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The Sporothriolides. A New Biosynthetic Family of Fungal Secondary Metabolites

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Electronic Supplementary Information

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S1 Experimental Details

S1.1 Sequencing and bioinformatics

S1.1.1 Genome sequencing

Hypomontagnella monticulosa MUCL 54604, *H. spongiphila* UP-CLL-205 (MUCL 57903) and *H. submonticulosa* DAOMC 242471 were grown in 250 ml Erlenmeyer flasks containing 50 ml YMG media (Table S2.4) for 5 to 10 days (depending on growth speed) at 150 rpm and 25 °C in a shaking incubator. Afterwards, mycelia were harvested by vacuum filtration using a Büchner funnel with filter paper (MN 640 w, Macherey-Nagel, Germany). The biomass was then frozen with liquid nitrogen and ground to a fine powder in a mortar. The DNA extraction and purification were performed with the GenElute® Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, USA) according to manufacturer's instructions.

MinION sequencing library with genomic DNA from the different fungal strains was prepared using the Nanopore Rapid DNA Sequencing kit (SQK-RAD04, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. Sequencing was performed on an Oxford Nanopore MinION Mk1b sequencer using a R9.5 flow cell, which was prepared according to the manufacturer's instructions. MinKNOW (v1.13.1, Oxford Nanopore Technologies) was used to control the run using the 48h sequencing run protocol; base calling was performed offline using albacore (v2.3.1, https://github.com/Albacore/albacore).

Whole-genome-shotgun PCR-free libraries were constructed from 5 µg of gDNA with the Nextera XT DNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's protocol. The libraries were quality controlled by analysis on an Agilent 2000 Bioanalyzer with Agilent High Sensitivity DNA Kit (Agilent Technologies, USA) for fragment sizes of 500–1000 bp. Sequencing was performed on the MiSeq platform (Illumina; 2x300 bp paired-end sequencing, v3 chemistry). Adapters and low-quality reads were removed by an in-house software pipeline prior to polishing as recently described.[1]

The assembly was performed using canu v1.6 and v1.7,[2] resulting in a few contigs representing the corresponding genome. These contigs were then polished with Illumina short read data using Pilon,[3] run for eight iterative cycles. BWA-MEM[4] was used for read mapping in the first four iterations and Bowtie2 v2.3.2[5] in the second set of four iterations. The respective sequences are stored on the ENA (European Nucleotide Archive) portal of the EMBL-EBI (https://www.ebi.ac.uk/) under the bioproject numbers PRJEB36647 (*H. monticulosa*), PRJEB37480 (*H. spongiphila*), and PRJEB36653 (*H. submonticulosa*). Genome details are listed in Table S1.1.

Strain	*H. monticulosa*	*H. spongiphila*	*H. submonticulosa*
Genome size [bp]	42,889,121 (Oxford nanopore/Illumina)	42,321,440 (Oxford nanopore/Illumina)	41,374,079 (Illumina)
Scaffolds/contigs	30	16	123
N_{50} value	3,439,634	5,056,634	657,615
Annotated genes	11,204	12,622	10,988

Table S1.1 Oxford Nanopore/Illumina-Sequencing results for *H. spongiphila*, *H. monticulosa and H. submonticulosa*.

S1.1.2 Gene prediction

Gene prediction was performed by applying Augustus version 3.2[6] and GeneMark-ES 4.3.6[7] using default settings. For Augustus, species parameter sets were established based on GeneMark-ES fungal version predictions. Predicted genes were functionally annotated using a modified version of the genome annotation platform GenDB 2.0[8] for eukaryotic genomes as previously described.[9] For automatic annotation within the platform, similarity searches against different databases including COG[10] KEGG[11] and SWISS-PROT[12] were performed.

S1.1.3 Transcriptome sequencing

Hypomontagnella monticulosa MUCL 54604 was grown in two 250 ml flasks each containing 50 ml of a different medium (DPY and PDB, Table S2.4) for 3 days at 25 °C and 150 rpm. Small quantities of mycelia (>100 µl) were removed with a sterile inoculating loop and RNA was extracted from the samples using the Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research, Germany). Samples were treated with DNase I (Zymo Research) according to the manufacturer's recommendations. The High Capacity RNA-to-cDNA™ kit (Applied Biosystems by Thermo Fisher Scientific, USA) was used to obtain cDNA. gDNA contamination of the extracted RNA was checked by PCR amplification of "Polymerase II subunit" (house-keeping gene) gene, the primers (P1 and P2) binding to the introns was used and cDNA as a template. PCR was conducted by using OneTaq® 2X Master Mix (New England BioLabs, USA), the manufacturer's protocol was followed and the annealing temperature was 60 °C (Table S2.2). In total, ~2 µg of RNA per sample was used for library preparation with the TruSeq mRNA Sample Preparation Kit (stranded, Illumina). Sequencing of the prepared cDNA libraries was carried out on the Illumina HiSeq 1500 platform (2 x 75 bp) using the 'Rapid Mode'. Data analysis and base calling were accomplished with in-house software[1]. The sequencing raw data for all libraries have been stored on the EBI ArrayExpress server, accession E-MTAB-8948.

S1.1.4 *spo* cluster identification

Gene clusters were initially screened with antiSMASH 5.1.2[13] fungal version, but did not result in the identification of a candidate cluster (Fig. S1.1). Therefore, manual BLASTp searches against the *Hypomontagnella* protein databases using the BfL2 citrate synthase[14] involved in byssochlamic acid biosynthesis (ANF07286) as template. Each genome contained four citrate synthase homologues with two of them being associated with other secondary metabolite genes. Both clusters contained fatty acid synthase homologues as core genes, however, only one cluster included the predicted 2-methylcitrate dehydratase (based on the predicted homology with the byssochlamic acid biosynthesis, Fig. S1.2, Table S1.2). The latter was therefore identified as the putative sporothriolide biosynthetic gene cluster (*spo*). Similarity of the putative *spo* cluster from the three genomes was investigated with the Artemis comparison tool (ACT)[15] using the tblastx search algorithm (Fig. S1.3). In addition, a synteny analysis between the *spo* cluster, oryzines (*ory*) cluster from *Aspergillus oryzae*[16] and the byssochlamic acid cluster from *Byssochlamis fulva* was also performed (Fig. S1.4). The transcriptome data was used to reannotate the *spo* gene cluster in *H. monticulosa* (GenBank MT889334) and the respective protein sequence are listed in Table S1.3.

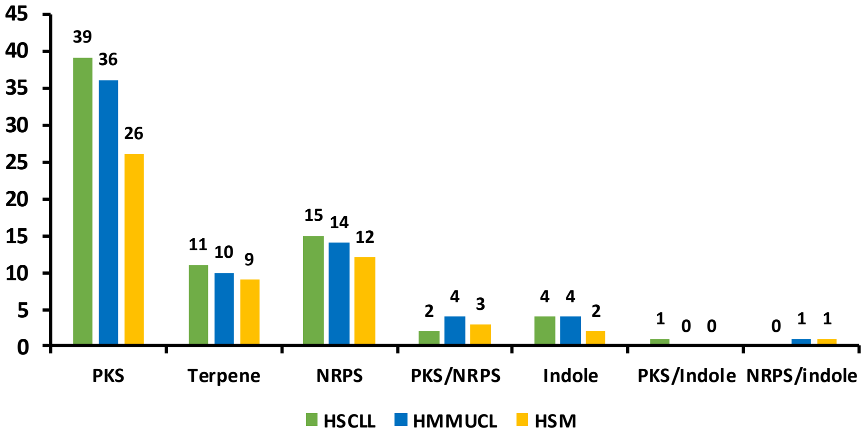


Figure S1.1 Bar chart of predicted biosynthetic gene clusters found in the genomes of *H. spongiphila* genome (**green**), *H. monticulosa* genome (**blue**) and *H. submonticulosa* genome (**yellow**) using fungiSMASH v 5.0.

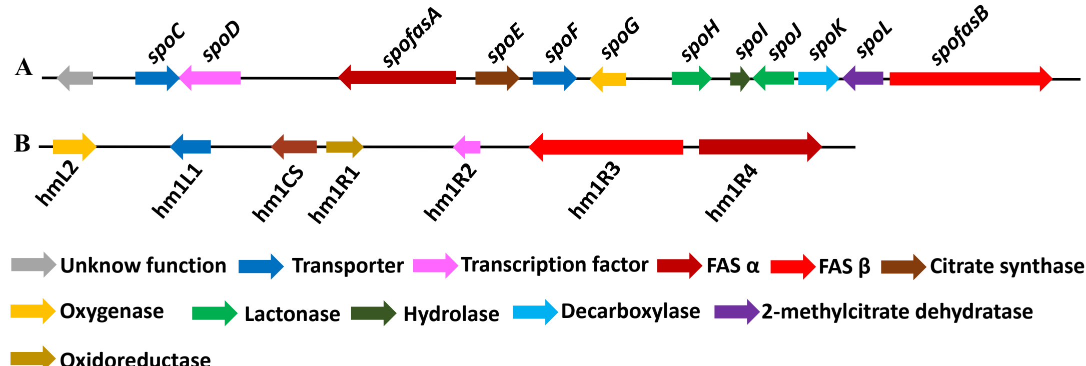


Figure S1.2 Two gene clusters from *H. monticulosa* MUCL 54604 which contain fatty acid synthases and citrate synthase. **A**, the *spo* BGC; **B**, BGC with unknown function.

Gene (locus_tag)	Gene	AA	Putative Function	BLASTp[a], PHYRE2[b]	Predicted Cofactor
HmMg6350	-	319	Unknown	Amino-acid permease[a], Membrane protein[b]	/
HmMg6351	*spoC*	508	Transporter	Citrinin biosynthesis cluster MFS transporter[a]	/
HmMg6352	*spoD*	819	Transcription factor	Transcriptional regulatory protein[a]	/
HmMg6353	*spofasA*	1619	Fatty acid synthase subunit alpha	Fatty acid synthase subunit alpha[a]	/
HmMg6354	*spoE*	460	Citrate synthase	Citrate synthase[a]	/
HmMg6355	*spoF*	493	Transporter	Efflux pump[a]	/
HmMg6356	*spoG*	373	Dioxygenase	Sulfonate dioxygenase[a], Oxidoreductase[b]	Alpha-ketoglutarate
HmMg6357	*spoH*	443	Lactonase	Gluconolactonase[a], Hydrolase[b]	/
HmMg6358	*spoI*	184	Putative hydrolase	Unknown[a, b]	/
HmMg6359	*spoJ*	441	Lactonase	Gluconolactonase[a], Hydrolase[b]	/
HmMg6360	*spoK*	508	Decarboxylase	Aconitate decarboxylase[a], Isomerase[b]	/
HmMg6361	*spoL*	491	Dehydratase	2-Methylcitrate dehydratase[a]	/
HmMg6362	*spofasB*	2060	Fatty acid synthase subunit beta	Fatty acid synthase subunit beta[a]	/
HmMg6363	-	408	Unknown	Cytosol aminopeptidase[a], Ribosomal protein[b]	/
HmMg6364	-	238	Unknown	Unknown[a], Sulfotransferase[b]	/
HmMg6365	-	516	Unknown	Methionyl-tRNA formyltransferase[a]	/

Table S1.2 Annotation of sporothriolide BGC and surrounding genes from *H. monticulosa* MUCL 54604 genome using BLASTp[17] and PHYRE-2[18].

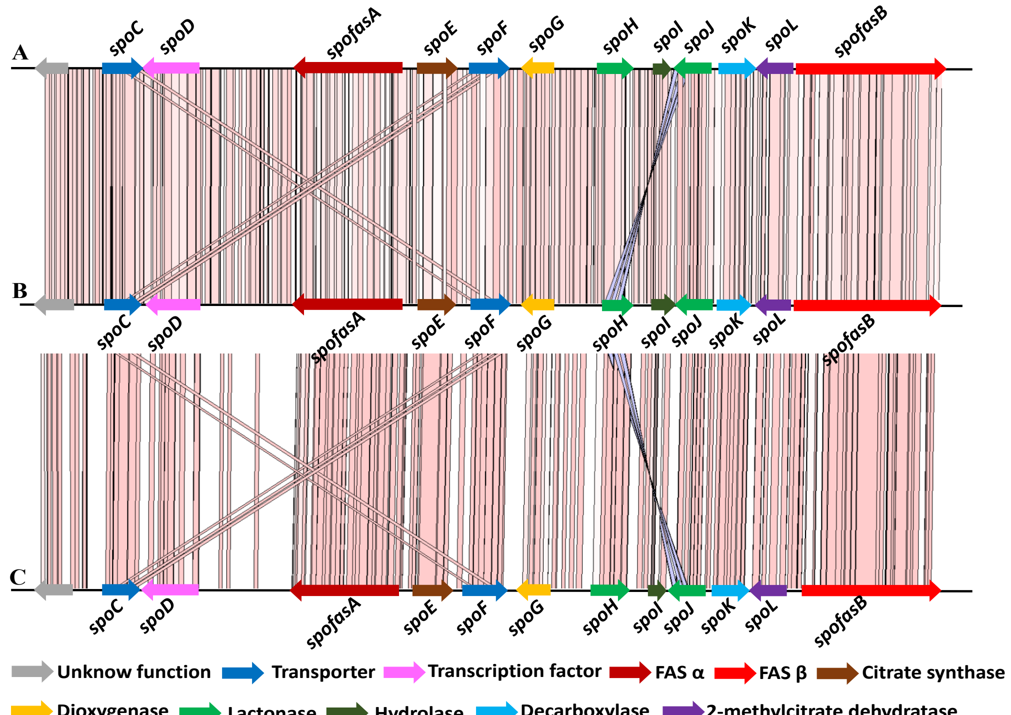


Figure S1.3 Artemis comparison tool (ACT) analysis of the *spo* cluster from : **A**, *H. monticulosa* MUCL 54604; **B**, *H. spongiphila* CLL 205; **C**, and *H. submonticulosa* DAOMC 242471. Areas (genes) with high similarity are marked in red (same gene orientation) or blue (inverted gene orientation).

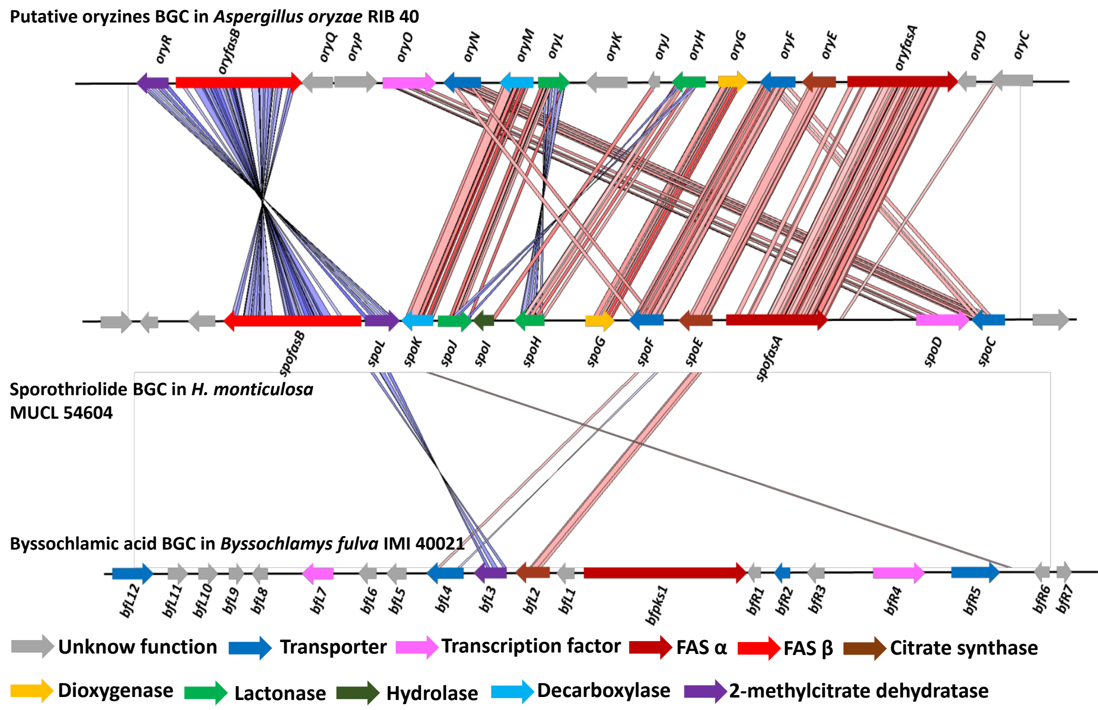


Figure S1.4 Artemis comparison tool (ACT) analysis between the putative oryzines cluster from *A. oryzae* RIB 40,[16] sporothriolide (*spo*) cluster from *H. monticulosa* and the byssochlamic acid cluster from *Byssochlamys fulva* IMI 40021.[14]

Protein Name	Protein sequence
SpoC	MSNDSDRTSVASNKDVDVEAATPPPEVVQEEKSKVEEEKDPNLVVWDGPDDPGNPQNFSNGYKVFITLIWVYG NLTTTIASSIWSSGAGAIAVEFDKSTIVVTLGVSLFLLGYAVGPPVWGPVSERFGRKYPMLAGMFLFTIFCIPAAVGH NMETLLIARFFQGAFGSAPLSLAGGGIVDIWSPAHRGVAIAACIGTIFGSPILAPIMGNFIAASYLGWRWTQWISCI MGGSCSILVLFGLPETLAPKILQGRAAALRKSGANPDAKTAFDGRKLAGPMDIVRIYLMRPFELLATEPILVLITIYQS FIYGILYLVFVSYPIAFREIRHWALGVSALPFLGLMVGVVLGAGAVIWHTKTKFMATIKANGGKIIPEQRLPMMIIG GCLLPVGLFIFAWTSHPETHWSGMVIGSIPTGMGMYMVFVQCFNYLVDVYAPIANSAIGGNTFIRSFFGAGFPLF APYMYHNLGVDWATSTLGFISIAMIPIPILFYKFGHRIRSWSKNSVNTN
SpoD	MYASAQTSPRRDAQPSQPDPDSDSASATRADGMRKRPRSGNDEPPTAACDQCRLRKVRCDRRQPECSNCRKA GVECNSSNTLKRVNHTKQLRDDFSVVLKHLNDVDHALGTLTELTRQIAARPCPHTVNPHAVCAPNNEIAPLPTPGS LDFMLPSAGDEDRVLNDPLALNGPLFETIEFDQGGERLYGYPAPLVLIKSLLRQATGALLESDKQGESHENGDSYIA RALQDMSARATLRQKLDDFPFNLPCRESVAVSDANPVTTPPRLMVNLFVDGYLHNINTRTPIFDDAGLRRAIDAH YGDEQPQESRAWALIINNIVLLELGLEIQAARASHSNSRGMNDDILPSFLRNCDRAIGNLEAFMGPSLVNVQALM TLTLAAREFYNNATAEKVCHAACQVGRAIGLHRSGARYPNEKGTLSQEPEQERERLFRVLYTMDKQRVFMTGQP CDLHMFDSDHRIGPDRNHEQAEPPISDAFDHMMTIWEEIYLNLYSLRAASSGGETRMRQIRLVTSSVEKFSQKHA GLMSPSCANGAADVDPLQIELLYGYRVSQVLILRCERGNEQSQEKMRELARSSLRLILEVCKTPLTTPRLALLASMFR NYPMVAFVELIAFHLASLFRRGECDPTAQADVSLLRAICDQLHILQHDNLTHIFYARLKLGLVWALETLEALGEILIRS SPQPRGMAGFSPQPQRDSRRSTESSRNPTNAPSPMAPDISTACGLHPSRGNQSLSSISSSRNGEEDFVQSGLAELT NFGFFTPGTDRMDLASRPLSAACQFNTSSSNSQSQSDLNSGPLTGSSNWGDFNMDFFQGVSA
SpofasA	MTATNSNGRVSEDRRASEQQIAYNLLIELLTYQFAFPVQWIDTQRELLTSECNVQRIIEIGPAKVLASMAKKSAKRL VGEQDLARSIEREFLNITDPEDARKIYYEYDENSSTAETISAKDGPAPPPVAPVNVVAAPIQAPVAAPIAVVSAPIAA ASTVDKDFTPTDVILSLVAQKLRRAFDEVSLSESIQNLSGGKSTLQNELIGDLAAEFGDLPDGSESTAMDALGEKLAS GFSGKLGKSSKKLVERFLSSKMPGGFGQTEMVAYLGSRWGLGSNSQTAVQCFCITIEPAARLSDVGQVHEFLDSA VARYAKHAGVSLPTQSAGGASQGASGAVMQVDKAGLDALKNEQNSVLRKQLEVLAQHLGVDITPNTASGVEGG DKLQEQLDRFYAELDEEFLSGVQGMFDPEKARSYSSWWNWVREDAARLLRHQDGTPASPQRLQALTNRWTAE LEEMLRYCAKAGPAKEAAESLLKLKPSTQGASPVFRFTEPAMAPHTSVDEEGQIHYTEKARQDDSGSSRTTTYYDV VSSTRRDGPSKSFVHCLHRRGGSWQYDGELTNTYLDALFAGNTSGISYAGKTALVTGAGTGSIGIEVVRGLLAGGA RVIVTTSRTAANAGAIMSQLYKEFGARGSELILLPFNAASKKDVENLVAHIYDSSKGIGADVDFVIPFAAIPEPGREID GIDARSEVAHRAMLTNVLRMLGCIKQEKEKRSYVGRPTTVVLPLSPNHGDFGGDGLYSESKIGLETLFNRYSSERW SGYLSIIGAVIGWTRGTGLMSANNIVAEGIENLGVMTFTAGEMAFNILALLYPSIIRKSDMEPIYADLSGGLMGFQN LKEEIMAIRTNITGKRRERQAIVAERQRHEEVLKGSKAASAQSQKKSSPQKKRSNIRQGFPRLSSHQEMTAGLESLT GMVDLSRTVVVVGFSELGPWGSSRTRWQMESQGKLAQDGLTEMAWMMGLVKHHDGLVDGKPYVGWLDVE SKKPVQEDEFSARYGEHIMNHSGIRIVEPEALDGFDPAKKELLHEVVLDDDLPAFDTSEALAQSFKLRHGDKVTIFQ KGSDADTWTVIVKRGATFIIPKSSTGHQTVAAQIPKGWNAATYGIPEDIISQVDPITLYVLCCVCEAMFSAGIEDPFE LYKYIHVSELENCIGSGAGGLKSMRDMYRHRYRDEPVQGDILQETFLNSMAAWTNMLLFGATGPIKTPTGTCATS VESLDNACEGIRSRRVKVALVGGTDDIQEEVAHEFSNMKATMVAEKELAKGYLPSQMSRPTATSRAGFVESAGC GVQIVMSAELAIQMGLPIYAVVAYTQMAGDSVGRSVPAPGQGVLTAARETPAASRSPLLDLRYRRSRLEQEIAEIE GWRLSQLASTSSHVGTHEAAHSQMIESAASRRKSDAQWMWNGDIRQLDPSIAPMRAALAVWGLSIDDIGIASF HGTSTKANDKNESSVINQQMTHLGRTSGNPLLVICQKFLTGHPKGAAGAWMLNGCMQVLESGLVPGNRNADD VDGALRAFPHLLYPSESLQVANIKAFMLTSFGFGQKGGIVIGVTPRALFAALAAPKFEAYREQVERRRRRADRAFQL AMMTNSVFKAKDQSAWIEAGRAAGAVFLDPTARI

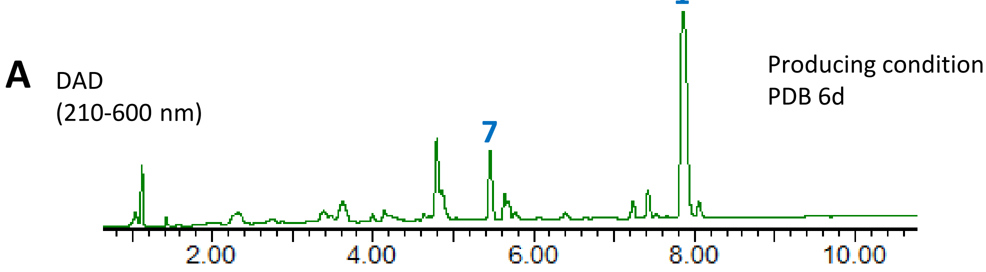
SpoE	MPSATVPKANGTNGTNGTNGVHKPVGDVLHVIDSRTGEYHAVKIHHNAINATDLKAIKAPKDLEHPEYQNEQGI RVYDPGFSNTVVSESKVTYIDGLEGTIQYRGYSIHDVIGKKKFVDLSYLLIWGEWPSAEEAQKYQERLNNVPLIDESV FNVIRSFPKNGSIVGMMVAGLSALQSCDMAAVPAHAAKNLYLGQPQNVDEQIIRVMSSLSMITAAAYCHHTGRT FTPPRKDFSYIENFLLMTGHVDESTGLPNPRYVNALERLWAVVADHEMTCSTAALLQTASALPDVISSLISAFSAGY GPLHGGAIEVAYKNIEEIGTVDDVPAKLARVKAGKERLYGYGHRVYRVTDPRFTYISEILGELTEEVNKDPLLQVAFA LDRAAAQDEYFISRKLRPNADLFAAFAYKALGFPPNFILPISMISRTQGFMAHWKEAMEGGPRIWRPGQIYTGKL NRKE
SpoF	MVSTTNNAEERSSLLPASNSSLNTFVLPENEKPRNWSRSYRWLCVGVISLYGLMSPVMAAAIVPALPAISDDLSISD EKTLGALVSIYLLSWSVTPVFLGPLSEVYGRVGLLQIGHGLFMVFNFLSVFAQTGPQLLVLRFLAGGVGSGPLSIGAG IIGDLWAPEERGVSISLYTLGPLLGPAIGPIAAAYISANFSWRWIFGFSSIYILITLILGLFVLQETLLPVITERKRAAFLSK FPQQGLVSDYGTVPVPEIPETEHKKDFKAVRQSLMRPFILLWTQPIIQVLAIFTGYQFGLNHLTITTFQSLWRDVYQ QDMLGASWNYIFIAVGFVFGSQATGMLNDRIYKRLDKKNSRSNPELRTYMMLPASLLVPLGLLLYGWSAERHMH WLIPDVGVCIYATGLIMSYQCTQAYIIDCYTSHAASSMSALMIVRSITGFTFPIFAPVLFSVWGYGLGSTWLAGCATI MGLGIPIMLKVYGPALRARSTYAVGE
SpoG	MSATNGTVQPLELSGALSAYEAIDMTPCIGTEFPTLDLAEALRAPNSDEIIRDLAITICRRGVVAFRSQTNMTNELQ KELTHRLGELSGKPAGHRLSKHPLHLIRKDDPEMGILDAGRQQALHGGDTTDKRQKASVEWHSDGSYEVCPPDFT SLRMTDIPRTGGDTLFASGYELYDRLSEPYQRFFESLTATHEVPALRKAAETMEGIYTGPRGAPANTDMLFKQSHP MVRTHPVTGWKTMFAGGLHCRRVNGVTEWESQELLEKILRLVADNHDLQVRIRWNTPCDMVIWDNRCVLHCP TQDHYGLGKRMGYRTMSVAEKPFLNLSSPSRLEANAVVGEKAGKKVSEVPVAAPVKIPAVAAPAAAPAVSAQA
SpoH	MRSLSPISDVTPSWGSSLVHLAGLLLLTQATTAAQVAKKCGNLDFPTVVCLDRYGTLLPGKFSRGALDVYSASSVD DTSFSNIKSASFLVFDQDKGLDVLGTAPVVEEMFDLDSAAPEAPVYVPDTNELWIGGLQTGVTSQTVVDLSQNPP KPVKRTLNPPIYAANGMRYRDGRVWVSAAGGNDTLAGGPYHPGIYSFDPKTGDSRVEANNYYGWFINSANDLD LDPSGRVWFTDPLYSRNMGVNTEAPLLQAAVYRYDPVSGQIQVMDDTLEFPNGIAFSPDGKILYLTNTAAGVGNI DPGTPWQNAGPLKYVSTNKRTLYAFDVGTDGLLRNRRPLYTAMDYVADCVKVASNGYLVTAAGHGVDILDPTG VHLMRIQLSFLAVSIEFAGPKRDSLWIIGHGKAARATINLTGQGASTASSRLRRHARTHAKRHVAPQSITE
SpoI	MADQQKEVPEKTLPVVHRFITTHNAEGKPTFETGIKEEIDFERSPLGGDMFLAYSGVEFPVALGHDSDLNQYKAHL EKKPESFMIPGGFLSRYIDYHPGCLPLWHRTITLDFGIVVEGQIQLELESGEKRILKKGDVAIQRGTNHAWSNPSKTE FARVFYVAMDAKPPVVNGQELGESLGVVSH
SpoJ	MCSRRPIRRFLPYLLISSQLSSALSFLGNDGLQIPLTVDADEVDEVAGEQSKLAAPCLGYEFPHVICIHRYGSLIHGDF ERKVRNVLGDTETYPSTHAPGESTFTHISDADFLIWDTDVGGQILGSNPSIDFMFEVAPVSHEAPVYSPTTNELYFS RLQQGFLPQIVVDLNQDPPVLTEKLASPPIYAAAGGRFYKGLIYFSTIGGNESLGGYTFRPGIYTLDPKTGESKTLLNN YYGWYFNAADDLDVDDQGQLWFTDNNYGRPVHVNTYAPQMGVATYRYNISSGLVAIVEDTLKEPNGVAFSPD RNTLYLSDTGAGSSIIDGRVNPAPSIHVNSTGPRLIYAYDVSKSRKGLSNKRPIYRAIDYAPDGVKISREGYIVTATGH GVDILSADGEPLVRVQTNFTVINIGWAGKESDELWVVGKGGVARVRWALRGPIVE
SpoK	MGKHGANVVFDETNDENSGVTAALCNWIAGLKKEDIPTPVLERAKHLILDGIACGLVGAHVRWSEKAADAVLDY EPEGQCSVIGYEEKLGPLAAAVLNGTFIQATELDDYHSVAPLHSASVVLPALLAAAQVKNKTRKSAQNGNGHSAN GSTRTVSGLDFLIAAVVGFETGPRSGSAMHGADLLLRGWHSGPVFGCPAAAAASSKLLGLSADDTESAIGIACTQA GGLMAAQYEGMIKRVQHAFAARNGLFGALLSRNGYVGIKKVYERNYGGFLNMFSQGNGKTPPYDVRKVTEGLG EVWQTTNIRVKLHACVGGCHGQIEAIEKLQKAHPERFAIGNLGHIKSIKVGLSGPIFAHDGWEPQERPLTETGAQ MNAAYIGAIQLVDGQVLIAEFANHKMDRDIVWDLVYKTKCHHDTQFDKPNHGCGAHIVVEFDDGFTVEETIQM PRGFDPPITDEEIRTKYRKLALSAIDQQRMEKIEELILGIDKLDDISEIFEVLAQPTRNVLG

SpoL	MHVPQPDDNSQRPYDEVINLIVDYAFDYEVKSEAAWARSKMALIDSFGVAIESLVKSKECESLIKPLLPGATNVTG GFRLPGTSYSLDVLQGAFNMGAMIRYLDHNDAFPGAEWGHPSDNLGAILVTADVLTRDALARGKPEEAITMKQV LIGLIKTYEIQGVFQIKNAFNRVGLDHTILVKIASTAMVSWMMGLSREQARAAVSHAWVDGHPLRIFRQAPNAGP RKGWAAGDACMRAVHLATLARSGQPGIRTPLTATRWGFYQVLNKDQEFQLPRPFGTWAVENVIFKVLTAEGHG LTTVEATMAASKELQARGLDPLKDIKNIHVRTQEAAMIIINKKGPLHNPADRDHCLRYMMGVILLKNGVEVEAED YQDDSPFATDPRVEALRSIISMEEDVQFTQDYHNPTIRSCGSSIEIFLKDGTTINVRQDFPLGHVVRDAETIPLVRKK AIHNLGLKFSQDEVTRIMETLEQPDFDTLPASKFIDLFQK
SpofasB	MESSGGSTSSFDEVNPTPGIADTPGIFTPGIPVTFTLNYEEVEVEFSLAPSDATHLDEHRRTFLTSLARSEGDNEEKK PMSAAALTFKFLEHLLRRSVSPGTLARFFYAVQSDLMEQKDIHDFISELPDGASTRKSALRTYMTLSSKLSCPLPSGP SALLTAARRERSSILVAFGGQSSSNPACVDDLAELYSLYRPLVEPLVSSLGAALLSLSRHPDTKAFFLGREIDLSAWLA DPSTRPAKNFIAGAAVSFPIIGLTGLLHYAIICKMLGKTPAELGQLLSGITGHSQGIVVAAAVAKSHSWESFFVEAR WAVELLFWMGYESQMAAPQSPISPAMVNDSVESGVGVPSHMLLVRGMRRQQLEAIVAASNKHLPKNERLYLSL INSARNYVIAGPPRSLRGLSLRLREICARDGLDQSRVPYSKRKPVILFQFLPVNAPFHSPYLNGAAERISARISGSWPE VTTISSLHVPVFYTENGADMTKSYKADVDVTQLLIDAVTTRVVDWPKTLQVGREKRLSHIITLGAGRFSSMIHENV DGYGVRVIDGARIDPVDSTIMGAKAEIFAQFLSRSTMSPSTWKDQFKPRLVQSSEGTFNIETRLNRILRAPPVITAG MTPTTVPWDFVSAVINAGYHIELAGGGYHNAEAMTTAIEKVAASIPTGRGITCNVIYVDPKAIGYQIPLIRQLIRKG VPIEGLTVGAGVPSPDVAAEYIQTTGIKHISFKPGSIAAIKEVIEIAKRHPTFPVILQWTGGRGGGHHSCEDFHEPLLE TYSEIRRCQNLYLVVGSGFGDGAGMFPYLTGSWSLQFGKPAMPCDGILLGSRMMIATDAHTSPGAKKLLLKAPG VDDAEWEKSYLKADAAGGVLTVTSEMGQPIHKLATRGVRLWKDMDDTIFSLPKPERKAALLKRKDEIIRRLNADY AKPWFGQDAAGQAVDVEDMTYADVLSRLVQLMYVKHQRRWVDQSYRELISEFAIQSLERLGSGDFEPSWLNSP ESFVDQVKEACPDVTEQLLHPEDVRFFIQCCKKRGRKPVNFVVALDDDFEHWFKKDSLWQSEDLDAVFDQDPER VCILQSPVSVRYATRDDQSSKEILDEIHRDLVVLMHAVEKPNGHVATRTNVTSRSRPMSENIMVDSMGDRIVFRP VPGEDLPSQEKWMECLDPYASSAILGLIREESLFEAASKRCRPNPFCRIFGPRHGYSLVLCRDYHEALLRDDSTGQTI VRVEARSAKDLRVEFTHRDSVPSGAATLVFQWEYDEHTRQLIDTTENRDKVIQDFYAHLWLPQNGTNRTGRLTD RFFADSFELTQQLQGALHSVVAHAFPSASPVGQTAVLPLESAVIAAWDVLMRPLLISDLDGDILRLVHQSIGVEYVP GVSPMQIGESVTTESSIRSITIEPSGKSVAVEARLIREGLHVATVTSEFFIKGKFSDYQNTFRHKEELPIELKIESSIDEAV LRDRSWLKLDDPSTPLVGKTIVFKVHTRSQWTNQTSAANLEILGTVEQKLWNDSKRRLGSVAFDASETHGNPVIEF LQRKGKTVDDKVPLKNPGWEGNSEVSIVAPPHTHLYAQVSGDCNPIHASPVFAELAELPGPIMHGMYTAAVCRK VVEDLAVPGEPERMRRFNASFVGMVRPGDKLTVGLSHVAMKNGRMILEVIARQEESGEEVLRGEAEVEQPSTAY LFTGQGSQSIGMGTALYESSPIAKALYDEMDKHLRDLFGWSILKIIRESPKELTVHFRGREGQRILENYLNMKTEIIG EDGIRRPAPIIPGLSRDSTSYTFSEARGLLHATQFAQPAIILLEKATLEHMRANGLIKEGAVFAGHSLGEYGALSSMA GFVDFKDMLSIGFYRGLLMQFAIPRDADGQTGYAMMAANPGRVGKHFDDSALRALVRHIAQESEELLEIVNFNIE GDQYVCAGHVRNLHCLTEILNAAAARKVHPESITEFVTASEPKTTTLGAIIAHSIAQSKTLPLSMQLQRGKATIPLNG IDVPFHSARLRSGVPTFRKFFHERVKAEDIRPERLVGSFIPNVVGKPFSIEKSFIQEVSKVTESPVLENLVC

Table S1.3 Protein sequence of *spo* (GenBank MT889334) gene cluster.

S1.1.5 *spo* cluster expression levels

The sequenced transcriptomes of *H. monticulosa*, which were obtained (see S1.1.3 for details) under producing (PDB medium, A) and non-producing (DPY medium, B) conditions (Fig. S1.5), were used to perform a differential expression sequence (DESeq) analysis.[19] Mean normalized expression levels (NEL) from conditions A and B were used to calculate the $\log_2$-fold change (B/A) to visualize differences in expression levels. If a $\log_2$-fold change of >2 was observed, genes were regarded as differentially expressed. Expression levels of the *spo* genes and surrounding genes are listed in Table S1.4.



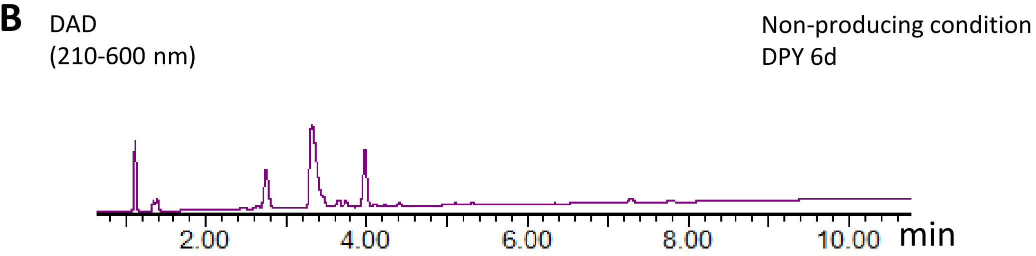


Figure S1.5 Diode array detector (DAD) chromatogram of *H. monticulosa* MUCL 54604 extract under: **A**, producing conditions (PDB media, 130 rpm, 28 °C, 6 days); and **B**, under non-producing conditions (DPY media, 130 rpm, 28 °C, 6 days). The production of sporothriolide **1** and trienylfuranol A **7** is only observed under producing conditions.

Gene (locus_tag)	Gene	NEL A (non-producing)	NEL B (producing)	Log_2-fold change (B/A)
HmMg6349	-	40.42	54.97	0.44
HmMg6350	-	92.24	246.13	1.42
HmMg6351	*spoC*	1176.34	11594.43	3.30
HmMg6352	*spoD*	35.24	174.58	2.31
HmMg6353	*spofasA*	9.33	771.14	6.37
HmMg6354	*spoE*	18.66	3936.16	7.72
HmMg6355	*spoF*	153.39	1263.85	3.04
HmMg6356	*spoG*	32.13	18022.20	9.13
HmMg6357	*spoH*	66.33	528.71	2.99
HmMg6358	*spoI*	5350.01	55269.24	3.37
HmMg6359	*spoJ*	69.44	2463.48	5.15
HmMg6360	*spoK*	2.07	1441.91	9.44
HmMg6361	*spoL*	3.11	820.59	8.04
HmMg6362	*spofasB*	21.76	2108.77	6.60
HmMg6363	-	0	3.72	-
HmMg6364	-	-	-	-
HmMg6365	-	3.11	0.88	-1.82

Table S1.4 Normalized expression level (NEL, BaseMean) for genes of the *spo* cluster and adjacent genes from *H. monticulosa* MUCL 54604 strain. Data calculated with DESeq.

S1.1.6 MultiGeneBLAST and other homology searches

In order to identify homologous *spo* clusters in other fungal genomes a MultiGeneBLAST analysis was conducted (http://multigeneblast.sourceforge.net/). SpofasB (FAS β), SpoL (2-Methyl citrate dehydratase), SpoK (Decarboxylase), SpoJ (Lactonase), SpoH (Lactonase), SpoG (Dioxygenase), SpoF (Transporter), SpoE (Citrate synthase) and SpofasA (FAS α) were utilized as MultiGeneBLAST architecture search templates. Due to an outdated version, only genomes released in NCBI GenBank until November 2015 were considered. The best hits of the search reveal the presence of various similar clusters in *Pestalotiopsis fici* and the genus *Aspergillus* including the previously published oryzine cluster from *A. oryzae* (Figure S1.7).[16]

Homology searches were also manually conducted within the genomes of other Hypoxylaceae and *Xylaria hypoxylon* obtained from an associated study[20] using the citrate synthase SpoE and the fatty acid synthase subunits (SpofasA, SpofasB) as template. Only *X. hypoxylon* contained a cluster with similar organisation as the *spo* cluster, which however lacked dioxygenase and lactonase genes. As *X. hypoxylon* is a known producer of piliformic acid

(2-hexylidene-3-methylsuccinic acid),[21] we assume that the compound is the most likely product of the cluster. To further verify this idea, a synteny analysis with the *spo* cluster was conducted utilizing the Artemic comparison tool (ACT, Fig. S1.6).

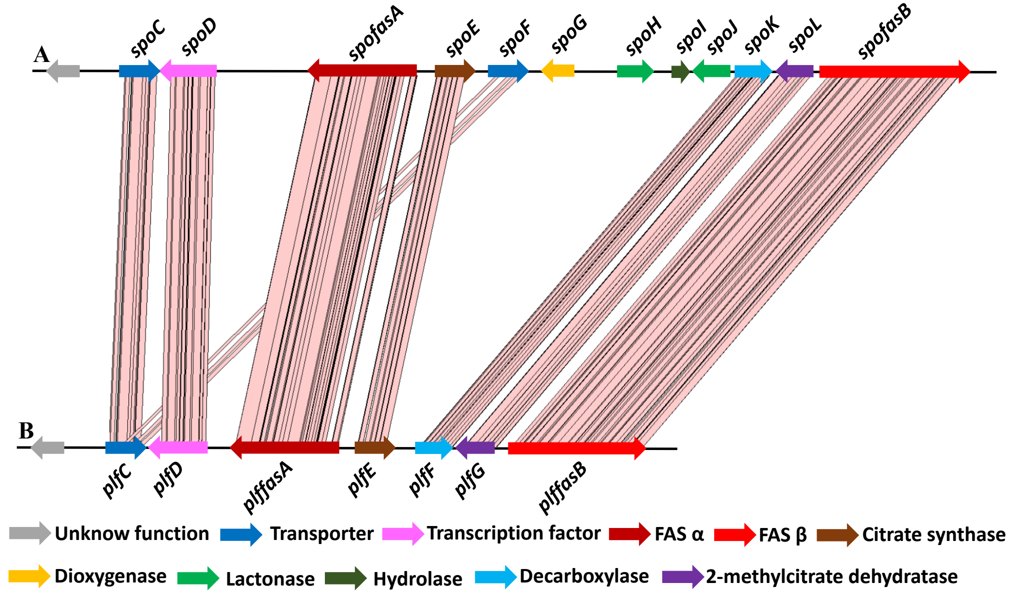


Figure S1.6 Artemis comparison tool (ACT) analysis: **A**, sporothriolide BGC of *H. monticulosa*; and **B**, the putative piliformic acid BGC of *Xylaria hypoxylon*.

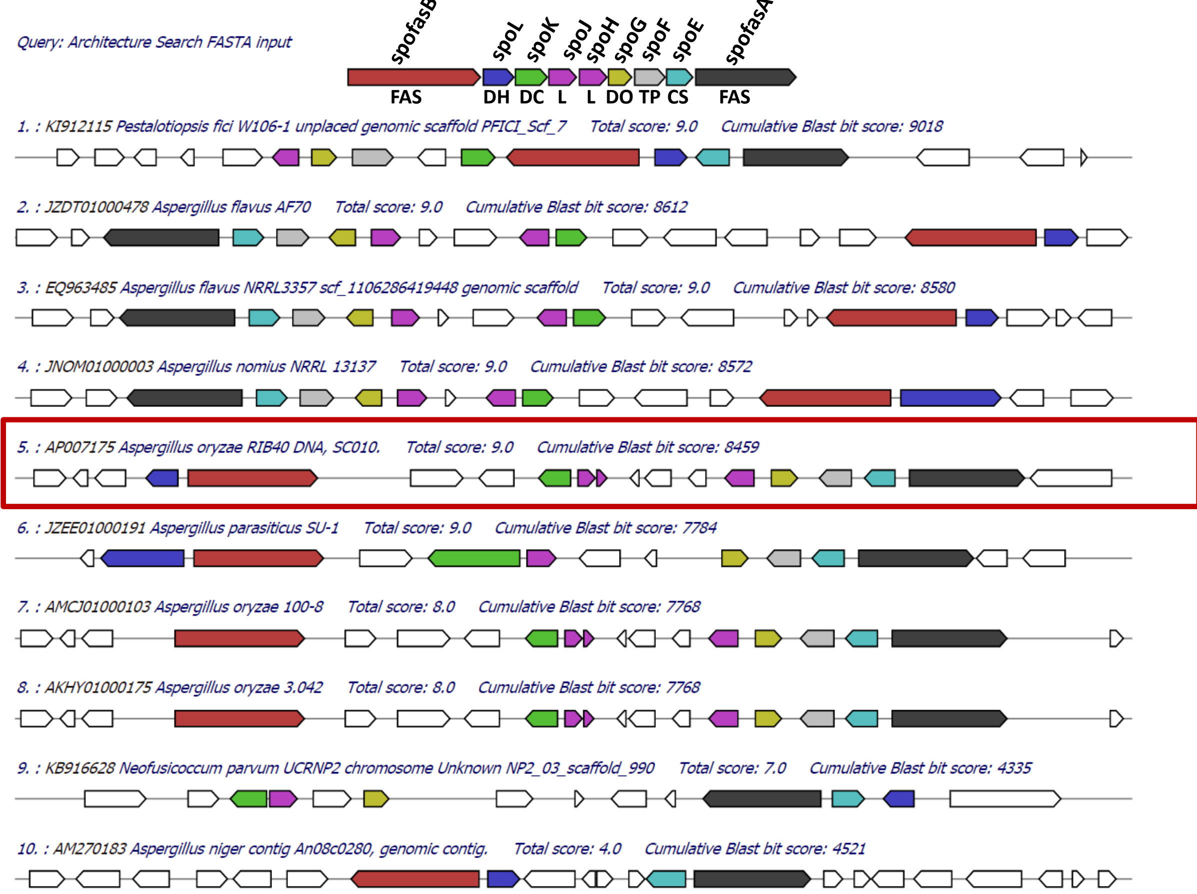


Figure S1.7 MultiGeneBlast (architecture search) of the sporothriolide biosynthetic enzymes of *H. monticulosa* MUCL 54604. There are six hits with 'Total score 9.0', and the putative biosynthetic gene cluster of the oryzines was included (red frame).

