro* studies with recombinant TrxA-His<sub>6</sub>-Mfr2 and TrxA-His<sub>6</sub>-Mfr1. **A**, LCMS analysis of *in vitro* assays with recombinant TrxA-His<sub>6</sub>-Mfr2 and TrxA-His<sub>6</sub>-Mfr1 and boiled TrxA-His<sub>6</sub>-Mfr2 and TrxA-His<sub>6</sub>-Mfr1 as control (arbitrary units, zoom is indicated by ( $\leftarrow \times$ ) where applied, ELSD trace smoothed in Excel); **B**, ES<sup>-</sup> (and ES<sup>+</sup>, DAD) spectra of products. See Fig. S75 for predicted structures.

## 11. EXPERIMENT 7 (*ctrR4* + *ctrR3* + *ctrACS* + *mfr1*) *in vivo*



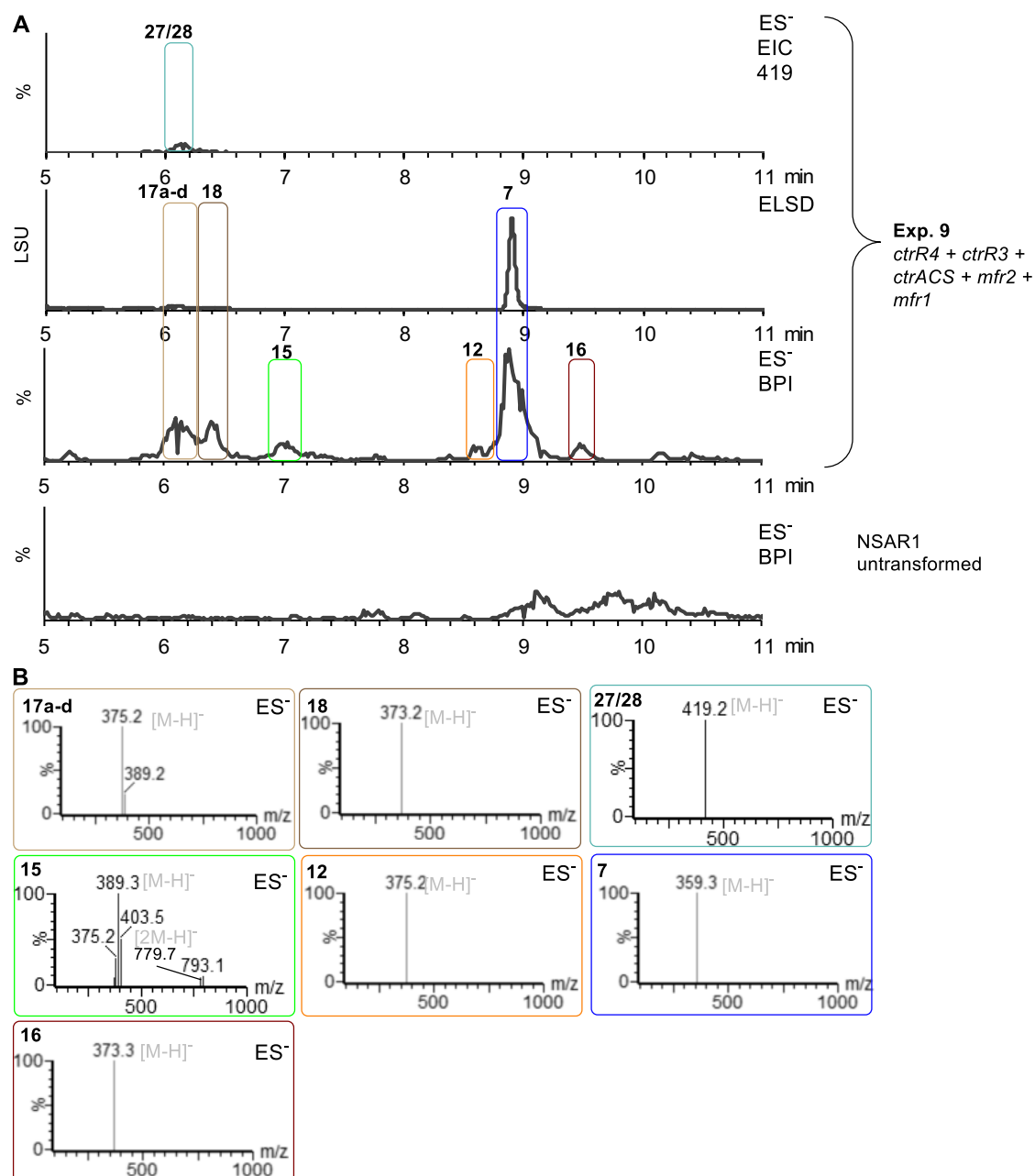
**Figure S59** Heterologous expression of the structural genes of the *ctr* BGC *ctrR4*, *ctrR3* and *ctrACS*, and SS1 BGC *mfr1*. **A**, ES<sup>-</sup> (and ELSD) chromatograms of extracts of *A. oryzae* NSAR1 transformants carrying genes from the SS1 BGC and *ctr* BGC and untransformed control (arbitrary units); **B**, ES<sup>-</sup> spectra of compounds detected in an *A. oryzae* NSAR1 transformants.

## 12. EXPERIMENT 8 (*ctrR4* + *ctrR3* + *ctrACS* + *mfr2*) *in vivo*



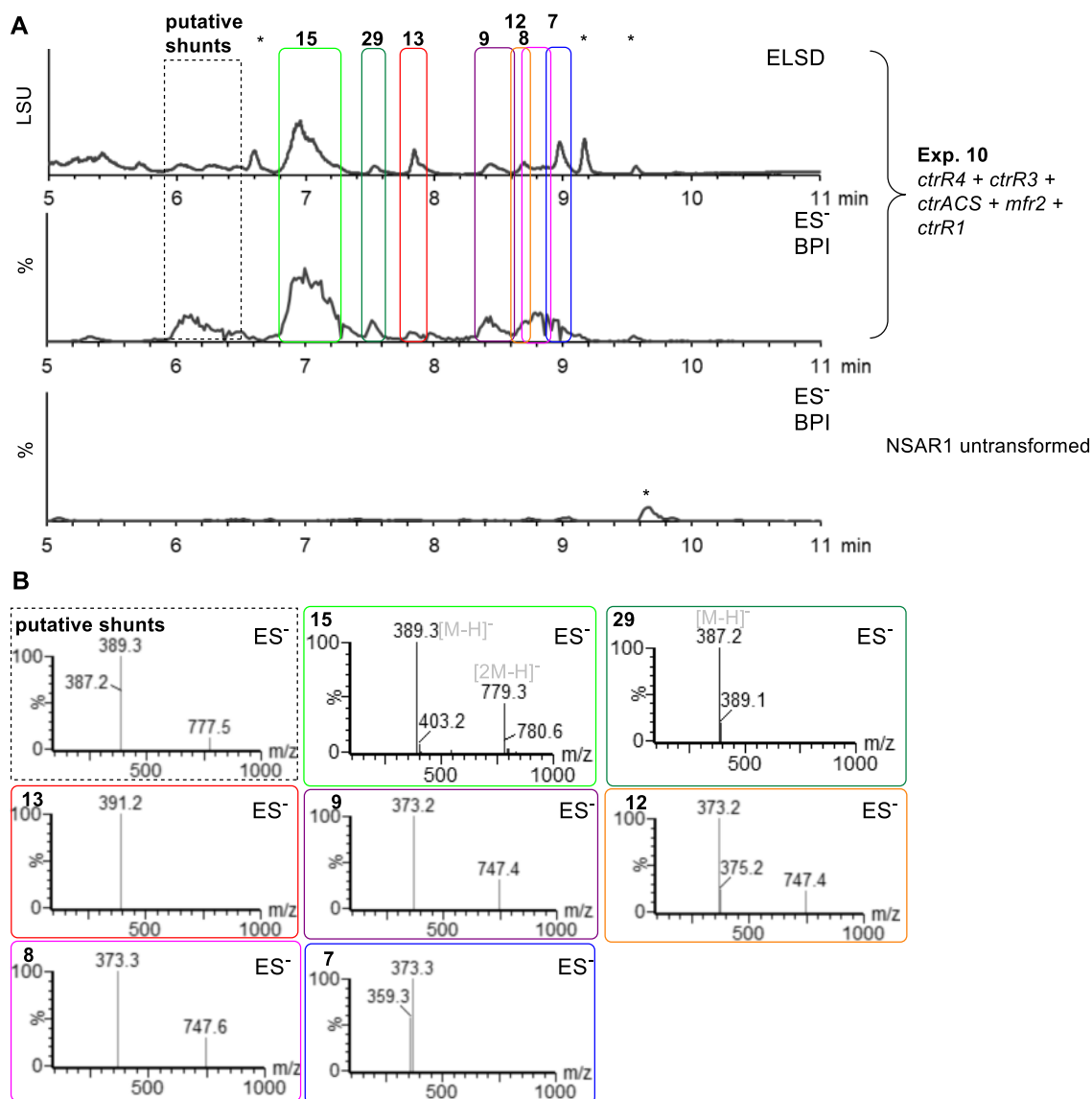
**Figure S60 A**, Heterologous expression of the structural genes of the *ctr* BGC *ctrR4*, *ctrR3* and *ctrACS*, and SS1 BGC *mfr2*. **A**, ES<sup>-</sup> (and ELSD) chromatograms of extracts of *A. oryzae* NSAR1 transformants carrying genes from the SS1 BGC and *ctr* BGC and untransformed control (arbitrary units); **B**, ES<sup>-</sup> spectra of compounds detected in an *A. oryzae* NSAR1 transformants. See Fig. S75 for predicted structures.