S1.2 Molecular biology

S1.2.1 *E. coli* transformation

To 50 µL of competent *E. coli* cells (Top10 or ccdB Survival ccdB Survival™ 2 T1R, Thermo Fisher Scientific, USA), either 1 µl of purified plasmid or 10 µl of a ligation mixture was added and incubated on ice for up to 20 min. After a 1 min heat shock at 42 °C, the cells were placed on ice for 3 min and 500 µL of LB medium (Table S2.4) was added. Cells were incubated at 37 °C, 350 rpm for 1 h and then distributed on LB agar (Table S2.5) supplemented with antibiotics for 12 h at 37 °C.

S1.2.2 *S. cerevisiae* transformation for yeast homologous recombination

A fresh (3 – 5 days old) single colony of *S. cerevisiae* was used to inoculate a 5 mL YPAD pre-culture and incubated at 30 °C, 200 rpm. After 18 h 20 mL of YPAD medium (Table S2.4) was added and incubated for 4 h at 30 °C, 200 rpm. Cells were collected by centrifugation (3000 x g, 5 min), washed with 25 mL ddH_2O and centrifuged (3000 x g, 5 min). The supernatant was discarded and the cells were suspended in ddH_2O before being transferred to a 1.5 mL microfuge tube. The mixture was centrifuged (21000 rpm, 15 s) and cells were resuspended in 400 µL 0.1 M lithium acetate. 50 µL aliquots were prepared, centrifuged (21000 rpm, 15 s) and

the supernatant discarded. 240 µL of a 50% PEG 3350 solution, 36 µL 1 M lithium acetate, 50 µL carrier DNA (denatured salmon sperm DNA, 2 mg / mL in TE buffer or ddH_2O), and up to 34 µL DNA were added to the cell pellet. The concentration of each linear DNA fragment was approximately 0.5-1 µg. Cells were suspended in the transformation mixture and incubated at 42 °C for 40 min. Cells were collected by centrifugation (21000 rpm, 15 s), the supernatant was discarded and the pellet was suspended in 500 µL ddH_2O. 250 µL were spread on SM-URA plates (Table S2.5) and incubated at 30 °C for 4-5 d.

S1.2.3 Vector construction for *A. oryzae* expression

The *A. oryzae* NSAR1 strain is auxotrophic in arginine (ΔargB), methionine (sC^-), adenine ($adeA^-$) and ammonium ($niaD^-$) metabolism, which enables its use as a host-vector system.[22] The two destination vectors pTYGSarg and pTYGSade[23] were used as a basis for plasmid construction with each including four insertion sites for the targeted genes. Genes were either inserted via yeast homologous recombination (YHR) or Gateway cloning. For the latter prior to the *in vitro* recombination, the targeted gene is cloned into the pE-YA[23] plasmid through YHR.

Genomic DNA of the *Hypomontagnella* strains was isolated from 5 days old liquid cultures using the GeneElute™ Plant Genomic DNA Miniprep Kit (Sigma Life Science, USA) was used following the manufacturer's instructions. For RNA extraction *H. spongiphila* and *H. monticulosa* were grown under sporothriolide producing conditions for 3 days in PDB medium. RNA was obtained by using the method in S1.1.3 and transcribed into cDNA using the High Capacity RNA-to-cDNA™ kit (Applied Biosystems by Thermo Fisher Scientific, USA). Genes of interest (*spofasA, spofasB, spoE, spoG, spoH, spoJ, spoK, spoL*) were either amplified from *H. monticulosa* cDNA or gDNA using the primer sets listed in Table S2.1 – S2.2 depending on the investigated combination of genes. PCR was conducted by using Q5® High-Fidelity 2X Master Mix according to the manufacturer's protocol, and PCR reaction was conducted with an annealing temperature of 60 °C if not stated otherwise (Table S2.2).

Prior to vector assembly, the pTYGS and pE-YA plasmids were digested with the restriction endonuclease: *Asc*I for pTYGS; *Asc*I and *NotI* for pE-YA. Targeted genes and digested destination vectors were then together transformed into yeast cells for YHR (see S1.2.2 for details). Plasmids were purified from yeast using the Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research, Germany), transformed into ccdB Survival ™ 2 T1R or Top10 *E. coli* cells for amplification, screened by colony PCR (for primer details see Table S2.2) and purified using the NucleoSpin™ Plasmid kit (Machery-Nagel, Germany). To transfer genes from the pE-YA to the pTYGS vectors a Gateway cloning kit (LR Clonase II Enzym-Mix, Invitrogen, USA) was applied following the manufacturers guidelines. A detailed list of the constructed vectors used in this work can be found in Figure S1.8.

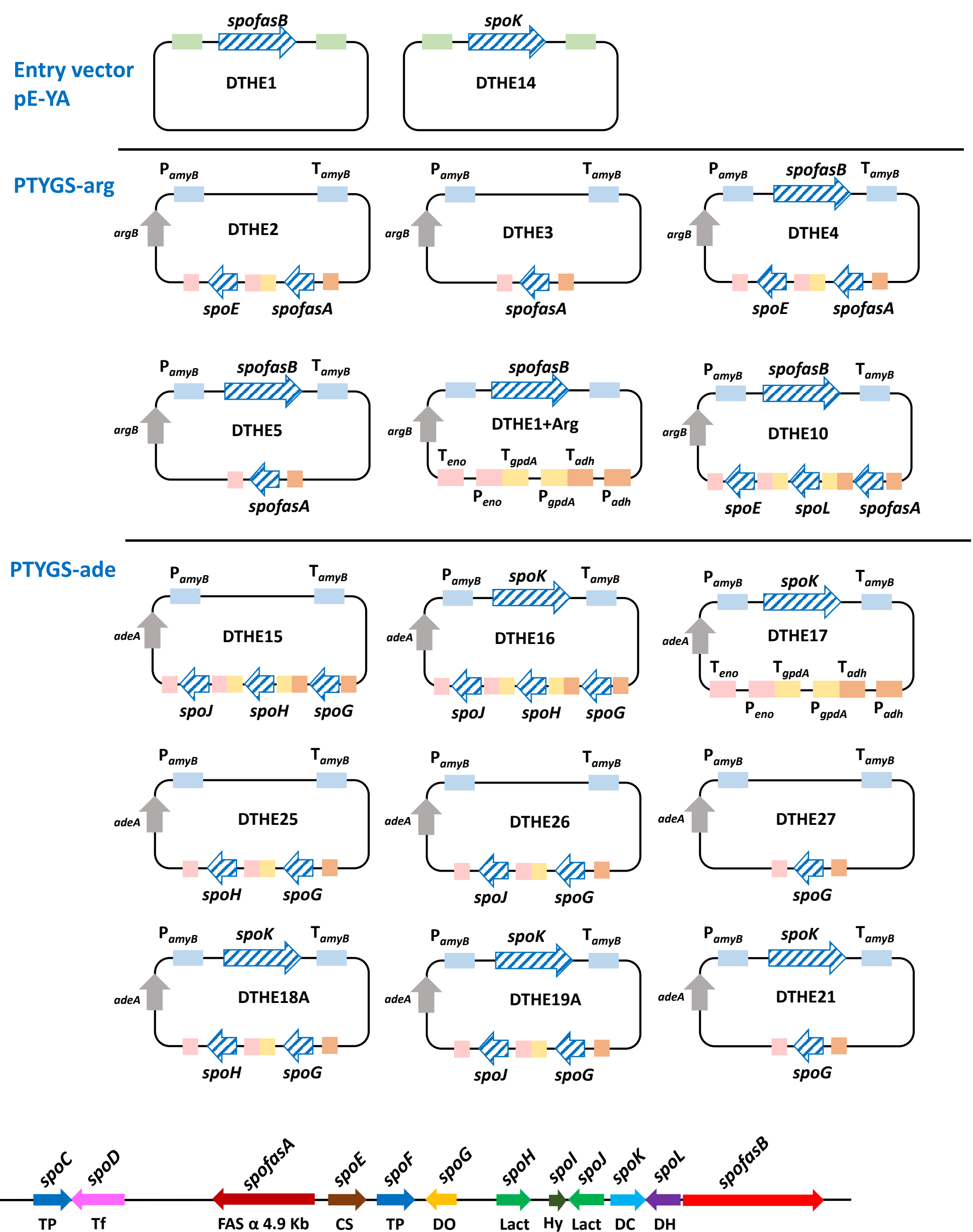


Figure S1.8 Constructed vectors for *A. oryzae* heterologous expression studies.

S1.2.4 *A. oryzae* NSAR1 transformation

1 mL spore suspension (1/10) from a fresh *A. oryzae* NSAR1 DPY plate was used to inoculate 50 mL (250 mL flask) of GN (Table S2.4) liquid medium. The culture was incubated for 24 h at 28 °C, 110 rpm. Cells were collected by filtration over sterile miracloth, washed with 0.8 M NaCl (50-100 mL) and suspended in 10 mL filter sterilised AO protoplast solution (10 mg/mL, Table S2.3) by inversion. The suspension was incubated at 30 °C and 50 rpm for 3.5 h. Protoplasts were released by repeated pipetting with a cut tip and gravity filtration through sterile miracloth removed remaining mycelia. Protoplasts were collected by centrifugation (3000 x g, 5 min) and the supernatant was discarded. The pellet was then directly suspended

in the required amount of fungal transformation solution I (100 µl per transformation, Table S2.3). After addition of the transformation vectors (≥ 1µg, in 10 µL ddH_2O) the mixture was incubated on ice for 5 min. Afterwards, 1 mL of fungal transformation solution II (Table S2.3) was added dropwise and the mixture was incubated at 25 °C for 20 min. 14 mL molten CZD+S 0.8% agar (Table S2.5, 50 °C) was added and the mixture was distributed over two plates containing CZD+S 1.5% agar (Table S2.5). Plates were incubated at 28 °C for 4-5 days until colonies became visible. These were transferred to new plates containing CZD 1.5% agar (without sorbitol, Table S2.5). For further selection well growing colonies were transferred onto a new CZD plate. Viable transformants were placed on DPY agar and incubated for 5-7 days before being used for subsequent experiments.

S1.2.5 Vector construction for *spofasA/spoE* knockout

In order to confirm that the *spo* BGC is responsible for the production of sporothriolide, a bipartite gene knockout strategy was applied.[24]

A vector based on the pE-YA plasmid including a 500 bp upstream fragment of *spofasA* and a 500 bp downstream fragment of *spoE* separated by a hygromycin resistance gene (*hph*) as selection marker was constructed (Figure S1.9). Homologous flanking arm1 (500 bp upstream fragment of *spofasA*) and arm2 (500 bp downstream fragment of *spoE*) were amplified by PCR from gDNA of *H. spongiphila* CLL205 using the primer sets P7+P8 and P9+P10 (Table S2.2). PCR reactions mixtures were prepared with the OneTaq® 2X Master Mix (New England BioLabs, USA) and PCR was performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.), the manufacturer's protocol was followed with annealing temperature 60 °C, 30 cycles. PCR products were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, USA). The pTH-GS-*egfp*[25] vector was used as the template to amplify the hygromycin resistance cassette (*hph*) with the primer sets P11+P12 (Table S2.2) using the above PCR setup. The pE-YA empty vector was linearized with the restriction enzymes *Asc*I and *Not*I (New England BioLabs). Recombination of the fragments was achieved with YHR (see S1.2.2 for details). Plasmids were purified from yeast using the Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research), transformed into Top10 *E. coli* cells for amplification, screened by colony PCR (for primer details see Table S2.1-S2.2) and purified using the NucleoSpin™ Plasmid kit (Machery-Nagel).

Two overlapping fragments of the constructed KO cassette (1946 bp and 3310 bp) were PCR amplified by OneTaq® 2X Master Mix (New England BioLabs, USA) using the primer set P7+P14 and P13+P10 (Table S2.1-S2.2) under the above conditions, but a cycle round of 35. Fragments were purified with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and used for transformation of *H. spongiphila* protoplasts.

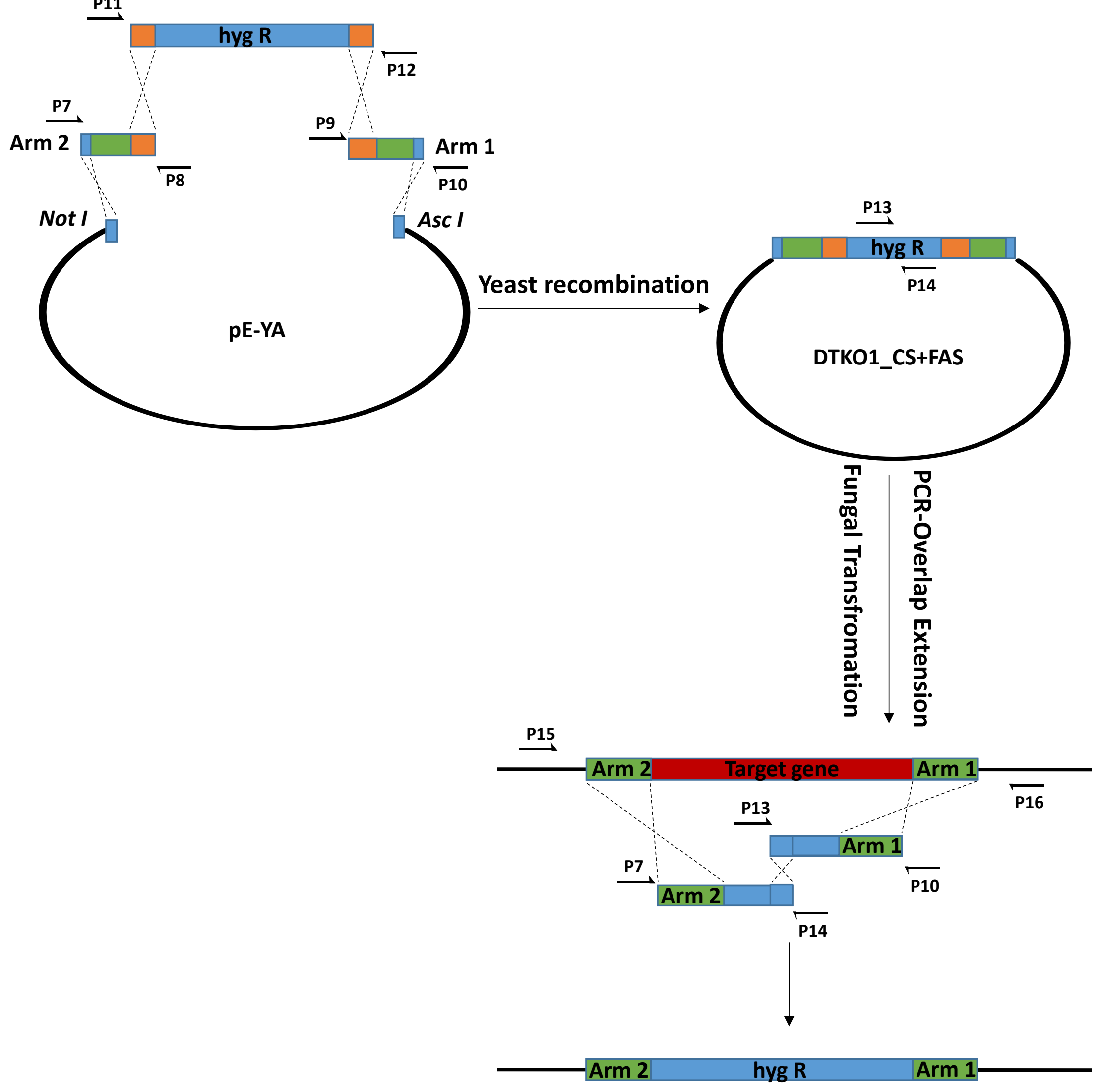


Figure S1.9 Bipartite knock-out[24] method to replace the targeted genes with a hygromycin resistance cassette *via* homologous recombination. Red: Target gene sequence (*spofasA* and *spoE* from *H. spongiphila* CLL 205). Blue: Hygromycin resistance cassette, containing the pgdpA promotor, the hygromycin resistance gene (*hph*) and the TrpC terminator.

S1.2.6 *Hypomontagnella spongiphila* transformation

200 µL mycelia from a cryo stock was used to inoculate 100 mL (500 mL flask) of DPY liquid culture and incubated for 24-48 h at 28 °C and 120 rpm. Cells were collected by filtration over sterile miracloth, washed with 0.7 M NaCl (50-100 mL) and resuspended in 11 mL filter-sterilised HYP (Table S2.3) protoplast solution (10 mg/mL) by inversion. The suspension was incubated at 28 °C and 110 rpm for 3 h. Protoplasts were released by repeated pipetting with a cut tip and gravity filtration through sterile miracloth removed remaining mycelia. Protoplasts were collected by centrifugation (4000 x g, 5 min) and directly resuspended in the required amount of HYP transformation solution I (100 µl per transformation, Table S2.3). Transformation vectors (≥ 1µg, in 10 µL ddH_2O) were added to the solution and incubated on ice for 50 min. 1.25 mL of HYP transformation solution II was added dropwise and the mixture was incubated at 25 °C for 30 min. Subsequently, 5 mL fungal transformation solution I was added to the mixture and a 1 mL aliquot was distributed on a DPY/S (1.2M sorbitol, Table S2.5)

agar plate containing 150 µg/mL hygromycin B. The plates were then air dried and thereafter incubated at 28 °C for 12 h. Each plate was overlaid with 5 mL DPY/S (Table S2.5) soft agar (0.8 %) containing 75 µg/mL hygromycin B. Plates were incubated at 28 °C for 8-10 days until colonies were observed. Colonies were transferred to new DPY plates (no sorbitol) containing 150 µg/mL hygromycin B. Well growing colonies were then again transferred to new DPY plates (no sorbitol) containing 150 µg/mL hygromycin B. Viable transformants were placed on DPY agar and incubated for 5-7 days before being used for subsequent experiments.

S1.2.7 Verification of transformants

Forty-six *H. spongiphila* transformants were screened for the deletion of the targeted genes by PCR. For this purpose, genomic DNA was isolated from six days old mycelia growing on plates using the GeneEluteTM Plant Genomic DNA Miniprep Kit (Sigma Life Science). Correct integration of the deletion cassette was checked with the primer sets P15+P14 and P13+P16 (Table S2.1-S2.2), which bind outside of the targeted genes and inside the *hph* cassette. PCR reactions mixtures were prepared by the OneTaq® 2X Master Mix (New England BioLabs, USA) and was conducted under the same conditions in S1.2.5. Gel electrophoresis revealed a single transformant with the expected fragments (Fig. S1.10).

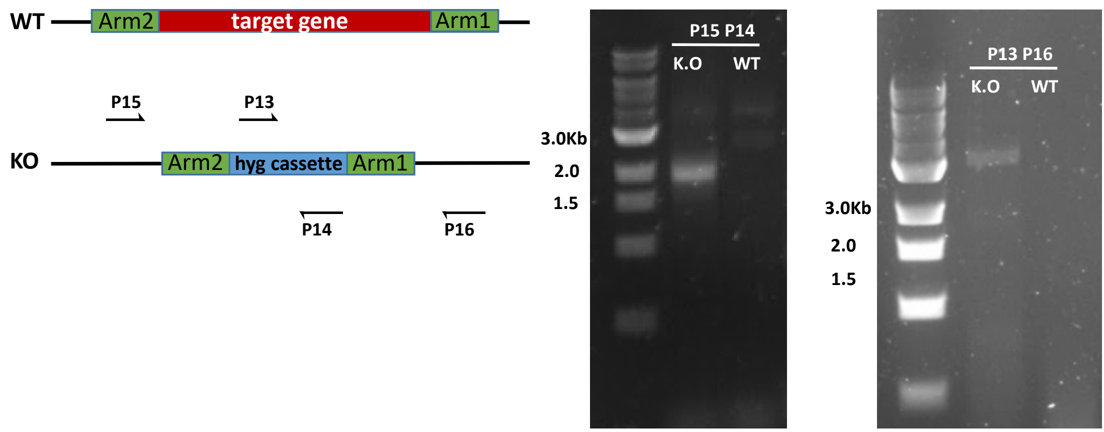


Figure S1.10 Verification of positive *H. spongiphila ΔspoE/spofasA* transformants using PCR.

S1.3 Chemistry

S1.3.1 Fermentation and extraction of compounds

Small scale

For product analysis of *Hypomontagnella* strains (wild-type and transformants), fungi were grown in 100 mL of DPY or PDB medium in 500 mL flasks for 6 to 7 days at 28 °C and 130 rpm. *Aspergillus oryzae* transformants were grown in 100 mL of DPY medium in 500 mL flasks for 5 to 7 days at 28 °C and 110 rpm. Culture broths were separated by Büchner filtration into supernatant and biomass. Cells were disrupted with a hand blender and stirred in 100 ml acetone for 1 h. The organic phase was filtered by vacuum filtration and the solvent was removed under reduced pressure in a rotary evaporator until a water phase remained. 100 ml H_2O was added to the aqueous phase and extracted twice with an equal amount of ethyl acetate. Combined organic layers were dried over $MgSO_4$ and solvent was removed under vacuum. Extracts were dissolved in methanol and adjusted to a concentration of 10 mg/ml,

filtered over glass wool and analysed by LCMS.

The supernatant was extracted twice with an equal amount of ethyl acetate. Combined organic layers were dried over $MgSO_4$ and solvent was removed under vacuum. Extracts were dissolved in methanol to a concentration of 10 mg/ml, filtered over glass wool and analysed by LCMS.

Large scale

For compound isolation fungi were grown in up to 1 L total volume of DPY or PDB medium (up to 10 flasks, each containing 100 mL medium) and grown for 6 to 7 days at 28 °C and 130 rpm in case of the *Hypomontagnella* strains and for 5 to 7 days at 28 °C and 110 rpm in case of *A. oryzae* transformants. Culture broths were separated by Büchner filtration into supernatant and biomass. The supernatant was extracted twice with an equal amount of ethyl acetate. Combined organic layers were dried over $MgSO_4$ and solvent was removed under vacuum. Extracts were dissolved in methanol to a concentration of 20-30 mg/ml, filtered over glass wool and processed by preparative LCMS.

S1.3.2 Analytical LCMS

A Waters 2545 binary gradient module with a Waters 515 HPLC pump coupled to a Waters 2767 autosampler, a Waters 2998 DAD, a Waters 2420 evaporative light scattering detector (ELSD) and a Waters single quadrupole mass detector 2 (SQ detector 2) were used for analytical LCMS. The DAD measured wavelengths from 210 to 600 nm and the mass detector was adjusted depending on the sample to measure in a range of 100-1000 *m/z*. A Phenomenex Kinetex column (2.6 µm, C_{18}, 100 Å, 4.6 x 100 mm) equipped with a Phenomenex Security Guard precolumn (Luna, C_5, 300 Å) served as stationary phase. The mobile phase was composed of HPLC-grade water mixed with 0.05 % formic acid (solvent A) and HPLC-grade acetonitrile mixed with 0.045 % formic acid (solvent B). A solvent gradient was run at a flow rate of 1 ml/min over 15 min starting at 10 % B and ramping up to 90 % B (Table S2.8).

S1.3.3 Preparative LCMS

Samples were dissolved in methanol or acetonitrile/water (9:1) mixture, adjusted to a concentration of 50 mg/mL and filtered over glass wool. For compound purification the same LCMS setup as above was used, but the stationary phase was replaced by a Phenomenex Kinetex Axia column (5 µm, C_{18}, 100 Å, 21.2 x 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna, C_5, 300 Å). The gradient was selected depending on the sample (Table S2.7-S2.8) and run with a flow rate of 20 mL/min. Between 50 and 600 µL of the crude extracts were injected for each run. Fractions were collected with the Waters Sample Manager 2767 by either mass directed or time-dependent trigger. Combined fractions were firstly evaporated under vacuum to remove the organic solvents, then the remaining aqueous phases were dried in a Freeze Dryers Rotational-Vacuum-Concentrators ALPHA 1-4 LDplus (Martin Christ Gefriertrocknungsanlagen GmbH, Germany), weighted, dissolved and analysed by HPLC and NMR.

S1.3.4 HRMS

High-resolution mass spectra were acquired on a Waters Acquity ultra-performance liquid

chromatography (UPLC) system coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF).

S1.3.5 Nuclear magnetic resonance (NMR) analysis

NMR data were recorded using either a Bruker Ascend 400, a Bruker Ultrashield 500 or a Bruker Ascend 600 instrument each equipped with a cryo-cooled probe at 400/ 500/ 600 MHz (^{1}H) and 100, 125 and 150 MHz (^{13}C). Chemical shifts are shown in parts per million (ppm) in comparison to the TMS (tetramethylsilane) standard. The coupling constants *J* are given in Hz. The software MestReNova 10.0 was used for the analysis of the data. For known compounds only 1D NMR spectra were recorded and compared to literature to confirm the structure. For new compounds complete structural elucidation was carried out by conducting 2D experiments in addition including Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear MultipleBond Correlation (HMBC) as well as Nuclear Overhauser Effect spectroscopy (NOESY).

S1.3.6 Calibration curve for sporothriolide, trienylfuranol A and sporochartines.

In order to quantify the production of compounds, the calibration curves for sporothriolide **1**, trienylfuranol A **7** and sporochartine B **6b** were first made based on a UV integration value calculation method. A dilution series of **1** (1.5, 1, 0.5, 0.25, 0.125, 0.0625, 0.03175 mg/ml) was measured and the corresponding signals for the extracted wavelength at 211 nm (λ_{max}) were integrated (Table S1.5). Integrated values were then plotted against the sample concentration (Figure S1.11) and fitted into a straight line described by Equation**:** UV= 91894C – 942.42, where UV is the dimensionless integration value of the UV peak (UV_{int}) and C is the corresponding concentration of sporothriolide **1** in $mg.ml^{-1}$. The relationship was linear within the 0.03 – 1.5 $mg.ml^{-1}$ concentration range. The equation was applied to quantify **1** across different samples. This quantification method was also used for trienylfuranol A **7** and sporochartine B **6b** (Table S1.6 - S1.7, Figure S1.12- S1.13).

211 nm	
Conc. / mg.ml^{-1}	UV_{int}
1.5	132848
1	96828
0.5	46285
0.25	21143
0.125	9113
0.0625	4340
0.03175	1650

Table S1.5 The integrated values of different dilutions of sporothriolide **1** solutions at 211 nm from LCMS analysis.

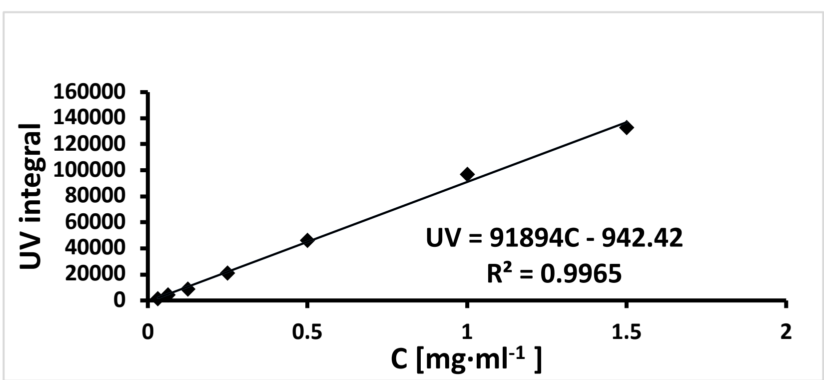


Figure S1.11 Calibration curve for sporothriolide **1** quantification.

211nm	
Conc. / mg.ml^{-1}	UV_{int}
0.25	31534
0.125	15507
0.0625	8410
0.03175	3906

Table S1.6 The integrated values of different dilutions of sporochartine B **6b** solution at 211 nm from LCMS analysis.

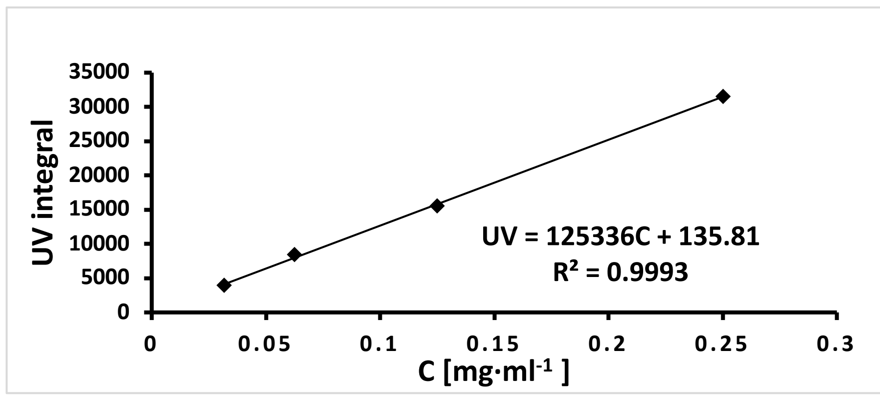


Figure S1.12 Calibration curve for sporochartine B **6b** quantification.

262nm	
Conc. / mg.ml^{-1}	UV_{int}
2	53526
1	31378
0.25	9303
0.125	4282

Table S1.7 The integrated values of different dilutions of trienylfuranol A **7** solution at 262 nm from LCMS analysis.

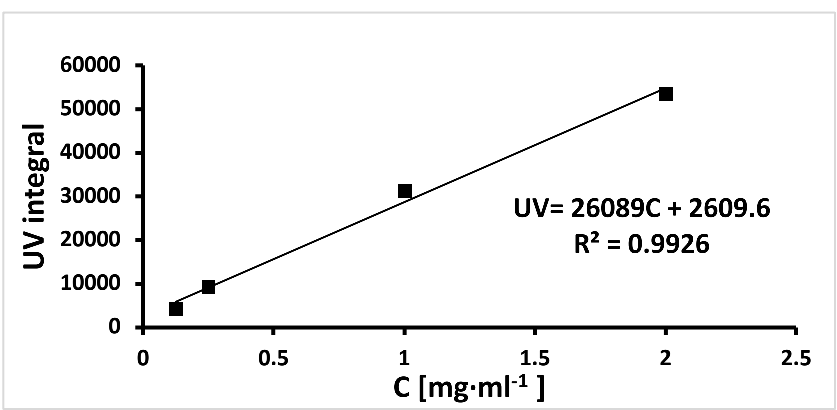


Figure S1.13 Calibration curve for trienylfuranol A **7** quantification.

S1.3.7 Production timecourse of 1, 6b and 7

Timecourse experiements for the three *Hypomontagnella* wild-type strains were conducted to monitor the production of **1**, **6b** and **7**. *H. monticulosa* and *H. spongiphila* were grown respectively in a flask containing 1 L PDB medium at 28 °C, 130 rpm for 14 d and 10 mL aliquots were taken daily. *H. submonticulosa* was grown in a flask containing 1 L PDB medium at 25 °C, 100 rpm for 9 d and 10 mL aliquots were taken daily. Aliquots were extracted with equal amounts of ethyl acetate and the organic phase was evaporated under vacuum. Crude extracts were analysed by LCMS and compound titers were calculated using the previously described equation (Table S1.8 – S1.10). Production kinetic of **1**, **6b** and **7** was visualized by graph (Figure S1.14).

	Sporothriolide	Sporochartine B	Trienylfuranol A
H. monticulosa MUCL 54604	Conc. / mg.ml^{-1}	Conc. / mg.ml^{-1})	Conc. / mg.ml^{-1})
1d	0	0	0
2d	0.071334	0	0
3d	0.164222	0	0
4d	0.190732	0	0
5d	0.136205	0	0
6d	0.141487	0	0
7d	0.032407	0.000333	0.052230
8d	0.038710	0	0
9d	0.022173	0	0
10d	0.007714	0	0
11d	0	0	0
12d	0.007392	0	0
13d	0	0	0
14d	0	0	0

Table S1.8 Kinetic production of sporothriolide **1**, sporochartine B **6b** and trienylfuranol A **7** from *H. monticulosa* MUCL 54604 cultivated in PDB media, 130 rpm and 28 °C.

	Sporothriolide	Sporochartine B	Trienylfuranol A
H. submonticulosa DAOMC 242471	Conc. / mg.ml^{-1}	Conc. / mg.ml^{-1}	Conc. / mg.ml^{-1}
1d	0	0	0
2d	0	0	0
3d	0	0	0.013686
4d	0	0	0.024935
5d	0.064536	0.000470	0.965539
6d	0.238332	0.004138	1.111585
7d	0.087014	0.000615	1.132046
8d	0.015634	0.000402	0.901220
9d	0	0	0.045082

Table S1.9 Kinetic production of sporothriolide **1**, sporochartine B **6b** and trienylfuranol A **7** from *H. submonticulosa* DAOMC 242471 cultivated in PDB media, 100 rpm and 25 °C.

	Sporothriolide	**Sporochartine B**	**Trienylfuranol A**
***H. spongiphila* CLL205**	**Conc. / mg.ml^{-1}**	**Conc. / mg.ml^{-1}**	**Conc. / mg.ml^{-1}**
1d	0	0	0
2d	0	0	0
3d	0.056434	0	0
4d	0.162090	0	0
5d	0.097996	0.004849	0
6d	0.181303	0.005883	0
7d	0.055589	0.002985	0
8d	0.039563	0.005768	0.188739
9d	0.008646	0.002970	0.41716
10d	0	0	0.325006
11d	0	0	0.323001
12d	0.002380	0	0.298783
13d	0	0	0.344887
14d	0	0	0.321181

Table S1.10 Kinetic production of sporothriolide **1**, sporochartine B **6b** and trienylfuranol A **7** from *H. spongiphila* CLL 205 cultivated in PDB media, 130 rpm and 28 °C.

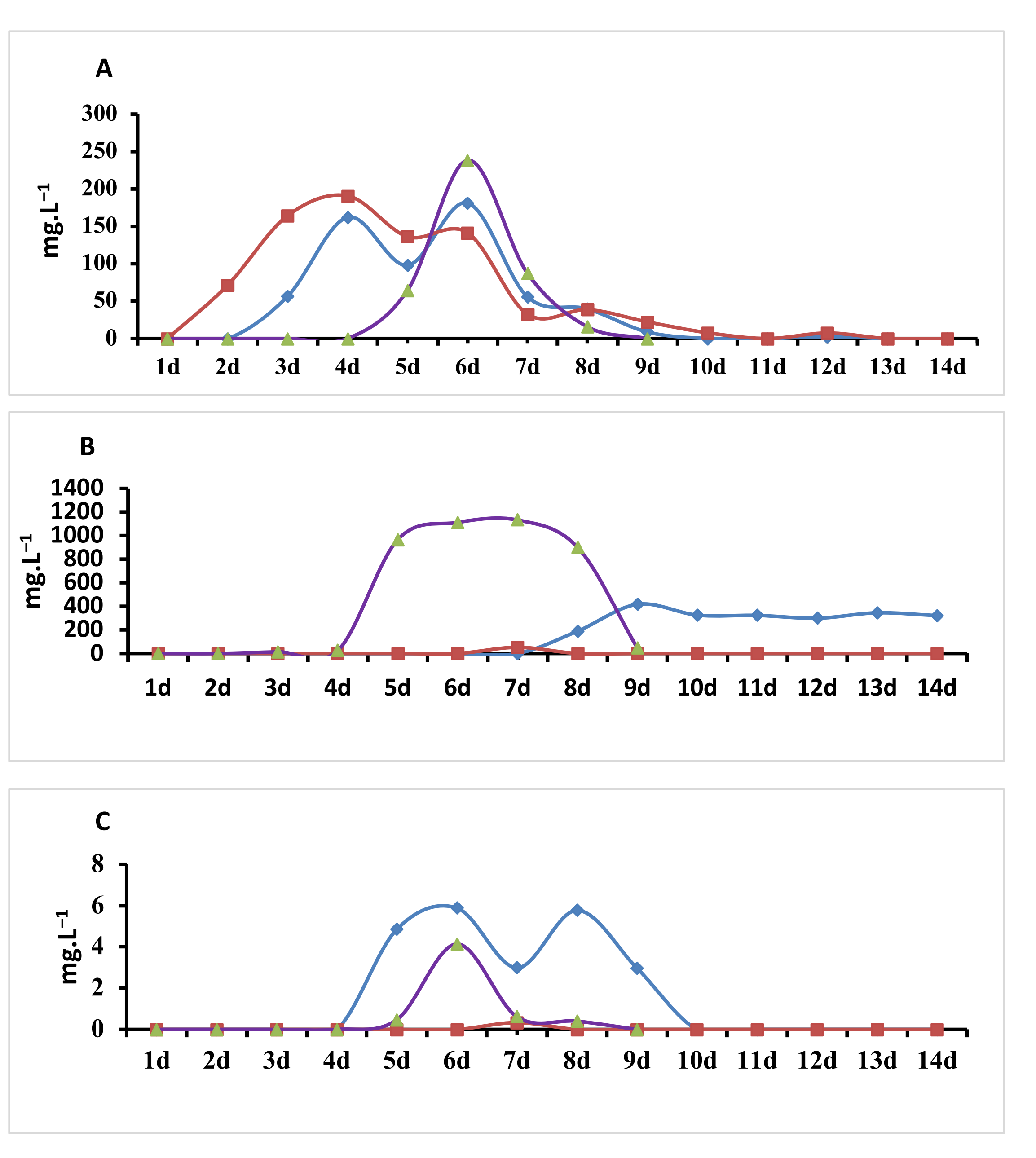


Figure S1.14 Kinetic production curve of: **A**, sporothriolide **1**; **B**, trienylfuranol A **7**; and **C**, sporochartine B **6b** from *H. spongiphila* CLL 205 (blue line), *H. monticulosa* MUCL 54604 (orange line) and *H. submonticulosa* DAOMC 242471 (purple line).

S1.3.8 Labelling experiment

To deduce the building blocks of **1**, **6** and **7**, feeding experiments with labelled sodium acetate were conducted based on the previously calculated production kinetics. Therefore, 415 mg of [1-^{13}C] and [2-^{13}C] sodium acetate dissolved in 3 mL ddH_2O were separately supplemented to 3 days old *H. spongiphila* cultures grown in 500 mL PDB medium (five 500 mL flasks each contain 100 mL medium) at 28 °C and 130 rpm. Feeding was repeated at day 4 and 5 to reach a final concentration of labelled acetate of 10 mM. Cultures were harvested on day 6. Extraction and purification of compounds was achieved as described in S1.3.1 and 1.3.3. ^{13}C NMR spectra were recorded for labelled sporothriolide **1** and sporochartine B **6b**. Peak enhancement was estimated by calculating the ratio between the normalised peak intensity

of each signal of the labelled compound and the normalised signal intensity of each carbon in natural abundance. C-12 was used as a reference for [1-^{13}C] sodium acetate feeding experiments, C-11 was used as a reference for [2-^{13}C] sodium acetate feeding experiments. (Figure S1.15 – S1.16, Table S1.11 – S1.12).

For **7**, 336 mg of [1,2-$^{13}C_2$] sodium acetate dissolved in 2.4 mL ddH_2O was fed to 3 days old *H. submonticulosa* cultures grown in 400 mL PDB medium (four 500mL flasks each contain 100 mL medium) at 25 °C and 100 rpm. Feeding was repeated at day 4 and 5 to reach a final concentration of labelled acetate of 10 mM. Labelled **7** was extracted and purified as previously described. ^{13}C NMR recordings revealed that only the signal of C-14 is enriched compared to unlabelled **7**. All other carbon signals displayed a doublet with coupling constants between 37 and 55 Hz, which is characteristic for intact acetate units. The neighbouring carbons from an intact acetate show an identical *J*-coupling constant (Table S1.13, Figure S1.17 – S1.18). Thus, C-14 probably resulted from a head carboxyl group decarboxylation.

Position	1-^{13}C-Sodium Acetate	2-^{13}C-Sodium Acetate
1	6.5	1
2	1	3.5
3	1.5	2.5
4	4.5	0.5
5	2.5	0.5
6	0.5	3
7	3	1
8	0.5	3
9	3.5	0.5
10	1	4
11 (reference)	5	1
12 (reference)	1	8
13	0.5	2

Table S1.11 NMR signal peak enhancement of labelled sporothriolide **1**. A significant incorporation threshold fold is set ≥ 2.

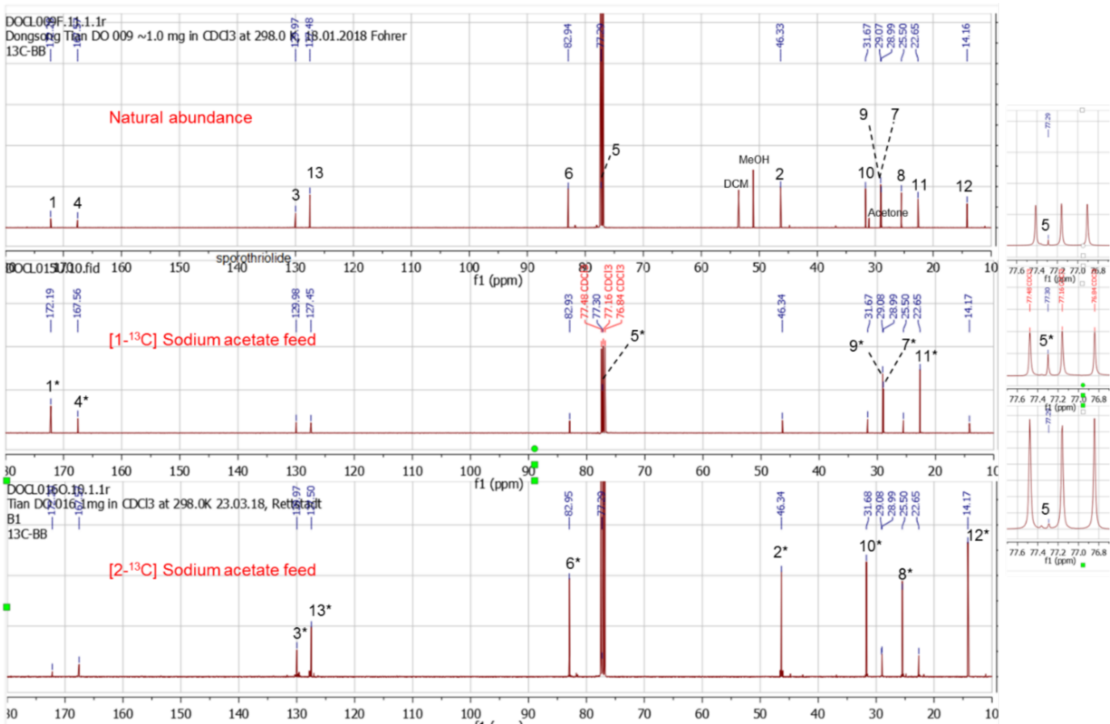
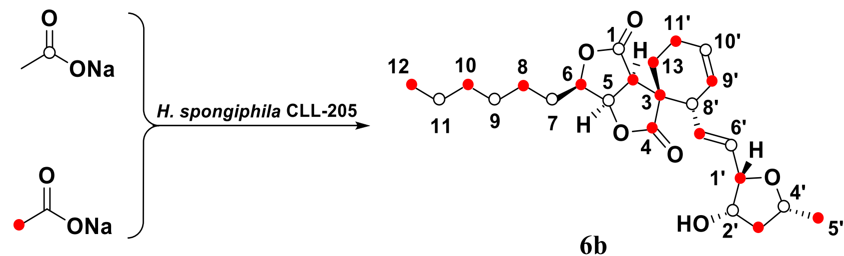


Figure S1.15 Natural abundance ^{13}C-NMR spectra of sporothriolide **1** compared to ^{13}C-NMR spectra of the two different feeding experiments ([1-^{13}C] and [2-^{13}C] sodium acetate). The enhanced peaks are marked with *.



Position	1-^{13}C-Sodium Acetate	2-^{13}C-Sodium Acetate
1	2.9	1.2
2	0.4	3.5
3	0.6	4.5
4	1.3	2
5	1.7	0.7
6	0.6	5
7	2	0.8
8	0.4	4.6
9	3	1.1
10	0.6	5.9
11 (reference)	2.9	1
12 (reference)	1	9.5
13	0.4	2.5
1'	0.5	4.3
2'	2.8	1.2
3'	0.5	5.4
4'	2.3	1.1
5'	0.5	4.6
6'	2	1
7'	0.5	4.7
8'	2.5	1
9'	0.5	4.8
10'	2.6	0.8
11'	0.4	4.3

Table S1.12 NMR signal peak enhancement of labelled sporochartine B **6b**. A significant incorporation threshold fold is set ≥ 1.5 fold.

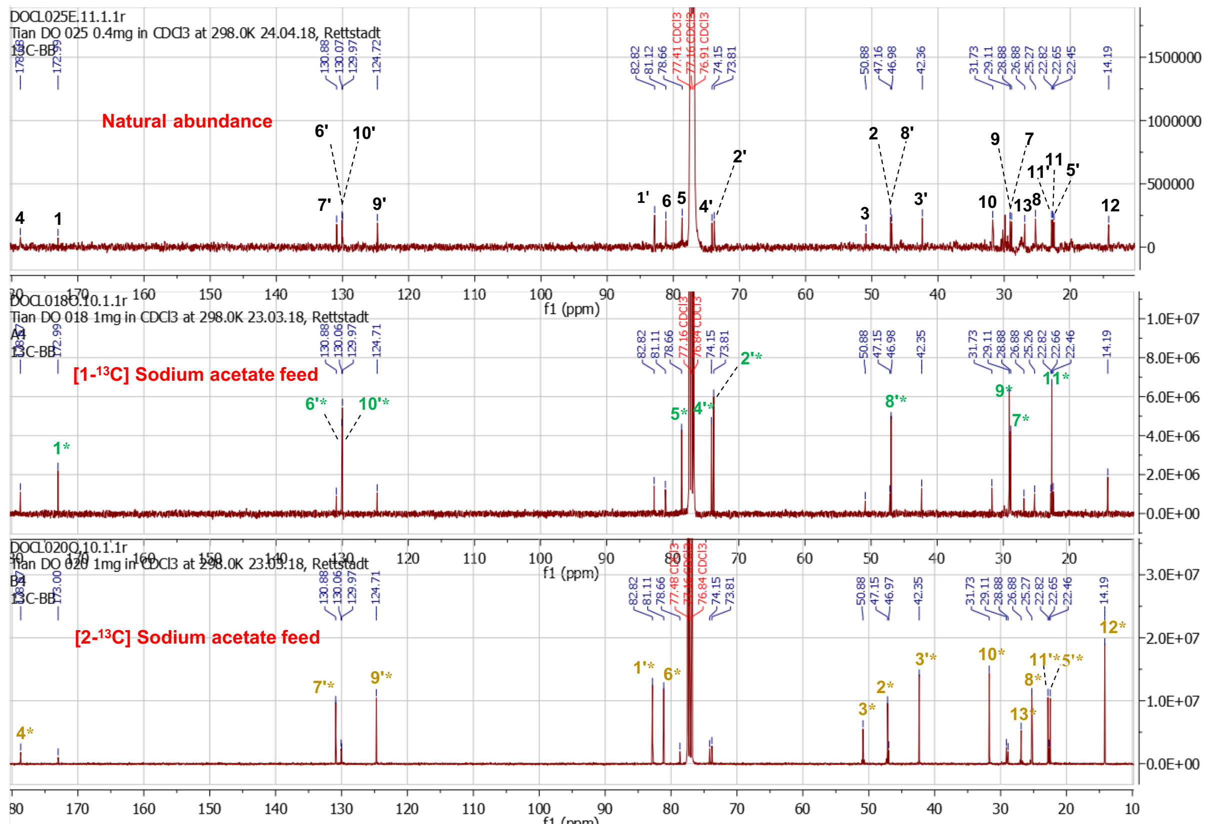


Figure S1.16 Natural abundance ^{13}C-NMR spectra of sporochartine B **6b** compared to ^{13}C-NMR spectra of the two different feeding experiments ([1-^{13}C] and [2-^{13}C] sodium acetate). The enhanced peaks are marked with *.

ONa → *H. submonticulosa* → OH

Atom numbers	J_{CC} (Hz)
C9'-C10'	55-55
C7'-C8'	overlap
C1'-C6'	51-51
C2'-C3'	37-37
C4'-C5'	40-40

Table S1.13 One bond ^{13}C-^{13}C spin-spin couplings constants observed in trienylfuranol A **7** derived from [1,2-$^{13}C_2$] acetate labelling experiment.

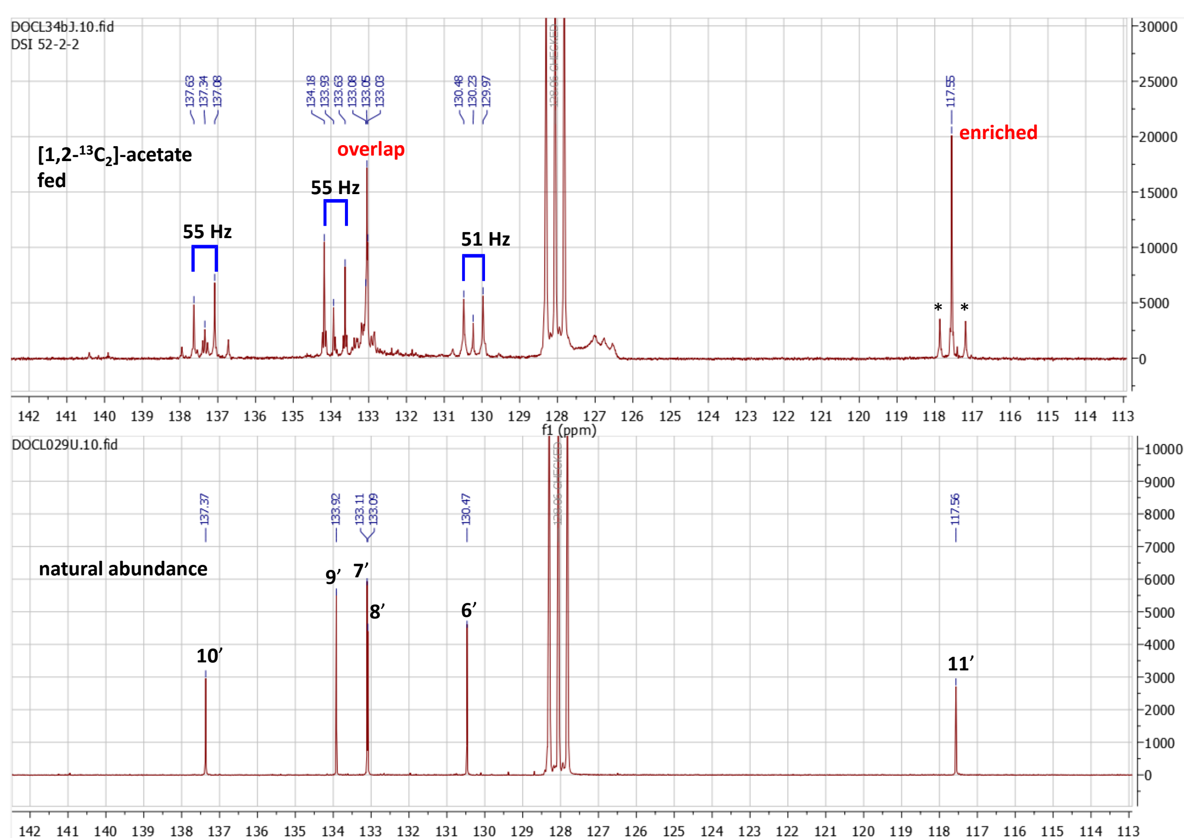


Figure S1.17 Natural abundance ^{13}C-NMR spectra (chemical shift between 113 and 142 ppm, 100 MHz in C_6D_6) of trienylfuranol A **7** compared to ^{13}C-NMR spectra of the feeding experiment ([1,2-$^{13}C_2$] sodium acetate).

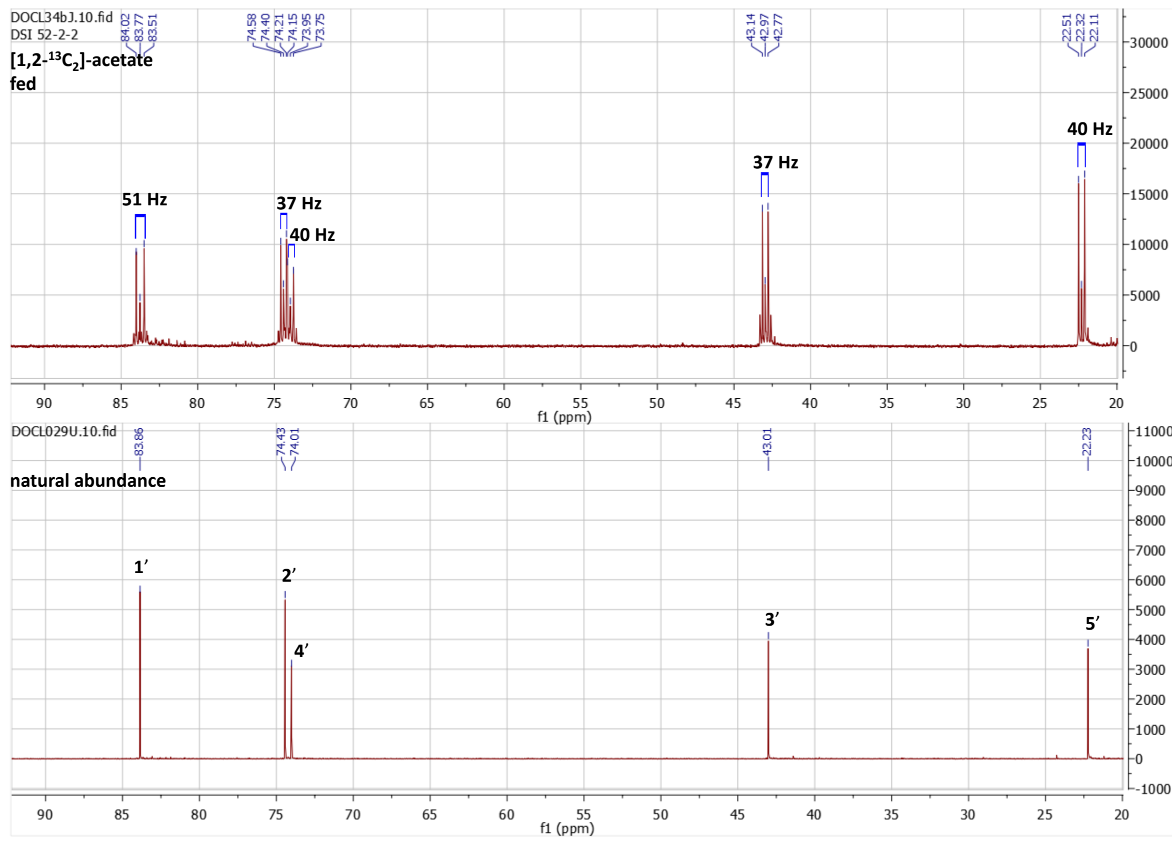


Figure S1.18 Natural abundance ^{13}C-NMR spectra (chemical shift between 20 and 90 ppm, 100 MHz in C_6D_6) of trienylfuranol A **7** compared to ^{13}C-NMR spectra of the feeding experiment ([1,2-$^{13}C_2$] sodium acetate).

S1.3.9 Stability assay of sporothriolide

0.5 mg of sporothriolide **1** was fed to *A. oryzae* NSAR1 grown in 20 mL DPY culture or incubated with 20 mL of various media (DPY, PDB, CMP, water) under normal fermentation conditions (28 °C , 110 rpm) for 24 h. Mixtures were extracted with equal amounts of ethyl acetate and analyzed by LCMS. Degradation of **1** to **13** was observed under all conditions except water (Figure S1.19). Additionally, pH value of each mixture was measured to investigate the possibility of pH-dependent elimination. Values between 5.65 and 6.50 indicate that the pH of the solutions has likely no influence on the degradation and that medium-specific ingredients are likely responsible for the conversion of **1** to **13**.

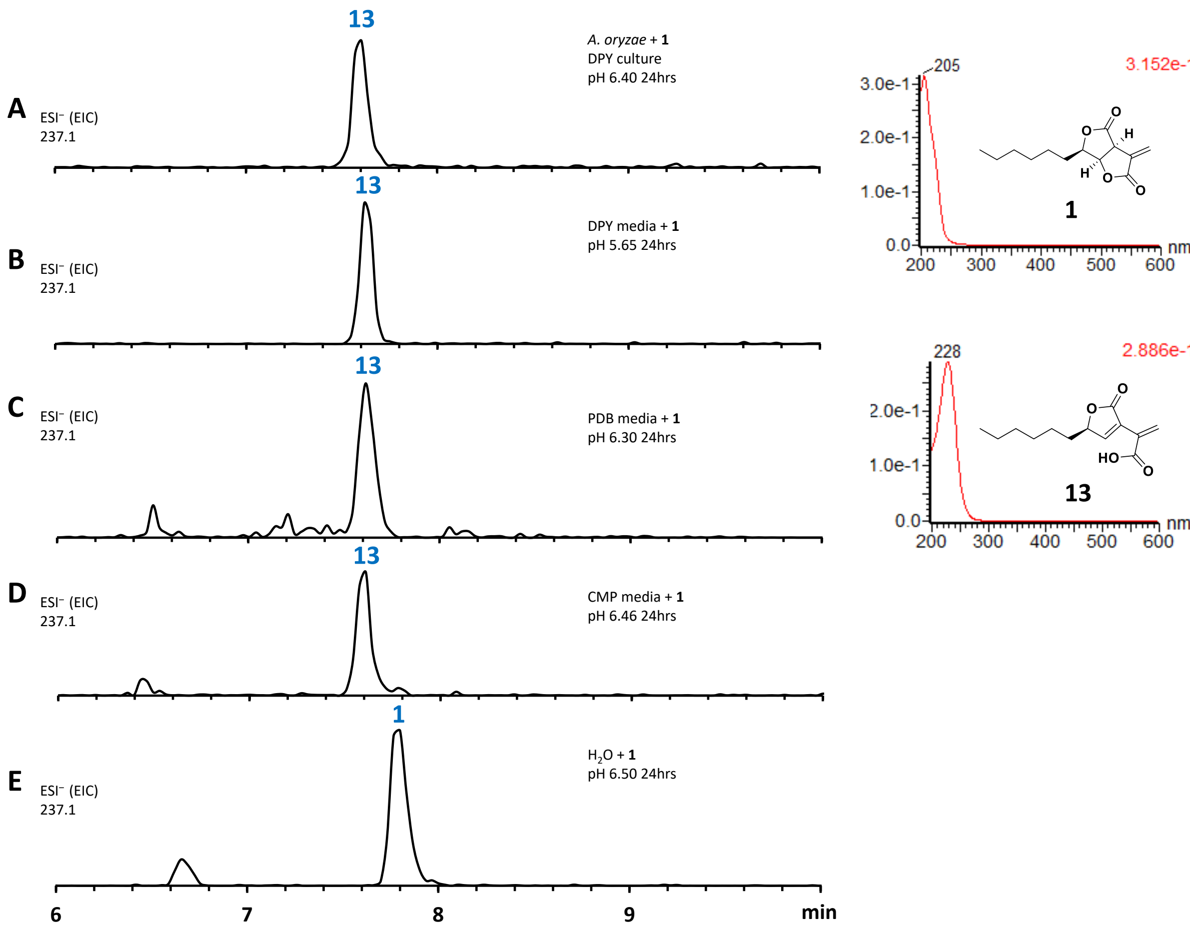


Figure S1.19 Extracted Ion chromatograms (237.1 [M–H]⁻) from the sporothriolide **1** stability assay with **1** being incubated under different conditions. *A. oryzae* NSAR1 DPY culture fed with **1** (**A**), DPY media (**B**), PDB media (**C**), CMP media (**D**) and water (**E**).

S1.2.11 *In vitro* Diels-Alder cycloaddition of 1 and 7

As sporochartines **6** occur as a mixture of various stereoisomers, it appeared possible that these compounds originate from a spontaneous cycloaddition of sporothriolide **1** and trienylfuranol A **7**. Therefore, various *in vitro* conditions were investigated to mimic the putative reaction between **1** and **7**.

To test if **6** can be formed spontaneously under fermentation conditions in the medium, the *H. spongiphila ΔspoE/spofasA* deficiency mutant was grown under producing conditions of **7** in 50 ml PDB medium for four days to accumulate **7**. The cells were then filtered off and the supernatant was supplemented with 10 mg of **1**. A control was conducted in parallel were the filtered supernatant was not supplied with **1**. Afterwards, the supernatant was incubated under fermentation conditions for 24 h before being lyophilized. The lyophilisates were partially dissolved in methanol and directly subjected to LCMS analysis. Extracted ion chromatogram searches with *m/z* 463.2 $[M+HCOO^-]^-$ showed that sporochartine A **6a** and B **6b** were detected when **1** was fed to the medium, but lacked in the control (Figure 5B) proofing that **1** can spontaneously react with **7** in the medium.

To test if the standard extraction process (ethyl acetate and evaporation of the organic phase at 40 °C) can influence the formation of **6** as well, reactions of **1** and **7** were investigated *in vitro*. Therefore, 1.5 mg of **1** and 1 mg of **7** were dissolved together in 1 mL ethyl acetate and incubated under nitrogen in the dark at either room temperature or 40 °C. LCMS was used to monitor the *in vitro* reaction. After two hours at room temperature, sporochartine A **6a** and B **6b** were formed in traces (Figure 5D). Reaction was proceeded over night, but did not result into increased concentrations of **6a/b**. Then heated reaction mixture, in contrast, exhibited significant higher concentrations of **6a/b** after two hours, which were comparable to the concentrations observed in the wild type (Figure 5E). Hence, it can be concluded that the observed sporochartines are likely formed spontaneous during fermentation and that this effect is enhanced through the extraction procedure.

S1.4 Biochemistry

S1.4.1 Cloning, expression and *in vitro* assay of SpoG

For expression of SpoG in *E. coli* BL21 (DE3), the expression plasmid pET-28a (+) was digested with *Bam*HI and *Xho*I (New England BioLabs) restriction enzymes. *H. monticulosa* MUCL 54604 cDNA was used as the DNA template to amplify the SpoG coding sequence with the primer pair P64+P65 (Table S2.1-S2.2). PCR conditions were as described in S1.2.5. T4 ligase (New England BioLabs) was utilized for ligation of the restriction digested vector and PCR fragments. Transformation of competent cells was performed based on a standard *E. coli* transformation protocol (see S1.2.1 for details).

A pre-culture was grown overnight in LB-media containing 50 µg/mL kanamycin at 37 °C 200 rpm. 900 µL of the pre-culture was used to inoculate three flasks containing 50 mL DPY-medium with 50 µg/mL kanamycin. Cells were grown at 37 °C and 200 rpm until an OD600 value between 0.4-0.6 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.25 M stock) was added to a final concentration of 0.3 mM, cells were incubated for another 16 h at 25 °C and 160 rpm. Cells were harvested by centrifugation (8000 × g, 3 min) at 4 °C and resuspended in loading buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 20 mM imidazole, 10% glycerol (v/v)) and lysed by sonication for 10 min (Pulse 10S/10S). Cell debris was removed from the total lysate by centrifugation (10.000 × g, 20 min, 4 °C).

SpoG containing a his6-tag (44.7 kDa) was purified by FPLC (Fast protein liquid

chromatography). The loaded Ni–NTA column with bound protein was eluted with elution buffer (loading buffer + 500 mM imidazole). The buffer was exchanged to storage buffer (50 mM Tris-HCL pH 7.5, 20% glycerol (v/v)) by ultrafiltration with a molecular weight cut-off of 30 KDa. Purity of the protein was assessed by SDS-PAGE (Figure S1.20).

In vitro assays were conducted by incubating SpoG (50 µM) with intermediate **20** (2.5 mM), tris buffer (50 mM, pH 7.5), ascorbate (4 mM), α-ketoglutarate (4 mM), and $FeSO_4$ (0.2 mM) at 30 °C for up to 16 h, the total volume of reaction mixture is 50 µL. After that, 100 µL of chloroform was added into the reaction mixture, vortex for 1 min, and centrifuge at 15,000 x g for 3 min, then the top layer of supernatant (aqueous phase) was directly subjected for analytical LCMS. The negative control was conducted by using deactivated SpoG (boiled at 95 °C, 20 min) under the same condition as above (Figure 7A-7C). Results showed a dihydroxylation of substrate **20**.

Cofactor dependence of SpoG were also studied by excluding ascorbate, α-ketoglutarate, and $FeSO_4$ individually. The reaction conditions and extraction method are the same as described above. Results (Figure S1.21) showed that α-ketoglutarate is essential for turnover *in vitro*, but iron and ascorbate can be omitted.

Additionally, the alternative substrates trans-2-hexenoic acid and 2-methylhexanoic acid were tested with SpoG. Reaction composition, conditions and extraction are as previously described. Results showed that SpoG does not accept these alternative substrates (Figure S1.22).

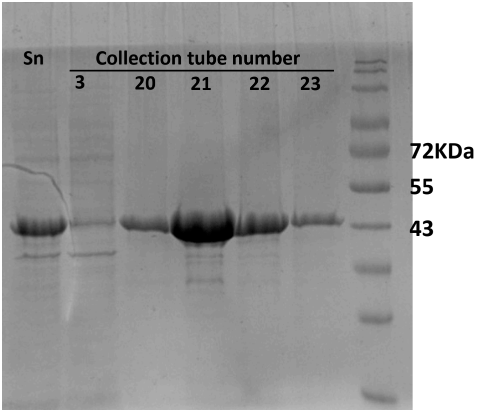


Figure S1.20 SDS-PAGE of SpoG after purification. 12% SDS gel run at 180V for 1h. Sn, centrifuge supernatant of the lysed *E. coli* cells.

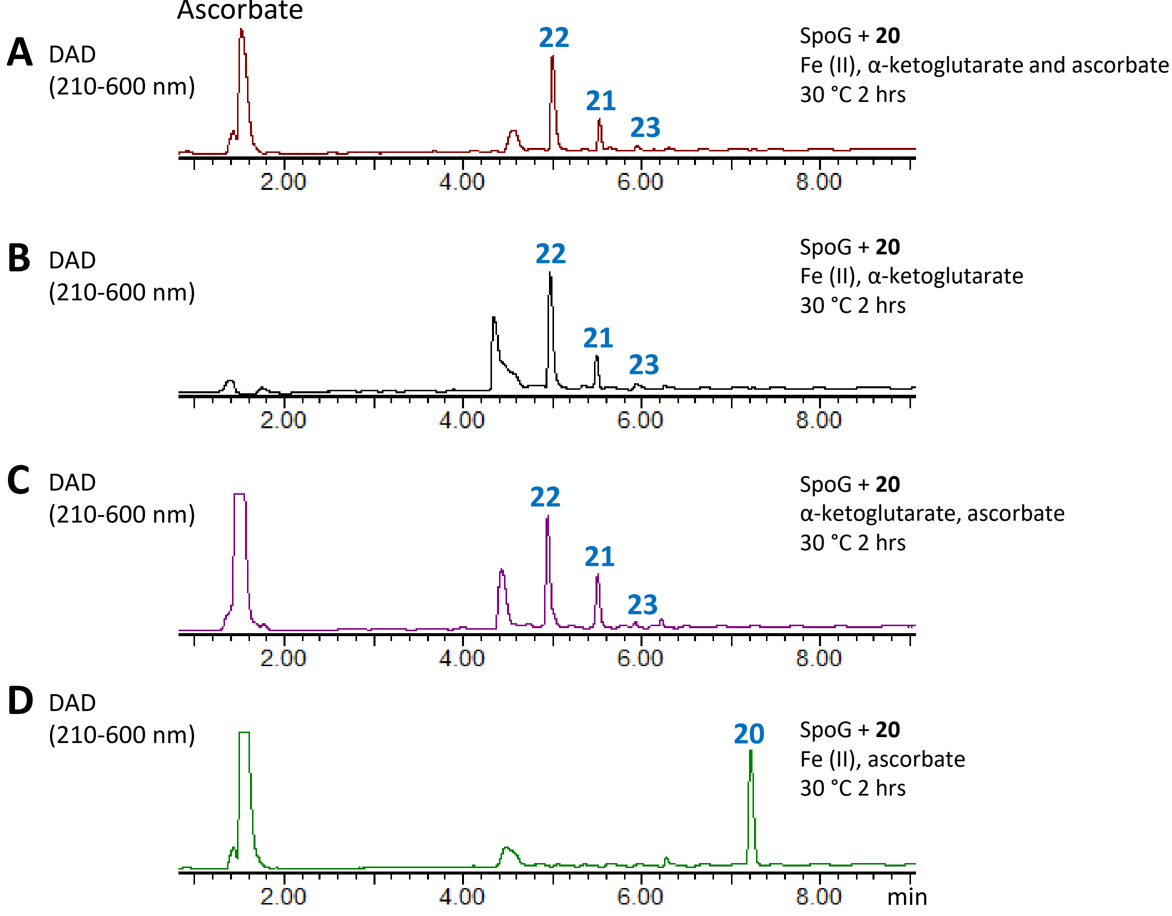


Figure S1.21 The cofactor dependence of SpoG (50 μm) incubated with **20** (2.5 mM), tris buffer (50 mM, pH 7.5), ascorbate (4 mM), α-ketoglutarate (4 mM), and $FeSO_4$ (0.2 mM) at 30 °C 2 hrs. DAD chromatograms of extracts from assays: **A**, including Fe (II), α-ketoglutarate and ascorbate; **B**, excluding ascorbate; **C**, excluding Fe (II); **D**, excluding α-ketoglutarate.

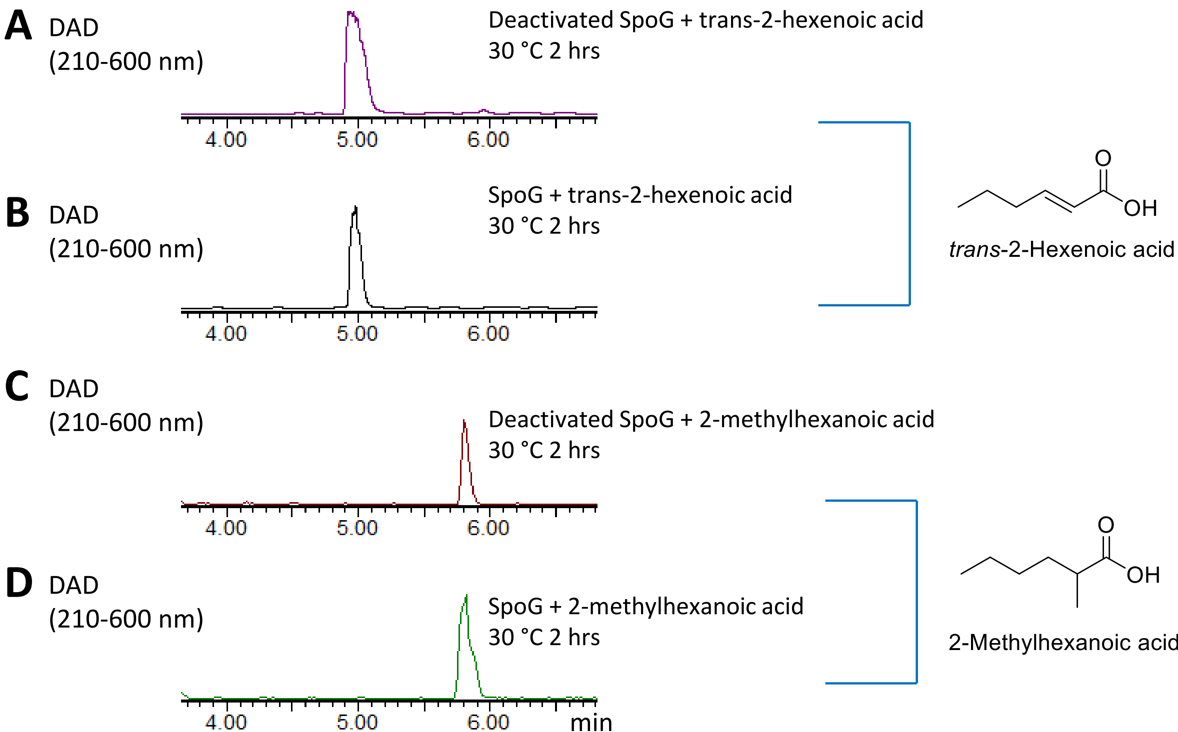


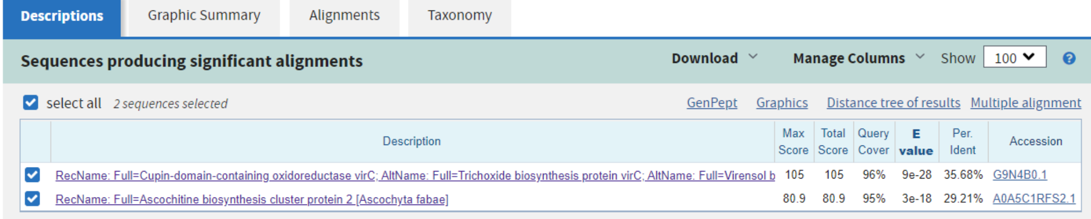
Figure S1.22 A, DAD chromatograms of extracts from alternative substrate assays with SpoG using trans-2-hexenoic acid (2.5 mM, **B**) and 2-methylhexanoic acid (2.5 mM, **D**). A negative control with heat-deactivated SpoG was conducted in parallel (**A**, **C**).

S1.4.2 Cloning, expression and *in vitro* assay of SpoI

SpoI function was predicted by using database BLASTp[17] and PHYRE-2[18], the hit results were shown in Figure S1.23 – S1.24. For expression of SpoI in *E. coli* BL21 (DE3), the expression plasmid pET-28a (+) was digested with *Bam*HI and *Eco*RI (New England BioLabs) restriction enzymes. *H. monticulosa* MUCL 54604 cDNA was used as the DNA template to amplify the SpoI coding sequence with the primer pair P66+P77 (Table S2.1-S2.2). PCR conditions were as described in S1.2.5. T4 ligase (New England BioLabs) was utilized for ligation of the restriction digested vector and PCR fragments. Transformation of competent cells was performed based on a standard *E. coli* transformation protocol (see S1.2.1 for details).

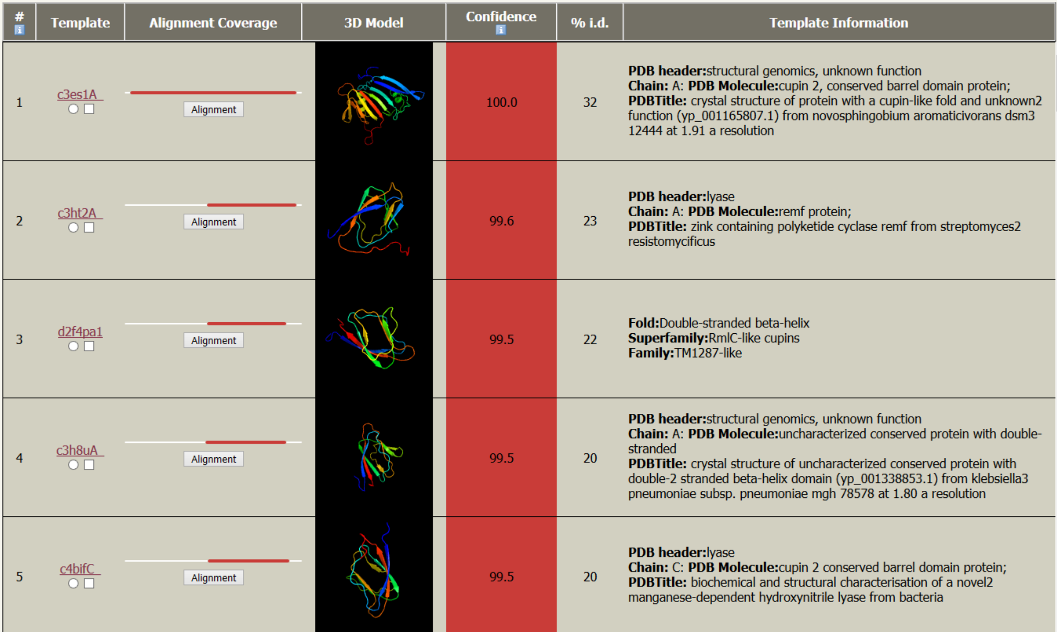
Expression and purification of SpoI was achieved as described in S1.4.1, except the molecular weight cut-off used is 10 KDa (SpoI size 24.1 kDa). Purity of the protein was assessed by SDS-PAGE (Figure S1.25).

In vitro assays were conducted by incubating SpoI (50 µM) either with sporothriolide **1** (2.5 mM) and **7** (2.5 mM) or solely **1** (2.5 mM) in PBS buffer (pH 7.5, Table S2.3) at 30 °C for up to 16 h, the total volume of reaction mixture is 50 µL. After that, 50 µL of acetonitrile was added into the reaction mixture, vortex for 1 min, and centrifuge at 15,000 x g for 3 min, then the supernatant was directly subjected for analytical LCMS. The negative control was conducted by using deactivated SpoI (boiled at 95 °C, 20 min) under the same condition as above (Fig. S1.26, Fig. 9A-9C).



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
RecName: Full=Cupin-domain-containing oxidoreductase virC; AltName: Full=Trichoxide biosynthesis protein virC; AltName: Full=Virensol b	105	105	96%	9e-28	35.68%	G9N4B0.1
RecName: Full=Ascochitine biosynthesis cluster protein 2 [Ascochyta fabae]	80.9	80.9	95%	3e-18	29.21%	A0A5C1RFS2.1

Figure S1.23 NCBI blast[17] results of SpoI.



#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c3es1A	Alignment		100.0	32	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**cupin 2, conserved barrel domain protein; **PDBTitle:** crystal structure of protein with a cupin-like fold and unknown2 function (yp_001165807.1) from novosphingobium aromaticivorans dsm3 12444 at 1.91 a resolution
2	c3ht2A	Alignment		99.6	23	**PDB header:**lyase **Chain:** A: **PDB Molecule:**remf protein; **PDBTitle:** zink containing polyketide cyclase remf from streptomyces2 resistomycificus
3	d2f4pa1	Alignment		99.5	22	**Fold:**Double-stranded beta-helix **Superfamily:**RmlC-like cupins **Family:**TM1287-like
4	c3h8uA	Alignment		99.5	20	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**uncharacterized conserved protein with double-stranded **PDBTitle:** crystal structure of uncharacterized conserved protein with double-2 stranded beta-helix domain (yp_001338853.1) from klebsiella3 pneumoniae subsp. pneumoniae mgh 78578 at 1.80 a resolution
5	c4bifC	Alignment		99.5	20	**PDB header:**lyase **Chain:** C: **PDB Molecule:**cupin 2 conserved barrel domain protein; **PDBTitle:** biochemical and structural characterisation of a novel2 manganese-dependent hydroxynitrile lyase from bacteria

Figure S1.24 Phyre2[18] search results of SpoI.

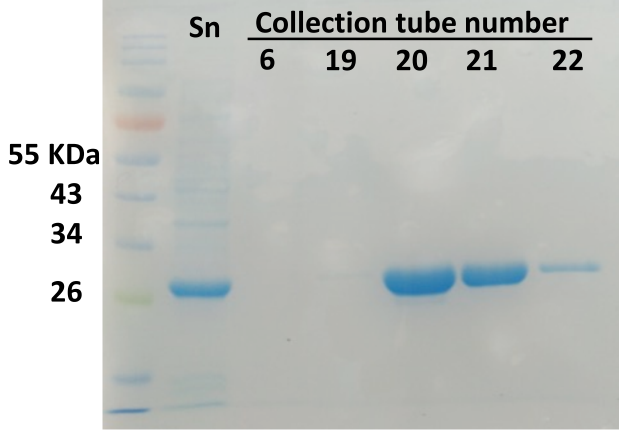


Figure S1.25 SDS-PAGE of SpoI after FPLC purification. 12% SDS gel run at 180 V for 50 min. Sn, centrifuge supernatant of the lysed *E. coli* cells.

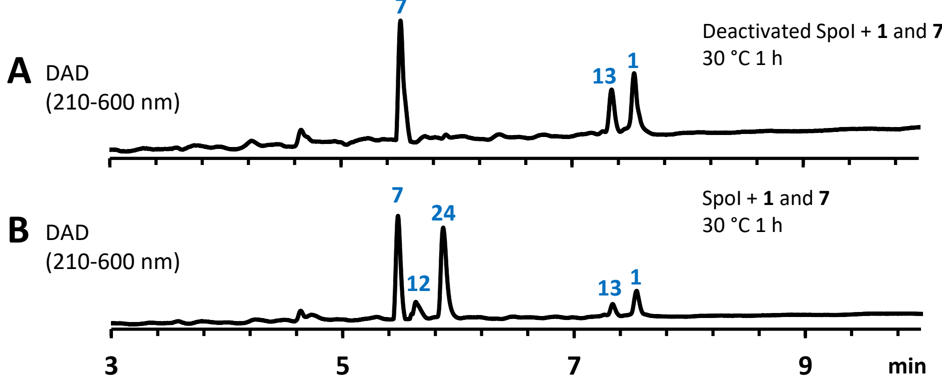


Figure S1.26 *In vitro* assay of SpoI using trienylfuranol A **7** and sporothriolide **1**: **A**, DAD chromatogram of deactivated SpoI (50 μM) incubated with **1** (2.5 mM) and **7** (2.5 mM) in PBS buffer (pH 7.5) at 30 °C for 1 h; **B**, DAD chromatogram of SpoI (50 μM) incubated with **1** (2.5 mM) and **7** (2.5 mM) in PBS buffer (pH 7.5) at 30 °C for 1 h.

S2 General Tables

Primer no.	Sequence (5’ to 3’)
P1	GGAACTGGGTCAGCAGCTG
P2	CATACGCCGCTCCGATCTAC
P3	CTTGGTCATTTAGAGGAAGTAA
P4	TCCTCCGCTTATTGATATGC
P5	TGTAAAACGACGGCCAGT
P6	CAGGAAACAGCTATGACC
P7	GCCAACTTTGTACAAAAAAGCAGGCTCCGCTTAAATACGGGCCGTGGGGT
P8	TCTCCACTCGACCTGCAGGCATGCAAGCTTCAACAGATGACGCACCTCGG
P9	ACGTATTTCAGTGTCGAAAGATCCACTAGAAAGGAACGTCTATACGGATA
P10	TGCCAACTTTGTACAAGAAAGCTGGGTCGGTTATTCCTTTCGGTTCAGCT
P11	AGACGTGCGTCCGAGGTGCGTCATCTGTTGAAGCTTGCATGCCTGCAGGT
P12	GCGGTGTCCATATCCGTATAGACGTTCCTTTCTAGTGGATCTTTCGACAC
P13	TCAGGACATTGTTGGAGCCG
P14	AGGAATCGGTCAATACACTA
P15	ACCCCATTGCTAGACGTTCC
P16	ATTCCGAGAGAGCCTCTCCC
P17	GCCAACTTTGTACAAAAAAGCAGGCTCCGCATGGAGAGCTCAGGAGGAAG
P18	CTAATGCTCTTAGGGCACTGTCGTCGAAGTGTTTTCCCACTCTACCCGGA
P19	TGGCGGCAAATCCGGGTAGAGTGGGAAAACACTTCGACGACAGTGCCCTA
P20	TGCCAACTTTGTACAAGAAAGCTGGGTCGGTTAACATACAAGATTCTCTA
P21	TTTCTTTCAACACAAGATCCCAAAGTCAAAATGACTGCCACCAACAGCAA
P22	CGATGAGCTCATTTTGGAGCGTGGATTTACCGCCGGAGAGATTTTGGATC
P23	TAAGCGAGTCGATCCAAAATCTCTCCGGCGGTAAATCCACGCTCCAAAAT
P24	ACGACAATGTCCATATCATCAATCATGACCTTAAATACGGGCCGTGGGGT
P25	GTCGACTGACCAATTCCGCAGCTCGTCAAAATGCCTTCTGCTACAGTCCC
P26	GGTTGGCTGGTAGACGTCATATAATCATACCTATTCCTTTCGGTTCAGCT
P27	GGTTGGCTGGTAGACGTCATATAATCATACTTAAATACGGGCCGTGGGGT
P28	TTCATTCTATGCGTTATGAACATGTTCCCTTTAAATACGGGCCGTGGGGT
P29	AACAGCTACCCCGCTTGAGCAGACATCACCATGCATGTCCCACAGCCAGA
P30	CTGCGGTCACCAAAATAGCTCCGAGGTTATCGGATGGATGGCCCCATTCT
P31	TCCCTGGTGCAGAATGGGGCCATCCATCCGATAACCTCGGAGCTATTTTG
P32	ACGACAATGTCCATATCATCAATCATGACCTTATTTTTGAAATAGGTCTA
P33	AACAGCTACCCCGCTTGAGCAGACATCACCATGCGTTCGCTATCGCCCAT
P34	ATCCAGAGCTCATTCGTGTCAGGGACATAAACAGGTGCCTCGG3GTGCGGC
P35	CCTCGATAGCGCCGCACCCGAGGCACCTGTTTATGTCCCTGACACGAATG
P36	CGGTGTTGACGCCCATGTTGCGGCTGTACAAGGGATCTGTGAACCATACC
P37	CAAGTGGACGGGTATGGTTCACAGATCCCTTGTACAGCCGCAACATGGGC
P38	ACGACAATGTCCATATCATCAATCATGACCTCATTCTGTAATAGATTGGG

P39	GTCGACTGACCAATTCCGCAGCTCGTCAAAATGTGTTCTCGACGACCGAT
P40	GGTTGGCTGGTAGACGTCATATAATCATACCTACTCCACAATAGGACCCC
P41	TTTCTTTCAACACAAGATCCCAAAGTCAAAATGAGCGCTACCAACGGAAC
P42	TTCATTCTATGCGTTATGAACATGTTCCCTCTAAGCCTGCGCGCTAACAG
P43	GCCAACTTTGTACAAAAAAGCAGGCTCCGCATGGGGAAACACGGCGCCAA
P44	TGCCAACTTTGTACAAGAAAGCTGGGTCGGCTATCCCAACACATTCCTTG
P45	GGTTGGCTGGTAGACGTCATATAATCATACTCATTCTGTAATAGATTGGG
P46	ACGACAATGTCCATATCATCAATCATGACCCTAAGCCTGCGCGCTAACAG
P47	GGTTGGCTGGTAGACGTCATATAATCATACCTAAGCCTGCGCGCTAACAG
P48	TGCTTGGAGGATAGCAACCG
P49	GGGGATGACAGCAGTAACGA
P50	ATTCACCACTATTATTCCCACCCTATAATA
P51	GAGACGAAACAGACTTTTTCATCGCTAAAA
P52	GAACTGTCCAGCGTCTCACC
P53	TCGTATCTTCTGTATCGGCG
P54	CTTTTCTTTTCTCTTTCTTTTCCCATCTTC
P55	TGACCTCCTAAAACCCCAGTG
P56	ACTTCATCGCAGCTTGACTA
P57	TCTTTCATTATCTTGCGAAC
P58	CTTCTTAAATATCGTTGTAACTGTTCCTGA
P59	CGAAGTATATTGGGAGACTATAGCTACTAG
P60	CTTCCGTCCTCCAAGTTAGT
P61	ACCATCTTTCGATAATGTGT
P62	TAATACGACTCACTATAGGG
P63	CTAGTTATTGCTCAGCGGT
P64	CGCGGATCCATGAGCGCTACCAACGGAACC
P65	CCGCTCGAGCTAAGCCTGCGCGCTAACAGC
P66	CGCGGATCCATGGCCGACCAACAGAAGGAA
P67	CCGGAATTCTTAGTGAGAGACAACACCCAA

Table S2.1 All oligonucleotides used in this work.

Primer set no.	Template	Target vector	Purpose	PCR condition*
P1+P2	cDNA of *H. monticulosa*	/	gDNA contamination check	OneTaq
P3+P4	gDNA	/	ITS sequencing	Q5
P7+P8	gDNA of *H. spongiphila*	DTKO1_CS+FAS	Arm2, amplification of flanking region 2 for KO vector construction	OneTaq
P9+P10	gDNA of *H. spongiphila*	DTKO1_CS+FAS	Arm1, amplification of flanking region 1 for KO vector construction	OneTaq
P11+P12	pTH-GS-*egfp*	DTKO1_CS+FAS	*hph*	OneTaq
P7+P14	DTKO1_CS+FAS	/	K.O Fragment 1 for bipartite KO method	OneTaq
P13+P10	DTKO1_CS+FAS	/	K.O Fragment 2 for bipartite KO method	OneTaq
P15+P14	gDNA of *H. spongiphila* transformants	/	5' side insertion verification	OneTaq
P13+P16	gDNA of *H. spongiphila* transformants	/	3' side insertion verification	OneTaq
P17+P18	gDNA of *H. monticulosa*	DTHE1	FASβ (*spofasB*), fragment 1	Q5, 65°C
P19+P20	cDNA of *H. monticulosa*	DTHE1	FASβ (*spofasB*), fragment 2	Q5, 55°C
P19+P6	*E.coli* transformants with DTHE1	DTHE1	Colony PCR	OneTaq
P43+P44	cDNA of *H. monticulosa*	DTHE14	Decarboxylase (*spoK*)	Q5, 65°C
P5+P6	*E.coli* transformants with DTHE14	DTHE14	Colony PCR	OneTaq
P23+P24	gDNA of *H. monticulosa*	DTHE2	FASα (*spofasA*), fragment 1	Q5, 65°C
P21+P22	cDNA of *H. monticulosa*	DTHE2	FASα (*spofasA*), fragment 2	Q5, 65°C
P25+P26	cDNA of *H. monticulosa*	DTHE2	Citrate synthase (*spoE*)	Q5
P50+P22	*E.coli* transformants with DTHE2	DTHE2	Colony PCR	OneTaq
P60+P61	*E.coli* transformants with DTHE2	DTHE2	Colony PCR	OneTaq
P21+P22	cDNA of *H. monticulosa*	DTHE3	FASα (*spofasA*) fragment 1	Q5, 65°C
P23+P27	gDNA of *H. monticulosa*	DTHE3	FASα (*spofasA*) fragment 2	Q5, 65°C
P50+P22	*E.coli* transformants with DTHE3	DTHE3	Colony PCR	OneTaq
P21+P28	DTHE2	DTHE10	FASα (*spofasA*)	Q5, 65°C
P25+P26	cDNA of *H. monticulosa*	DTHE10	Citrate synthase (*spoE*)	Q5
P29+P30	gDNA of *H. monticulosa*	DTHE10	Dehydratase (*spoL*) fragment 1	Q5

P31+P32	gDNA of *H. monticulosa*	DTHE10	Dehydratase (*spoL*) fragment 2	Q5
P19+P49	*E.coli* transformants with DTHE10	DTHE10	Colony PCR	OneTaq
P52+P22	*E.coli* transformants with DTHE10	DTHE10	Colony PCR	OneTaq
P56+P57	*E.coli* transformants with DTHE10	DTHE10	Colony PCR	OneTaq
P60+P61	*E.coli* transformants with DTHE10	DTHE10	Colony PCR	OneTaq
P33+P34	gDNA of *H. monticulosa*	DTHE15	Lactonase (*spoH*) fragment 1	Q5, 65°C
P35+P36	gDNA of *H. monticulosa*	DTHE15	Lactonase (*spoH*) fragment 2	Q5
P37+P38	gDNA of *H. monticulosa*	DTHE15	Lactonase (*spoH*) fragment 3	Q5
P39+P40	cDNA of *H. monticulosa*	DTHE15	Lactonase (*spoJ*)	Q5
P41+P42	cDNA of *H. monticulosa*	DTHE15	Dioxygenase (*spoG*)	Q5
P52+P53	*E.coli* transformants with DTHE15	DTHE15	Colony PCR	OneTaq
P56+P57	*E.coli* transformants with DTHE15	DTHE15	Colony PCR	OneTaq
P60+P61	*E.coli* transformants with DTHE15	DTHE15	Colony PCR	OneTaq
P41+P42	DTHE15	DTHE25	Dioxygenase (*spoG*)	Q5
P33+P45	DTHE15	DTHE25	Lactonase (*spoH*)	Q5
P50+P53	*E.coli* transformants with DTHE25	DTHE25	Colony PCR	OneTaq
P56+P61	*E.coli* transformants with DTHE25	DTHE25	Colony PCR	OneTaq
P41+P46	DTHE15	DTHE26	Dioxygenase (*spoG*)	Q5
P39+P40	DTHE15	DTHE26	Lactonase (*spoJ*)	Q5
P50+P57	*E.coli* transformants with DTHE26	DTHE26	Colony PCR	OneTaq
P60+P61	*E.coli* transformants with DTHE26	DTHE26	Colony PCR	OneTaq
P41+P47	DTHE15	DTHE27	Dioxygenase (*spoG*)	Q5
P50+P61	*E.coli* transformants with DTHE27	DTHE27	Colony PCR	OneTaq
P64+P65	DTHE15	DSPE1	Dioxygenase (*spoG*)	Q5
P62+P63	*E.coli* transformants with DSPE1	DSPE1	Colony PCR	OneTaq
P66+P67	cDNA of *H. monticulosa*	DSPE7	Hydrolase (*spoI*)	Q5
P62+P63	*E.coli* transformants with DSPE7	DSPE7	Colony PCR	OneTaq
DTHE4 (LR clone of DTHE1 + DTHE2)				
DTHE5 (LR clone of DTHE1 + DTHE3)				
DTHE1 + Arg (LR clone of DTHE1 + pTYGSarg)				
DTHE16 (LR clone of DTHE14 + DTHE15)				
DTHE17 (LR clone of DTHE14 + pTYGSade)				
DTHE18A (LR clone of DTHE14 + DTHE25)				
DTHE19A (LR clone of DTHE14 + DTHE26)				
DTHE21 (LR clone of DTHE14 + DTHE27)				

Table S2.2 Primer sets used in this study. * Deviating PCR annealing temperatures (standard is 60 °C) are stated under PCR condition.