### 13. EXPERIMENT 9 (*ctrR4* + *ctrR3* + *ctrACS* + *mfr1* + *mfr2*) *in vivo*



**Figure S61** LCMS analysis of extracts of a representative transformant of the *ctr* BGC *ctrR4*, *ctrR3* and *ctrACS*, and *SS1* BGC *mfr2* and *mfr1*. **A**, ES<sup>-</sup>, ELSD and EIC chromatograms of extracts of *A. oryzae* NSAR1 transformants carrying indicated genes and untransformed control (arbitrary units); **B**, ES<sup>-</sup> spectra of compounds detected in extracts of *A. oryzae* NSAR1 transformant from *A. oryzae* + *ctrR4* + *ctrR3* + *ctrACS* + *mfr2* + *mfr1*. See Fig. S75 for proposed structures.

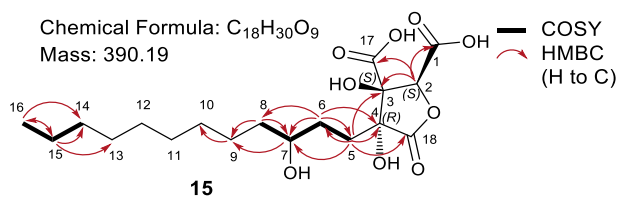
# 14. EXPERIMENT 10 (*ctrR4 + ctrR3 + ctrACS + ctrR1 + mfr2*) *in vivo*



**Figure S62** LCMS analysis of extracts of a representative transformant of indicated experiments. **A**, ES<sup>-</sup> and ELSD chromatograms of extracts of *A. oryzae* NSAR1 transformants carrying indicated genes and untransformed control (arbitrary units, \* = unrelated compounds); **B**, ES<sup>-</sup>, ES<sup>+</sup> and DAD spectra of new compounds detected in extracts of *A. oryzae* NSAR1 transformant from indicated peaks. See Fig. S75 for predicted structures.

## 14.1 Characterization of 7-Hydroxy-Cinatriin C<sub>3</sub>, 15

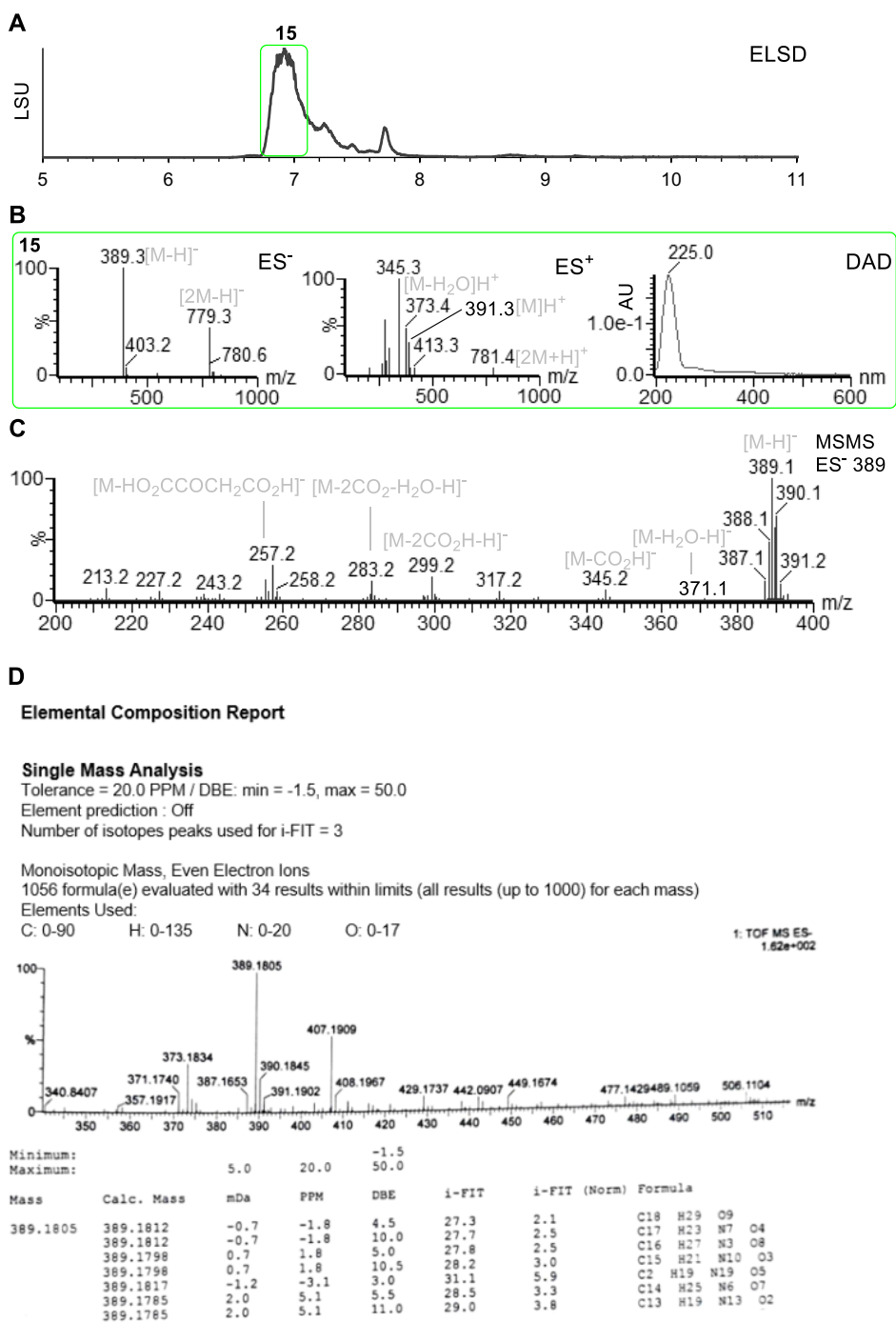
Table S25 Chemical shifts of **15** in CD<sub>3</sub>OD (600 MHz).



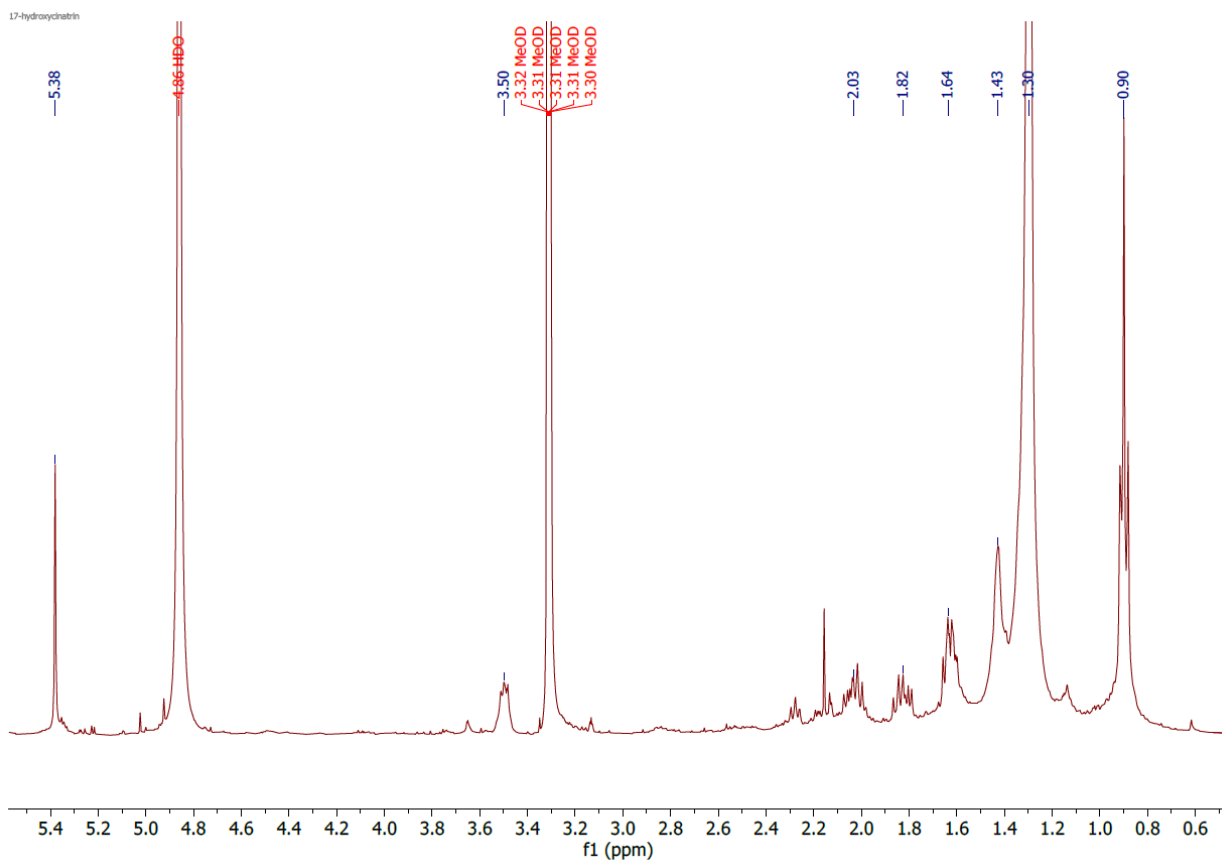
7-hydroxy-cinatriin C<sub>3</sub>

| Pos.                  | $\delta_H$ / ppm (mult, J in Hz) | $\delta_C$ / ppm | C-type          | HMBC H to C    | COSY      |
|-----------------------|----------------------------------|------------------|-----------------|----------------|-----------|
| <b>1</b>              | -                                | 169.8            | CO              | -              | -         |
| <b>2</b>              | 5.38 (s)                         | 82.2             | CH              | 1, 3, 17       | -         |
| <b>3</b>              | -                                | 81.7             | C               | -              | -         |
| <b>4</b>              | -                                | 80.1             | C               | -              | -         |
| <b>5a</b>             | 2.06 (m)                         | 28.2             | CH <sub>2</sub> | 3, 4, 6, 7, 18 | 5b, 6     |
| <b>5b</b>             | 1.83 (ddd, 14, 9.8, 6.5)         | 28.2             | CH <sub>2</sub> | 4, 6, 7, 18    | 5a, 6     |
| <b>6</b>              | 1.64 (ddt, 14, 9.8, 7.5)         | 30.1             | CH <sub>2</sub> | 4, 5, 7, 8     | 5a, 5b, 7 |
| <b>7</b>              | 3.53 (m)                         | 72.8             | CH              | 9              | 6, 8      |
| <b>8</b>              | 1.45 (m)                         | 38.3             | CH <sub>2</sub> | 7, 9           | 7         |
| <b>9</b>              | 1.45 (m)                         | 26.8             | CH <sub>2</sub> | 10             | 10-15     |
| <b>10</b>             | 1.32 (m)                         | 30.7             | CH <sub>2</sub> | 11-15          | 11-15     |
| <b>11<sup>†</sup></b> | 1.32 (m)                         | 30.8             | CH <sub>2</sub> | 10-15          | 10-15     |
| <b>12<sup>†</sup></b> | 1.32 (m)                         | 30.9             | CH <sub>2</sub> | 10-15          | 10-15     |
| <b>13<sup>†</sup></b> | 1.32 (m)                         | 30.5             | CH <sub>2</sub> | 10-15          | 10-15     |
| <b>14</b>             | 1.32 (m)                         | 33.1             | CH <sub>2</sub> | 10-15          | 10-15     |
| <b>15</b>             | 1.32 (m)                         | 23.7             | CH <sub>2</sub> | 13, 14, 16     | 16        |
| <b>16</b>             | 0.92 (t, 7)                      | 14.4             | CH <sub>3</sub> | 14, 15         | 15        |
| <b>17</b>             | -                                | 173.6            | CO              | -              | -         |
| <b>18</b>             | -                                | 176.4            | CO              | -              | -         |

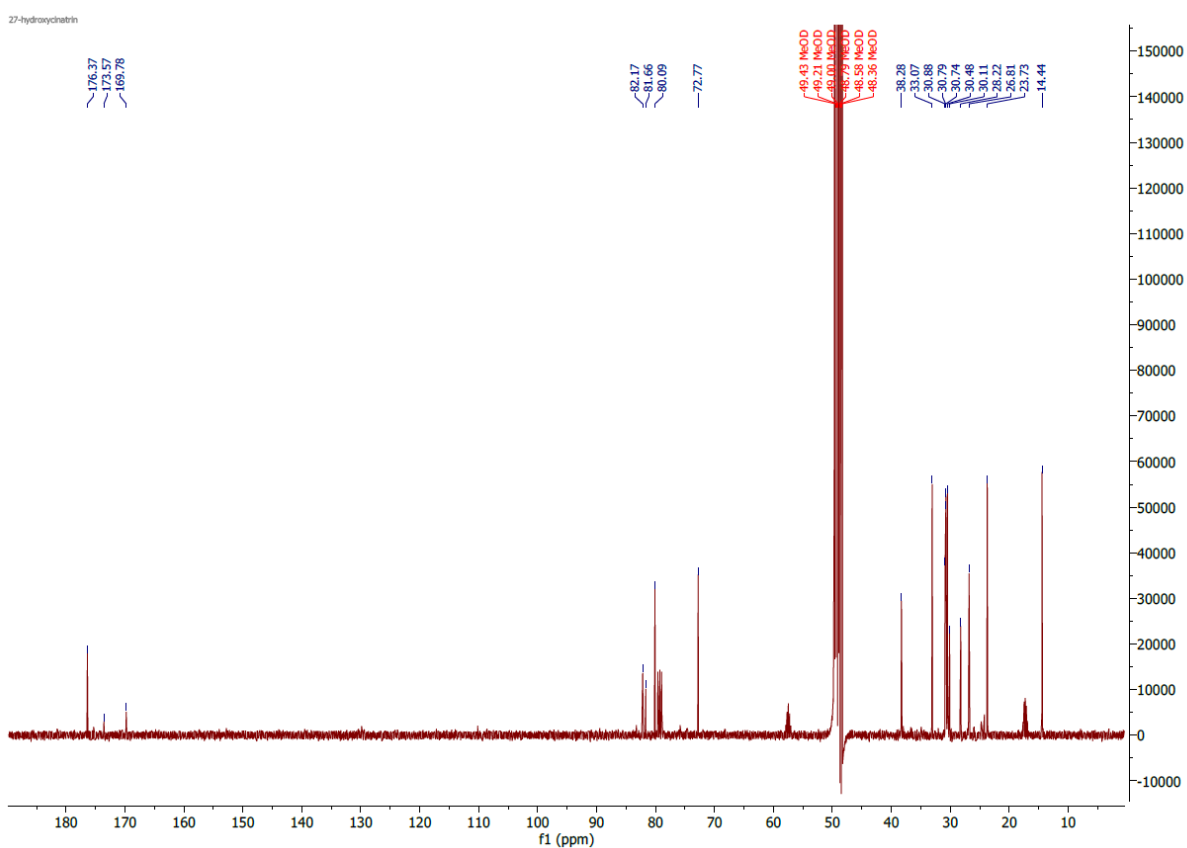
<sup>†</sup> These signals are indistinguishable from each other



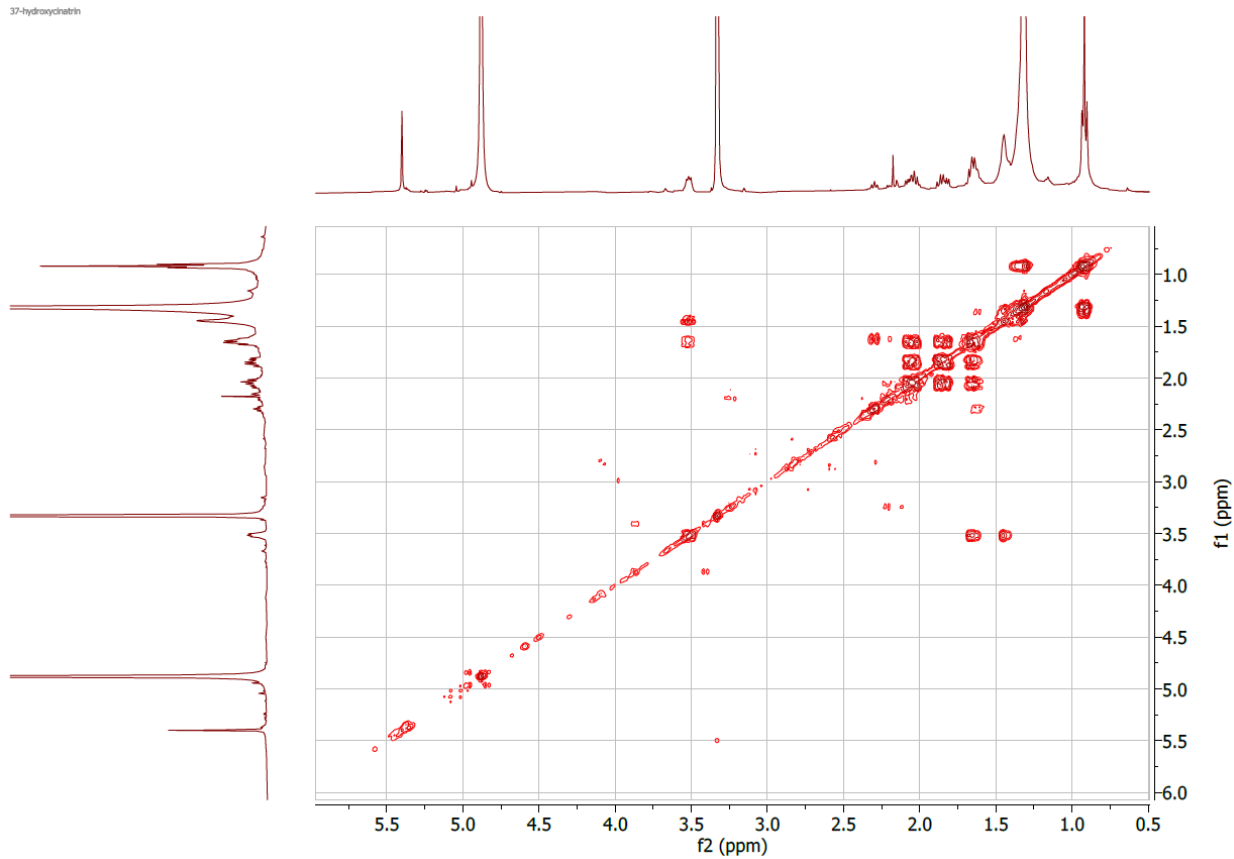
**Figure S63** LCMS characterization of **15**. **A**, ELSD chromatogram of **15**; **B**, ES<sup>-</sup>, ES<sup>+</sup> and DAD spectra of **15**; **C**, MSMS data obtained for **15**; **D**, HRMS analysis and calculated molecular formula of **15**.