Buffer	Conc.	Components
1x TAE buffer	40 mM	Tris–HCl
	20 mM	Acetic acid
	1 mM	EDTA
HYP protoplasting solution	0.7 M	NaCl
	10 mg/mL	lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich)
HYP transformation solution I	1.2 M	Sorbitol
	50 mM	$CaCl_2$
	10 mM	Tris–HCl, pH 7.5
HYP transformation solution II	60%	PEG 6000 (w/v)
	50 mM	$CaCl_2$
	10 mM	Tris–HCl, pH 7.5
A. oryzae NSAR1 protoplasting solution	0.8 M	NaCl
	10 mg/mL	lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich)
A. oryzae NSAR1 transformation solution I	10 mM	$CaCl_2$
	0.8 M	NaCl
	50 mM	Tris–HCl, pH 7.5
A. oryzae NSAR1 transformation solution II	60%	PEG 3350 (w/v)
	10 mM	$CaCl_2$
	0.8 M	NaCl
	50 mM	Tris–HCl, pH 7.5
Protein loading buffer	50 mM	Tris–HCl, pH 8.0
	150 mM	NaCl
	20 mM	Imidazole
	10 %	Glycerol (v/v)
Protein elution buffer	50 mM	Tris–HCl, pH 8.0
	150 mM	NaCl
	500 mM	Imidazole
	10 %	Glycerol (v/v)
Protein storage buffer	50 mM	Tris–HCl, pH 7.5
	20 %	Glycerol (v/v)

Tris buffer (SpoG *in vitro* assay use)	50 mM	Tris–HCl, pH 7.5
PBS buffer (SpoI *in vitro* assay use)	0.80 %	NaCl (w/v)
	0.02%	KCl (w/v)
	0.27%	$Na_2HPO_4 \cdot 7H_2O$ (w/v)
	0.027%	KH_2PO_4 (w/v)

Table S2.3 Components of buffers and solutions used in this work.

Media	Conc. [%(w/v)]	Ingredient
LB	0.50	Yeast extract
	1.00	Tryptone
	0.50	NaCl
YPAD	1.00	Yeast extract
	2.00	Tryptone
	2.00	D(+)-glucose monohydrate
	0.03	Adenine
GN	2.00	D(+)-glucose Monohydrate
	3.00	Nutrient broth Nr.2 from Oxoid (Fisher Scientific)
DPY	2.00	Dextrin from potato starch
	1.00	Polypeptone
	0.50	Yeast extract
	0.50	KH_2PO_4
	0.05	$MgSO_4$
PDB	2.40	Potato dextrose broth
YMG	0.40	D(+)-glucose Monohydrate
	0.40	Yeast extract
	1.00	Malt extract

Table S2.4 Liquid medium used in this work.

Agar	Conc.[%(w/v)]	Ingredient
LB agar	0.50	Yeast extract
	1.00	Tryptone
	0.50	NaCl
	1.50	Agar
YPAD agar	1.00	Yeast extract
	2.00	Tryptone
	2.00	D(+)-glucose monohydrate
	0.03	Adenine
	1.50	Agar
SM–URA agar	0.17	Yeast nitrogen base
	0.50	Ammonium sulfate
	2.00	D(+)-glucose monohydrate
	0.077	Complete supplement mixture minus uracil
	1.50	Agar
DPY agar	2.00	Dextrin from potato starch
	1.00	Polypeptone
	0.50	Yeast extract
	0.50	KH_2PO_4
	0.05	$MgSO_4$
	2.50	Agar
PD agar	2.40	Potato dextrose broth
	1.50	Agar
DPY/S agar	2.00	Dextrin from potato starch
	1.00	Polypeptone
	0.50	Yeast extract
	0.50	KH_2PO_4
	0.05	$MgSO_4$
	21.86	Sorbitol (1.2 M)
	1.50	Agar
DPY/S soft agar	2.00	Dextrin from potato starch

	1.00	Polypeptone
	0.50	Yeast extract
	0.50	KH_2PO_4
	0.05	$MgSO_4$
	21.86	Sorbitol (1.2 M)
	0.80	Agar
CZD/S agar	3.50	Czapek Dox broth
Used in transform pTYGSarg vector	18.22	Sorbitol (1.0 M)
	0.10	Ammonium sulphate
	0.05	Adenine
	0.15	L – methionine
	1.50	Agar
CZD/S soft agar	3.50	Czapek Dox broth
Used in transform pTYGSarg vector	18.22	Sorbitol (1.0 M)
	0.10	Ammonium sulphate
	0.05	Adenine
	0.15	L – methionine
	0.80	Agar
CZD/S1 agar (CZD/S agar w/o adenine)	3.50	Czapek Dox broth
Used in co-transform pTYGSarg and pTYGSade vectors	18.22	Sorbitol (1.0 M)
	0.10	Ammonium sulphate
	0.15	L – methionine
	1.50	Agar
CZD/S1 soft agar (CZD/S soft agar w/o adenine)	3.50	Czapek Dox broth
Used in co-transform pTYGSarg and pTYGSade vectors	18.22	Sorbitol (1.0 M)
	0.10	Ammonium sulphate
	0.15	L – methionine
	0.80	Agar

Table S2.5 Agar used in this work.

Strain	Genotype	Phylum	Origin
Hypomontagnella monticulosa MUCL 54604	wildtype	*Ascomycetes*	Ref.[20,26]
Hypomontagnella spongiphila CLL 205	wildtype	*Ascomycetes*	Ref. [20,26]
Hypomontagnella sub*monticulosa* DAOMC 242471	wildtype	*Ascomycetes*	Ref. [20,26]
Saccharomyces cerevisiae CEN.PK	MATa/α ura3-52/ura3-52 trp1-289/trp1-289 leu2-3_112/leu2-3_112 his3 Δ1/his3 Δ1 MAL2-8C /MAL2-8C SUC2/SUC2	*Ascomycetes*	Lazarus group Bristol
Aspergillus oryzae NSAR1	*Δ*argB, sC^-, $adeA^-$, $niaD^-$	*Ascomycetes*	Lazarus group Bristol
Escherichia coli OneShot TOP10	F-*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	*Proteobacteria*	Thermo Fisher Scientific
Escherichia coli OneShot *ccd*B survival $2T1^R$	F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G *fhu*A::*IS2*	*Proteobacteria*	Thermo Fisher Scientific
E. coli BL21 (DE3)	F˘ompT *hsd*SB (r˘*B* m˘*B*) *gal dcm* (DE3)	*Proteobacteria*	Thermo Fisher Scientific

Table S2.6 Strains and origin.

Extract	Gradient (Preparation LCMS)	Targeted compound	Retention time* (min) 10-90%, 15 min Ana.	Amount (exp.) / mg/1L media
H. spongiphila	10-60-90%, 15 min Prep.	1	7.70-7.90	14
H. spongiphila	10-60-90%, 15 min Prep.	6a	8.85-8.95	1.2
H. spongiphila	Flash Chromatogram (Petroleum ether/Ethyl acetate: 9/1 – 8/1)	7	5.30-5.40	23
H. spongiphila	10-60-90%, 15 min Prep.	8	7.90-7.95	12
H. spongiphila	10-60-90%, 15 min Prep.	9	7.40-7.50	9
H. spongiphila	10-60-90%, 15 min Prep.	10	6.20-6.40	4
H. spongiphila	10-60-90%, 15 min Prep.	11	6.00-6.20	4
A. H. spongiphila	10-60-90%, 15 min Prep.	12	5.70-5.80	5.5
B. A. oryzae EXP8 (Table 1)	10-30-80%, 15 min Prep.	13	7.60-7.70	8
C. A. oryzae EXP5 (Table 1)	10-30-80%, 15 min Prep.	14	6.40-6.60	3
D. A. oryzae EXP5 (Table 1)	10-30-80%, 15 min Prep.	15	7.00-7.20	7.5
Originated from compound 23	-	16	7.40-7.60	7
E. A. oryzae EXP4 (Table 1)	10%-90%, 15 min Prep.	20	7.65-7.80	4
SpoG *in vitro* assay	-	21	5.40-5.60	/
F. A. oryzae EXP5 (Table 1)	10-30-80%, 15 min Prep.	22	4.95-5.15	6
G. A. oryzae EXP5 (Table 1)	10-30-80%, 15 min Prep.	23	5.90-6.30	7
SpoI *in vitro* assay	10%-90%, 15 min Prep.	24	5.82-5.92	/

Table S2.7 Compounds LCMS purification details. Exp., indicates experimentally purified amounts.

*Retention times slightly over time.

	Time / min	Flow / mL/min	%A (Water)	%B (Acetonitrile)
10-60-90%, 15 min Prep.	Initial	20	90	10
	9	20	40	60
	13	20	40	60
	14	20	10	90
	15	20	90	10
10-30-80%, 15 min Prep.	Initial	20	90	10
	2	20	70	30
	13	20	20	80
	14	20	90	10
	15	20	90	10
10-90%, 15 min Prep.	Initial	20	90	10
	10	20	10	90
	12	20	10	90
	13	20	90	10
	15	20	90	10
10-90%, 15 min Ana.	Initial	1	90	10
	10	1	10	90
	12	1	10	90
	13	1	90	10
	15	1	90	10

Table S2.8 LCMS gradient details.

S3 Compounds Physical Data and Structure Elucidation

Compound 1 (known from literature[27])

1

Sporothriolide

Chemical formula: $C_{13}H_{18}O_4$

Colorless oil; $[\alpha]_D^{26}$ –104 (*c* = 0.5, CH_3OH); UV (λ_{max}): 206 nm. ^{13}C NMR data ($CDCl_3$, 125 MHz): δ_C 172.2 (C-4), 167.6 (C-1), 130.0 (C-3), 127.5 (C-13), 82.9 (C-6), 77.3 (C-5), 46.3 (C-2), 31.7 (C-10), 29.1 (C–9), 29.0 (C-7), 25.5 (C-8), 22.6 (C-11), 14.2 (C-12); ^1H NMR data ($CDCl_3$, 500 MHz): δ_H 6.47 (1H, d, *J* = 2.1Hz, H-13a), 6.16 (1H, d, *J* = 2.1 Hz, H-13b), 5.15 (1H, dd, *J* = 6.8, 4.7 Hz, H-5), 4.65 (1H, ddd, *J* = 7.9, 6.3, 4.7 Hz, H-6), 4.01 (1H, dt, *J* = 6.8, 2.0 Hz, H-2), 1.88 (2H, m, H-7), 1.49 (2H, m, H-8), 1.38 (2H, m, H-9), 1.30 (2H, m, H-10), 1.30 (2H, m, H-11), 0.89 (3H, t, *J* = 7.0 Hz, H-12); NMR data are consistent with those previously reported,[27] see table S3.1; ESI-MS *m/z* 237 $[M–H]^–$, 239 $[M+H]^+$, 477 $[2M+H]^+$; HR-ESI-MS *m/z* 237.1127 $[M–H]^–$ (calcd. for $C_{13}H_{17}O_4$, 237.1127).

pos.	δ_C / ppm	δ_H / ppm (*J* / Hz)	δ_C / ppm literature[27]	δ_H / ppm (*J* / Hz) literature[27]
1	167.6	-	167.5	-
2	46.3	4.01, 1H, dt (6.8, 2.0)	46.2	4.00, 1H, dt (6.7, 2.1)
3	130.0	-	129.9	-
4	172.2	-	172.1	-
5	77.3	5.15, 1H, dd (6.8, 4.7)	77.2	5.14, 1H, dd (6.7, 4.6)
6	82.9	4.65, 1H, ddd (7.9, 6.3, 4.7)	82.8	4.64, 1H, ddd (8.0, 6.3, 4.6)
7	29.0	1.88, 2H, m	28.9	1.86, 2H, m
8	25.5	1.49, 2H, m	25.4	1.50, 2H, m
9	29.1	1.38, 2H, m	29.0	1.37, 2H, m
10	31.7	1.30, 2H, m	31.6	1.30, 2H, m
11	22.6	1.30, 2H, m	22.5	1.31, 2H, m
12	14.2	0.89, 3H, t (7.0)	14.1	0.88, 3H, t (7.0)
13	127.5	6.47, 1H, d (2.1) 6.16, 1H, d (2.1)	127.4	6.46, 1H, d (2.1) 6.15, 1H, d (2.1)

Table S3.1 ^{1}H NMR (500 MHz) data and ^{13}C NMR (125 MHz) data for **1** in $CDCl_3$. Literature[27] data were measured at 700 MHz in $CDCl_3$.

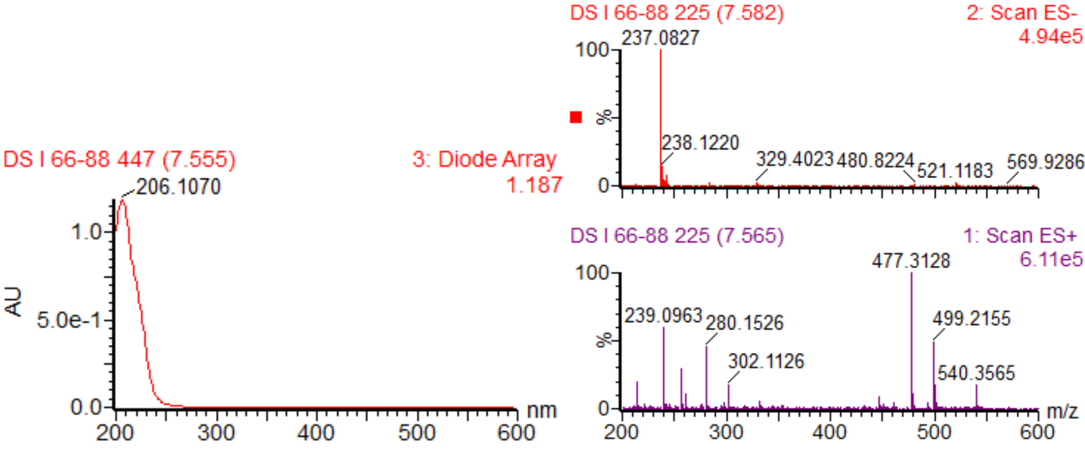
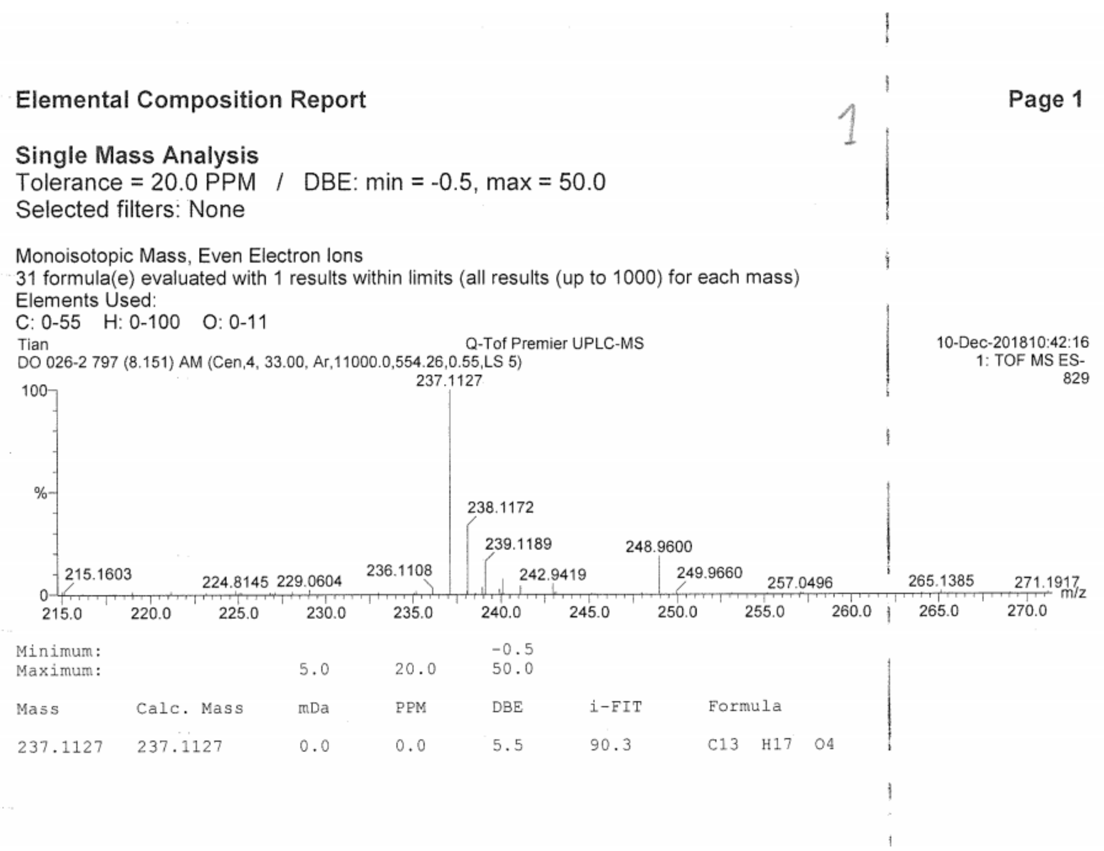


Figure S3.1 UV and mass spectra for compound **1**.



Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
31 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-55 H: 0-100 O: 0-11

Minimum:				-0.5		
Maximum:		5.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
237.1127	237.1127	0.0	0.0	5.5	90.3	C13 H17 O4

Figure S3.2 HRMS data for compound **1**.

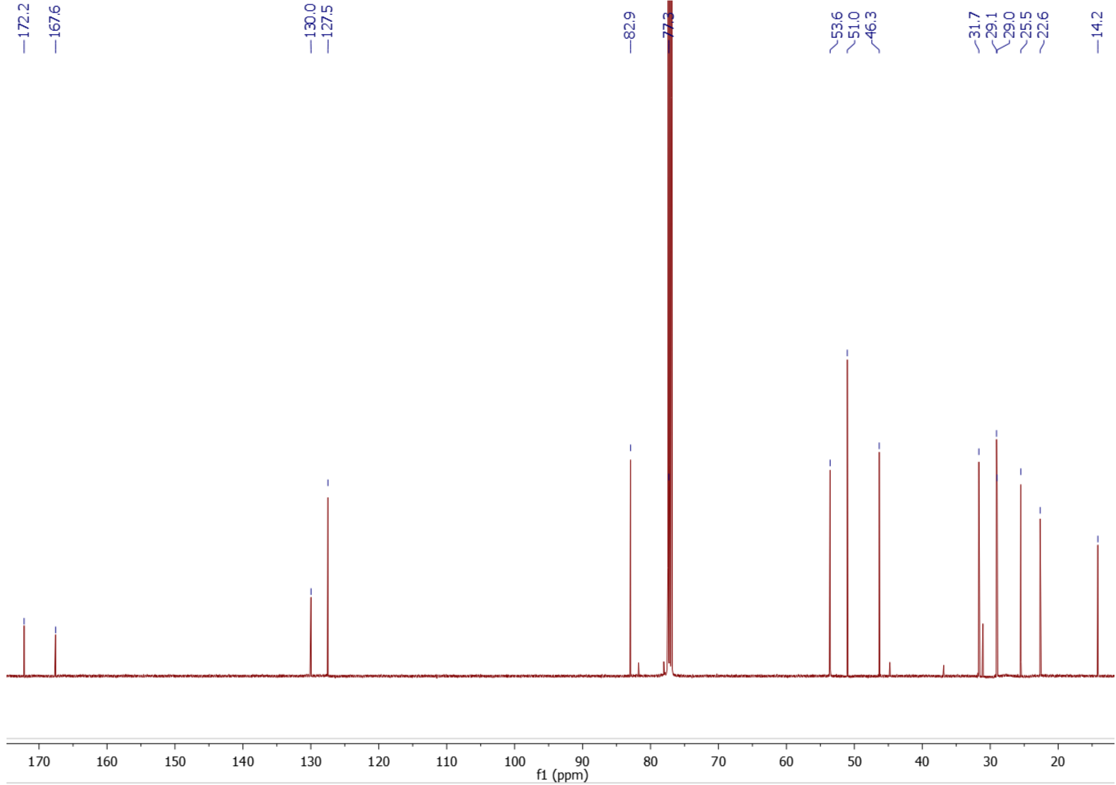


Figure S3.3 ^{1}H NMR of compound **1**.

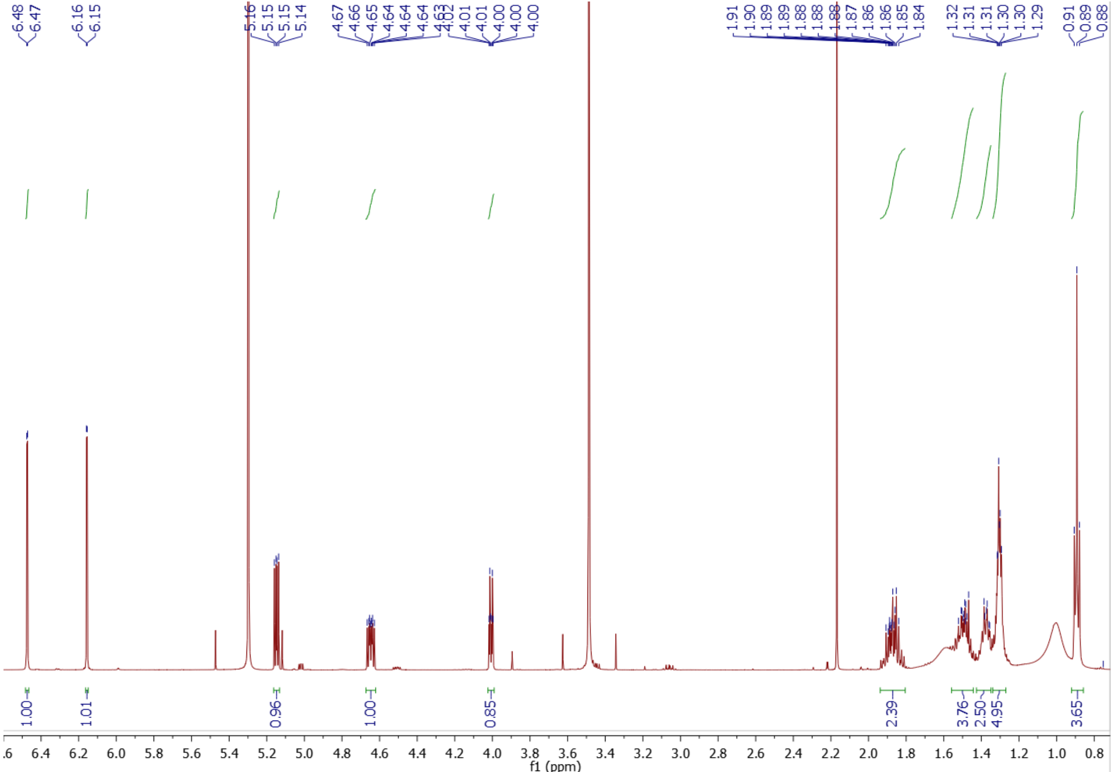


Figure S3.4 ^{13}C NMR of compound **1**.

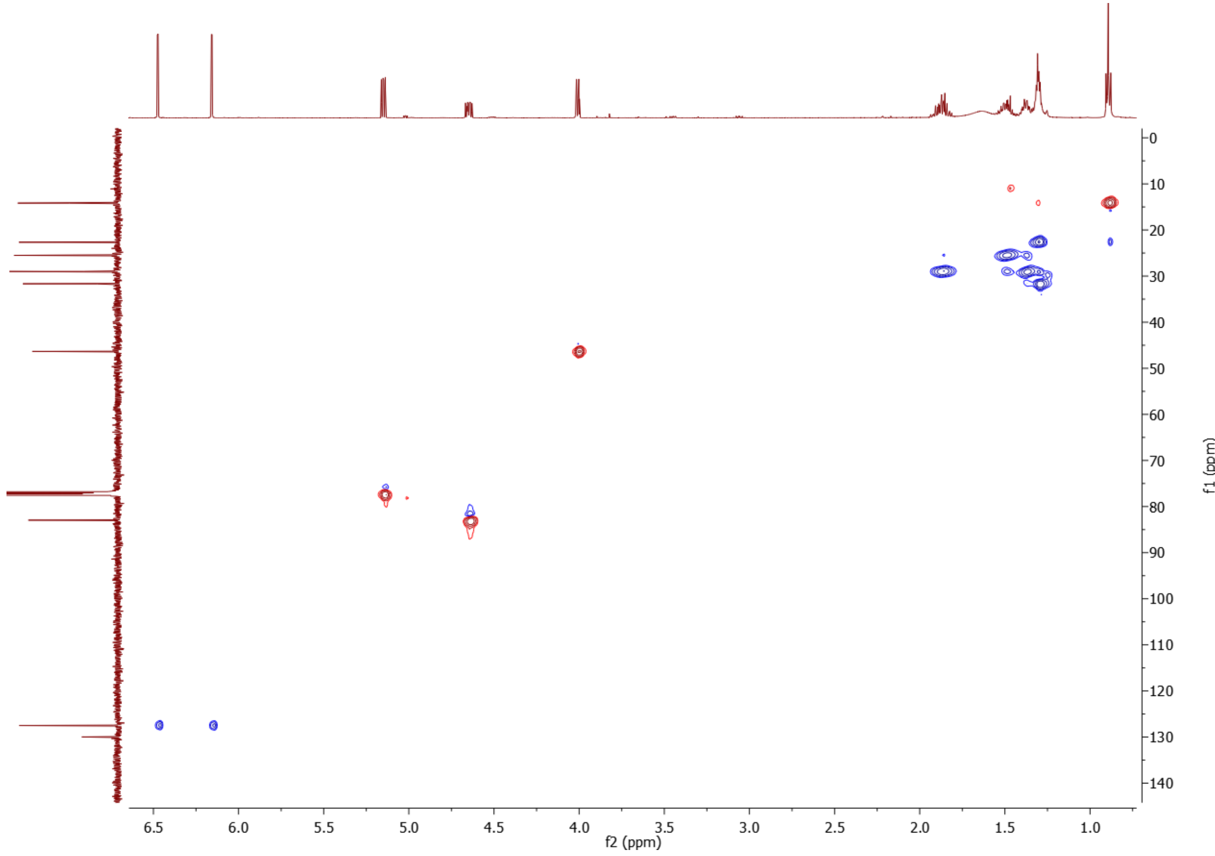


Figure S3.5 HSQC of compound **1**.

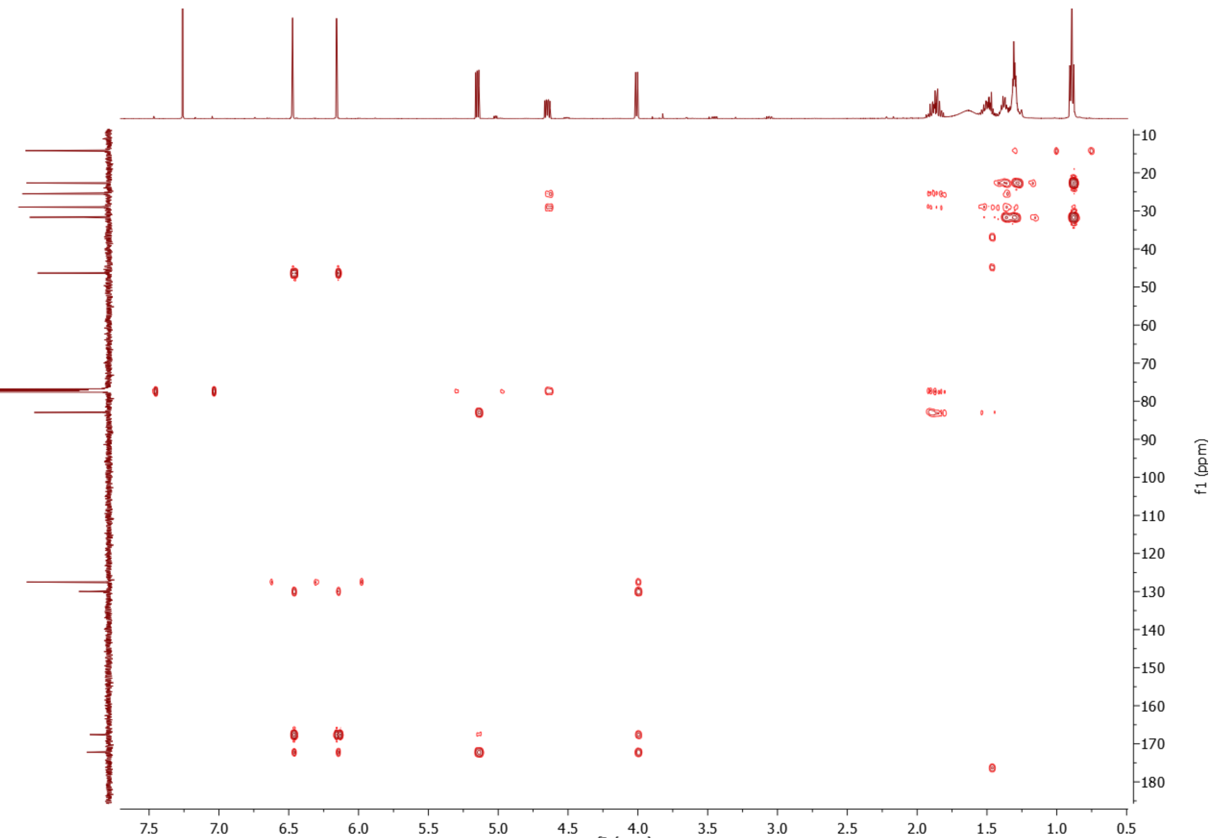


Figure S3.6 HMBC of compound **1**.

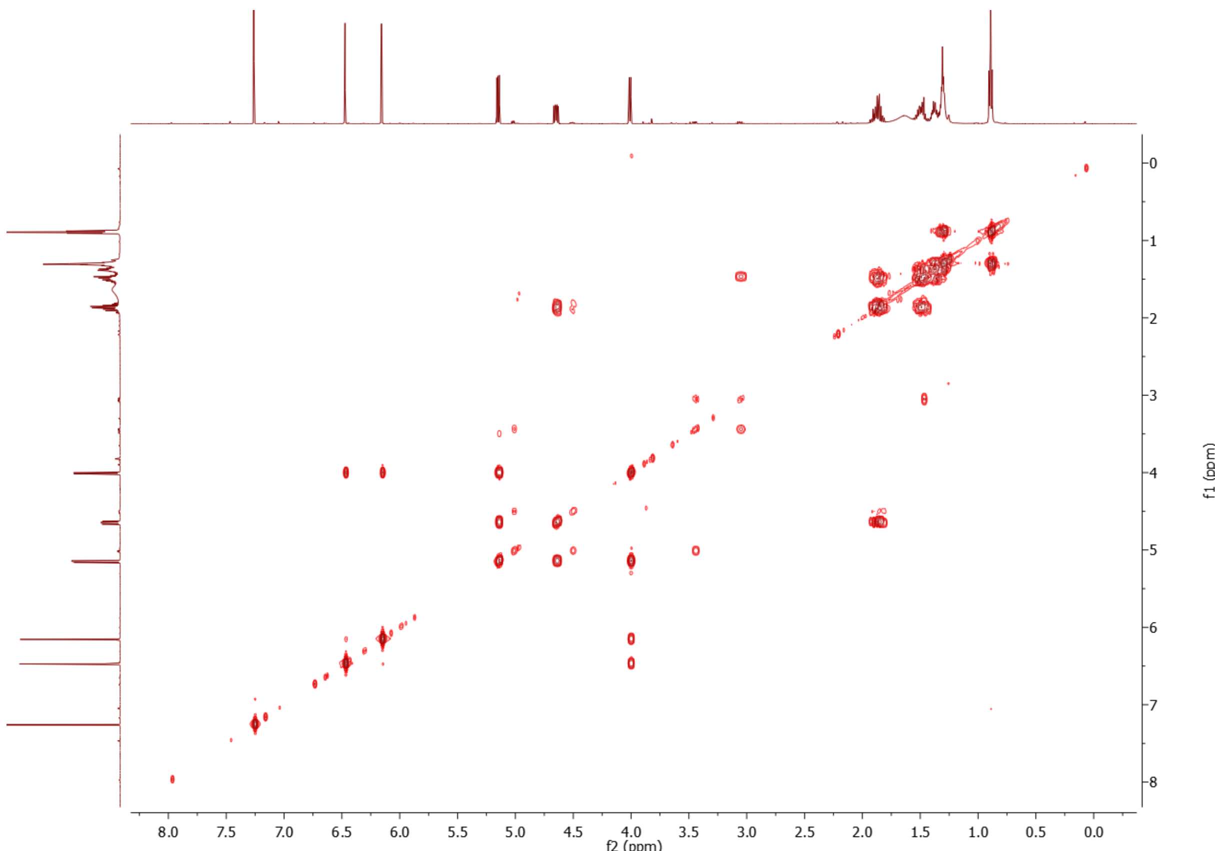


Figure S3.7 ^{1}H-^{1}H COSY of compound **1**.

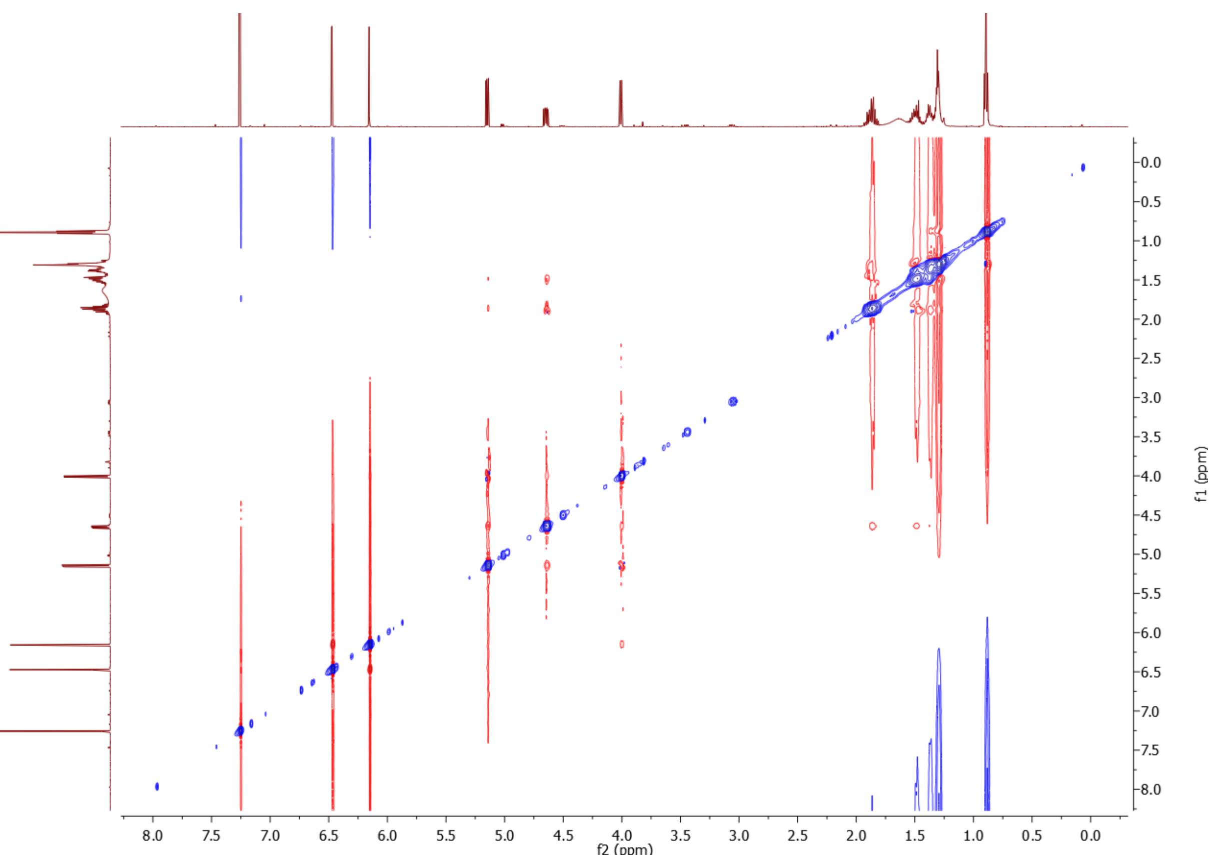


Figure S3.8 NOESY of compound **1**.

Compound 6b (known from literature[28])

6b

Sporochartine B

Chemical formula: $C_{24}H_{34}O_6$

White powder; $[\alpha]_D^{25}$ + 57 (*c* = 0.8, $CHCl_3$); UV (λ_{max}): 211 nm. ^{13}C NMR data ($CDCl_3$, 125 MHz): δ_c 178.7 (C-4), 173.0 (C-1), 130.8 (C-7’), 130.1 (C-6’), 129.9 (C-10’), 124.7 (C-9’), 82.8 (C-1’), 81.1 (C-6), 78.7 (C-5), 74.1 (C-4’), 73.8 (C-2’), 50.8 (C-3), 47.2 (C-2), 46.9 (C-8’), 42.4 (C-3’), 31.7 (C-10), 29.1 (C–9), 28.9 (C-7), 26.9 (C-13), 25.3 (C-8), 22.6 (C-11’), 22.8 (C-11), 22.4 (C-5’), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 500 MHz): δ_H 5.95 (1H, m, H-10’), 5.84 (1H, ddd, *J* = 1.6, 9.0, 15.5 Hz, H-7’), 5.75 (1H, dd, *J* = 4.0, 15.5 Hz, H-6’), 5.54 (1H, m, H-9’), 5.14 (1H, dd, *J* = 6.0, 4.1 Hz, H-5), 4.40 (1H, m, H-6), 4.28 (1H, m, H-2’), 4.19 (1H, m, H-1’), 4.06 (1H, m, H-4’), 3.31 (1H, d, *J* = 5.9 Hz, H-2), 3.23 (1H, m, H-8’), 2.25 (1H, m, H-11’), 2.03 (1H, m, H-13), 1.76 (1H, m, H-7), 1.59 (1H, m, H-3’), 1.45 (2H, m, H-8), 1.34 (2H, m, H-9), 1.33 (3H, d, *J* = 6.2 Hz, H-5’), 1.29 (2H, m, H-11), 1.28 (2H, m, H-10), 0.88 (3H, m, H-12); NMR data are consistent with those previously reported,[28] see table S3.2; ESI-MS *m/z* 463 $[M+HCOOH–H]^-$, 419 $[M+H]^+$, 436 $[M+H_2O]^+$; HR-ESI-MS *m/z* 417.2278 $[M–H]^-$ (calcd. for $C_{24}H_{33}O_6$, 417.2277).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	δ_C / ppm literature[28]	δ_H / ppm (J / Hz) literature[28]
1	173.0	-	173.1	-
2	47.2	3.31, 1H, d (5.9)	47.2	3.30, 1H, d (5.8)
3	50.8	-	51.0	-
4	178.7	-	178.7	-
5	78.7	5.14, 1H, dd (4.1, 6.0)	78.7	5.13, 1H, dd (4.3, 5.9)
6	81.1	4.40, 1H, m	81.7	4.39, 1H, m
7	28.9	1.76, 1H, m	28.9	1.76, 1H, m
8	25.3	1.45, 2H, m	25.3	1.45, 2H, m
9	29.1	1.34, 2H, m	29.1	1.34, 2H, m
10	31.7	1.28, 2H, m	31.7	1.29, 2H, m
11	22.8	1.29, 2H, m	22.9	1.29, 2H, m
12	14.2	0.88, 3H, m	14.3	0.88, 3H, t (6.9)
13	26.9	2.03, 1H, m	26.9	2.04, 1H, m
1’	82.8	4.19, 1H, m	82.8	4.19, 1H, m
2’	73.8	4.28, 1H, m	73.9	4.27, 1H, m
3’	42.4	1.59, 1H, m	42.4	1.59, 1H, m
4’	74.1	4.06, 1H, m	74.2	4.07, 1H, m
5’	22.4	1.33, 3H, d (6.2)	22.5	1.34, 3H, d (6.1)
6’	130.1	5.75, 1H, dd (4.0, 15.5)	130.1	5.76, 1H, dd (3.9, 15.4)
7’	130.8	5.84, 1H, ddd (1.6, 9.0, 15.5)	130.9	5.82, 1H, ddd (1.5, 8.8, 15.4)
8’	46.9	3.23, 1H, m	47.0	3.23, 1H, brm
9’	124.7	5.54, 1H, m	124.8	5.54, 1H, brd (10.9)
10’	129.9	5.95, 1H, m	130.0	5.95, 1H, brd (10.9)
11’	22.6	2.25, 1H, m	22.7	2.25, 1H, m

Table S3.2 ^{1}H NMR (500 MHz) data and ^{13}C NMR (125 MHz) data for **6b** in $CDCl_3$. Literature[28] data was measured at 500 MHz in $CDCl_3$.