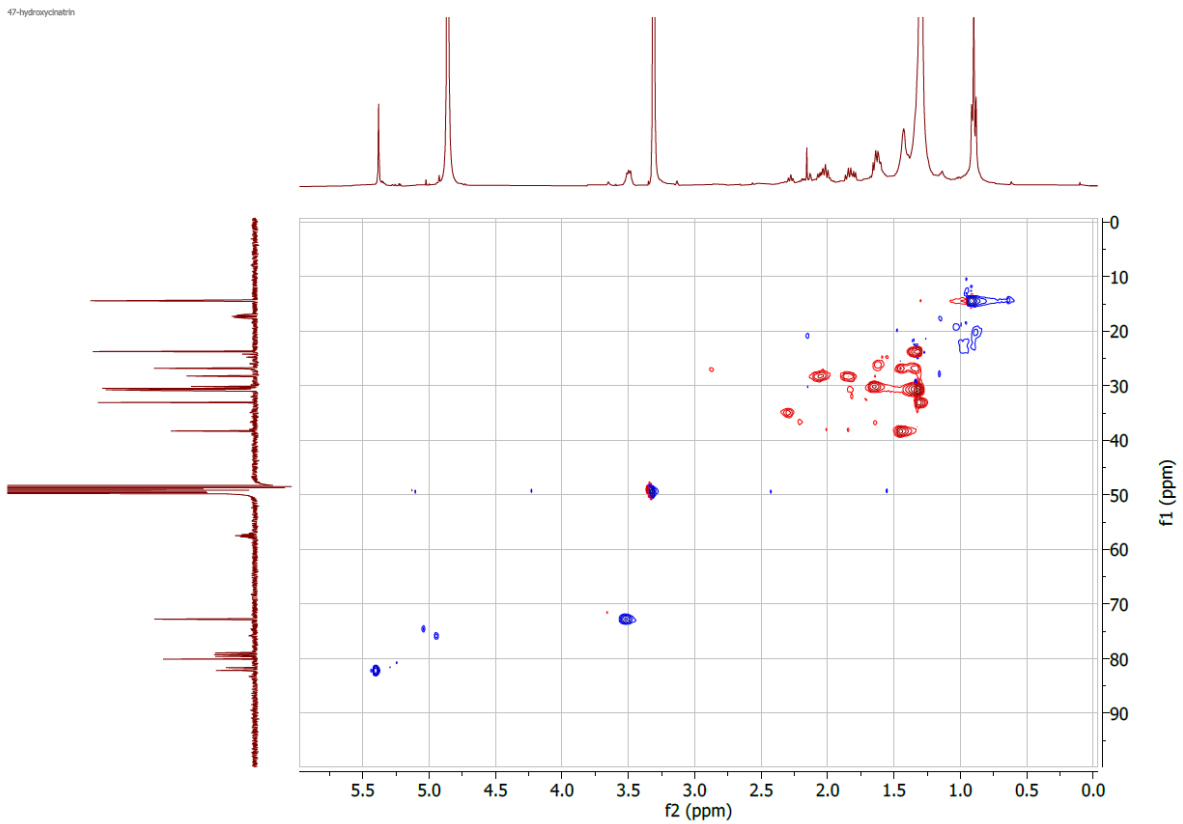
**Figure S64**  $^1\text{H}$  NMR of **15** in  $\text{CD}_3\text{OD}$  (600 MHz). See Table S25 for assignment.



**Figure S65**  $^{13}\text{C}$  NMR of **15** in  $\text{CD}_3\text{OD}$  (150 MHz). See Table S25 for assignment.



**Figure S66**  $^1\text{H}$ ,  $^1\text{H}$  COSY NMR spectrum of **15** in  $\text{CD}_3\text{OD}$ .



**Figure S67** HSQC NMR spectrum of **15** in  $\text{CD}_3\text{OD}$ .

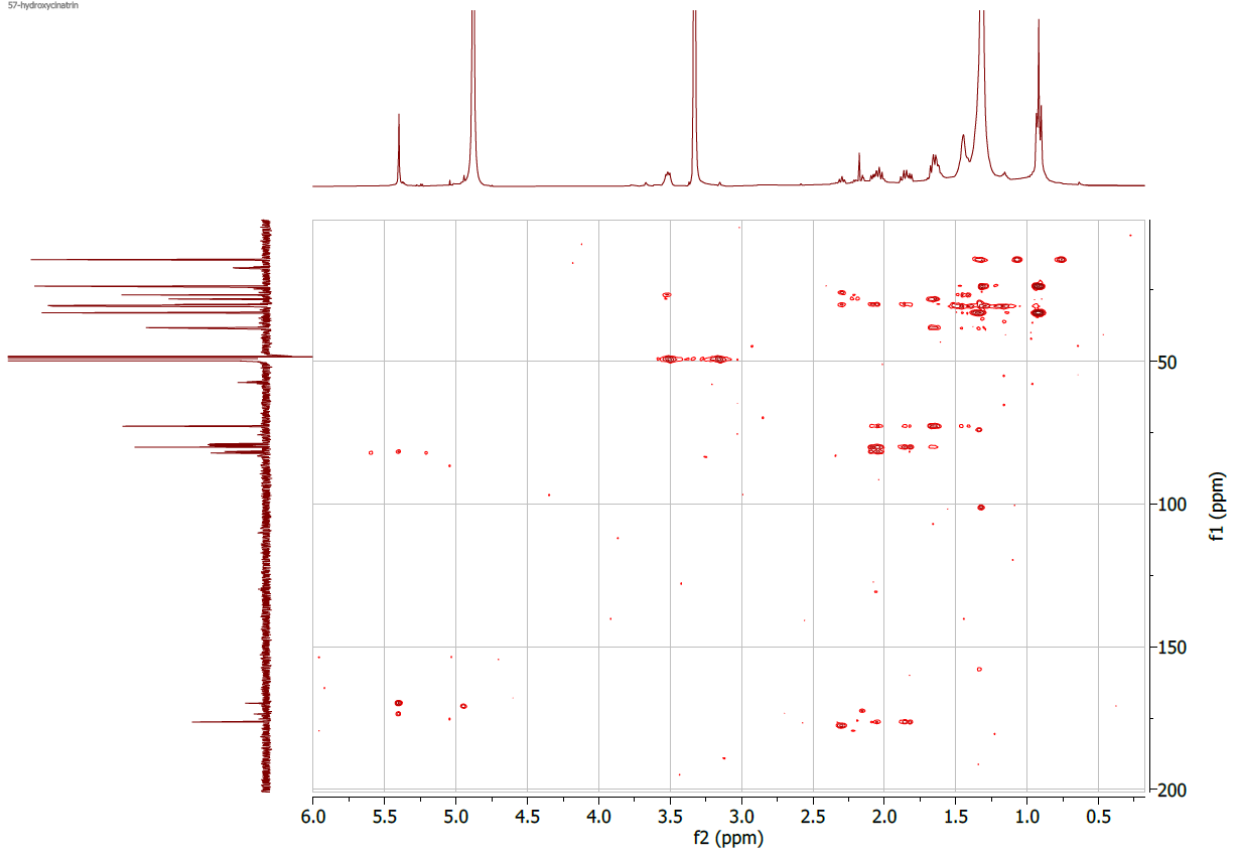


Figure S68 HMBC NMR of 15 in CD<sub>3</sub>OD.

## 15. Chemical Synthesis

### 15.1 Tris(tetrabutylammonium)hydrogenphosphate 19

Tris(tetrabutylammonium)hydrogenphosphate **19** was synthesized according to Oberhauser et al.<sup>22</sup> Disodium-dihydrogenpyrophosphate (3.13 g, 14 mmol) was dissolved in 25 mL deionized H<sub>2</sub>O containing 1 mL concentrated ammonia (25 % in H<sub>2</sub>O) and eluted with water through a column containing ion exchange resin (Amberchrom® 50WX8, H<sup>+</sup>-form, 100-200 mesh). The solution was then titrated with tetra-*n*-butylammoniumhydroxide to pH 7.3. Lyophilization afforded the compound as a flocculant white solid material (12.25 g, 13.6 mmol, 97 %).

<sup>1</sup>H-NMR (D<sub>2</sub>O, 400 MHz):  $\delta$  = 3.16-3.22 (m, 24H), 1.50-1.62 (m, 24H), 1.27 (sex,  $J$  = 7.4 Hz, 24H), 0.86 (t,  $J$  = 7.4 Hz, 36H) ppm. <sup>31</sup>P-NMR (D<sub>2</sub>O, 162 MHz):  $\delta$  = -7.35 (s, 2P) ppm.

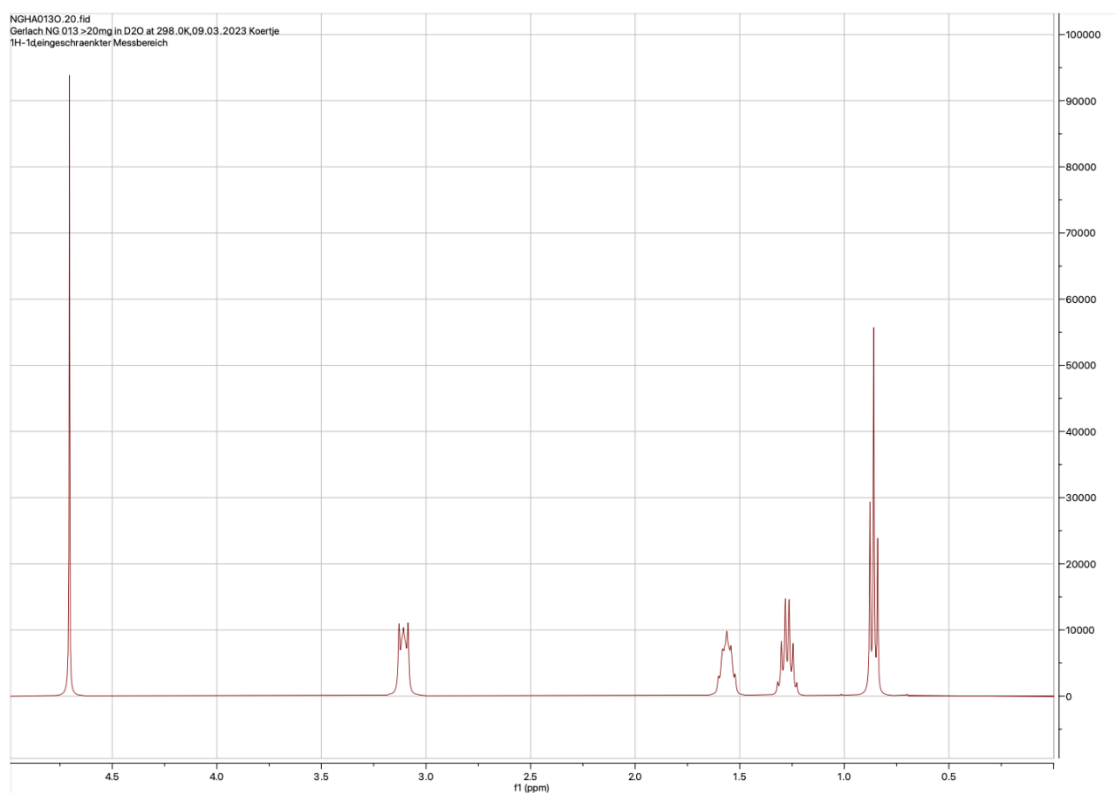
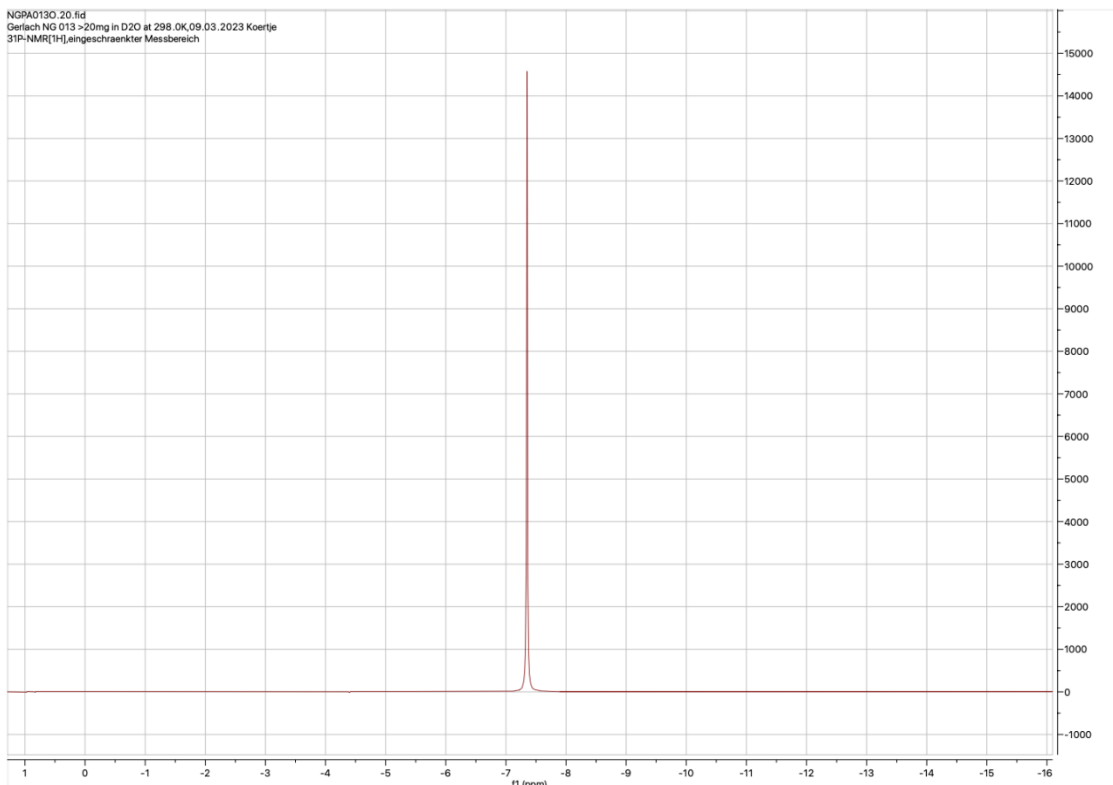


Figure S69 <sup>1</sup>H NMR of **19** in D<sub>2</sub>O (400 MHz).



**Figure S70**  $^{31}\text{P}$ -NMR of **19** in  $\text{D}_2\text{O}$  (162 MHz).

## 15.2 Farnesylpyrophosphate 20

Farnesylpyrophosphate **20** was synthesized following Oberhauser et al.<sup>22</sup> The reaction was carried out under  $\text{N}_2$  atmosphere. To tris(tetrabutylammonium)hydrogenphosphate **19** (1.0 g, 105 mmol, 2.5 eq.) in acetonitrile (5.0 mL) was added dropwise a solution of *trans, trans*-farnesylbromide (120 mg, 42 mmol, 1.0 eq.) in acetonitrile (3.5 mL). The reaction mixture was stirred at 24 °C for 19 h. After removing the solvent under reduced pressure, the residue was dissolved in 5 mL buffer 1 (2 g  $\text{NH}_4\text{HCO}_3$  in 2 % (v/v) isopropylalcohol). This solution was eluted with buffer through a column containing ion exchange resin (Amberchrom ® 50WX8,  $\text{NH}_4^+$ -form, 100-200 mesh). After lyophilization, the crude residue was dissolved in 2 mL of buffer 2 (50 mM  $(\text{NH}_4)_2\text{CO}_3$ ), mixed with 8 mL acetonitrile:isopropylalcohol (1:1) and vortexed. After centrifugation ( $5000 \times g$ , 10 min) the supernatant was removed, and the extraction was repeated two more times. Removal of the solvent under reduced pressure and lyophilization afforded farnesylpyrophosphate **20** as a light yellowish material (83.7 mg, 19.3 mmol, 46 %).

For equilibrating the ion exchange resin from  $\text{H}^+$  to  $\text{NH}_4^+$  form, the resin was washed with 2.5 times column volume 3M aqueous hydrochloric acid to achieve a pH of 1. After washing it with 4 times column volume with deionized  $\text{H}_2\text{O}$  the flow through should have a nearly neutral pH. To set the pH to 11, the column got washed with 3.5 times column volume 25 % ammonia solution (diluted 1:4 with  $\text{H}_2\text{O}$ ). After using 8 times column volume deionized  $\text{H}_2\text{O}$  to adjust the column to pH 7, it was equilibrated with 150 mL buffer 1.

$^1\text{H}$ -NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta$  = 5.47 (t,  $J$  = 4.1 Hz, 1H), 5.14-5.26 (m, 2H), 4.49 (t,  $J$  = 6.7 Hz, 2H), 2.00-2.20 (m, 8H), 1.73 (s, 3H), 1.70 (s, 3H), 1.63 (s, 6H) ppm.  $^{31}\text{P}$ -NMR ( $\text{D}_2\text{O}$ , 162 MHz):  $\delta$  = -10.30 (d,  $J$  = 20.9 Hz, 1P), -9.34 (d,  $J$  = 20.9 Hz, 1P) ppm.

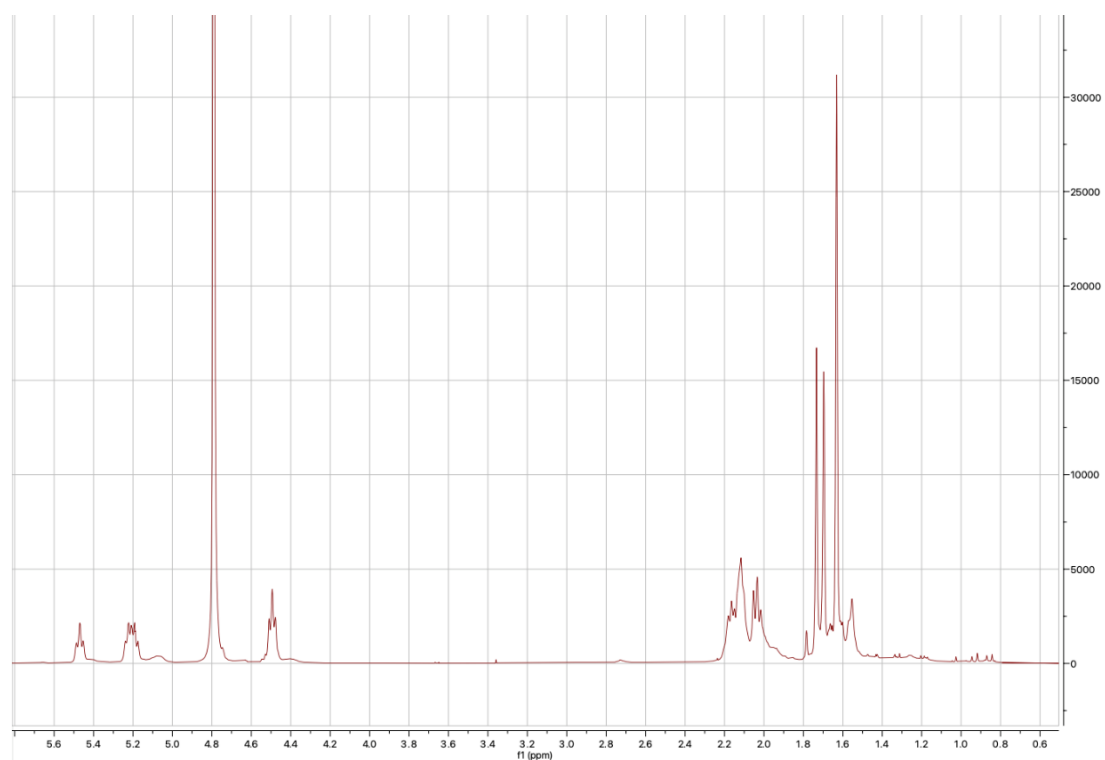


Figure S71  $^1\text{H}$  NMR of **20** in  $\text{D}_2\text{O}$  (400 MHz).