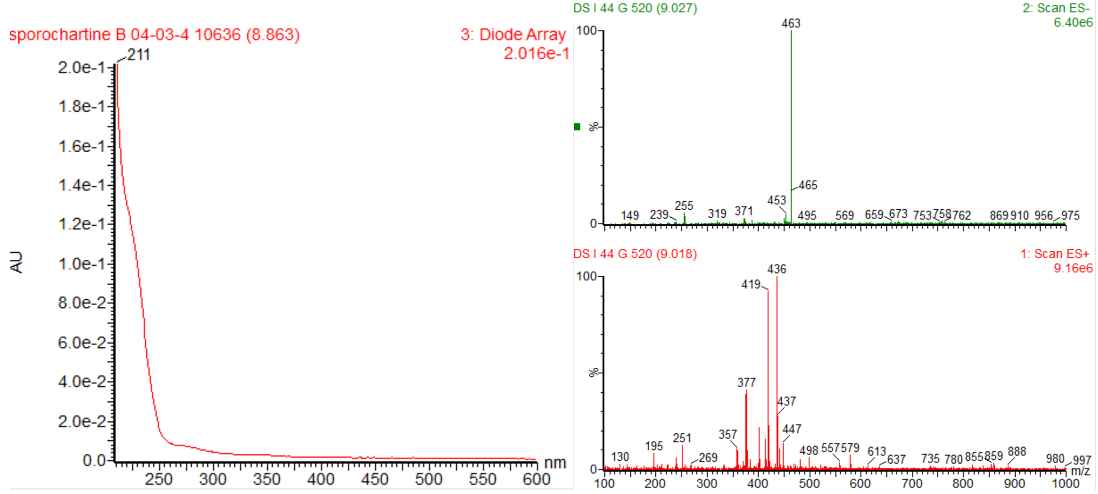
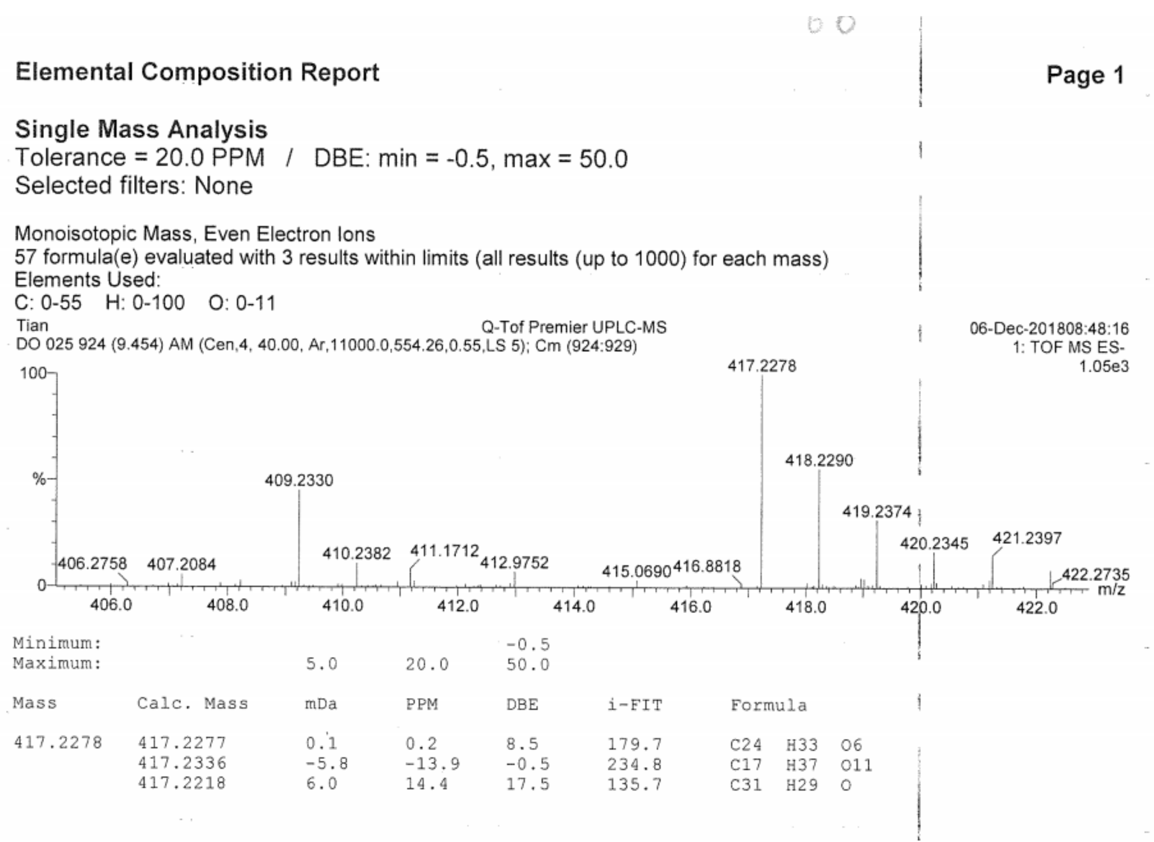


Figure S3.9 UV and mass spectra for compound **6b**.



Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
57 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-55 H: 0-100 O: 0-11

Tian Q-Tof Premier UPLC-MS 06-Dec-201808:48:16
DO 025 924 (9.454) AM (Cen,4, 40.00, Ar,11000.0,554.26,0.55,LS 5); Cm (924:929) 1: TOF MS ES- 1.05e3

		mDa	PPM	DBE		
Minimum:				-0.5		
Maximum:		5.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
417.2278	417.2277	0.1	0.2	8.5	179.7	C24 H33 O6
	417.2336	-5.8	-13.9	-0.5	234.8	C17 H37 O11
	417.2218	6.0	14.4	17.5	135.7	C31 H29 O

Figure S3.10 HRMS data for compound **6b.**

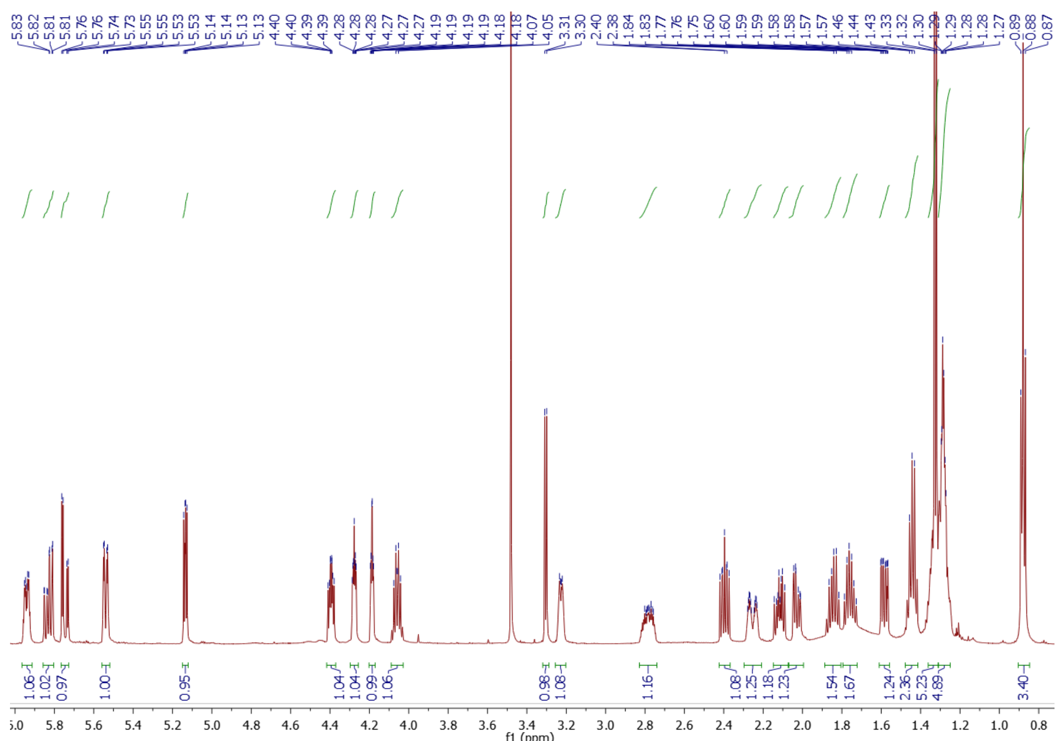


Figure S3.11 ^{1}H NMR of compound **6b**.

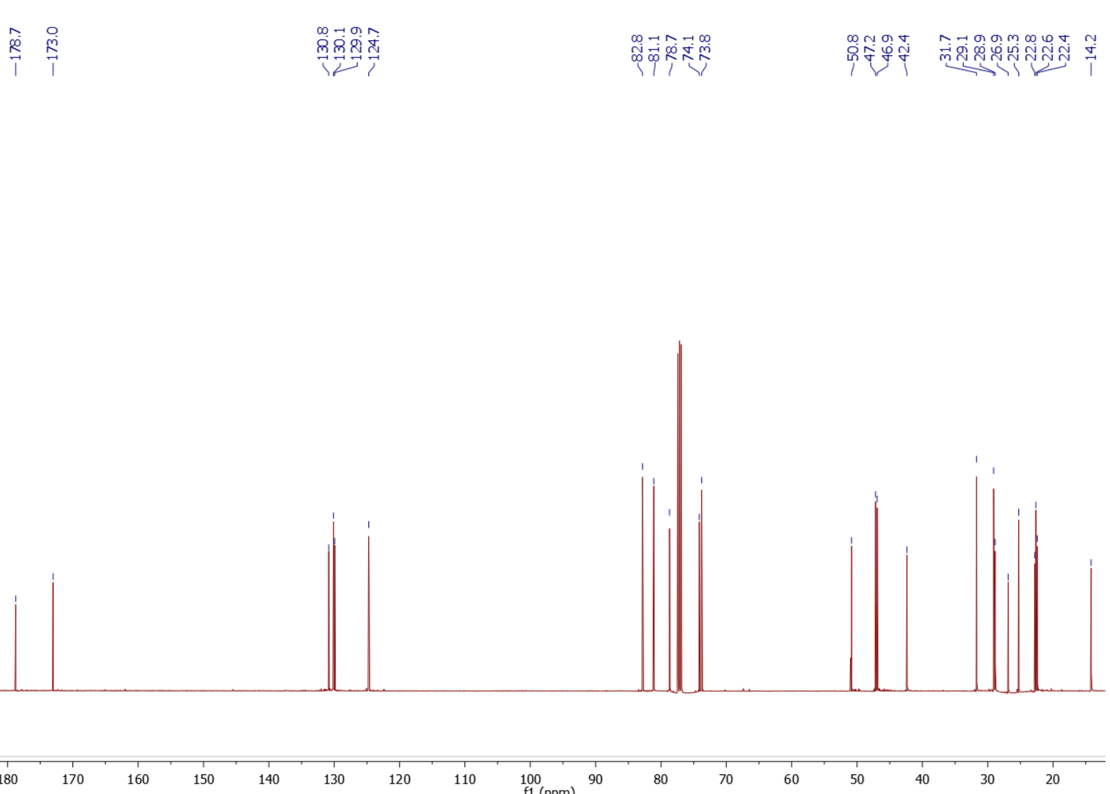


Figure S3.12 ^{13}C NMR of compound **6b**.

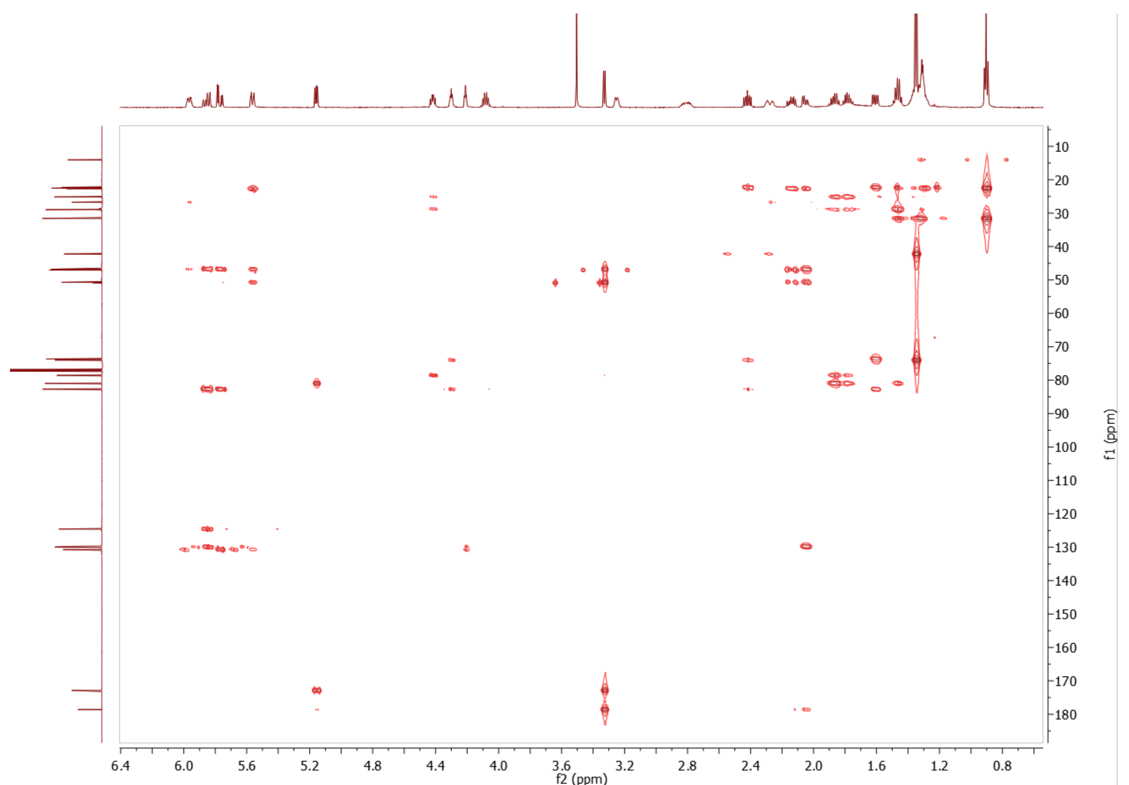


Figure S3.13 HMBC of compound **6b**.

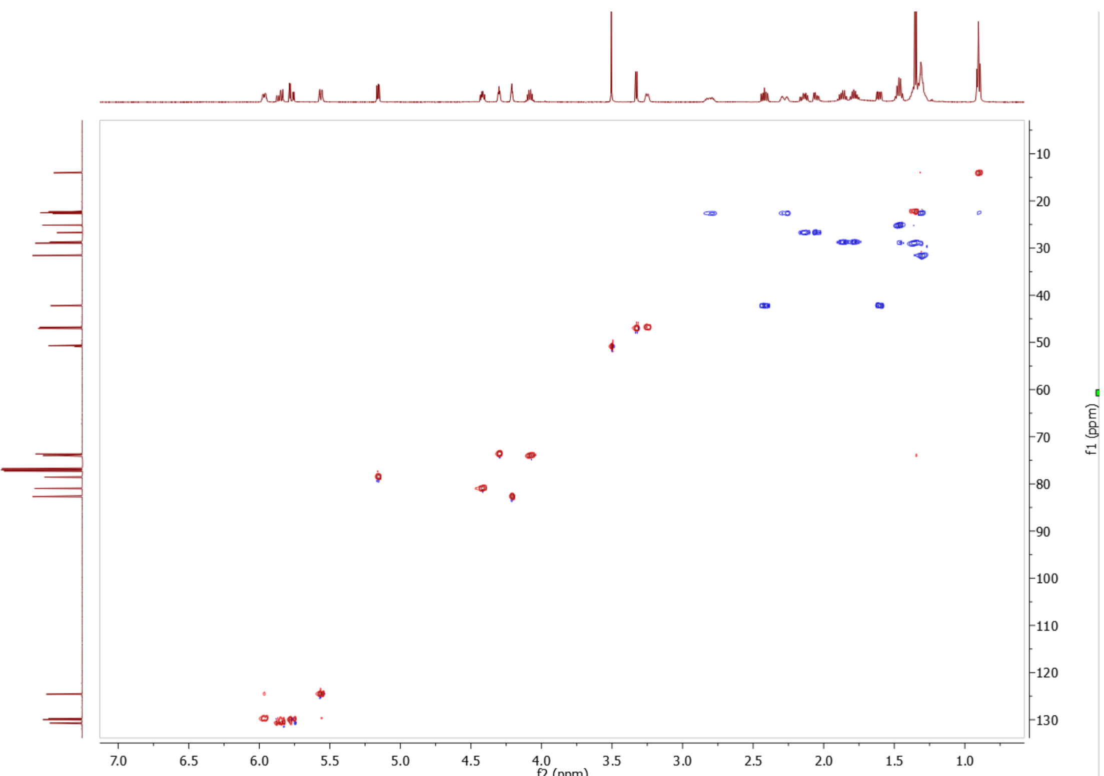


Figure S3.14 HSQC of compound **6b**.

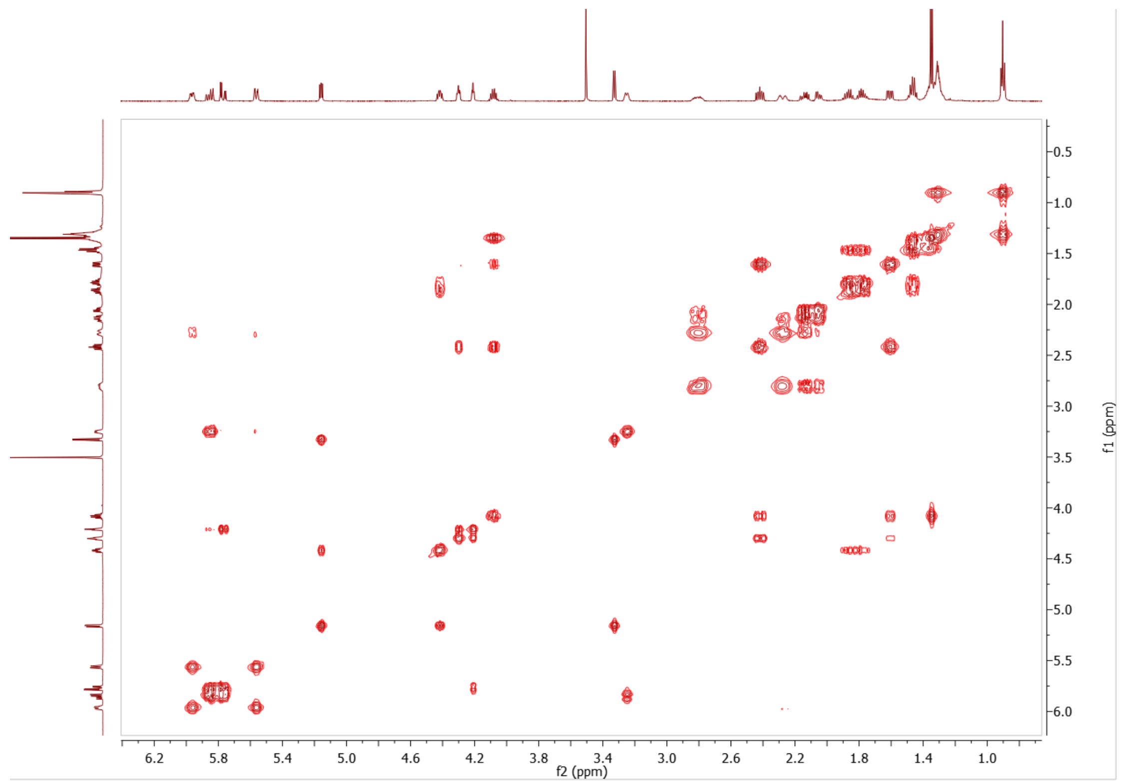


Figure S3.15 ^{1}H-^{1}H COSY of compound **6b**.

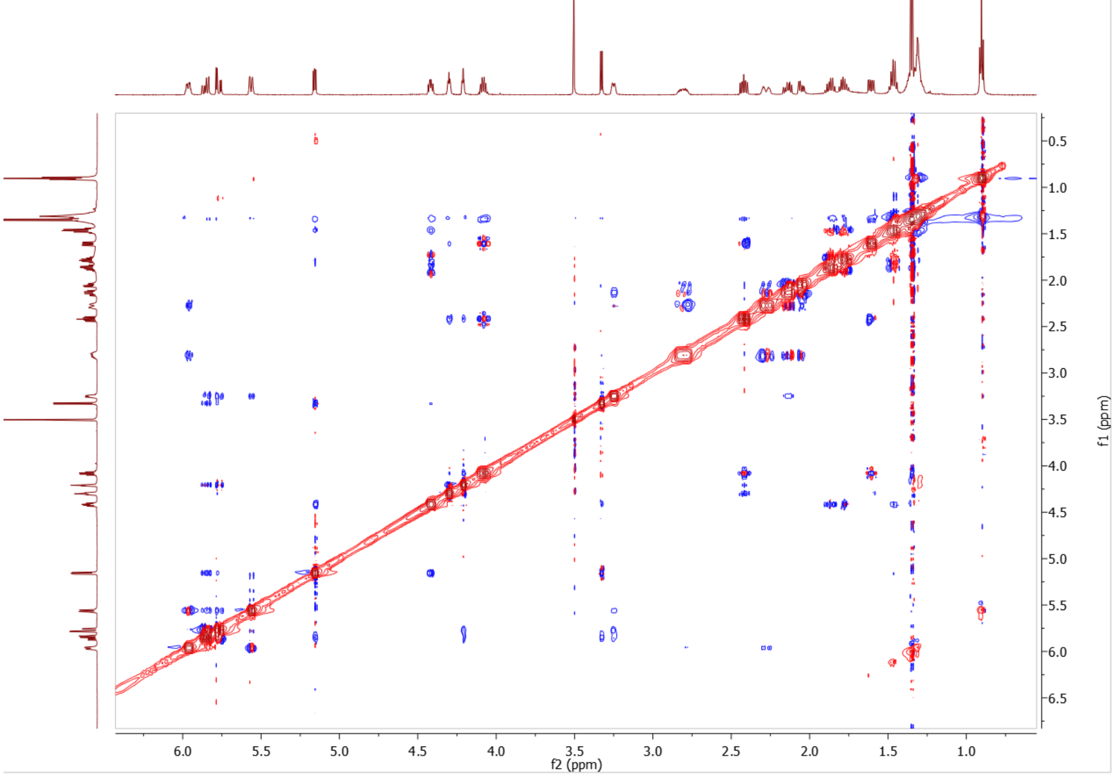


Figure S3.16 NOESY of compound **6b**.

Compound 7 (known from literature[29])

7

Trienylfuranol A

Chemical formula: $C_{11}H_{16}O_2$

White powder; $[\alpha]_D^{25}$ + 3 (*c* = 0.1, MeCN); UV (λ_{max}): 263 nm. ^{13}C NMR data (C_6D_6, 100 MHz): δ_C 137.4 (C-10'), 133.9 (C-9'), 133.1 (C-7'), 133.1 (C-8'), 130.5 (C-6'), 117.6 (C-11'), 83.9 (C-1'), 74.4 (C-2'), 74.0 (C-4'), 43.0 (C-3'), 22.2 (C-5'); ^{1}H NMR data (C_6D_6, 400 MHz): δ_H 6.42 (1H, m, H-7'), 6.27 (1H, m, H-10'), 6.16 (1H, m, H-8'), 6.13 (1H, m, H-9'), 5.80 (1H, dd, *J* = 15.4, 6.1 Hz, H-6'), 5.10 (1H, dd, *J* = 16.8, 1.7 Hz, H-11'b), 4.99 (1H, dd, *J* = 10.0, 1.7 Hz, H-11'a), 3.96 (1H, ddd, *J* = 5.9, 4.1, 1.3 Hz, H-1'), 3.91 (1H, m, H-2'), 3.78 (1H, m, H-4'), 1.97 (1H, ddd, *J* = 13.3, 7.7, 6.3 Hz, H-3'b), 1.44 (1H, ddd, *J* = 13.3, 6.8, 2.8 Hz, H-3'a), 1.29 (3H, d, *J* = 6.2 Hz, H-5'); NMR data are consistent with those previously reported,[29] see table S3.3; ESI-MS *m/z* 225 $[M+HCOOH-H]^-$, 181 $[M+H]^+$, 361 $[2M+H]^+$; HR-ESI-MS *m/z* 203.1049 $[M+Na]^+$ (calcd. for $C_{11}H_{16}O_2Na$, 203.1048).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	δ_C / ppm literature[29]	δ_H / ppm (J / Hz) literature[29]
1’	83.9	3.96, 1H, ddd (5.9, 4.1, 1.3)	83.8	3.95, 1H, ddd (5.7, 4.0, 1.4)
2’	74.4	3.91, 1H, m	74.4	3.86, 1H, m
3’	43.0	1.44, 1H, ddd (13.3, 6.8, 2.8) 1.97, 1H, ddd (13.3, 7.7, 6.3)	43.0	1.42, 1H, ddd (13.3, 6.6, 2.7) 1.93, 1H, ddd (13.3, 7.8, 6.3)
4’	74.0	3.78, 1H, m	74.0	3.77, 1H, ddq (7.7, 6.3, 6.3)
5’	22.2	1.29, 3H, d (6.2)	22.3	1.28, 3H, d (6.2)
6'	130.5	5.80, 1H, dd (15.4, 6.1)	130.3	5.75, 1H, dd (15.4, 6.0)
7'	133.1	6.42, 1H, m	133.1	6.43, 1H, ddd (15.4, 9.9, 1.5)
8'	133.1	6.16, 1H, m	133.1	6.18, 1H, dd (15.4, 10.4)
9'	133.9	6.13, 1H, m	133.9	6.12, 1H, dd (15.1, 10.3)
10'	137.4	6.27, 1H, m	137.4	6.27, 1H, ddd (16.9, 10.1, 10.1)
11'	117.6	4.99, 1H, dd (10.0, 1.7) 5.10, 1H, dd (16.8, 1.7)	117.6	4.98, 1H, dd (10.1, 1.7) 5.09, 1H, dd (16.9, 1.7)

Table S3.3 ^{1}H NMR (400 MHz) data and ^{13}C NMR (100 MHz) data for **7** in C_6D_6. Literature[29] data was measured at 600 MHz in C_6D_6.

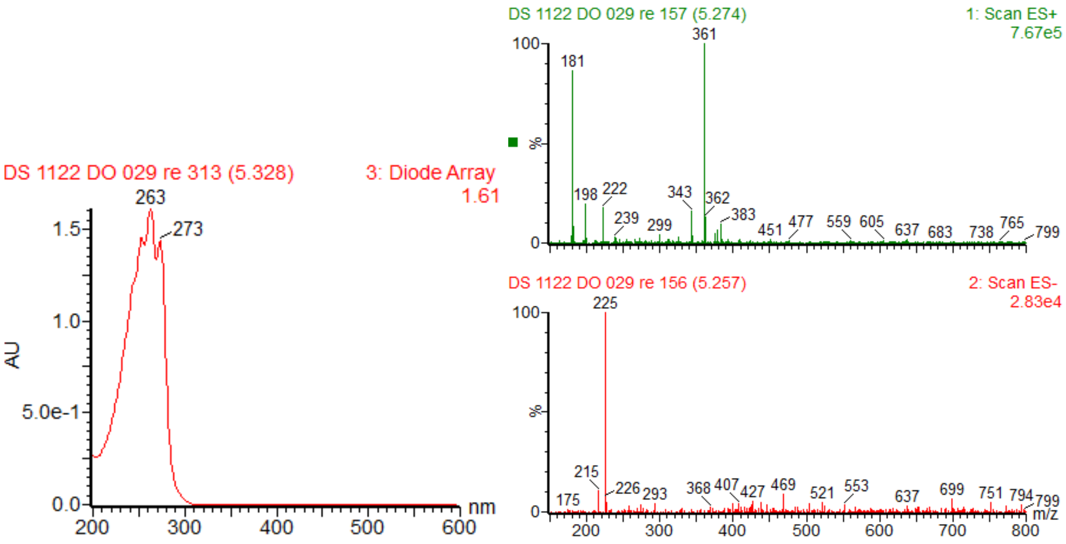
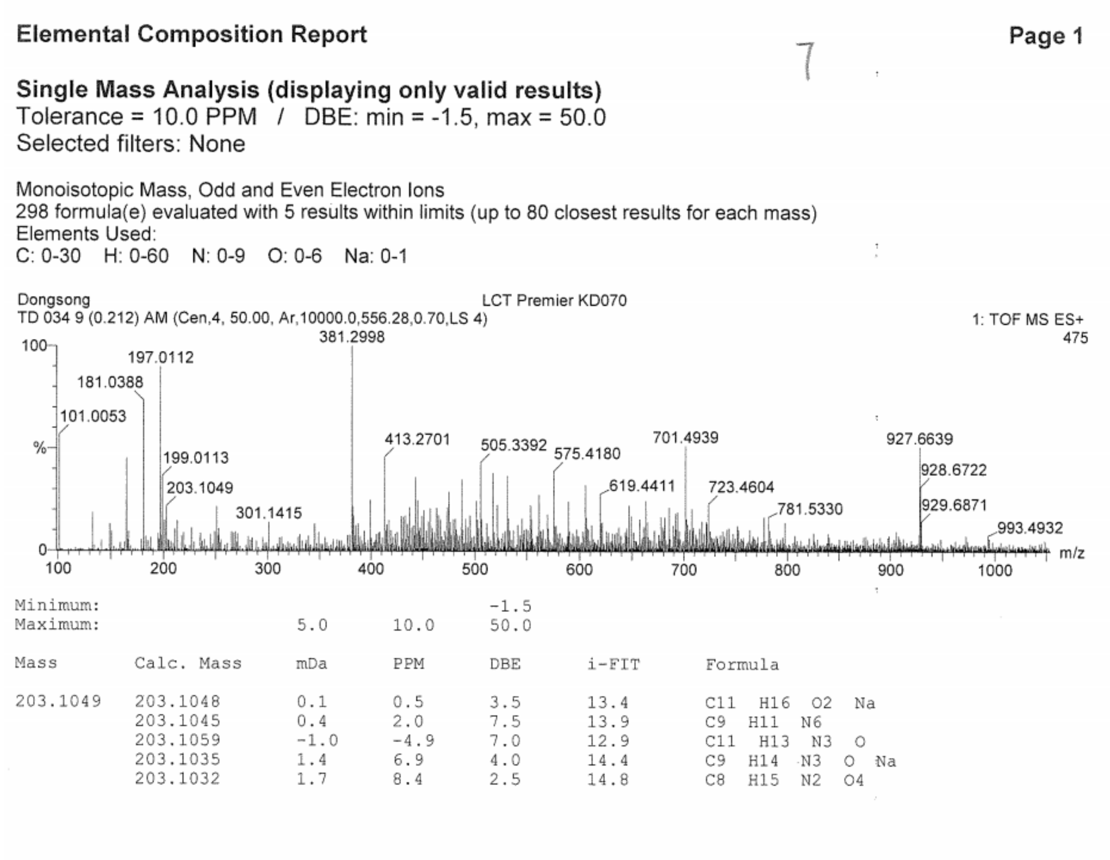


Figure S3.17 UV and mass spectra for compound **7**.



Elemental Composition Report 7 **Page 1**

Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Odd and Even Electron Ions
298 formula(e) evaluated with 5 results within limits (up to 80 closest results for each mass)
Elements Used:
C: 0-30 H: 0-60 N: 0-9 O: 0-6 Na: 0-1

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
Minimum:				-1.5		
Maximum:		5.0	10.0	50.0		
203.1049	203.1048	0.1	0.5	3.5	13.4	C11 H16 O2 Na
	203.1045	0.4	2.0	7.5	13.9	C9 H11 N6
	203.1059	-1.0	-4.9	7.0	12.9	C11 H13 N3 O
	203.1035	1.4	6.9	4.0	14.4	C9 H14 N3 O Na
	203.1032	1.7	8.4	2.5	14.8	C8 H15 N2 O4

Figure S3.18 HRMS data for compound **7**.

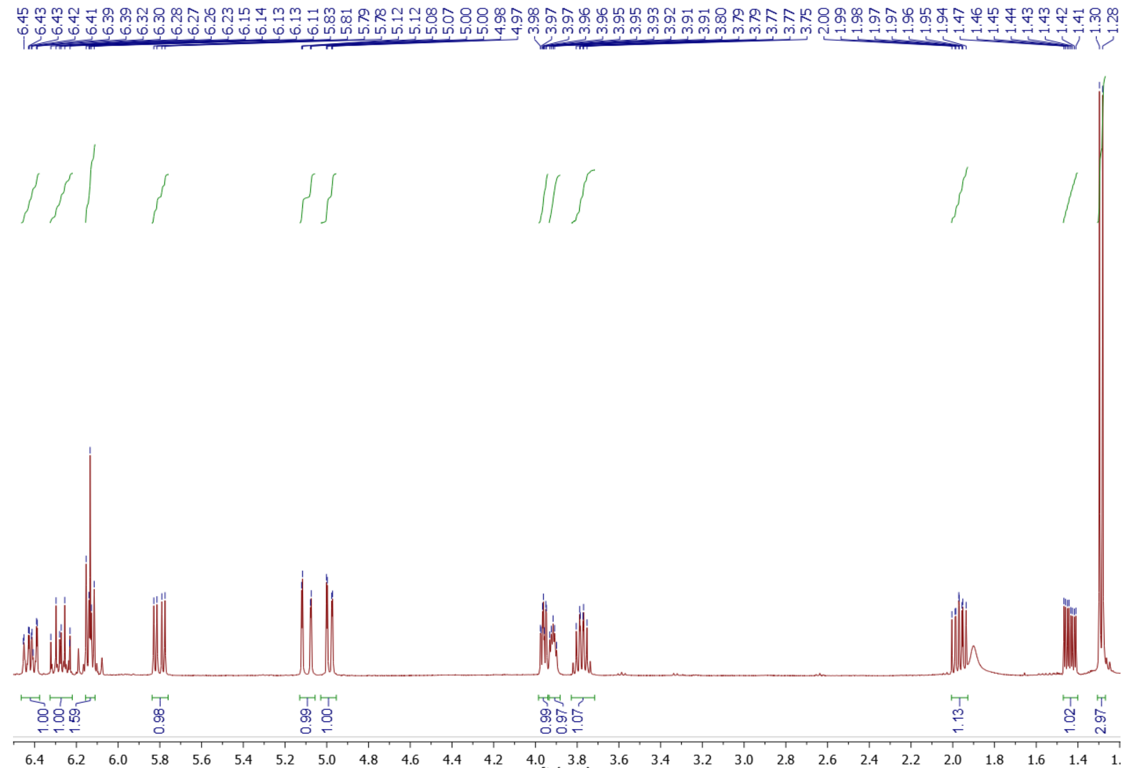


Figure S3.19 ^{1}H NMR of compound **7**.

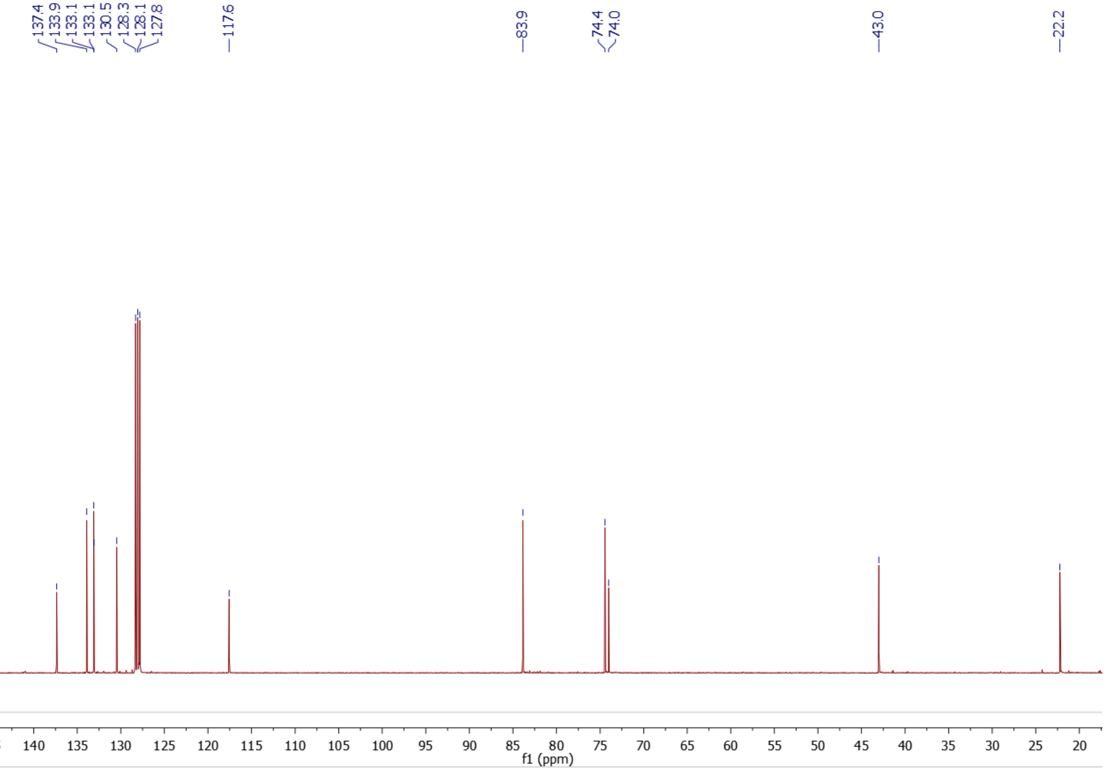


Figure S3.20 ^{13}C NMR of compound **7**.

Compound 8 (known from literature[27])

Dihydrosporothriolide

Chemical formula: $C_{13}H_{20}O_4$

Colorless oil; $[\alpha]^{25}_{D}$ + 94 (*c* = 0.1, $CHCl_3$); UV (λ_{max}): 227 nm. ^{13}C NMR data ($CDCl_3$, 100 MHz): δ_C 176.3 (C-1), 172.2 (C-4), 81.8 (C-6), 78.1 (C-5), 44.8 (C-2), 36.8 (C-3), 31.7 (C-10), 29.1 (C–9), 28.9 (C-7), 25.5 (C-8), 22.6 (C-11), 14.2 (C-12), 11.1 (C-13); ^{1}H NMR data ($CDCl_3$, 400 MHz): δ_H 5.02 (1H, dd, *J* = 6.0, 4.0 Hz, H-5), 4.51 (1H, ddd, *J* = 8.3, 6.1, 4.0 Hz, H-6), 3.45 (1H, dd, *J* = 10.1, 6.1 Hz, H-2), 3.06 (1H, dq, *J* = 10.2, 7.6 Hz, H-3), 1.91 (1H, m, H-7a), 1.81 (1H, m, H-7b), 1.50 (2H, m, H-8), 1.47 (3H, d, *J* = 7.5 Hz, H-13), 1.37 (2H, m, H-9), 1.31 (2H, m, H-11), 1.30 (2H, m, H-10), 0.89 (3H, t, *J* = 7.0 Hz, H-12); NMR data are consistent with those previously reported,[27] see table S3.4; ESI-MS *m/z* 239 $[M–H]^-$, 479 $[2M–H]^-$, 241 $[M+H]^+$; HR-ESI-MS *m/z* 241.1429 $[M+H]^+$ (calcd. for $C_{13}H_{21}O_4$, 241.1434).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	δ_C / ppm literature[27]	δ_H / ppm (J / Hz) literature[27]
1	176.3	-	176.2	-
2	44.8	3.45, 1H, dd (10.1, 6.1)	44.7	3.44, 1H, dd (10.1, 6.0)
3	36.8	3.06, 1H, dq (10.2, 7.6)	36.8	3.05, 1H, dq (10.1, 7.5)
4	172.2	-	172.1	-
5	78.1	5.02, 1H, dd (6.0, 4.0)	78.1	5.01, 1H, dd (6.0, 3.9)
6	81.8	4.51, 1H, ddd (8.3, 6.1, 4.0)	81.7	4.50, 1H, ddd (8.0, 6.2, 3.9)
7	28.9	1.91, 1H, m 1.81, 1H, m	28.9	1.92, 1H, m 1.81, 1H, m
8	25.5	1.50, 2H, m	25.3	1.50, 2H, m
9	29.1	1.37, 2H, m	29.0	1.37, 2H, m
10	31.7	1.30, 2H, m	31.6	1.30, 2H, m
11	22.6	1.31, 2H, m	22.6	1.31, 2H, m
12	14.2	0.89, 3H, t (7.0)	14.2	0.88, 3H, t (7.0)
13	11.1	1.47, 3H, d (7.5)	11.1	1.47, 3H, d (7.5)

Table S3.4 ^{1}H NMR (400 MHz) data and ^{13}C NMR (100 MHz) data for **8** in $CDCl_3$. Literature[27] data was measured at 700 MHz in $CDCl_3$.

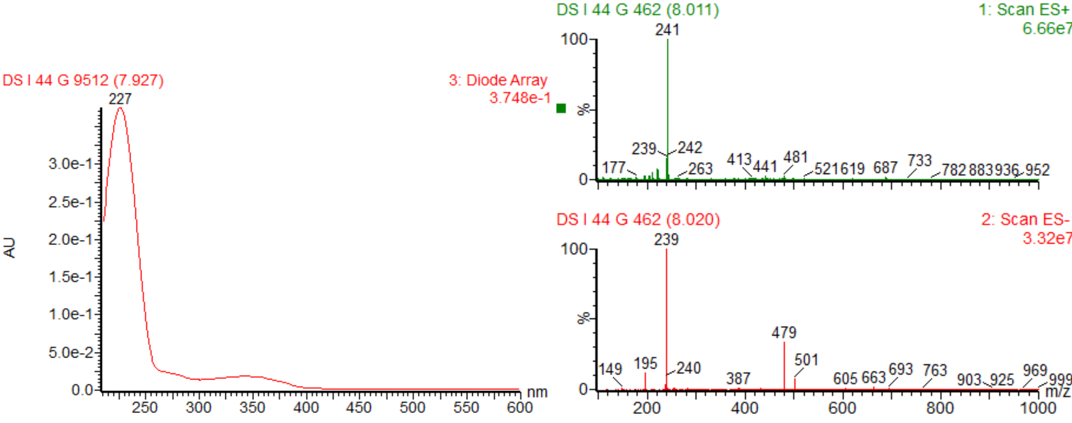


Figure S3.21 UV and mass spectra for compound **8**.

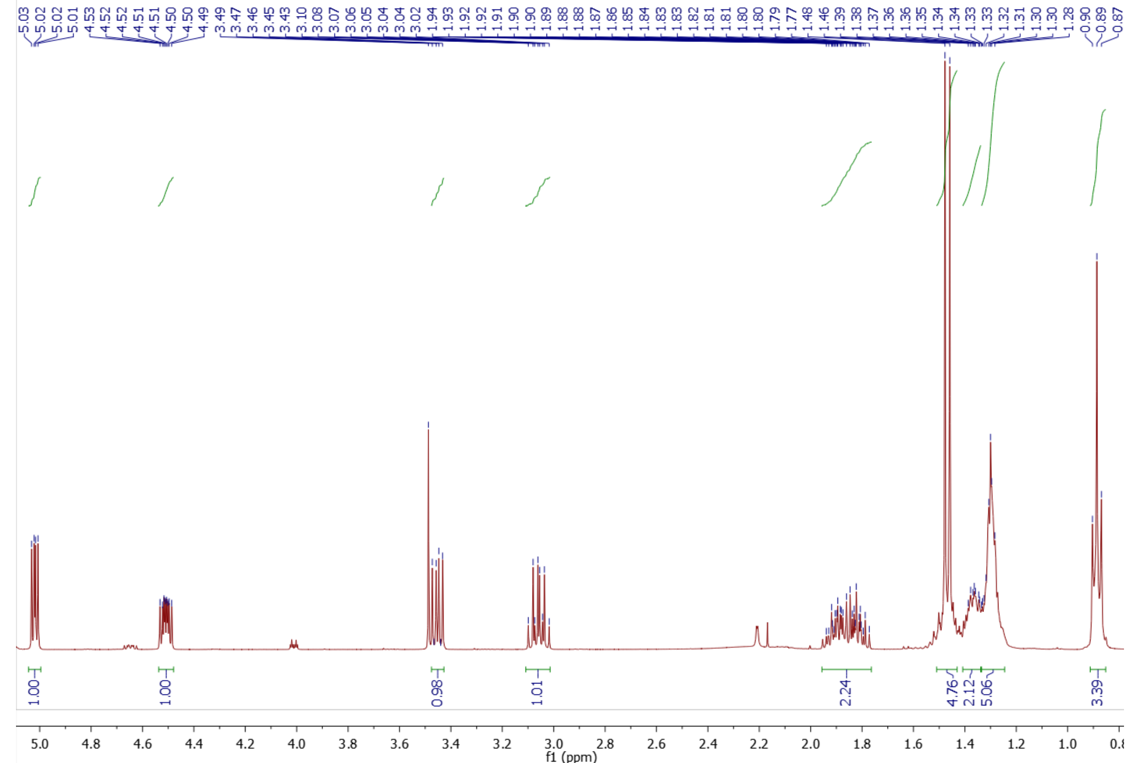


Figure S3.22 ^{1}H NMR of compound **8**.

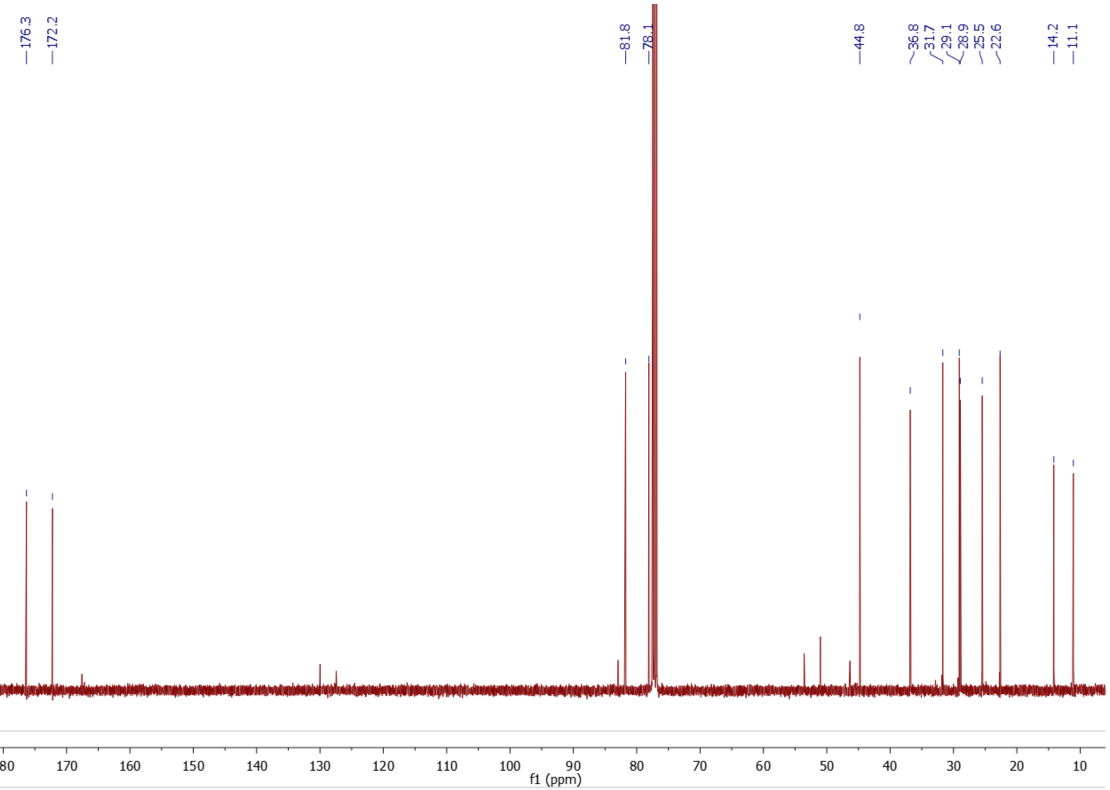


Figure S3.23 ^{13}C NMR of compound **8**.