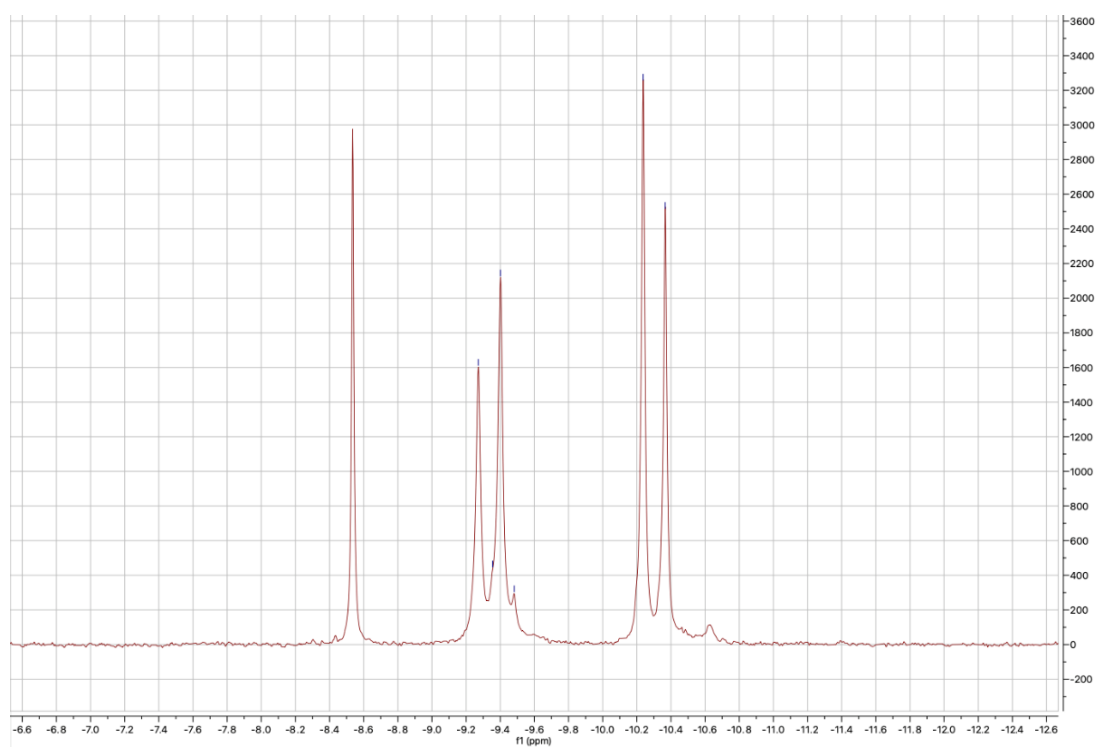


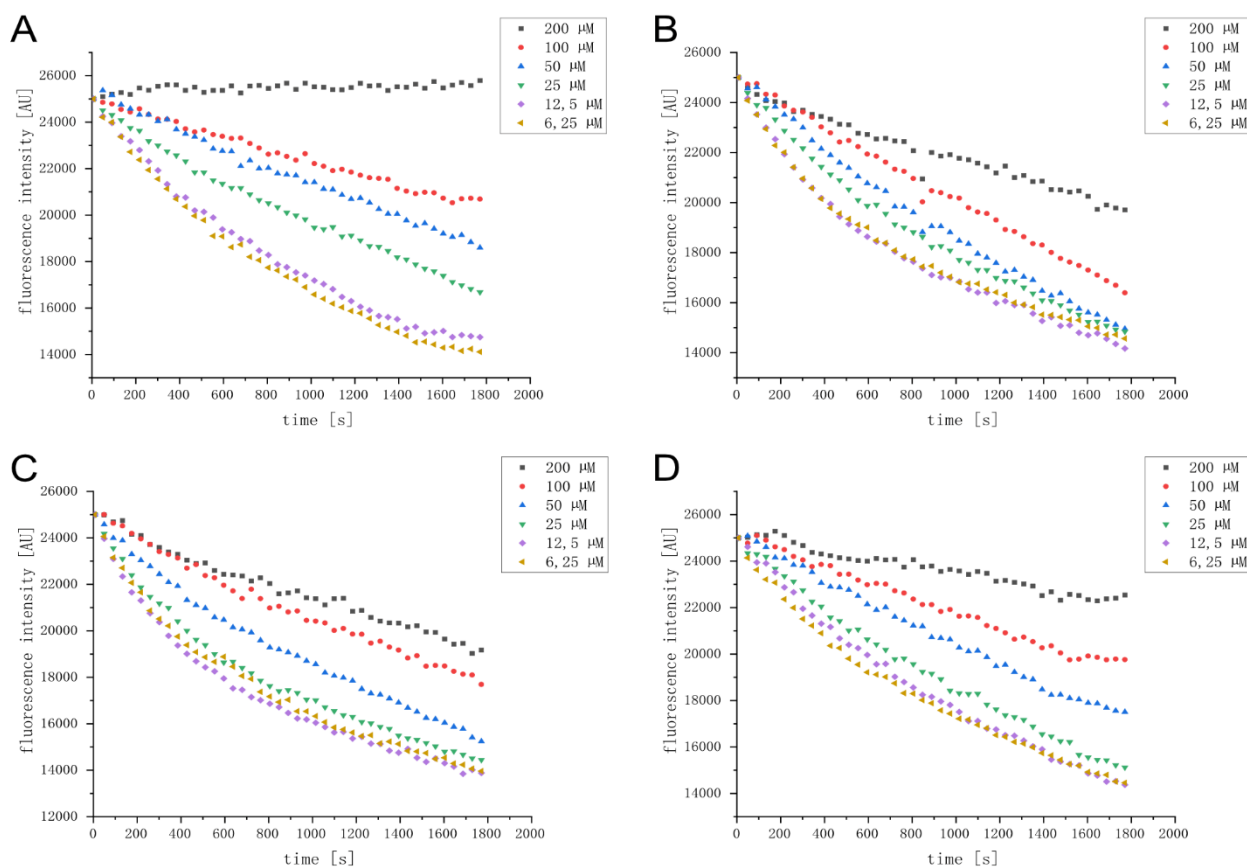
Figure S72  $^{31}\text{P}$ -NMR of **20** in  $\text{D}_2\text{O}$  (162 MHz).

## 16. Bioactivity Assays

The inhibitory activity of the alkyl citrates was determined using a squalene synthase (SQS) assay based on the consumption of NADPH.<sup>23</sup> and a phospholipase A2 (PLA<sub>2</sub>) assay based on Holzer et al.<sup>24</sup> SQS fluorescence assays were performed on an Agilent BioTek Synergy HTX Multimode Reader. PLA<sub>2</sub> assays were performed on a SpectraMax Plus 384 Microplate Reader.

### 16.1 Squalene Synthase Assay

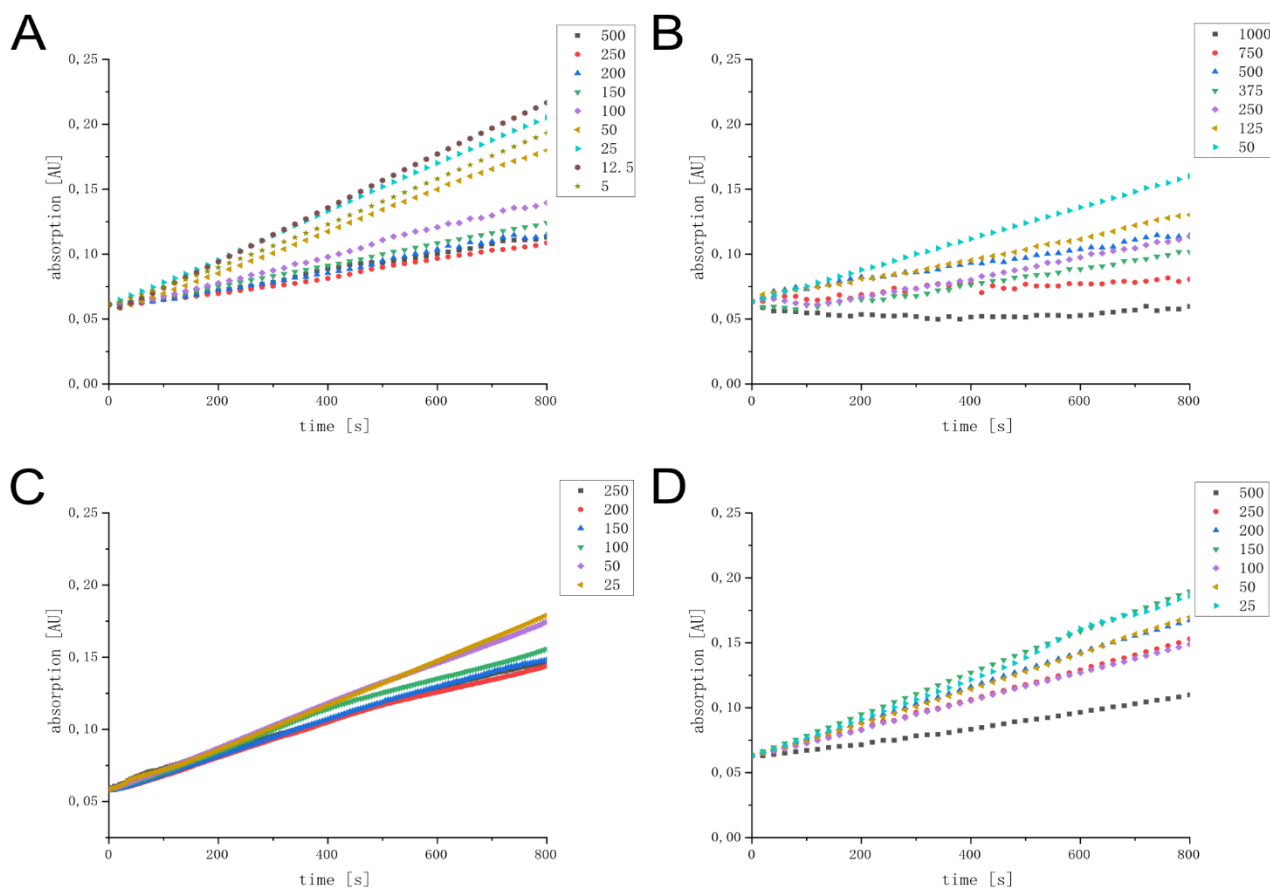
The synthesis of squalene by *Aspergillus oryzae* Squalene Synthase (SQSAo) was monitored in a fluorescence based assay by using an excitation wavelength of 360 nm.<sup>23</sup> The emission at 460 nm was measured. SQSAo was purified from *E. coli* after heterologous expression of the corresponding gene. The reactions were performed in a black walled 96-well plate in triplicates in 200  $\mu$ L reaction volume containing 25 mM Tris-HCl (pH 7.4), 1 mM MgCl<sub>2</sub>, 1 mM Triton X-100, 25  $\mu$ M NADPH, 25  $\mu$ M FPP and 5  $\mu$ M SQSAo. Tested substrates were dissolved in DMSO; final concentration of DMSO in the assay was 5 % (v/v).



**Figure S73** Raw data of squalene synthase inhibitory assays. **A**, concentration dependent inhibition of squalene synthase by the mixture of **9** and **8**. **B**, concentration dependent inhibition of squalene synthase by **7**. **C**, concentration dependent inhibition of squalene synthase by **14**. **D**, concentration dependent inhibition of squalene synthase by **15**.

## 16.2 Phospholipase A<sub>2</sub> Assay

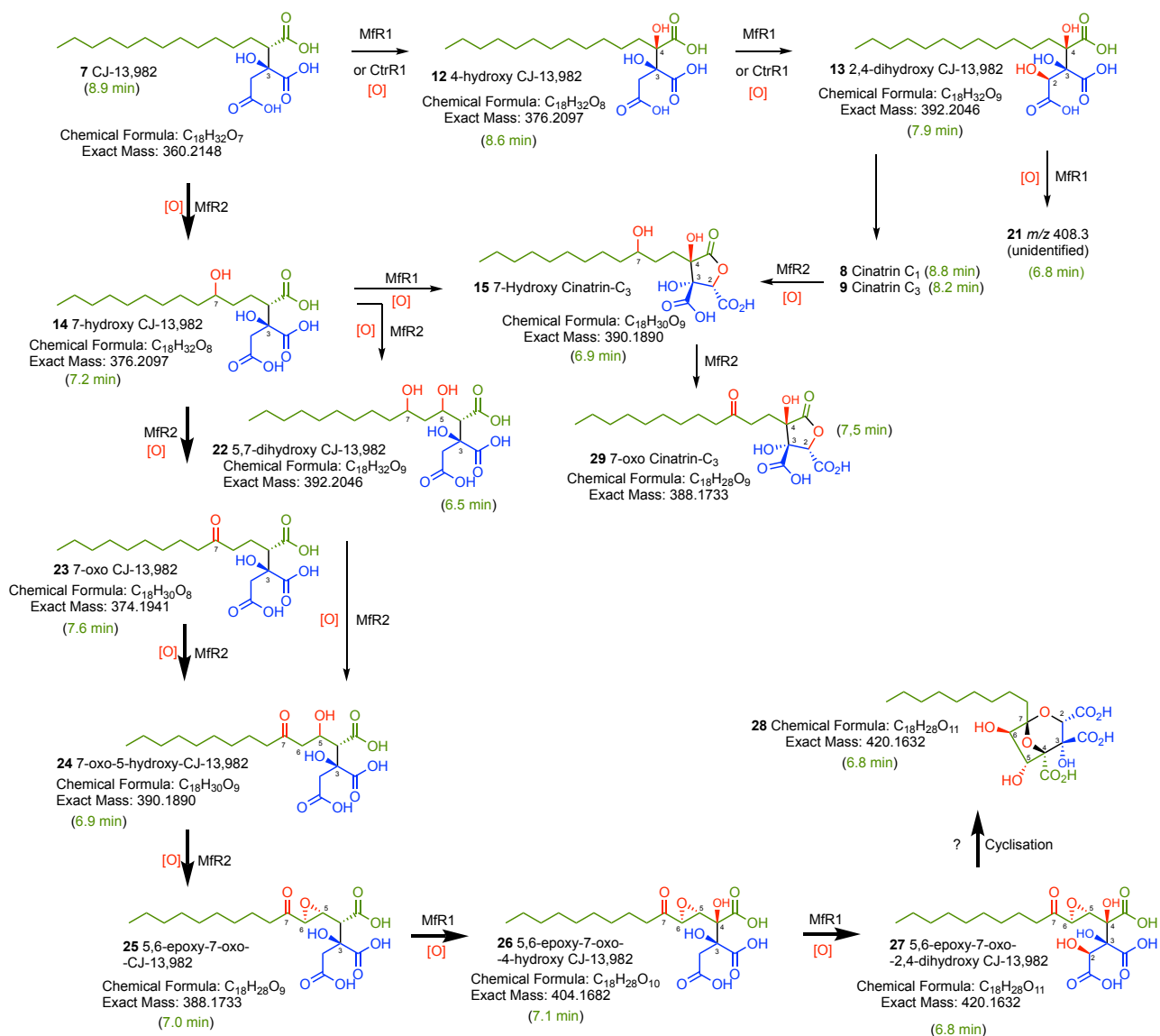
The PLA<sub>2</sub> assay was performed by using the chromogenic substrate 4-nitro-3-(octanoyloxy)benzoic acid (NOBA), which undergoes hydrolysis to form 3-hydroxy-4-nitrobenzoic acid.<sup>24</sup> The absorption at 425 nm was monitored continuously to track the hydrolysis of NOBA. PLA<sub>2</sub> from porcine pancreas was purchased from Sigma-Aldrich. The reactions were performed in a 96-well plate in triplicates in 200  $\mu$ L reaction volume containing 10 mM Tris-HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, 100 mM NaCl, 10  $\mu$ g porcine pancreas PLA<sub>2</sub> and 50  $\mu$ M NOBA. Tested substrates were dissolved in DMSO, final concentration of DMSO in the assay was 5 % (v/v).



**Figure S74** Raw data of phospholipase A<sub>2</sub> inhibitory assays. **A**, concentration dependent inhibition of phospholipase A<sub>2</sub> by the mixture of **9** and **8**. **B**, concentration dependent inhibition of phospholipase A<sub>2</sub> by **7**. **C**, concentration dependent inhibition of phospholipase A<sub>2</sub> by **14**. **D**, concentration dependent inhibition of phospholipase A<sub>2</sub> by **15**.

# 17. Comparison and Interpretation of CtrR1 and Mfr1/2 Activity *in vitro*

## 17.1 Proposed Oxidative Pathway for *in vitro* Reactions of CJ-13,982 with Trx-Mfr1 and Trx-Mfr2

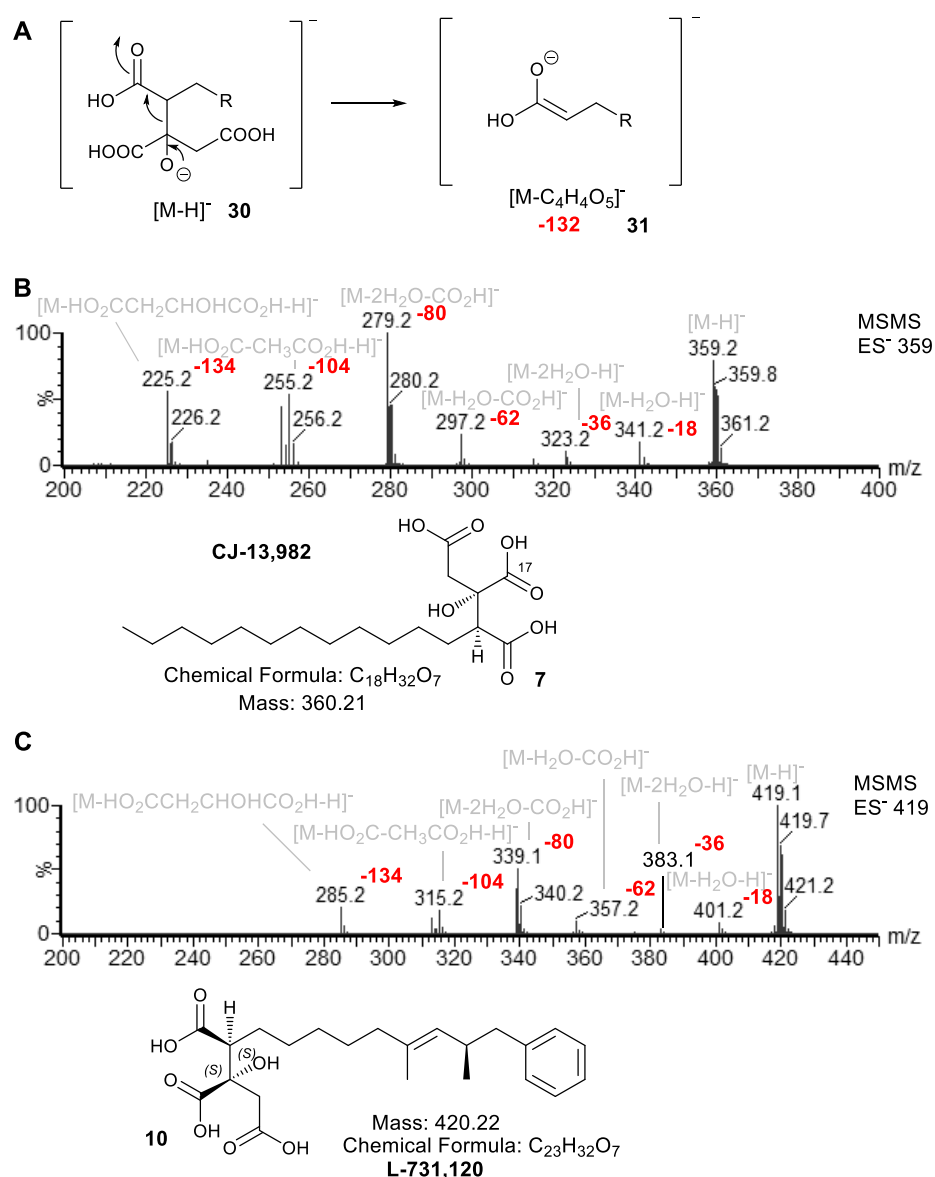


**Figure S75** Oxidative pathway consistent with observed LCMS masses for *in vitro* reactions of CJ-13,982 with TrxA-His<sub>6</sub>-Mfr1 and TrxA-His<sub>6</sub>-Mfr2. See Figs S48 - S50, Fig S58 & Fig S62 for observed retention times and masses after LCMS analysis of *in vitro* reactions. Bold arrows indicate proposed main pathway.