Compound 9 (Known from literature[30])

9

Deoxysporothric acid

Chemical formula: $C_{13}H_{20}O_4$

White powder; $[\alpha]^{25}_{D}$ + 9 (c = 0.1, $CHCl_3$); UV (λ_{max}): 211 nm. ^{13}C NMR data ($CDCl_3$, 100 MHz): δ_C 176.0 (C-1), 169.8 (C-4), 135.5 (C-3), 131.8 (C-13), 79.3 (C-6), 44.8 (C-2), 35.7 (C-5), 35.5 (C-7), 31.8 (C-10), 29.1 (C-9), 25.3 (C-8), 22.7 (C-11), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 400 MHz): δ_H 6.56 (1H, s, H-13a), 5.98 (1H, s, H-13b), 4.44 (1H, m, H-6), 3.68 (1H, dd, J = 12.2, 8.9 Hz, H-2), 2.57 (1H, ddd, J = 11.5, 9.3, 5.7 Hz, H-5a), 2.00 (1H, q, J = 10.3 Hz, H-5b), 1.82 (1H, m, H-7a), 1.65 (1H, m, H-7b), 1.47 (2H, m, H-8), 1.33 (2H, m, H-9), 1.30 (2H, m, H-11), 1.28 (2H, m, H-10), 0.89 (3H, t, J = 6.8 Hz, H-12); NMR data are consistent with those previously reported,[30] see table S3.5; ESI-MS m/z 239 $[M–H]^-$, 479 $[2M–H]^-$, 241 $[M+H]^+$, 481 $[2M+H]^+$; HR-ESI-MS m/z 241.1436 $[M+H]^+$ (calcd. for $C_{13}H_{21}O_4$, 241.1440).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	δ_C / ppm literature[30]	δ_H / ppm (J / Hz) literature[30]
1	176.0	-	176.1	-
2	44.8	3.68, 1H, dd (12.2, 8.9)	44.8	3.68, 1H, dd (12.0, 8.9)
3	135.5	-	135.8	-
4	169.8	-	169.8	-
5	35.7	2.57, 1H, ddd (11.5, 9.3, 5.7) 2.00, 1H, q (10.3)	35.7	2.56, 1H, m 2.01, 1H, q (10.5)
6	79.3	4.44, 1H, m	79.3	4.43, 1H, m
7	35.5	1.82, 1H, m 1.65, 1H, m	35.5	1.82, 1H, m 1.65, 1H, m
8	25.3	1.47, 2H, m	25.3	1.48, 2H, m
9	29.1	1.33, 2H, m	29.1	1.33, 2H, m
10	31.8	1.28, 2H, m	31.8	1.28, 2H, m
11	22.7	1.30, 2H, m	22.7	1.30, 2H, m
12	14.2	0.89, 3H, t (6.8)	14.2	0.89, 3H, t (6.9)
13	131.8	6.56, 1H, s 5.98, 1H, s	131.4	6.54, 1H, s 5.95, 1H, s

Table S3.5 ^{1}H NMR (400 MHz) data and ^{13}C NMR (100 MHz) data for **9** in $CDCl_3$. Literature[30] data was measured at 500 MHz in $CDCl_3$.

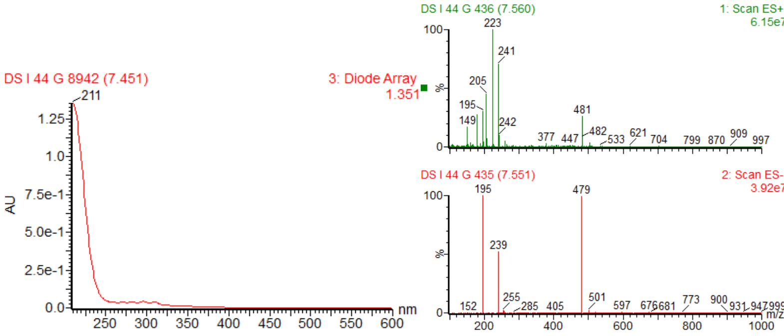


Figure S3.24 UV and mass spectra for compound **9**.

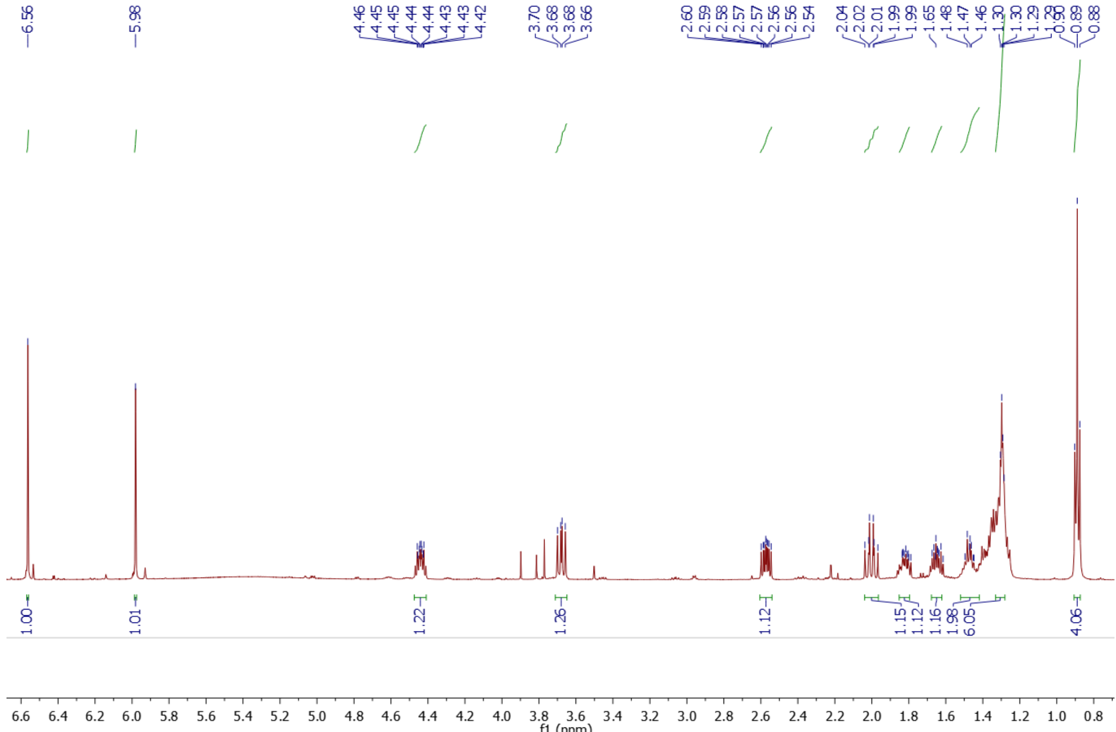


Figure S3.25 ^{1}H NMR of compound **9**.

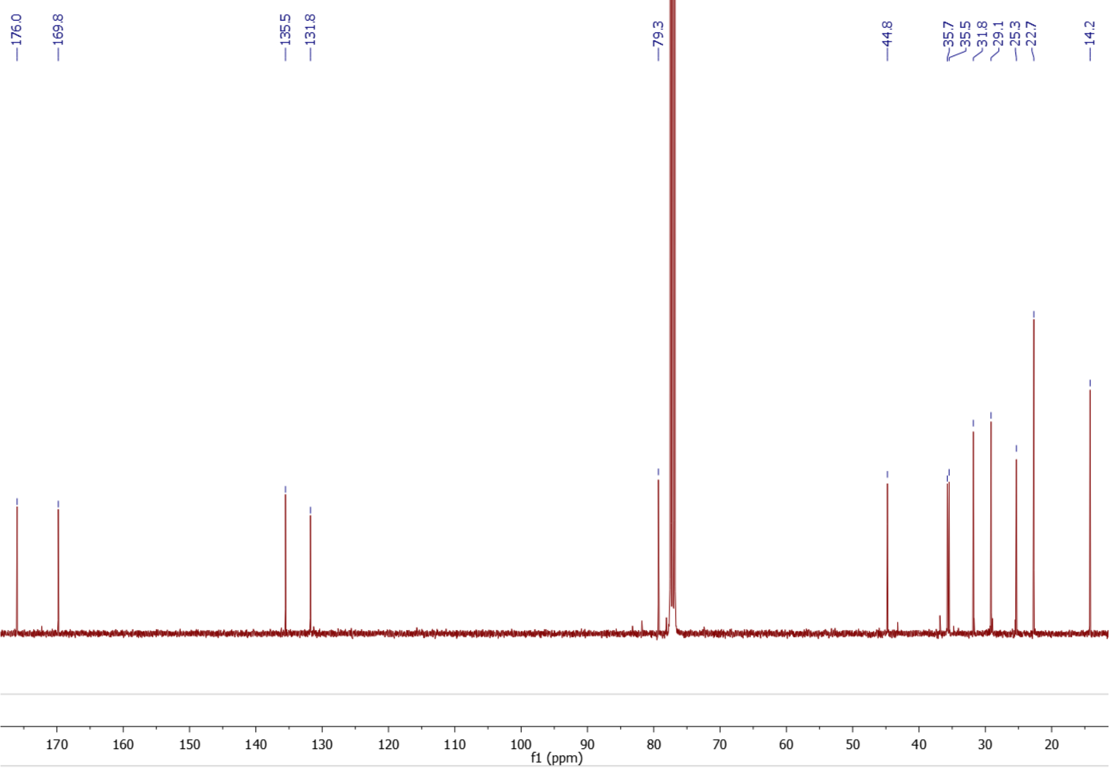


Figure S3.26 ^{13}C NMR of compound **9**.

Compound 10 (known from literature[27])

Isosporothric acid

Chemical formula: $C_{13}H_{20}O_5$

Colorless oil; $[\alpha]_D^{25}$ – 7 (c = 0.1, $CHCl_3$); UV (λ_{max}): 236 nm. ^{13}C NMR data ($CDCl_3$, 125 MHz): δ_C 173.2 (C-4), 165.0 (C-1), 144.8 (C-2), 140.4 (C-3), 83.1 (C-5), 70.2 (C-6), 34.5 (C-7), 31.8 (C-10), 29.2 (C-9), 25.9 (C-8), 22.7 (C-11), 14.2 (C-12), 11.3 (C-13); ^1H NMR data ($CDCl_3$, 500 MHz): δ_H 5.08 (1H, m, H-5), 4.26 (1H, m, H-6), 2.24 (3H, d, J = 2.0 Hz, H-13), 1.74 (2H, m, H-7), 1.49 (2H, m, H-8), 1.36 (2H, m, H-9), 1.30 (2H, m, H-11), 1.29 (2H, m, H-10), 0.89 (3H, t, J = 6.9 Hz, H-12); NMR data are consistent with those previously reported,[27] see table S3.6; ESI-MS m/z 255 $[M–H]^–$, 511 $[2M–H]^–$, 257 $[M+H]^+$, 513 $[2M+H]^+$, HR-ESI-MS m/z 257.1384 $[M+H]^–$ (calcd. for $C_{13}H_{21}O_5$, 257.1384).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	δ_C / ppm literature[27]	δ_H / ppm (J / Hz) literature[27]
1	165.0	-	164.8	-
2	144.8	-	144.7	-
3	140.4	-	140.2	-
4	173.2	-	173.1	-
5	83.1	5.08, 1H, m	83.1	5.06, 1H, qd (2.1, 1.5)
6	70.2	4.26, 1H, m	70.1	4.25, 1H, dt (7.0, 1.5)
7	34.5	1.74, 2H, m	34.4	1.73, 2H, m
8	25.9	1.49, 2H, m	25.8	1.50, 2H, m
9	29.2	1.36, 2H, m	29.1	1.34, 2H, m
10	31.8	1.29, 2H, m	31.7	1.28, 2H, m
11	22.7	1.30, 2H, m	22.6	1.29, 2H, m
12	14.2	0.89, 3H, t (6.9)	14.1	0.88, 3H, t (7.0)
13	11.3	2.24, 3H, d (2.0)	11.2	2.24, 3H, d (2.1)

Table S3.6 ^{1}H NMR (500 MHz) data and ^{13}C NMR (125 MHz) data for **10** in $CDCl_3$. Literature[27] data was measured at 700 MHz in $CDCl_3$.

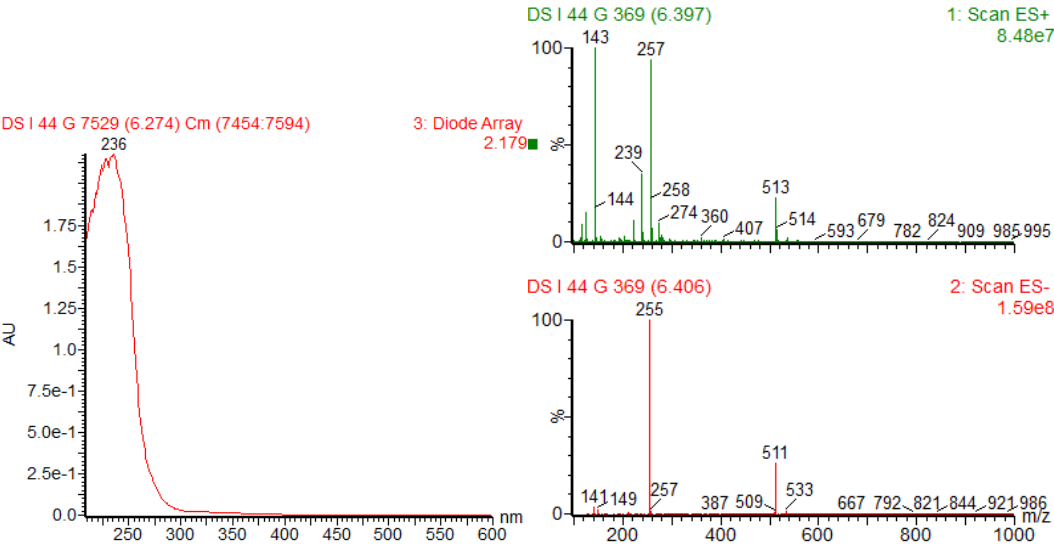


Figure S3.27 UV and mass spectra for compound **10**.

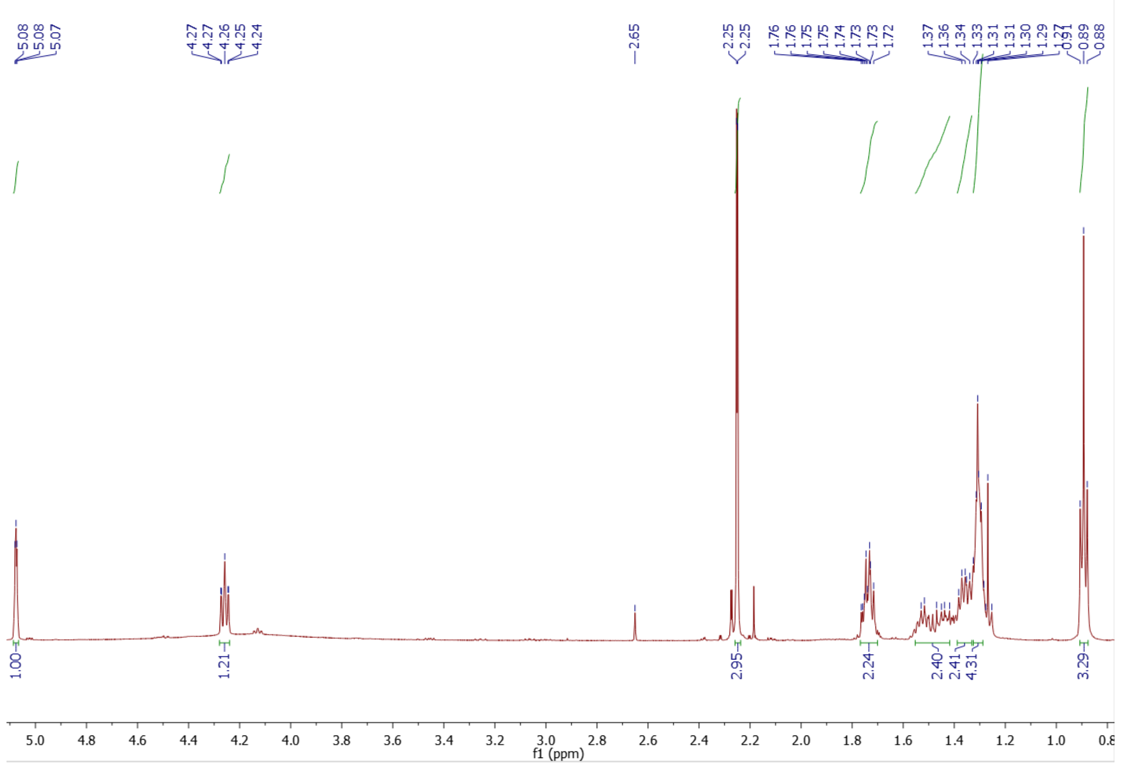


Figure S3.28 ^{1}H NMR of compound **10**.

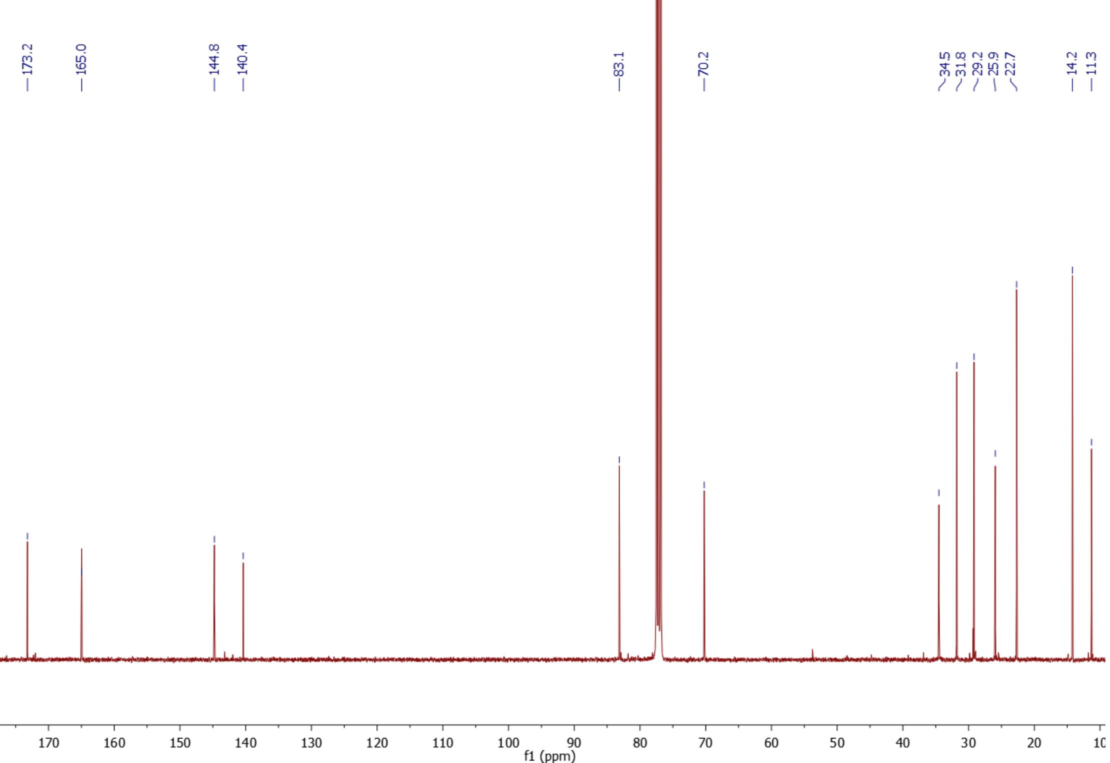


Figure S3.29 ^{13}C NMR of compound **10**.

Compound 11 (known from literature[27])

Dihydroisosporothric acid

Chemical formula: $C_{13}H_{22}O_5$

Colorless oil; $[\alpha]_D^{25}$ + 12 (c = 0.1, $CHCl_3$); UV (λ_{max}): 329 nm, 213 nm. ^{13}C NMR data ($CDCl_3$, 125 MHz): δ_C 176.8 (C-4), 174.1 (C-1), 80.6 (C-5), 71.1 (C-6), 48.4 (C-2), 39.2 (C-3), 34.2 (C-7), 31.8 (C-10), 29.2 (C-9), 25.8 (C-8), 22.7 (C-11), 14.9 (C-13), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 500 MHz): δ_H 4.48 (1H, dd, J = 9.0, 2.1 Hz, H-5), 3.72 (1H, m, H-6), 3.25 (1H, dd, J = 11.0, 9.0 Hz, H-2), 2.99 (1H, dq, J = 11.0, 7.0 Hz, H-3), 1.65 (2H, m, H-7), 1.64 (1H, m, H-8b), 1.50 (1H, m, H-8a), 1.41 (3H, d, J = 7.1 Hz, H-13), 1.32 (2H, m, H-9), 1.29 (2H, m, H-11), 1.28 (2H, m, H-10), 0.89 (3H, t, J = 6.9 Hz, H-12); NMR data are consistent with those previously reported,[27] see table S3.7; ESI-MS m/z 257 $[M-H]^-$, 515 $[2M-H]^-$, 259 $[M+H]^+$, 281 $[M+Na]^+$, 241 $[M-H_2O+H]^+$, 517 $[2M+H]^+$; HR-ESI-MS m/z 259.1544 $[M+H]^+$ (calcd. for $C_{13}H_{23}O_5$, 259.1540).

pos.	δ_C / ppm	δ_H / ppm (*J* / Hz)	δ_C / ppm literature[27]	δ_H / ppm (*J* / Hz) literature[27]
1	174.1		171.9	
2	48.4	3.25, 1H, dd (11.0, 9.0)	48.2	3.23, 1H, dd (11.0, 9.0)
3	39.2	2.99, 1H, dq (11.0, 7.0)	39.2	2.99, 1H, dq (11.0, 7.1)
4	176.8		176.7	
5	80.6	4.48, 1H, dd (9.0, 2.1)	80.6	4.47, 1H, dd (9.0, 2.2)
6	71.1	3.72, 1H, m	71.0	3.71, 1H, m
7	34.2	1.65, 2H, m	34.0	1.82, 2H, m
8	25.8	1.50, 1H, m 1.64, 1H, m	25.7	1.46, 2H, m
9	29.2	1.32, 2H, m	29.0	1.29, 2H, m
10	31.8	1.28, 2H, m	31.8	1.25, 2H, m
11	22.7	1.29, 2H, m	22.6	1.26, 2H, m
12	14.2	0.89, 3H, t (6.9)	14.2	0.88, 3H, t (7.0)
13	14.9	1.41, 3H, d (7.1)	14.8	-

Table S3.7 ^{1}H NMR (500 MHz) data and ^{13}C NMR (125 MHz) data for **11** in $CDCl_3$. Literature[27] data was measured at 700 MHz in $CDCl_3$.

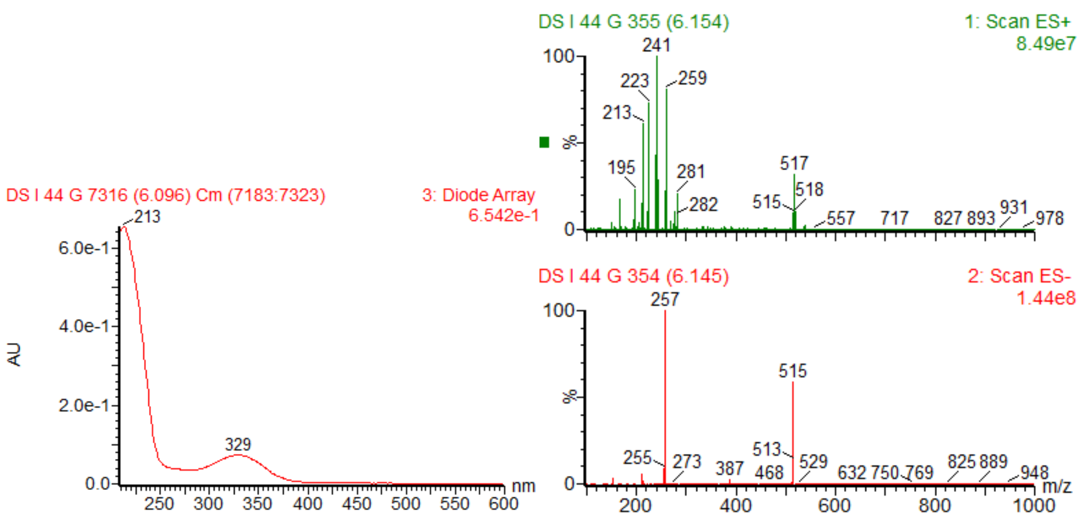


Figure S3.30 UV and mass spectra for compound **11**.

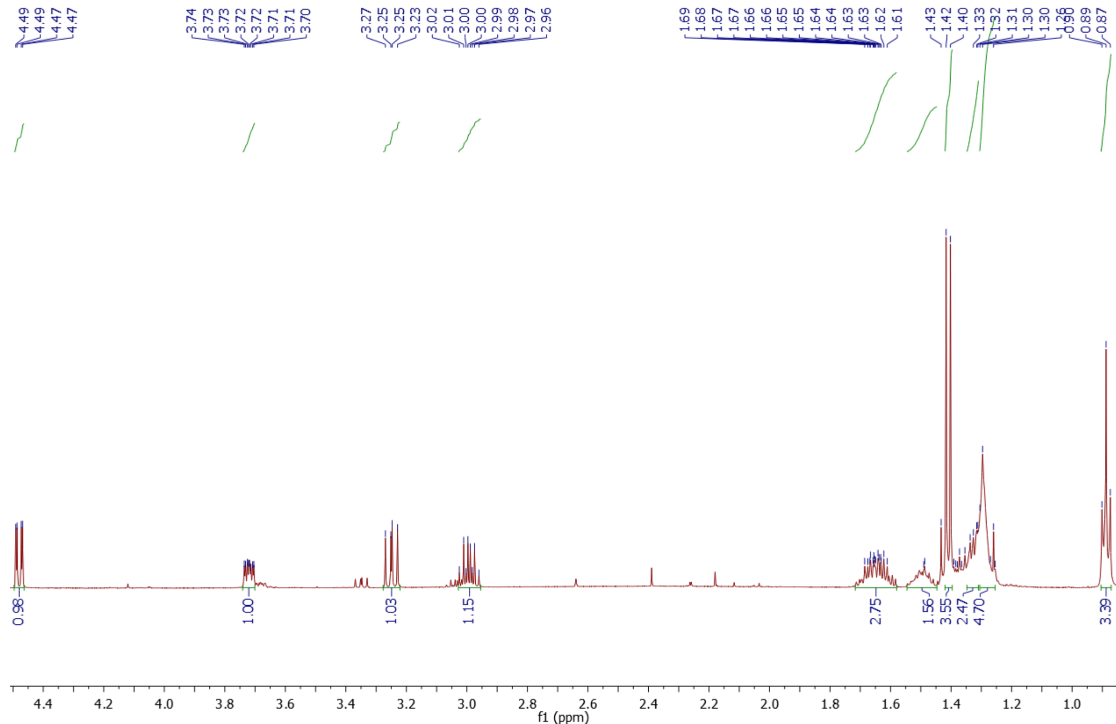


Figure S3.31 ^{1}H NMR of compound **11**.

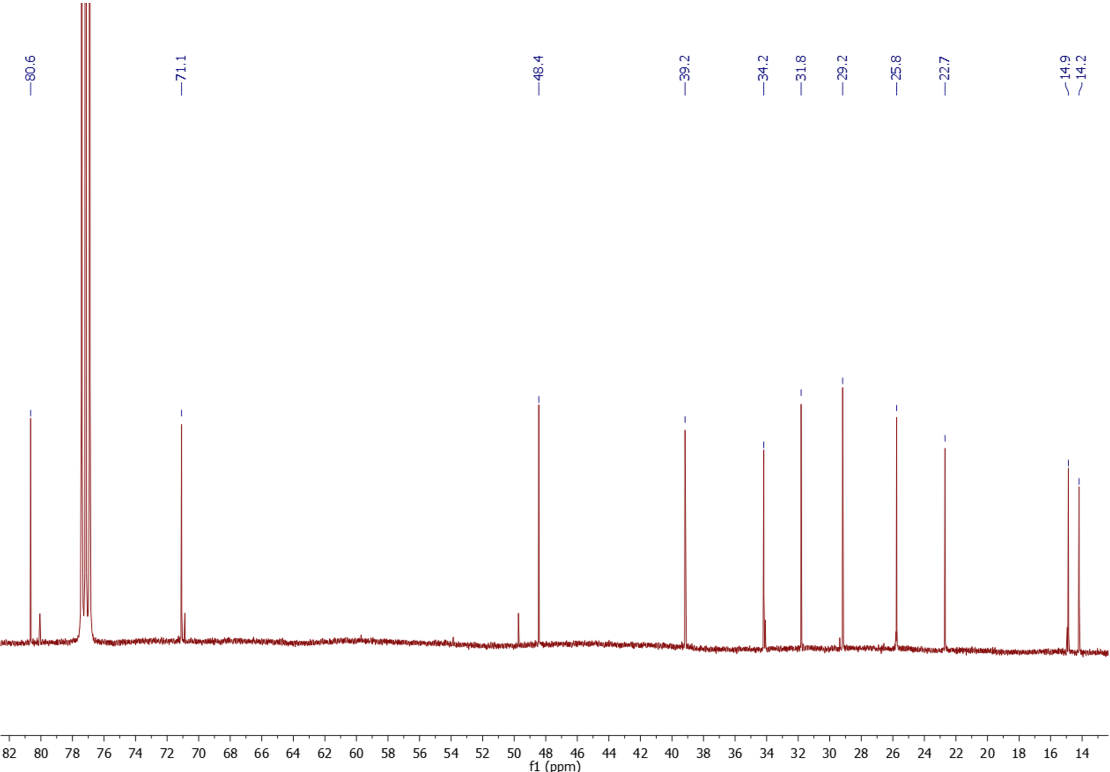


Figure S3.32 ^{13}C NMR of compound **11**.

Compound 12 (known from literature[27])

12

Sporothric acid

Chemical formula: $C_{13}H_{20}O_5$

Colorless oil; $[\alpha]_D^{25}$ + 5 (c = 0.1, $CHCl_3$); UV (λ_{max}): 208 nm. ^{13}C NMR data ($CDCl_3$, 100 MHz): δ_C 171.3 (C-4), 163.6 (C-1), 134.7 (C-13), 128.6 (C-3), 79.2 (C-6), 66.5 (C-5), 50.1 (C-2), 31.7 (C-10), 31.0 (C-7), 29.1 (C-9), 25.2 (C-8), 22.6 (C-11), 14.1 (C-12); ^{1}H NMR data ($CDCl_3$, 400 MHz): δ_H 6.79 (1H, s, H-13a), 5.96 (1H, s, H-13b), 4.51 (1H, m, H-6), 4.32 (1H, m, H-5), 3.83 (1H, m, H-2), 1.80 (1H, m, H-7a), 1.70 (1H, m, H-7a), 1.56 (1H, m, H-8a), 1.40 (1H, m, H-8b), 1.27-1.34 (6H, m, H-9/H-10/H-11), 0.88 (3H, t, J = 6.5 Hz, H-12); NMR data are consistent with those previously reported,[27] see table S3.8; ESI-MS m/z 255 $[M–H]^–$, 511 $[2M–H]^–$, 257 $[M+H]^+$, 513 $[2M+H]^+$. HR-ESI-MS m/z 255.1232 $[M–H]^–$ (calcd. for $C_{13}H_{19}O_5$, 255.1232).

pos.	δ_H / ppm (*J* / Hz)	δ_C / ppm	δ_H / ppm (*J* / Hz) literature[27]	δ_C / ppm literature[27]
1	-	163.6	-	163.5
2	3.83, 1H, m	50.1	3.82, 1H, dd (3.9, 1.4)	50.0
3	-	128.6	-	128.5
4	-	171.3	-	171.3
5	4.32, 1H, m	66.5	4.30, 1H, dd (3.9, 1.3)	66.5
6	4.51, 1H, m	79.2	4.51, 1H, ddd (8.6, 5.2, 1.4)	79.1
7	1.80, 1H, m 1.70, 1H, m	31.0	1.80, 1H, m 1.69, 1H, m	31.0
8	1.56, 1H, m 1.40, 1H, m	25.2	1.54, 1H, m 1.38, 1H, m	25.1
9	1.27-1.34, 2H, m	29.1	1.32, 2H, m	29.0
10	1.27-1.34, 2H, m	31.7	1.27, 2H, m	31.6
11	1.27-1.34, 2H, m	22.6	1.28, 2H, m	22.6
12	0.88, 3H, t (6.5)	14.1	0.88, 3H, t (7.0)	14.1
13	6.79, 1H, s 5.96, 1H, s	134.7	6.78, 1H, s 5.95, 1H, s	134.6

Table S3.8 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data for **12** in $CDCl_3$. Literature[27] data was measured at 700 MHz in $CDCl_3$.

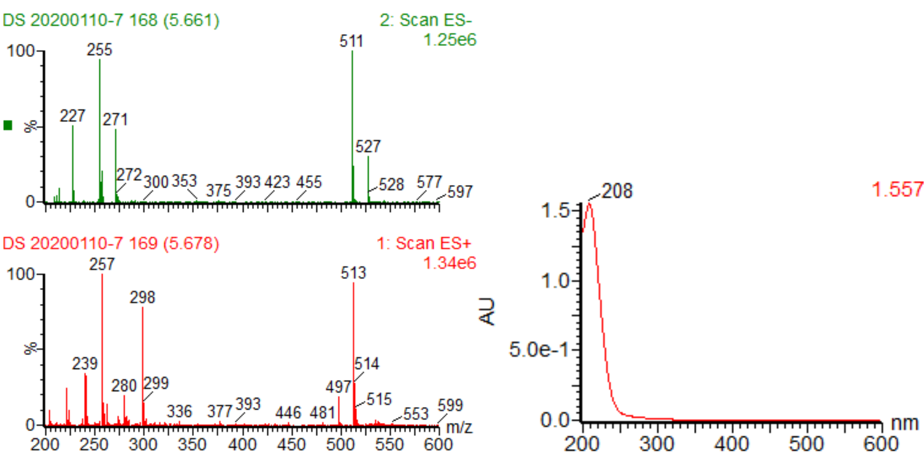


Figure S3.33 UV spectrum and ESI spectrum for **12**.

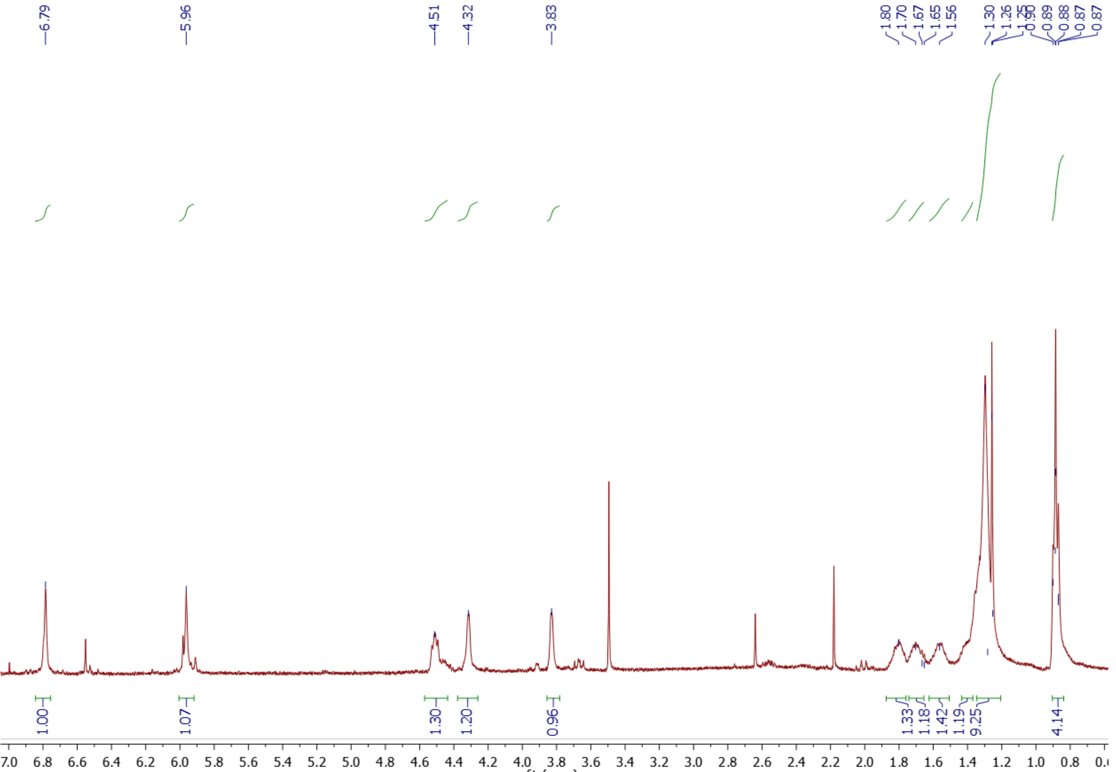


Figure S3.34 ^{1}H NMR of compound **12.**

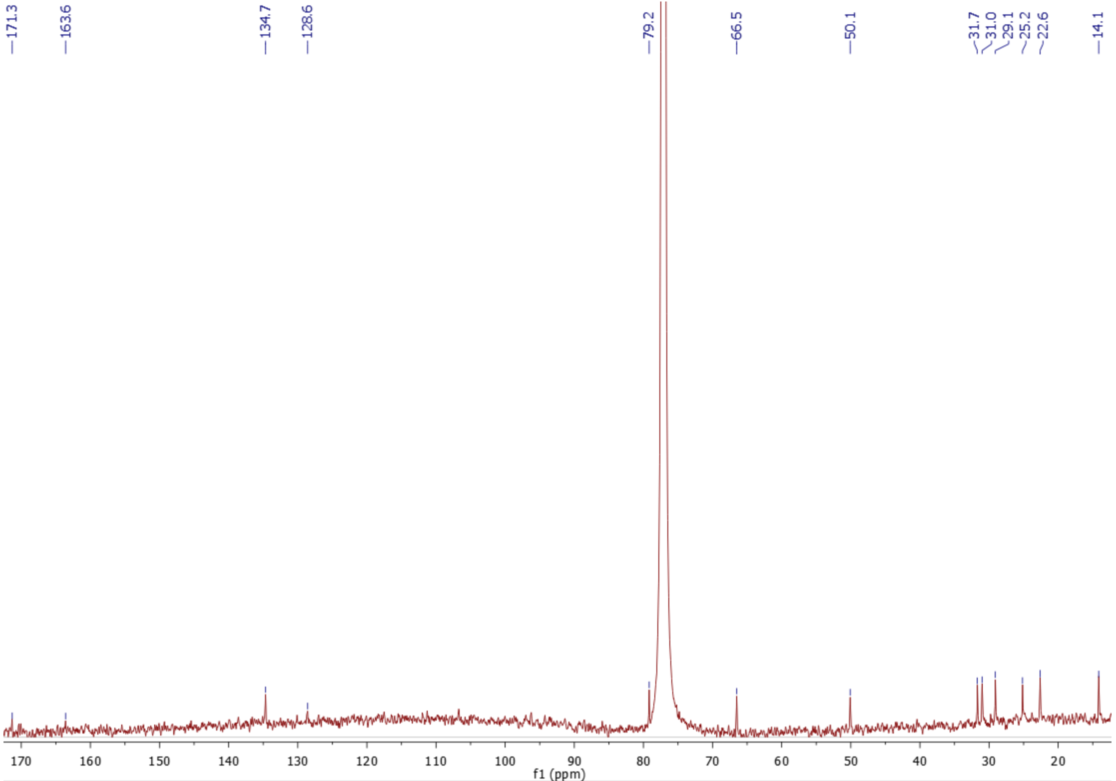
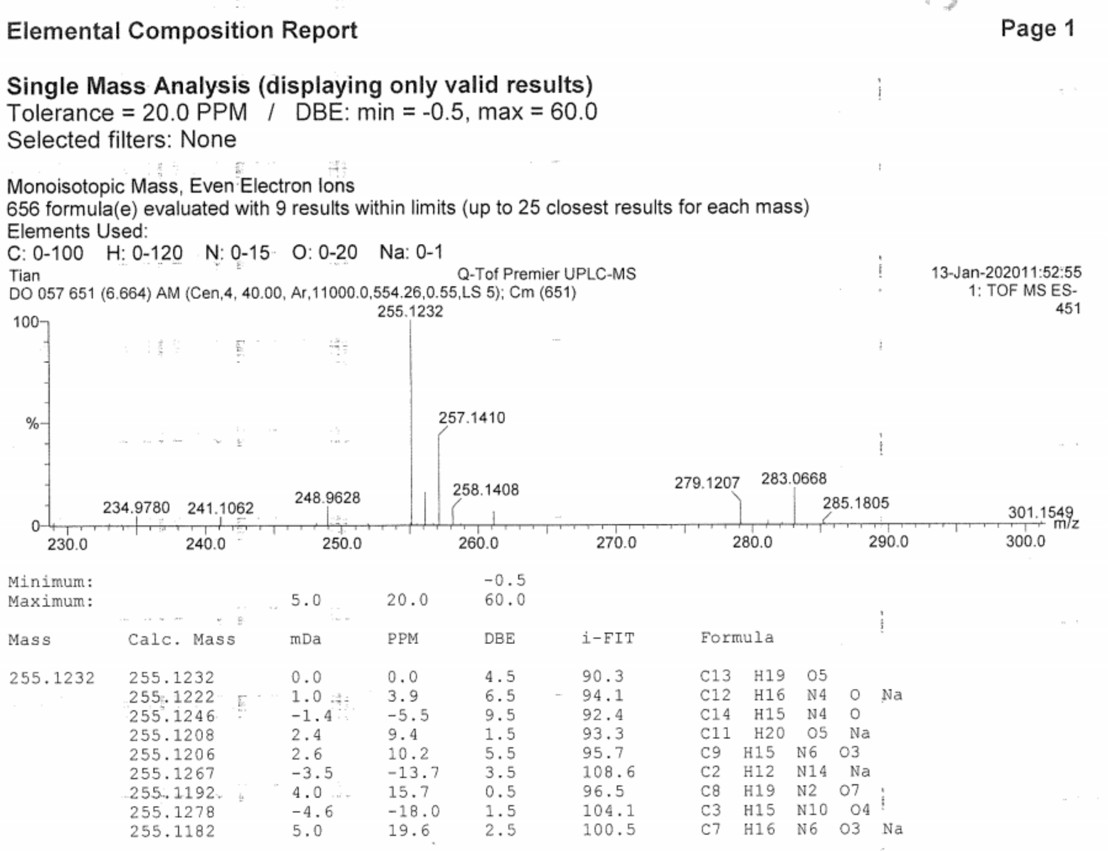


Figure S3.35 ^{13}C NMR of compound **12.**



Elemental Composition Report Page 1

Single Mass Analysis (displaying only valid results)
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 60.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
656 formula(e) evaluated with 9 results within limits (up to 25 closest results for each mass)
Elements Used:
C: 0-100 H: 0-120 N: 0-15 O: 0-20 Na: 0-1

	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		
Mass						
255.1232	255.1232	0.0	0.0	4.5	90.3	C13 H19 O5
	255.1222	1.0	3.9	6.5	94.1	C12 H16 N4 O Na
	255.1246	-1.4	-5.5	9.5	92.4	C14 H15 N4 O
	255.1208	2.4	9.4	1.5	93.3	C11 H20 O5 Na
	255.1206	2.6	10.2	5.5	95.7	C9 H15 N6 O3
	255.1267	-3.5	-13.7	3.5	108.6	C2 H12 N14 Na
	255.1192	4.0	15.7	0.5	96.5	C8 H19 N2 O7
	255.1278	-4.6	-18.0	1.5	104.1	C3 H15 N10 O4
	255.1182	5.0	19.6	2.5	100.5	C7 H16 N6 O3 Na

Figure S3.36 HRMS data for compound **12**.

Compound 13

13

Dehydrodeoxysporothric acid

Chemical formula: $C_{13}H_{18}O_4$

White powder; $[\alpha]^{25}_{D}$ – 25 (c = 0.83, MeOH); UV (λ_{max}): 229 nm. ^{13}C NMR data ($CDCl_3$, 150 MHz): δ_C 171.7 (C-1), 170.4 (C-4), 153.4 (C-5), 133.7 (C-13), 128.6 (C-3), 124.9 (C-2), 80.8 (C-6), 33.4 (C-7), 31.7 (C-10), 29.1 (C-9), 25.1 (C-8), 22.6 (C-11), 14.1 (C-12); ^{1}H NMR data ($CDCl_3$, 600 MHz): δ_H 7.95 (1H, d, J = 1.7 Hz, H-5), 7.19 (1H, s, H-13a), 6.81 (1H, s, H-13b), 4.99 (1H, m, H-6), 1.79 (1H, m, H-7a), 1.69 (1H, m, H-7b), 1.46 (2H, m, H-8), 1.34 (2H, m, H-9), 1.29 (2H, m, H-11), 1.28 (2H, m, H-10), 0.88 (3H, t, J = 7.0 Hz, H-12); NMR data see table S3.9; ESI-MS *m/z* 237 $[M–H]^–$, 475 $[2M–H]^–$, 239 $[M+H]^+$, 261 $[M+Na]^+$. HR-ESI-MS *m/z* 237.1127 $[M–H]^–$ (calcd. for $C_{13}H_{17}O_4$, 237.1127).

pos.	δ_C / ppm	δ_H / ppm (*J* / Hz)	HMBC (H to C)	^{1}H-^{1}H COSY
1	171.7	-	-	-
2	124.9	-	-	-
3	128.6	-	-	-
4	170.4	-	-	-
5	153.4	7.95, 1H, d (1.7)	1, 2, 3, 6, 7	6
6	80.8	4.99, 1H, m	1, 2, 5, 7, 8,	5, 7
7	33.4	1.79, 1H, m 1.69, 1H, m	5, 6, 8, 9	6, 8
8	25.1	1.46, 2H, m	6, 7, 9, 10	7, 9
9	29.1	1.34, 2H, m	7, 8, 10, 11	8, 10
10	31.7	1.28, 2H, m	8, 9, 11, 12	9, 11
11	22.6	1.29, 2H, m	9, 10, 12	10, 12
12	14.1	0.88, 3H, t (7.0)	10, 11	11
13	133.7	7.19, 1H, s 6.81, 1H, s	2, 3, 4	-

Table S3.9 ^{1}H NMR (600 MHz) data and ^{13}C NMR (150 MHz) data for **13** in $CDCl_3$.

Compound **13** was isolated as a white powder. The molecular formula $C_{13}H_{18}O_4$ in accordance with its HR-MS data, indicating 5 degrees of unsaturation. The 1D and 2D NMR data (Table S3.9) revealed that scaffold of compound **13** shares a high similarity with deoxysporothric acid **9**, except the absent of one aliphatic methylene group and one aliphatic methine group, but the presence of two sp2-hybridized carbons. Together with the molecular weight of **13** is 2 Da less than **9**, which suggests a double bond was formed to lose two hydrides. The ^{1}H-^{1}H COSY correlations of H5, H6 and H7, as well as the HMBC correlations from H5 to C1, C2, C3, C6, and C7, and the correlations from H6 and H13 to C2 indicate the double bond located at C2 and C5 (Figure S3.39). Thus the planar structure of **13** was solved. The absolute configuration of **13** was confirmed to be 6*R* by chemical conversion to isosporothric acid **10** (Figure S3.37). We designated **13** as dehydrodeoxysporothric acid.

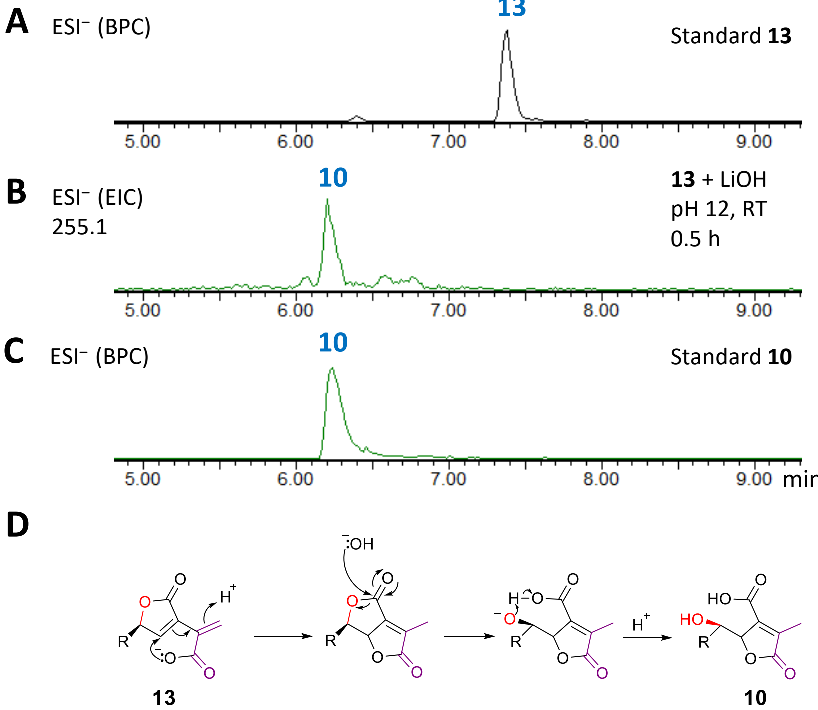


Figure S3.37 Structure transformation of **13**. ESI⁻ spectrum of standard **13** (**A**) and **10** (**C**); **B**, ESI⁻ spectrum of 1 mg **13** dissolved in 2 mL methanol then treated with 0.3 mL LiOH (1 M) to pH 12, RT for 0.5 h; **D**, Proposed chemical conversion steps from **13** to **10**.

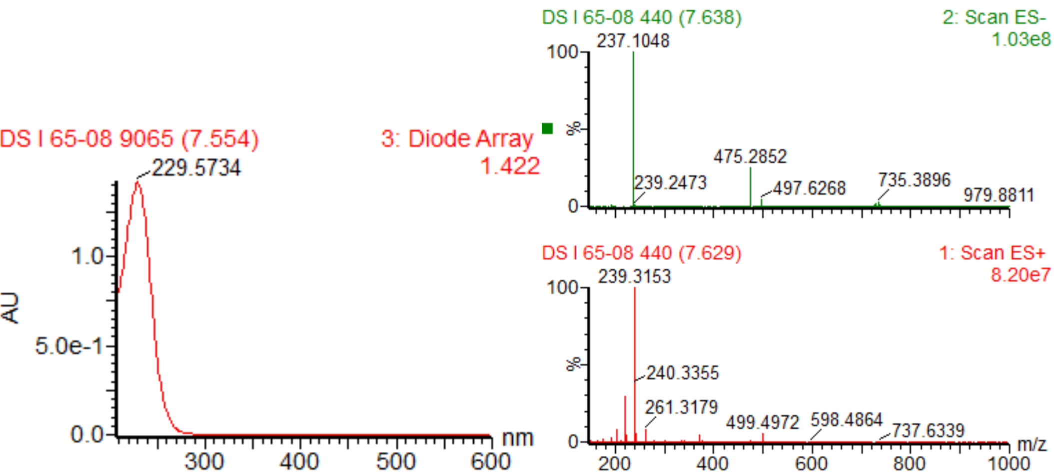


Figure S3.38 UV spectrum and ESI spectrum for **13**.

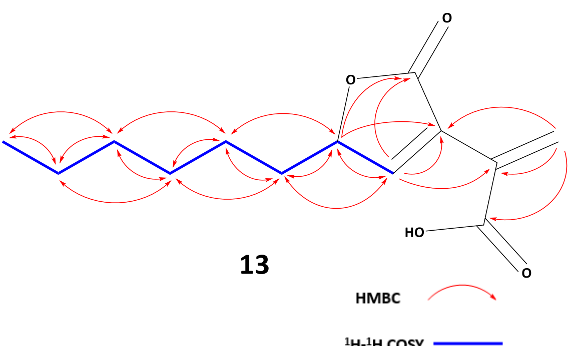


Figure S3.39 Key HMBC and ^{1}H-^{1}H COSY correlations of **13**.

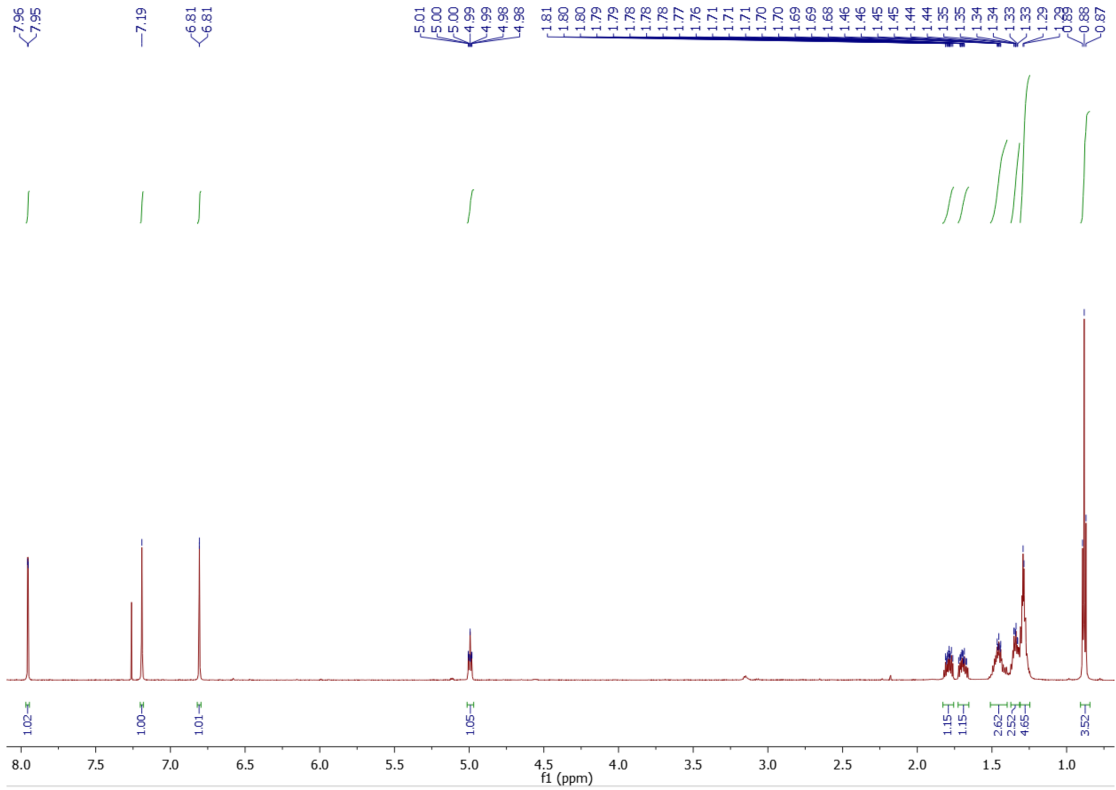


Figure S3.40 ^{1}H NMR of compound **13**.

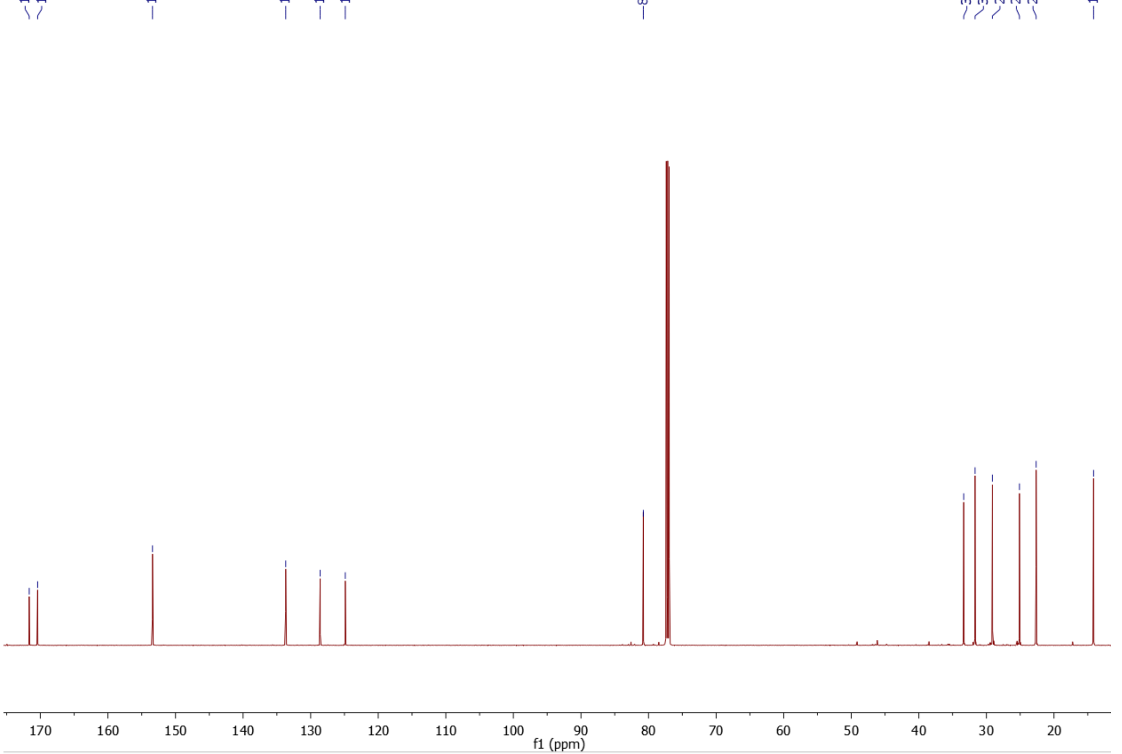


Figure S3.41 ^{13}C NMR of compound **13**.

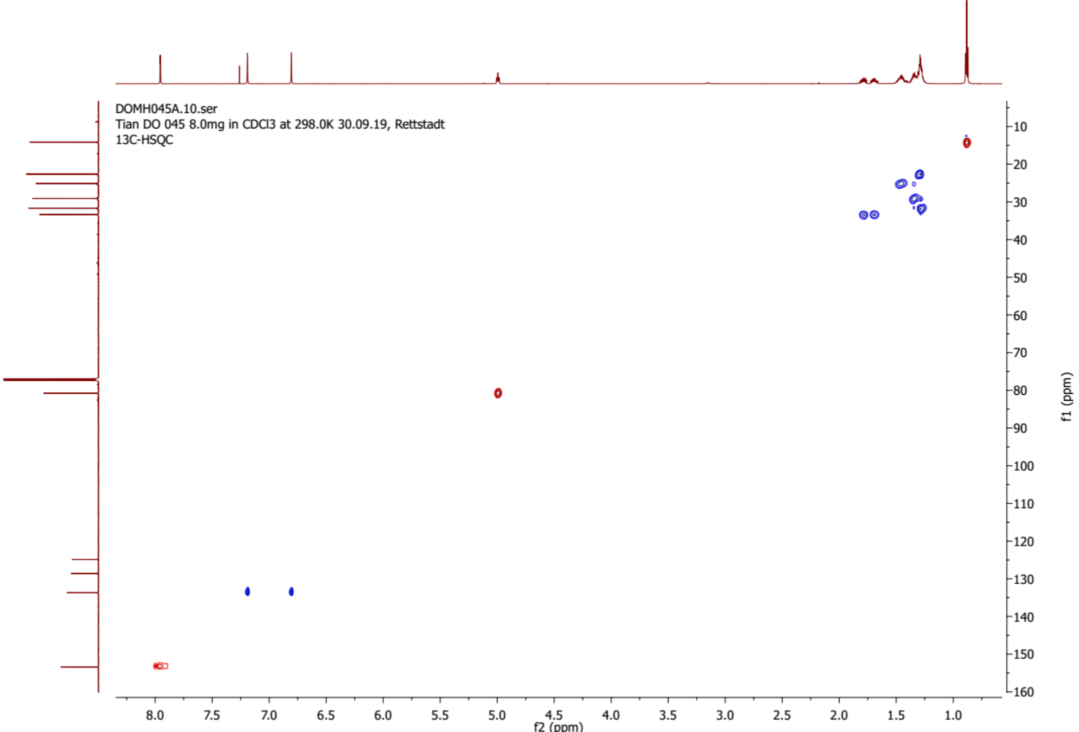


Figure S3.42 HSQC of compound **13**.

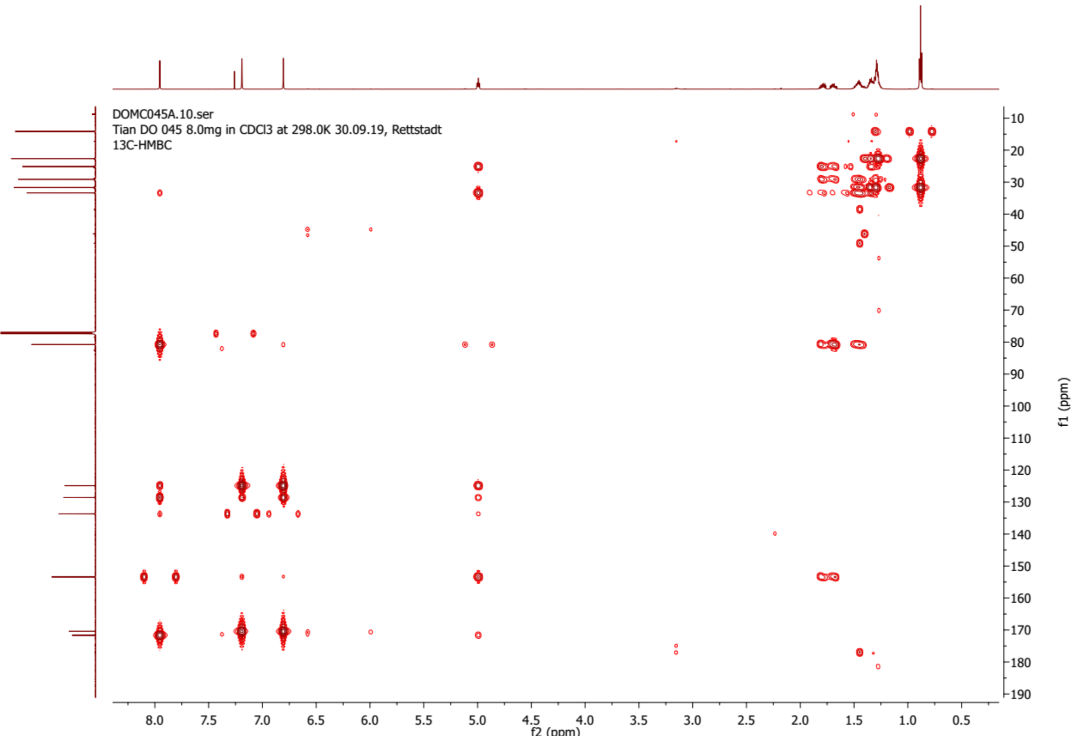


Figure S3.43 HMBC of compound **13**.

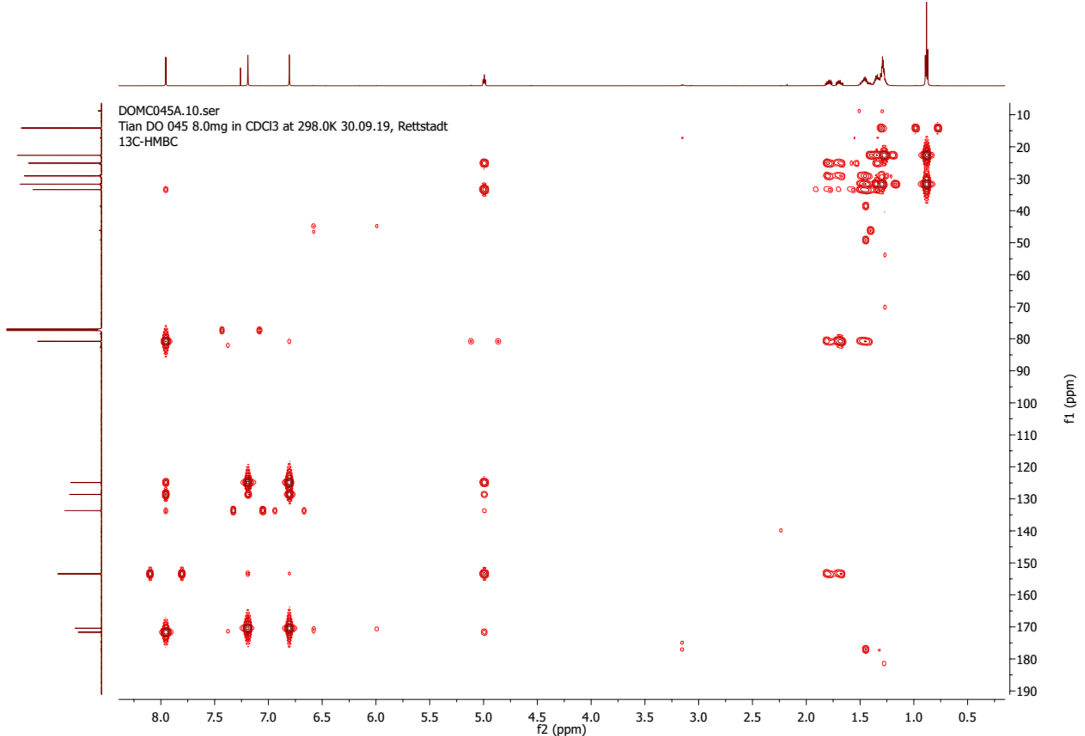
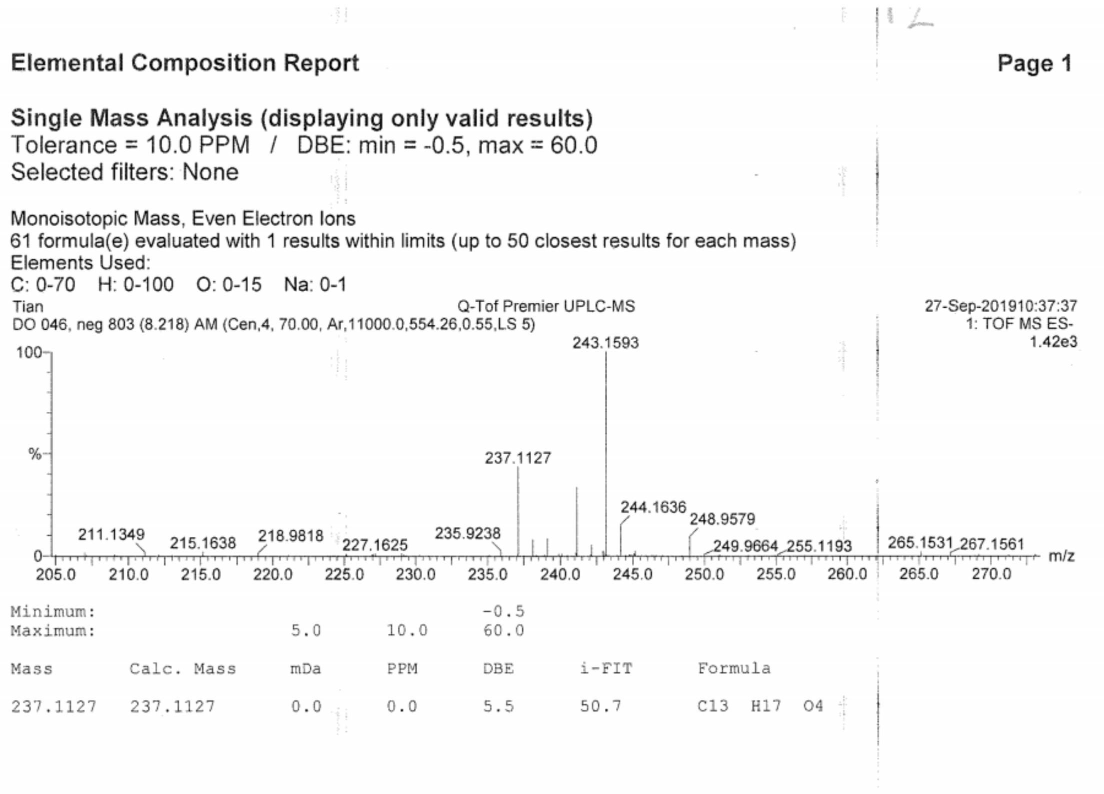


Figure S3.44 ^{1}H-^{1}H COSY of compound **13**.



Elemental Composition Report **Page 1**

Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 PPM / DBE: min = -0.5, max = 60.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
61 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-70 H: 0-100 O: 0-15 Na: 0-1

Tian Q-Tof Premier UPLC-MS 27-Sep-201910:37:37
DO 046, neg 803 (8.218) AM (Cen,4, 70.00, Ar,11000.0,554.26,0.55,LS 5) 1: TOF MS ES- 1.42e3

Minimum:				-0.5		
Maximum:		5.0	10.0	60.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
237.1127	237.1127	0.0	0.0	5.5	50.7	C13 H17 O4

Figure S3.45 HRMS data for compound **13**.

Compound 14 (proposed structure)

14

Chemical formula: $C_{13}H_{20}O_5$

UV (λ_{max}): 203 nm. ESI-MS *m/z* 237 $[M–H_2O–H]^–$, 255 $[M–H]^–$, 511 $[2M–H]^–$, 257 $[M+H]^+$, 513 $[2M+H]^+$. HR-ESI-MS *m/z* 255.1234 $[M–H]^–$ (calcd. for $C_{13}H_{19}O_5$, 255.1232).

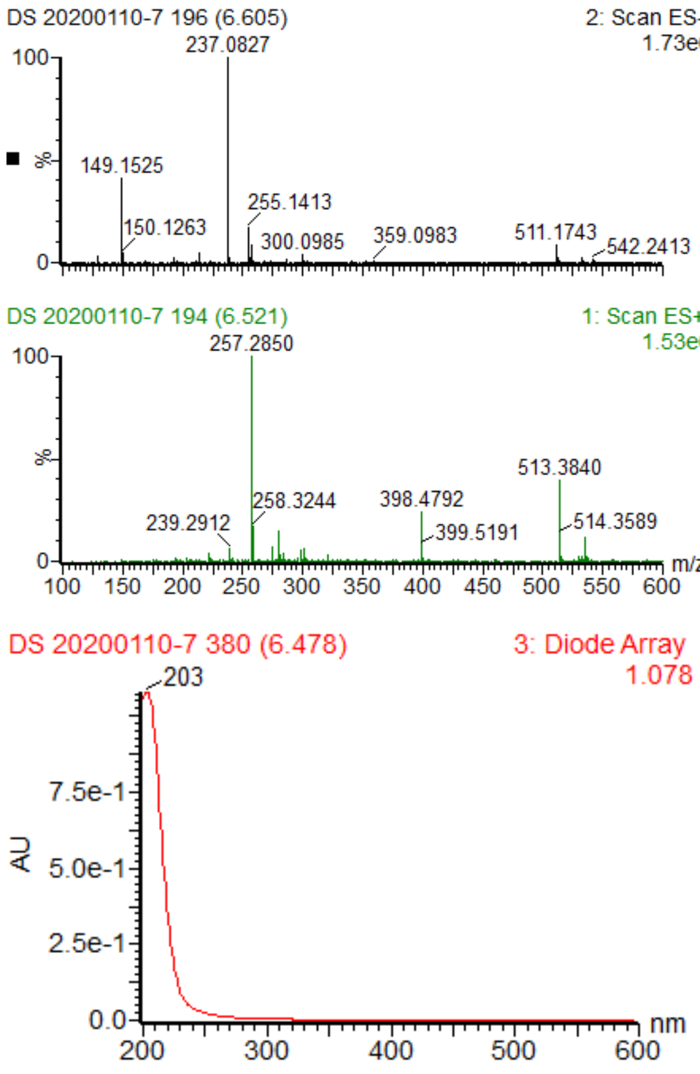


Figure S3.46 UV spectrum and ESI spectrum for **14**.

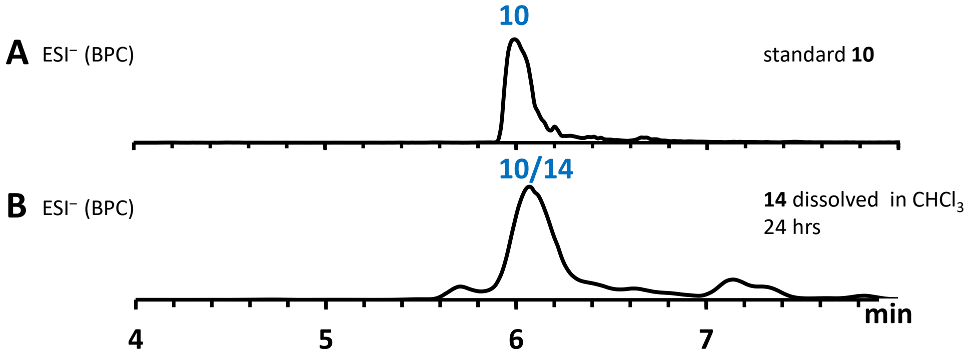
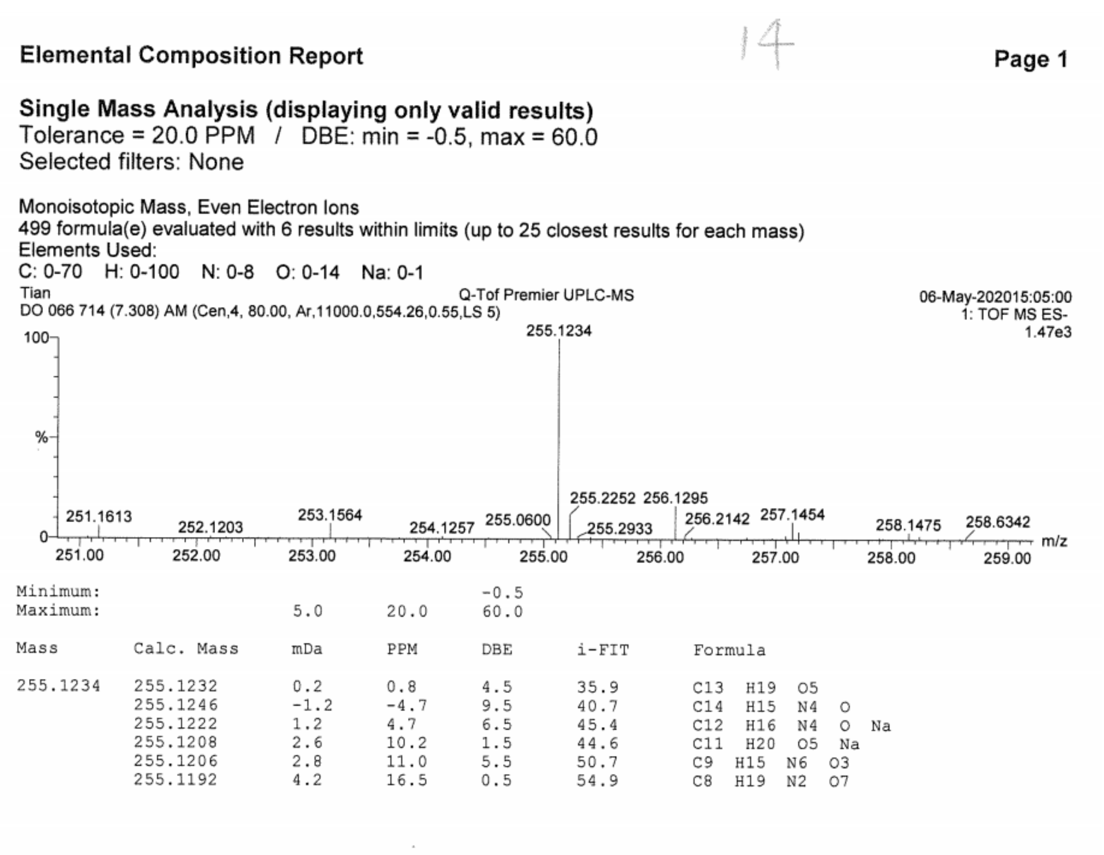


Figure S3.47 **A**, ESI spectrum of standard **10**; **B**, ESI spectrum of standard **14** dissolved in $CHCl_3$ for 24 hrs.



Elemental Composition Report 14 **Page 1**

Single Mass Analysis (displaying only valid results)
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 60.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
499 formula(e) evaluated with 6 results within limits (up to 25 closest results for each mass)
Elements Used:
C: 0-70 H: 0-100 N: 0-8 O: 0-14 Na: 0-1
Tian Q-Tof Premier UPLC-MS 06-May-202015:05:00
DO 066 714 (7.308) AM (Cen,4, 80.00, Ar,11000.0,554.26,0.55,LS 5) 1: TOF MS ES-
1.47e3

		mDa	PPM	DBE		
Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
255.1234	255.1232	0.2	0.8	4.5	35.9	C13 H19 O5
	255.1246	-1.2	-4.7	9.5	40.7	C14 H15 N4 O
	255.1222	1.2	4.7	6.5	45.4	C12 H16 N4 O Na
	255.1208	2.6	10.2	1.5	44.6	C11 H20 O5 Na
	255.1206	2.8	11.0	5.5	50.7	C9 H15 N6 O3
	255.1192	4.2	16.5	0.5	54.9	C8 H19 N2 O7

Figure S3.48 HRMS data for compound **14**.

Compound 15

15

Sporodride A

Chemical formula: $C_{13}H_{20}O_4$

White powder; $[\alpha]^{21}_{D}$ – 6 (*c* = 0.32, $CHCl_3$); UV (λ_{max}): 211 nm. ^{13}C NMR data ($CDCl_3$, 100 MHz): δ_C 175.9 (C-1), 164.6 (C-4), 132.5 (C-13), 130.9 (C-3), 77.4 (C-6), 42.3 (C-2), 35.7 (C-7), 31.8 (C-10), 30.7 (C-5), 29.2 (C-9), 24.8 (C-8), 22.7 (C-11), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 400 MHz): δ_H 6.59 (1H, s, H-13a), 5.87 (1H, s, H-13b), 4.55 (1H, m, H-6), 3.72 (1H, m, H-2), 2.31 (1H, dt, *J* = 2.6, 14.4 Hz, H-5b), 1.90 (1H, ddd, *J* = 5.8, 11.1, 14.4 Hz, H-5a), 1.71 (1H, m, H-7b), 1.60 (1H, m, H-7a), 1.50 (1H, m, H-8a), 1.39 (1H, m, H-8b), 1.29 (2H, m, H-11), 1.28 (2H, m, H-9), 1.27 (2H, m, H-10), 0.88 (3H, t, *J* = 6.8 Hz, H-12); NMR data see table S3.10; ESI-MS *m/z* 239 $[M–H]^–$, 479 $[2M–H]^–$, 241 $[M+H]^+$, 263 $[M+Na]^+$. HR-ESI-MS *m/z* 239.1282 $[M–H]^–$ (calcd. for $C_{13}H_{19}O_4$, 239.1283).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	HMBC (H to C)	^{1}H-^{1}H COSY	NOESY
1	175.9	-	-	-	-
2	42.3	3.72, 1H, m	1, 3, 4, 5, 6, 13	5	6
3	130.9	-	-	-	-
4	164.6	-	-	-	-
5	30.7	1.90, 1H, ddd (5.8, 11.1, 14.4) 2.31, 1H, dt (2.6, 14.4)	1, 2, 6, 7	2, 6	6, 13
6	77.4	4.55, 1H, m	2, 7, 8	5, 7	2, 5, 13
7	35.7	1.60, 1H, m 1.71, 1H, m	5, 6, 8, 9	6, 8	-
8	24.8	1.50, 1H, m 1.39, 1H, m	7, 9, 10	7, 9	-
9	29.2	1.28, 2H, m	8, 10, 11	8, 10	-
10	31.8	1.27, 2H, m	9, 11	9, 11	-
11	22.7	1.29, 2H, m	10, 12	10, 12	-
12	14.2	0.88, 3H, t (6.8)	10, 11	11	-
13	132.5	6.59, 1H, s 5.87, 1H, s	2, 4	-	5, 6

Table S3.10 ^{1}H NMR (400 MHz) data and ^{13}C NMR (100 MHz) data for **15** in $CDCl_3$.

Compound **15** was isolated as a white powder**,** with molecular formula $C_{13}H_{20}O_4$ (calc. $[M-H]^-$ HRMS 239.1283, measured 239.1282) which is same as deoxysporothric acid **9**, also indicating 4 degrees of unsaturation. Analysis the 1D and 2D NMR data (Table S3.10) revealed that **15** contains the same number of methyl, methylene, methine, and quaternary carbons as **9**, in addition the proton and carbon chemical shifts are just slightly shift compared with **9**. This suggests **15** to be an isomer of **9**. However, the key evidence of the lactone scaffold with HMBC correlations from H6 to C1 and C4 was not shown in **15** (Figure S3.50). This information suggest the only possibility of an anhydride scaffold for **15**. NOESY correlations of H13 and H5, as well H13 and H6 show the rotation of the five-membered anhydride ring. In addition the NOESY correlation of H2 and H6 establish the relative configuration of **15** (Figure S3.51). We designated **15** as sporodride A, to serve as the first sporothriolides anhydride structure.

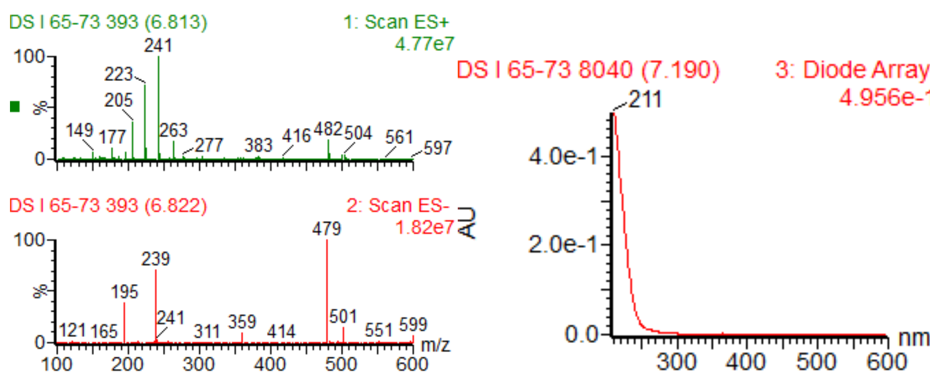


Figure S3.49 UV spectrum and ESI spectrum for **15**.

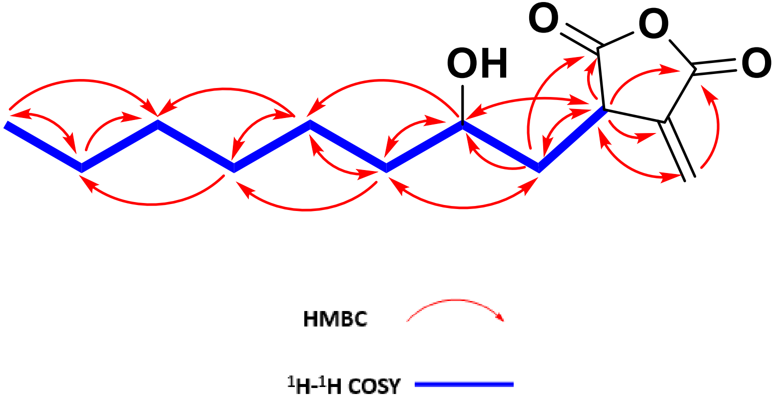


Figure S3.50 Key HMBC and ^{1}H-^{1}H COSY correlations of **15**.

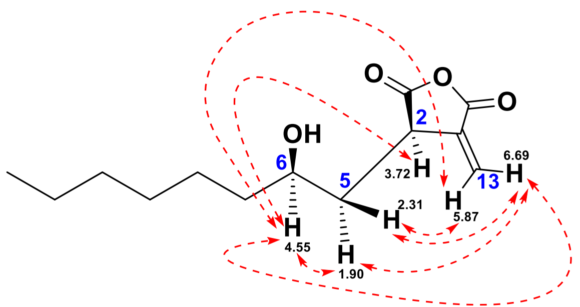


Figure S3.51 Key NOESY correlations of **15**.

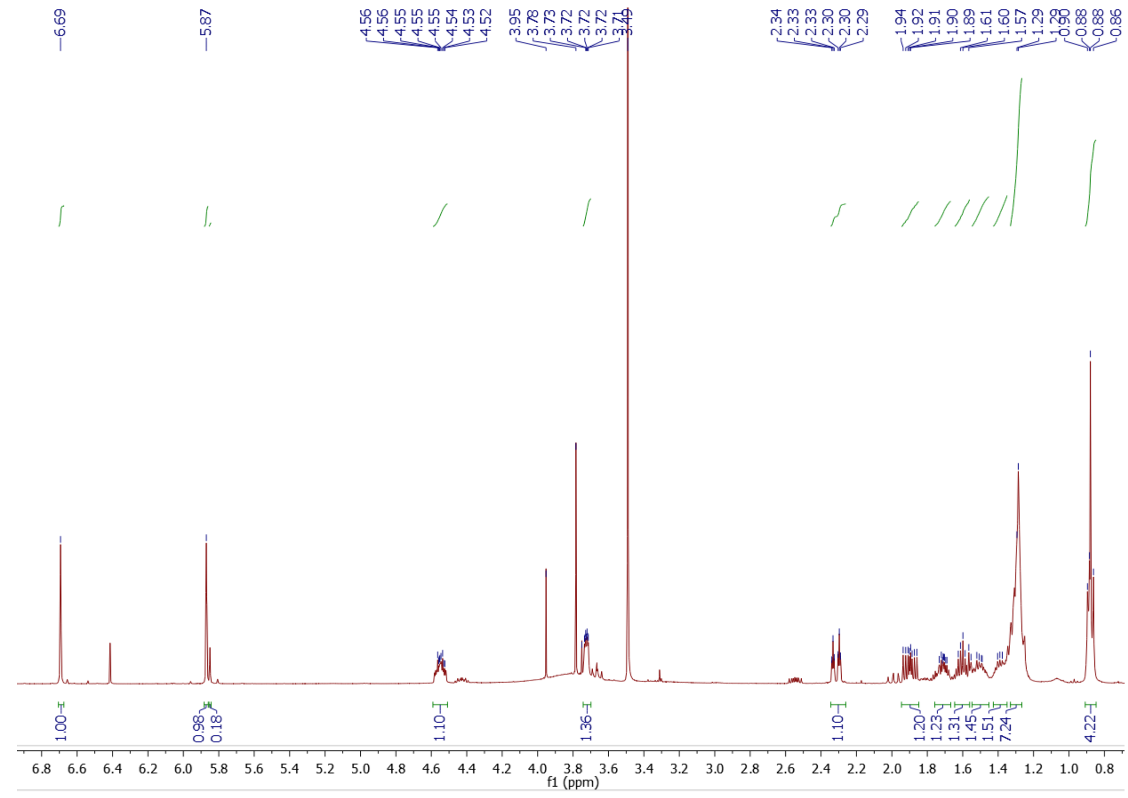


Figure S3.52 ^{1}H NMR of compound **15**.

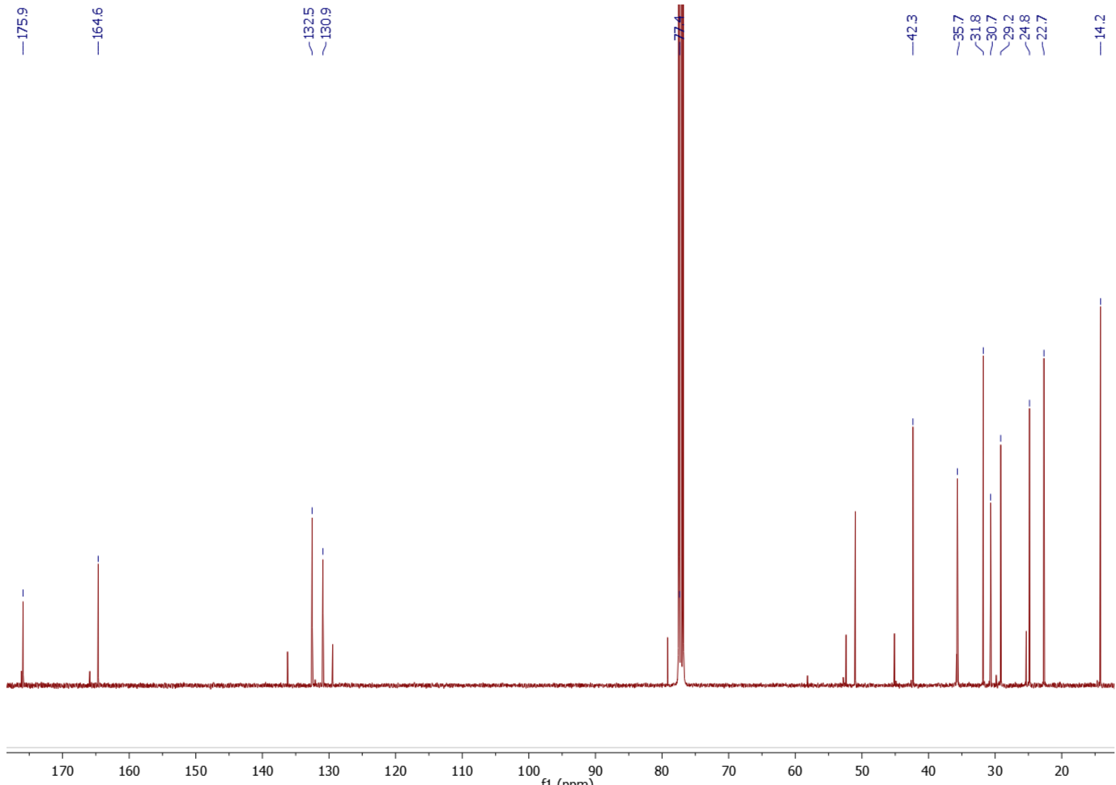


Figure S3.53 ^{13}C NMR of compound **15**.

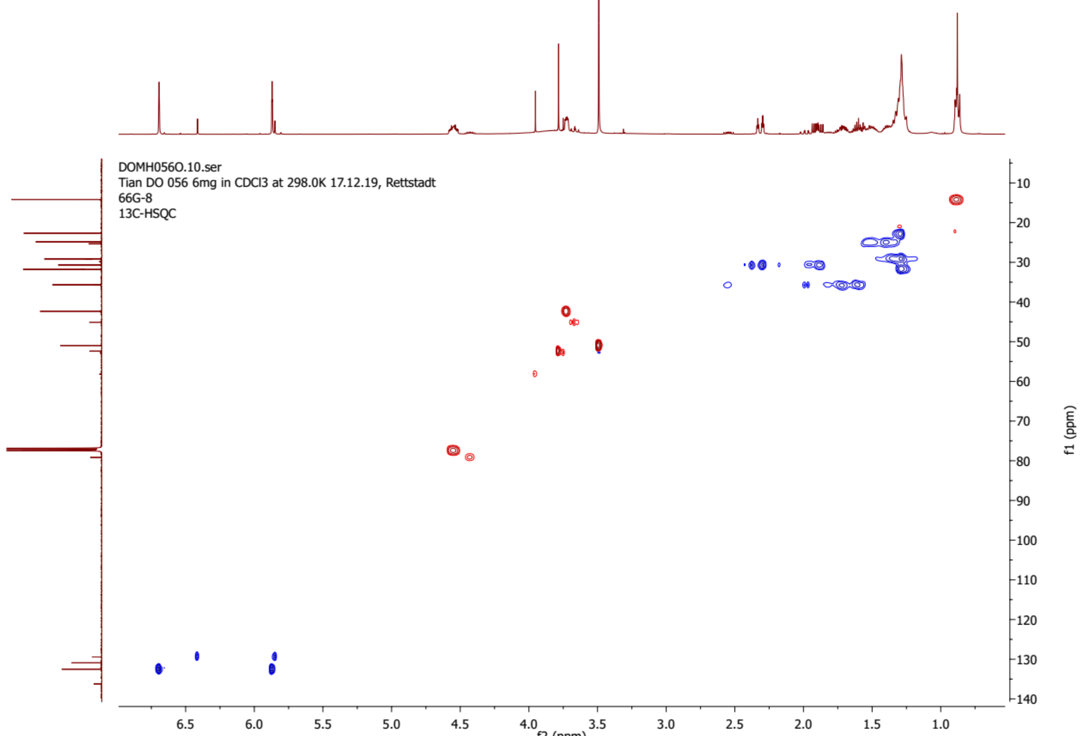


Figure S3.54 HSQC of compound **15**.