## 17.2 Revision of Oxidative Steps during the Biosynthesis of Squalstatin S1

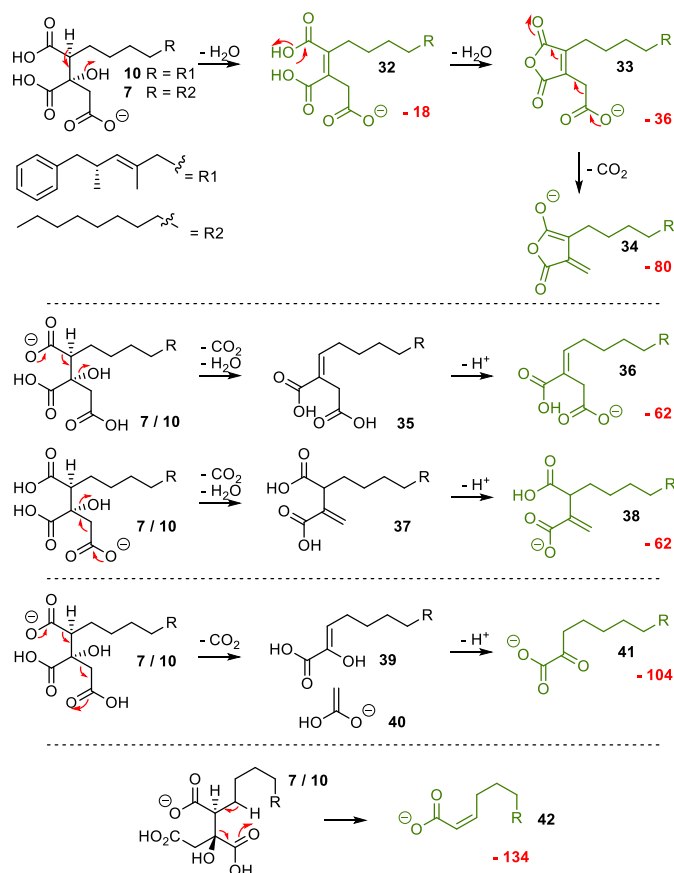
In previous work, studies of the activity of Mfr1 and Mfr2 from the squalstatin S1 pathway could not be performed *in vitro* because the native proteins could not be obtained in soluble or active form.<sup>13</sup> Evidence for their activity was inferred from LC-HR-MS studies of *in vivo* reconstruction of partial squalstatin biosynthetic pathways in *A. oryzae*. Observation of an  $[M - 132 - H]^-$  fragment ion in the MSMS data had been suggested to be linked to loss of oxaloacetate **2** from the oxidatively-modified alkyl citrates *via* the mechanism shown in Fig. S76A. This, in turn, had been suggested to indicate that oxidative modifications must have occurred at the polyketide backbone and not in the oxalic-acid-derived moiety.

However, it must be noted that neither in this study for CJ-13,982 **7** (Fig. S76B) nor previously for squalstatin S1 intermediate L-731,120 **10** (Fig. S76C), was loss of 132 Da observed for **7** ( $359 - 132 = 227$  Da) or for **10**: ( $419 - 132 = 287$  Da). This would be expected if the assumptions made in the Squalstatin S1 **11** study were correct. Hence, observation of specific ions in the MSMS data appears to be an inadequate method for localization of the oxidative modifications for the alkyl citrates. Consequently, incorrect assumptions on the oxidations catalyzed by Mfr1 and Mfr2 during the biosynthesis of squalstatin S1 **11** were made previously.



**Figure S76** Comparison of MSMS data of unmodified alkyl citrates. **A**, previously proposed mechanism for loss of oxaloacetate; **B**, MSMS data of CJ-13,982 **7** obtained in this study; **C**, MSMS data of L-731,120 **10** obtained in previous studies.<sup>13</sup> Red numbers indicate loss in mass compared to  $[M - H]^-$  ion.

Notably, for CJ-13,982 **7** and L-731,120 **10** the same fragmentation patterns were observed (Fig. S76, compare red numbers). Fig. S77 summarizes fragmentation reactions we now suggest for the formation of the observed fragments **32** [ $M - H_2O - H$ ]<sup>-</sup>, **33** [ $M - 2H_2O - H$ ]<sup>-</sup>, **34** [ $M - 2H_2O - CO_2H$ ]<sup>-</sup>, **36** or **38** [ $M - H_2O - CO_2H$ ]<sup>-</sup>, **41** [ $M - HO_2C - CH_3CO_2H - H$ ]<sup>-</sup> and **42** [ $M - HO_2CCH_2CHOHCO_2H - H$ ]<sup>-</sup> starting from the [ $M - H$ ]<sup>-</sup> ion **7** or **10**.

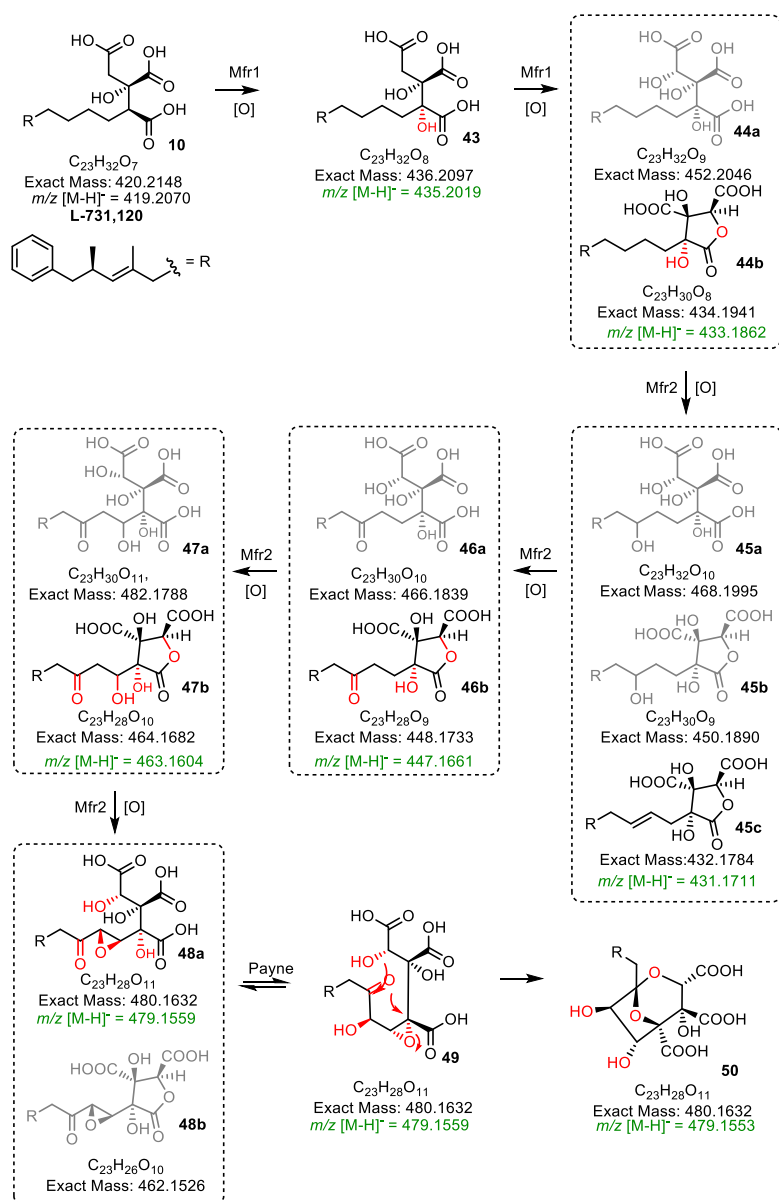


**Figure S77** Observed fragmentations of **7** and **10** under  $ES^-$  conditions. Proposed fragment ions are shown in green and mass loss in red. Mass losses match those observed in Fig. S76.

In the course of this study, we confirmed by NMR that Mfr1 catalyzes modifications at C-2 and C-4 of CJ-13,982 **7** and that Mfr2 modifies the alkyl chain. The investigations on the *ctr* BGC revealed lactonization of the 2,4-dihydroxylated alkyl citrate **13**. We used this knowledge to reinterpret the HRMS data obtained in the previous study on the oxidative steps during squalestatin S1 **10** biosynthesis.<sup>13</sup>

In a targeted knockout (KO) experiment in *Phoma* MF5453, *mfr2* has been deleted. In the knockout strain, a compound **43** with  $m/z$   $ES^-$  [ $M - H$ ]<sup>-</sup> = 435.2019 was observed.<sup>13</sup> We now suggest that compound **43** is 4-hydroxy-L-731,210 as our *in vitro* study demonstrated that Mfr1 hydroxylates C-4 (Fig. S78). Another compound **44b** with  $m/z$   $ES^-$  [ $M - H$ ]<sup>-</sup> = 433.1862 Da was also observed,<sup>13</sup> which we suggest to be a lactonized derivative of 2,4-dihydroxy-L-731,210 **44a** (Fig. S78).

For heterologous expression studies, *A. oryzae* was transformed with genes for the production of L-731,120 **10** (*sqhks*, *mfr3*, *mfm8*) and *mfr1* and *mfr2*. In the respective transformant  $m/z$  [ $M - H$ ]<sup>-</sup> = 479.1553 Da was observed. This ion was identified as highly oxygenated compound **50** by NMR in the previous study.<sup>13</sup> In addition, a compound **47b** with  $m/z$  [ $M - H$ ]<sup>-</sup> = 463.1604 Da was observed.<sup>13</sup> Here, we propose that this compound could be a derivative of 2,4-dihydroxy-7-oxo-L-731,210 **46a** hydroxylated at C-5 if designated for epoxide formation as in **48a/b** (Fig. S78). The keto-function at C-7 could possibly be introduced by Mfr2, *via* hydroxylation at C-7 first (e.g. **45a/b** Fig. S78).



**Figure S78** Reinterpretation of HRMS data obtained in studies on the oxidative steps during the biosynthesis of squalestatin S 11 **11**.<sup>13</sup> For 2,4-dihydroxylated compounds the lactonized (only one possible lactonization product is shown) and opened form are shown in dashed boxes. Compounds in grey were not directly observed. Stereochemistry of intermediates is based on known stereochemistry of squalestatin S1 **11**.

## 18. Literature

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