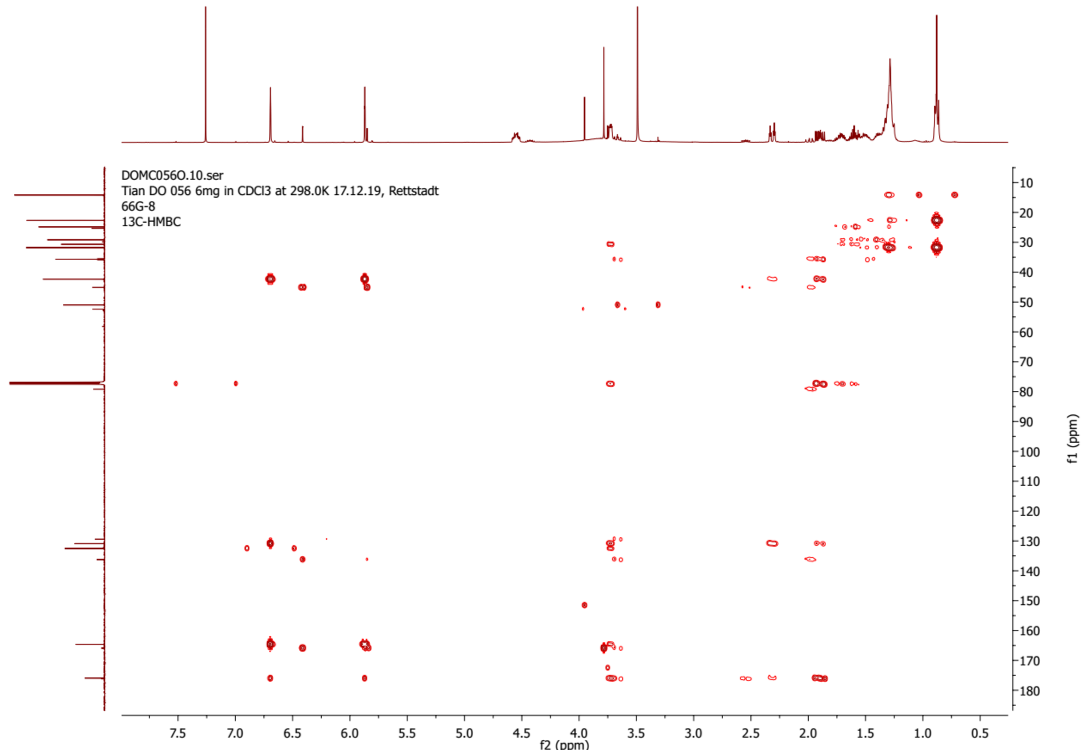


Figure S3.55 HMBC of compound **15**.

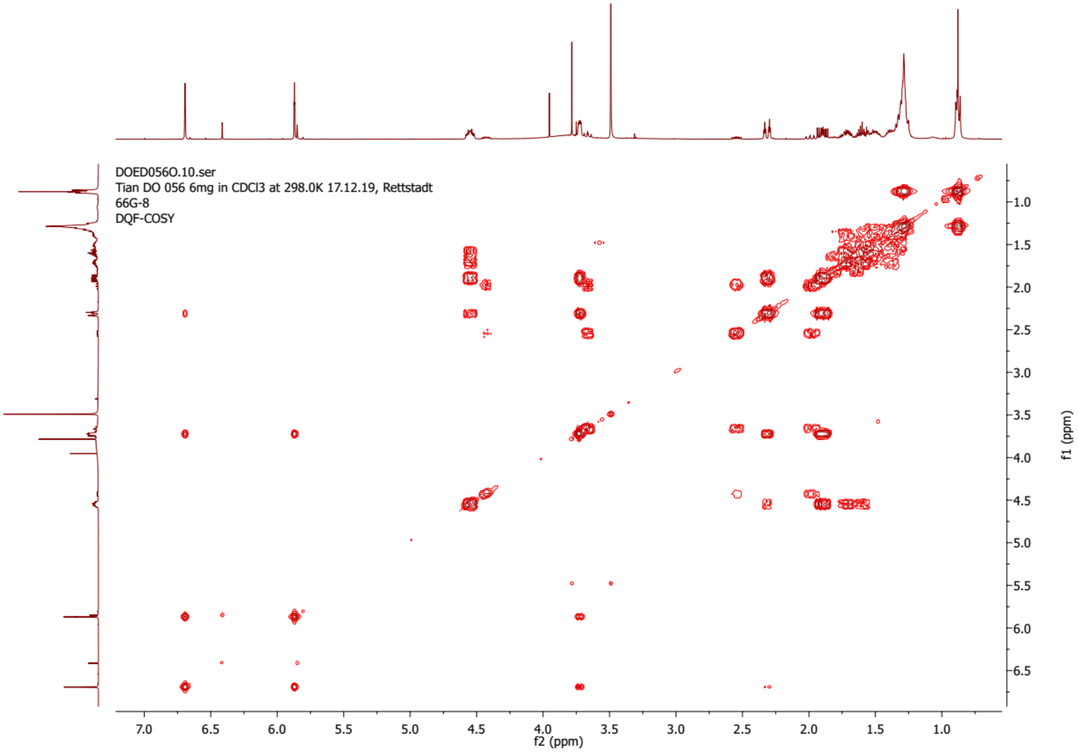


Figure S3.56 ^{1}H-^{1}H COSY of compound **15**.

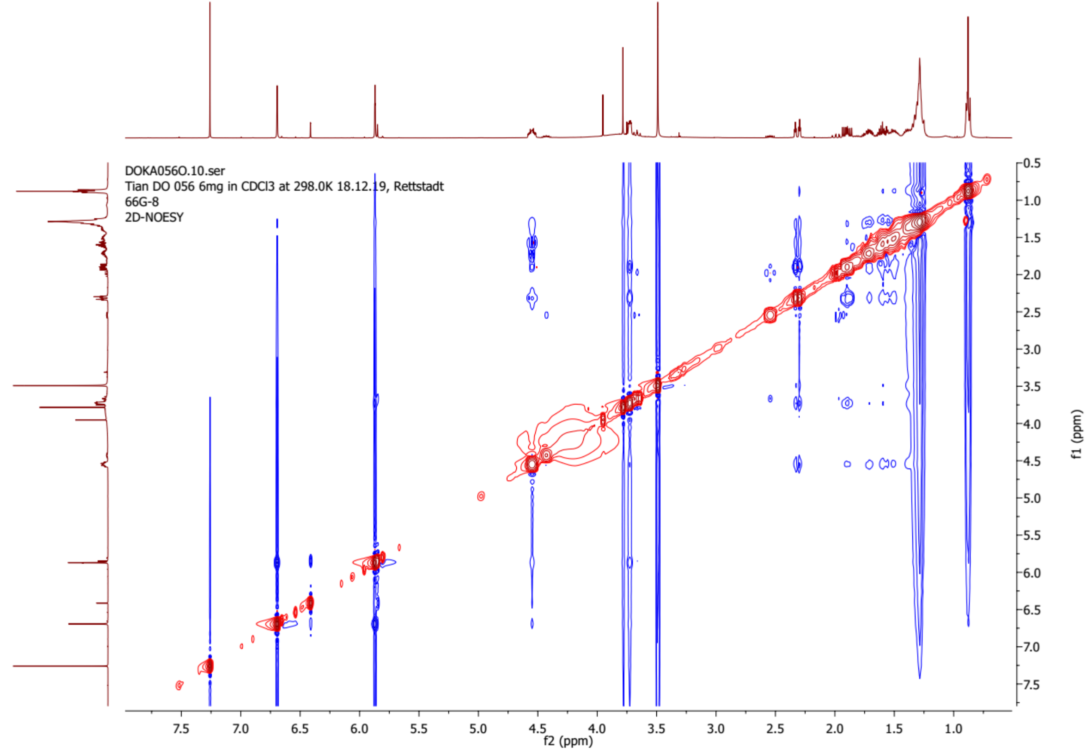
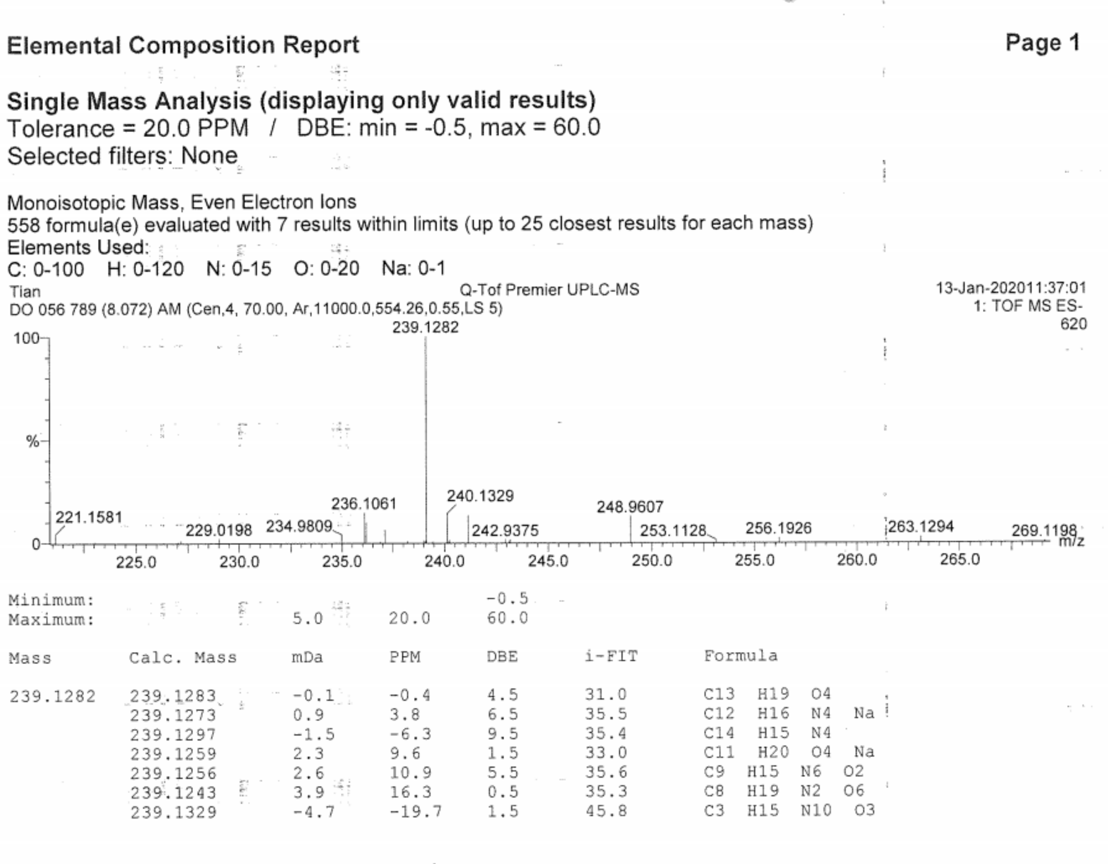


Figure S3.57 NOESY of compound **15**.



Elemental Composition Report Page 1

Single Mass Analysis (displaying only valid results)

Tolerance = 20.0 PPM / DBE: min = -0.5, max = 60.0

Selected filters: None

Monoisotopic Mass, Even Electron Ions

558 formula(e) evaluated with 7 results within limits (up to 25 closest results for each mass)

Elements Used:

C: 0-100 H: 0-120 N: 0-15 O: 0-20 Na: 0-1

Tian Q-Tof Premier UPLC-MS 13-Jan-202011:37:01

DO 056 789 (8.072) AM (Cen,4, 70.00, Ar,11000.0,554.26,0.55,LS 5) 1: TOF MS ES- 620

	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
239.1282	239.1283	-0.1	-0.4	4.5	31.0	C13 H19 O4
	239.1273	0.9	3.8	6.5	35.5	C12 H16 N4 Na
	239.1297	-1.5	-6.3	9.5	35.4	C14 H15 N4
	239.1259	2.3	9.6	1.5	33.0	C11 H20 O4 Na
	239.1256	2.6	10.9	5.5	35.6	C9 H15 N6 O2
	239.1243	3.9	16.3	0.5	35.3	C8 H19 N2 O6
	239.1329	-4.7	-19.7	1.5	45.8	C3 H15 N10 O3

Figure S3.58 HRMS data for compound **15**.

Compound 16 and 23

23

Hydroxyalkylitaconic acid A
Chemical formula: $C_{13}H_{22}O_5$

16

Epideoxyisosporothric acid
Chemical formula: $C_{13}H_{20}O_4$

Compound **23**

White powder; $[\alpha]^{21}_{D}$ – 55 (*c* = 0.12, $CHCl_3$); UV (λ_{max}): 203 nm; ^{13}C NMR data ($CDCl_3$, 100 MHz): δ_C 177.6 (C-1), 169.1 (C-4), 132.0 (C-13), 135.4 (C-3), 34.7 (C-6), 53.5 (C-2), 25.5 (C-7), 31.9 (C-10), 73.8 (C-5), 29.5 (C-9), 29.4 (C-8), 22.8 (C-11), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 400 MHz): δ_H 6.56 (1H, s, H-13a), 5.98 (1H, s, H-13b), 1.43-1.51 (2H, m, H-6), 3.54 (1H, d, *J* = 6.8 Hz, H-2), 4.13 (1H, m, H-5), 1.21-1.35 (10H, m, H-7/H-8/H-9/H-10/H-11), 0.87 (3H, t, *J* = 6.5 Hz, H-12); NMR data see table S3.11; ESI-MS *m/z* 257 $[M–H]^–$, 515 $[2M–H]^–$, 259 $[M+H]^+$, 517 $[2M+H]^+$. HR-ESI-MS *m/z* 257.1386 $[M–H]^–$ (calcd. for $C_{13}H_{21}O_5$, 257.1389).

Compound **16**

White powder; $[\alpha]^{21}_{D}$ – 91 (*c* = 0.35, $CHCl_3$); UV (λ_{max}): 211 nm; ^{13}C NMR data ($CDCl_3$, 125 MHz): δ_C 170.8 (C-1), 169.0 (C-4), 133.7 (C-3), 125.4 (C-13), 78.2 (C-5), 48.9 (C-2), 31.8 (C-10), 31.6 (C-6), 29.3 (C-9), 29.2 (C-8), 25.7 (C-7), 22.7 (C-11), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 500 MHz): δ_H 6.43 (1H, s, H-13a), 5.87 (1H, s, H-13b), 4.65 (1H, m, H-5), 4.00 (1H, dt, *J* = 2.2, 7.7 Hz, H-2), 1.71 (2H, m, H-6), 1.55 (1H, m, H-7b), 1.41 (1H, m, H-7a), 1.32 (2H, m, H-9), 1.29 (2H, m, H-8), 1.28 (2H, m, H-11), 1.26 (2H, m, H-10), 0.88 (3H, t, *J* = 7.2 Hz, H-12); NMR data see table S3.11; ESI-MS *m/z* 239 $[M–H]^–$, 479 $[2M–H]^–$, 241 $[M+H]^+$, 481 $[2M+H]^+$, 503 $[2M+Na]^+$. HR-ESI-MS *m/z* 239.1282 $[M–H]^–$ (calcd. for $C_{13}H_{19}O_4$, 239.1283).

pos.	23		16			
	δ_C /ppm	δ_H / ppm (*J* / Hz)	δ_C / ppm	δ_H / ppm (*J* / Hz)	HMBC	^{1}H-^{1}H COSY
1	177.6	-	170.8	-	-	-
2	53.5	3.54, 1H, d (6.8)	48.9	4.00, 1H, dt (2.2, 7.7)	1, 3, 5, 6, 13	5
3	135.4	-	133.7	-	-	-
4	169.1	-	169.0	-	-	-
5	73.8	4.13, 1H, m	78.2	4.65, 1H, m	1, 2, 3, 6, 7	2, 6
6	34.7	1.43-1.51, 2H, m	31.6	1.71, 2H, m	2, 5, 7, 8	5, 7
7	25.5	1.21-1.35, 2H, m	25.7	1.41, 1H, m 1.55, 1H, m	5, 6, 8, 9	6, 8
8	29.4	1.21-1.35, 2H, m	29.2	1.29, 2H, m	6, 7, 10	7, 9
9	29.5	1.21-1.35, 2H, m	29.3	1.32, 2H, m	7, 8	8, 10
10	31.9	1.21-1.35, 2H, m	31.8	1.26, 2H, m	8, 9, 11	9, 11
11	22.8	1.21-1.35, 2H, m	22.7	1.28, 2H, m	10, 12	10, 12
12	14.2	0.87, 3H, t (6.5)	14.2	0.88, 3H, t (7.2)	10, 11	11
13	132.0	6.56, 1H, s 5.98, 1H, s	125.4	6.43, 1H, d (2.1) 5.87, 1H, d (2.1)	2, 3, 4	-

Table S3.11 ^{1}H NMR (400 MHz) data and ^{13}C NMR (100 MHz) data for **23** in $CDCl_3$. ^{1}H NMR (500 MHz) data and ^{13}C NMR (125 MHz) data for **16** in $CDCl_3$.

Mono-hydroxyl **23** were purified and submitted for NMR measurement, it was interesting that **23** always cyclize spontaneously to **16** in a short time (Figure S3.60), although the NMR measuring was operated right away. We could obtain the clean 1H-NMR of **23**, but the 13C-NMR of **23** was a mixture of **23** and **16**, the afterwards 2D-NMR measurement resulted in pure **16**, which suggest a fully convert from **23** to **16**.

Compound **23** was isolated as a white powder**,** with molecular formula $C_{13}H_{22}O_5$ (calc. [M – H]$^-$ HRMS 257.1389, measured 257.1386), indicating 3 degrees of unsaturation. Compound **16** was a white powder**,** with molecular formula $C_{13}H_{20}O_4$ (calc. [M – H]$^-$ HRMS 239.1283, measured 239.1282), indicating **16** was a dehydrate form result from **23**. Extensive analysis the 1D and 2D NMR (Table S3.11) of **16** showed that the structure was quite similar with deoxyisosporothric acid[31], while there is a loss of one methyl and one aromatic methine, but the addition of one aromatic quaternary carbon and one aromatic methylene. The molecular weight of **16** is also identical with deoxyisosporothric acid, indicating the intramolecular double bond shifted outside the lactone ring led to **16**. HMBC correlations from H13 to C2, C3, and C4, from H2 to C1, C3, C5, C6, and C13, as well correlations from H2, H5 and H13 to C3 support this speculation (Figure S3.60). The ^{1}H-^{1}H COSY correlations of H5, H6, and H7 make a further confirmation (Figure S3.60). The relative configuration of **16** was assigned by NOESY correlations of H2 and H5, suggesting two protons are located on the same orientation (Figure S3.61). Compound **23** is the precursor of **16**, thus a reasonable scaffold for **23** was proposed. The carbon and proton signals for **16** could be easily assigned based on **23**. In addition the relative configuration of **16** should keep consistent with **23**.

16 was designated as epideoxyisosporothric acid as its double bond isomer of deoxyisosporothric acid. **23** is defined as hydroxyalkylitaconic acid A.

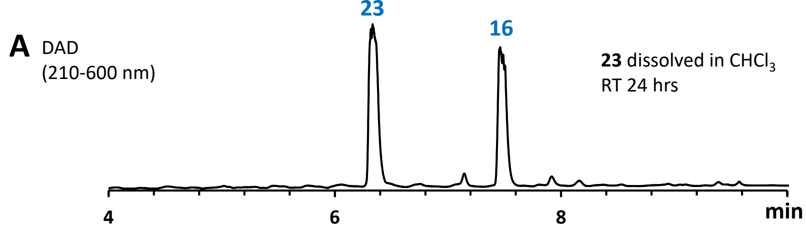


Figure S3.59 HPLC chromatogram (DAD) of compound **23** transformed to compound **16** spontaneously.

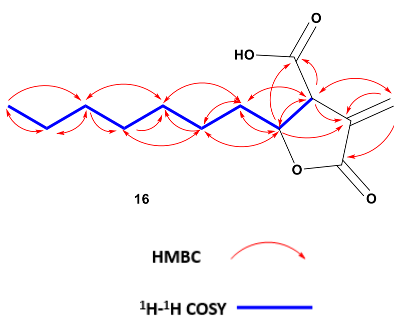


Figure S3.60 Key HMBC and ^{1}H-^{1}H COSY correlations of **16**.

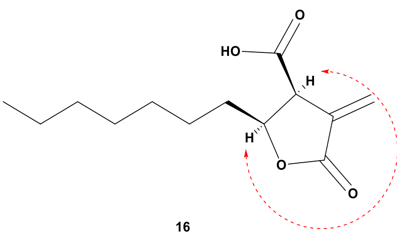


Figure S3.61 Key NOESY correlations of **16**.

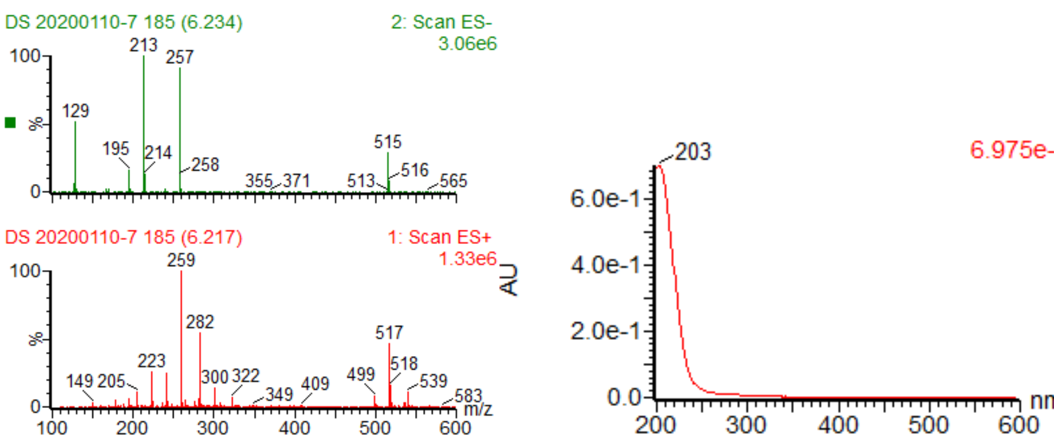


Figure S3.62 UV spectrum and ESI spectrum for **23**.

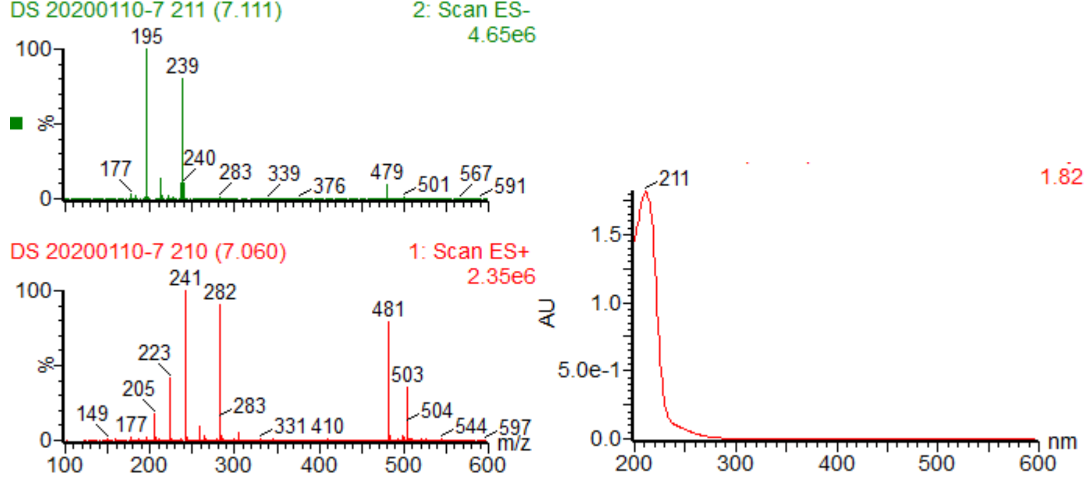


Figure S3.63 UV spectrum and ESI spectrum for **16**.

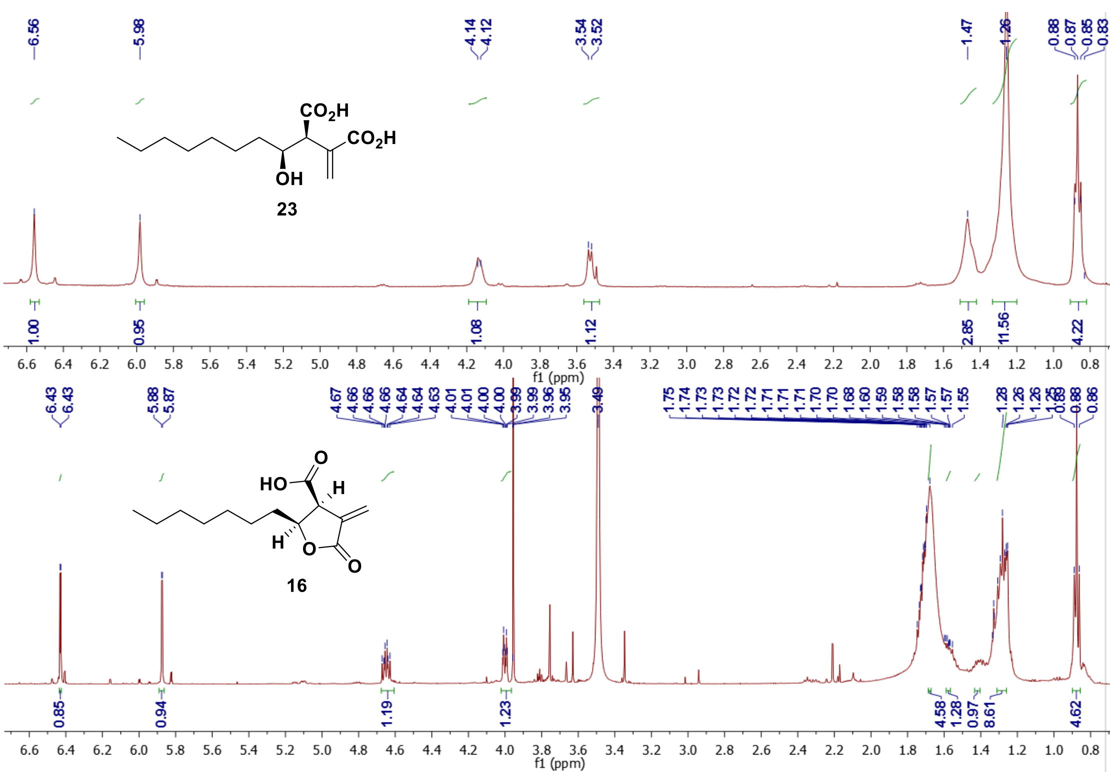


Figure S3.64 ^{1}H NMR of compound **23** and **16.**

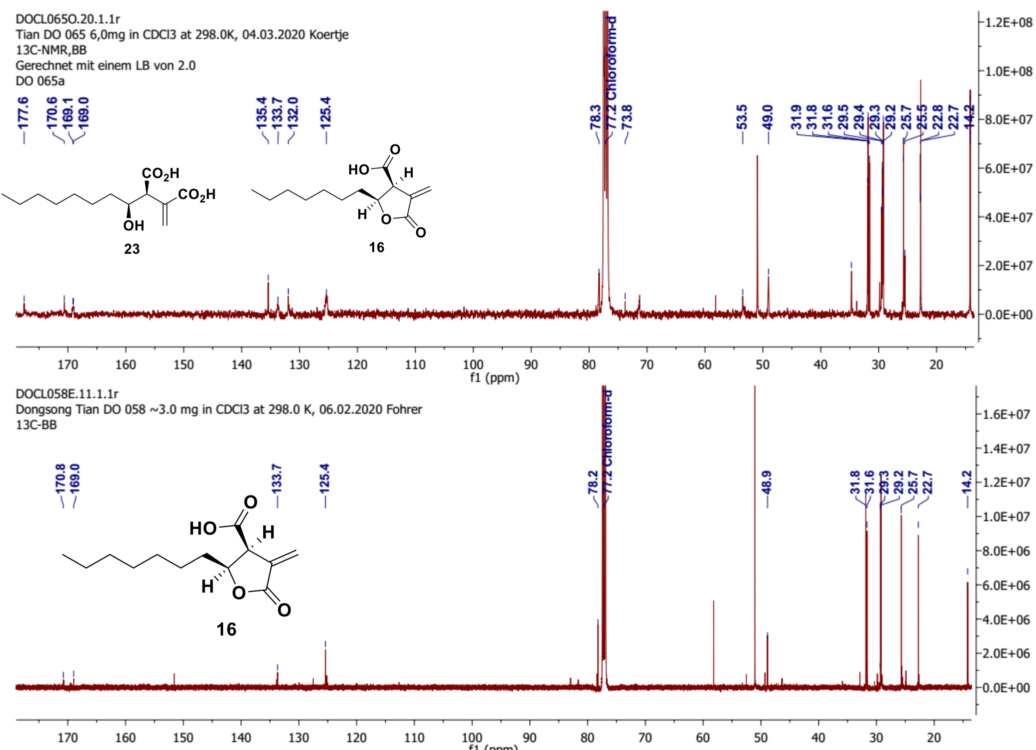


Figure S3.65 ^{13}C NMR of compound **23** and **16.**

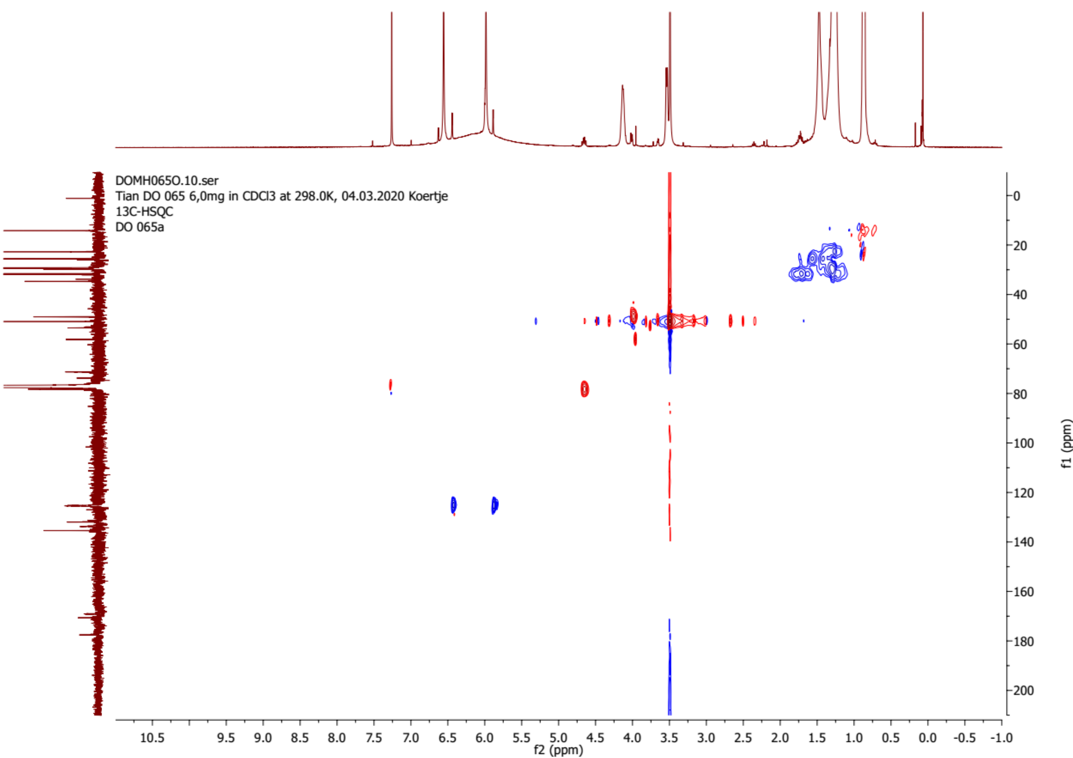


Figure S3.66 HSQC of compound **16**.

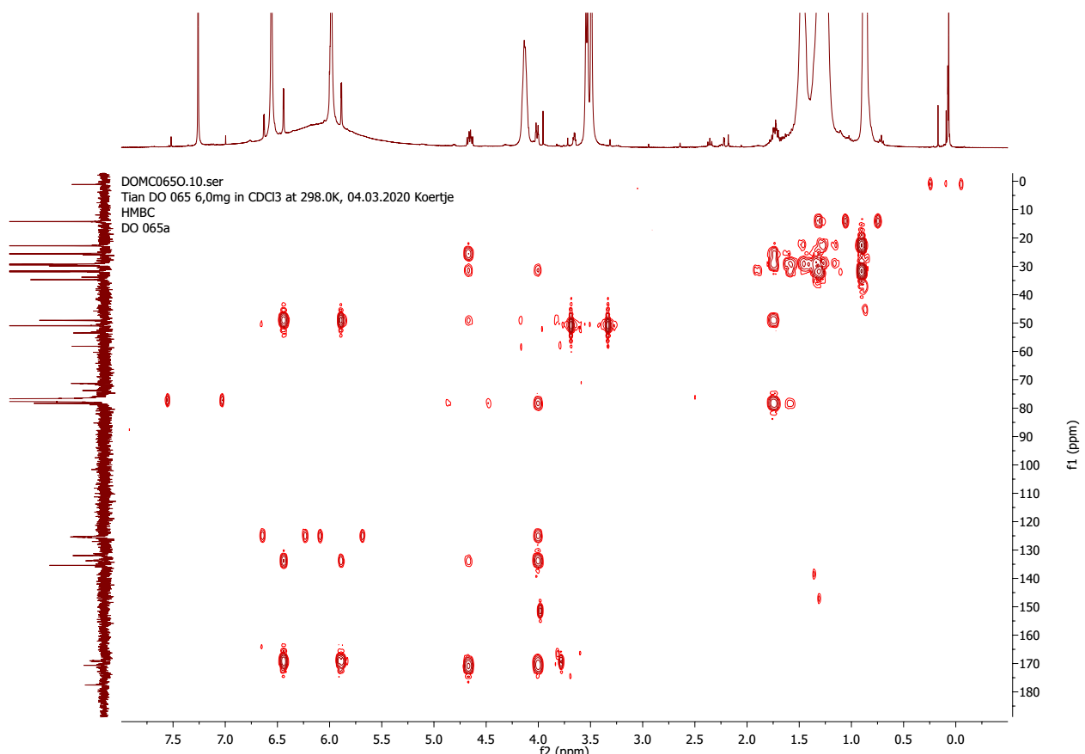


Figure S3.67 HMBC of compound 16.

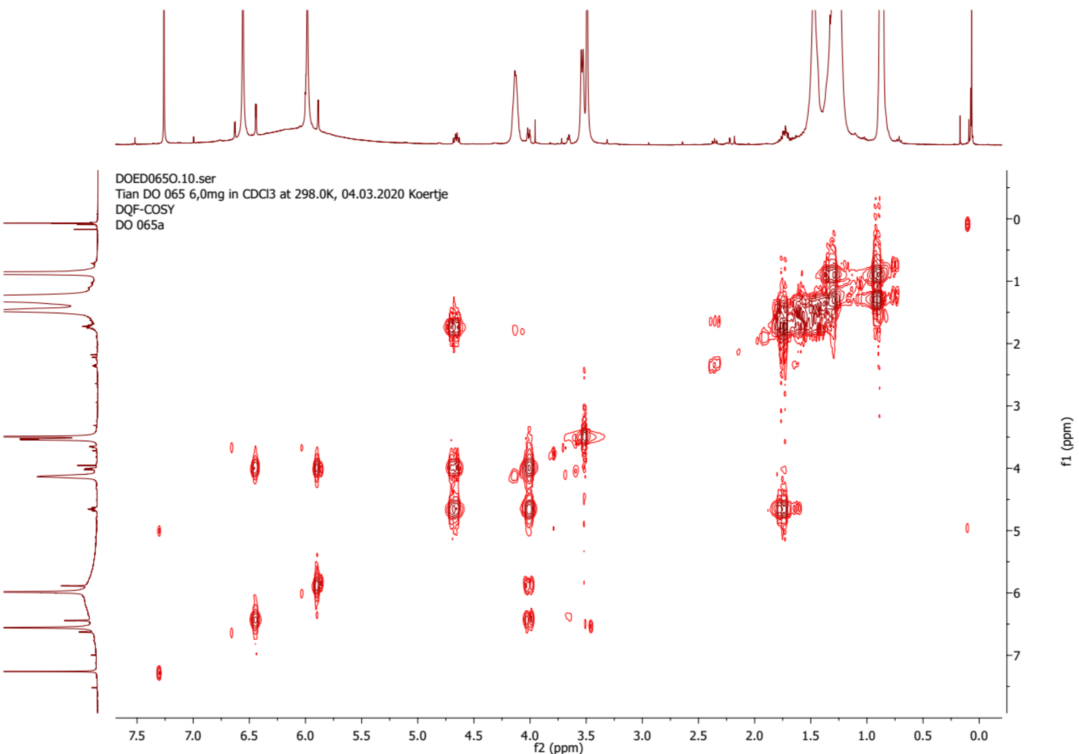


Figure S3.68 ^{1}H-^{1}H COSY of compound **16**.

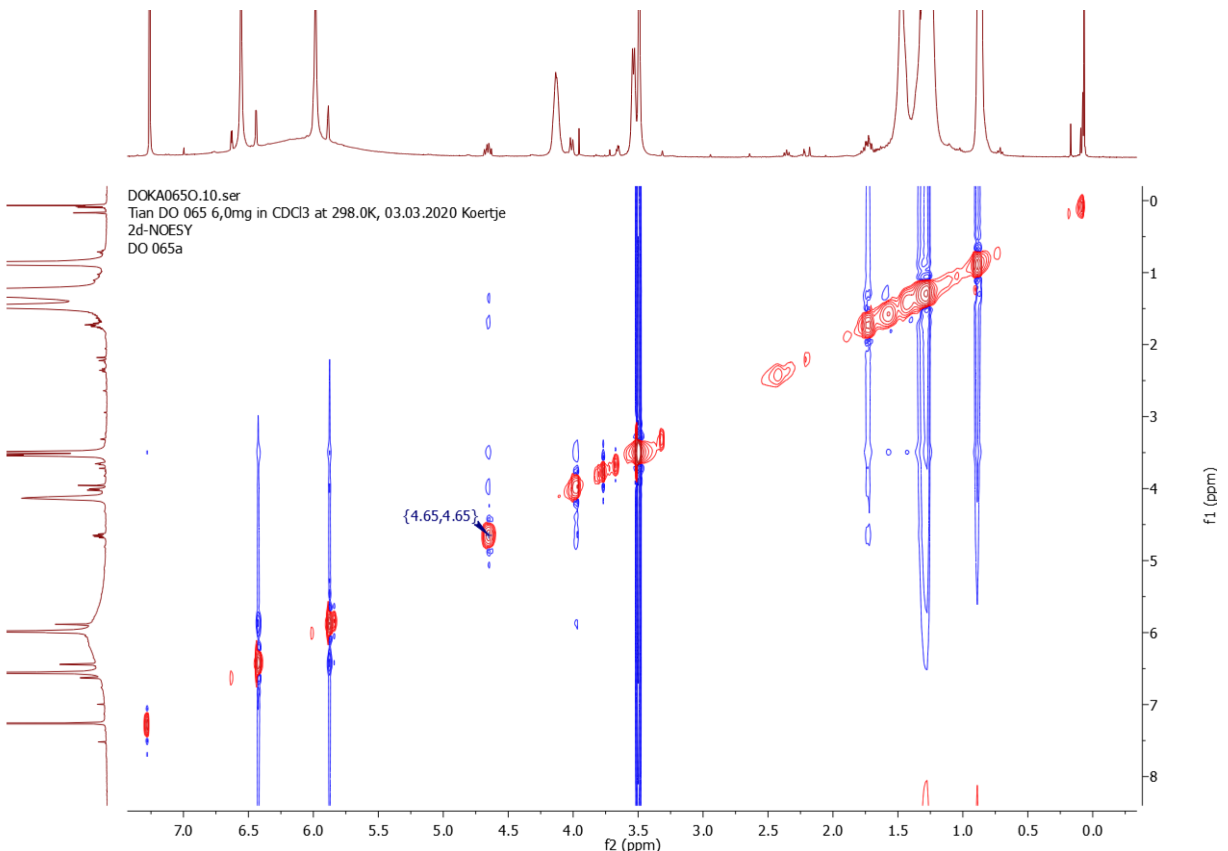
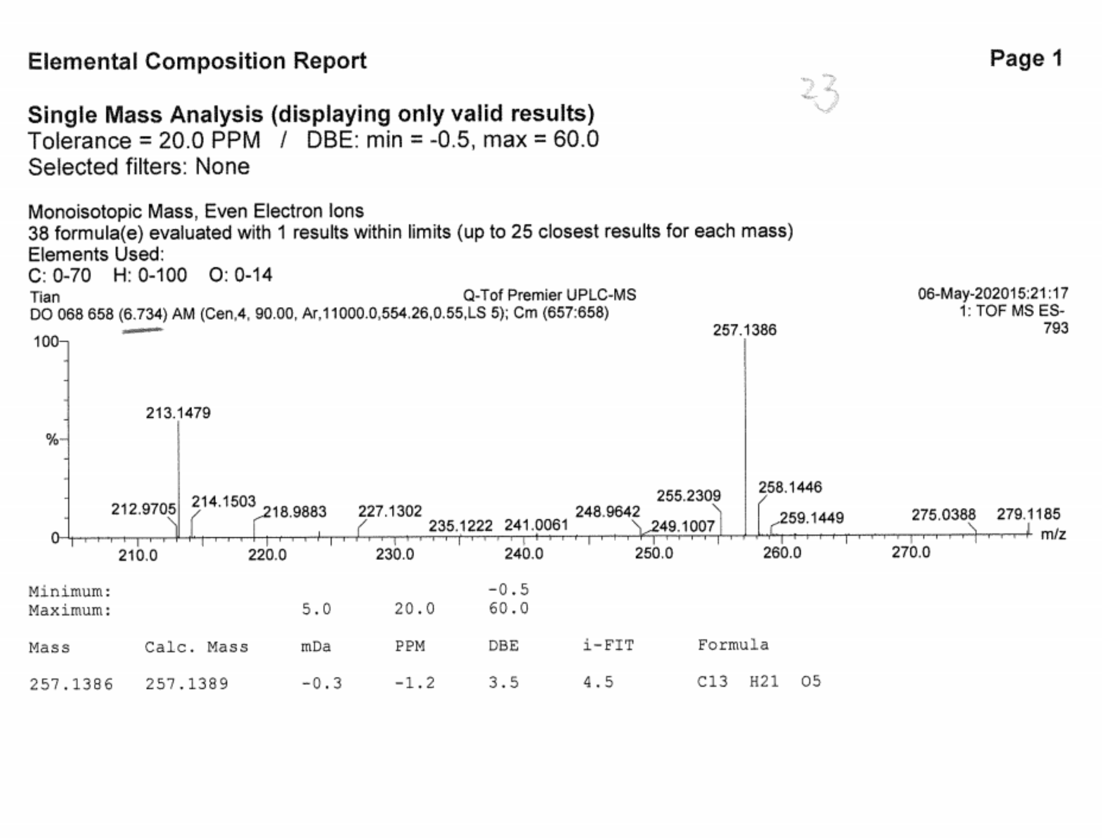


Figure S3.69 NOESY of compound **16**.



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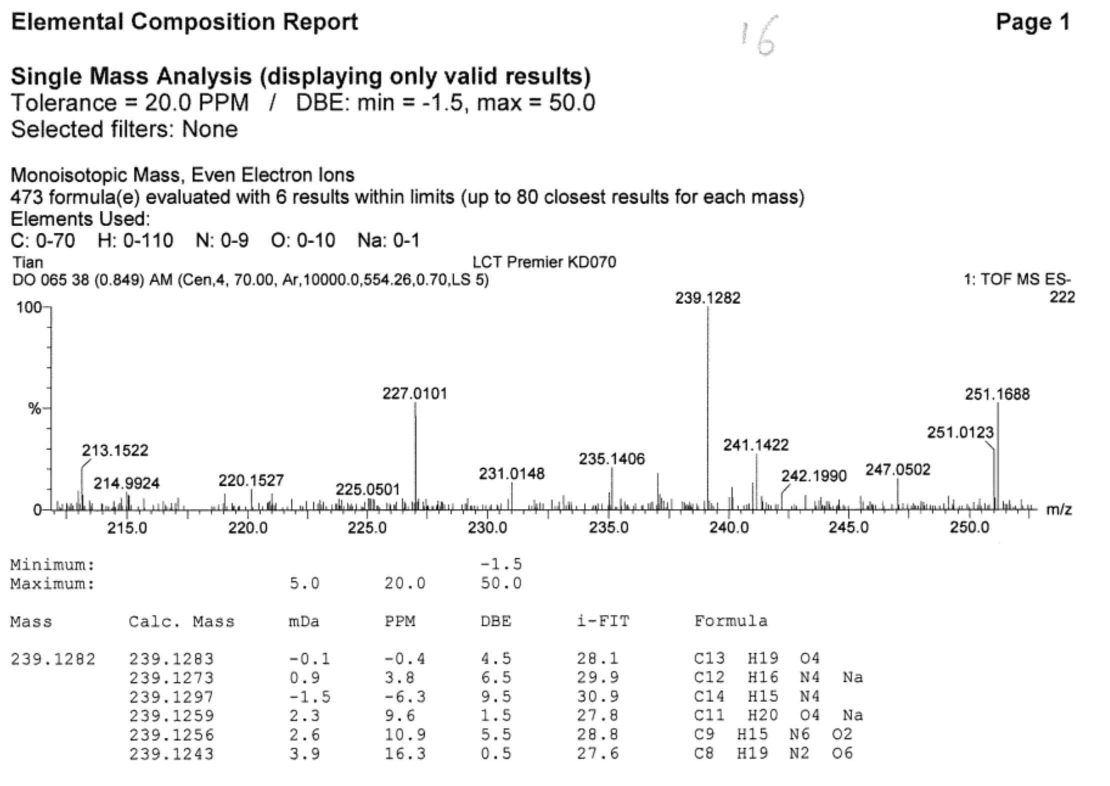
Single Mass Analysis (displaying only valid results)
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 60.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
38 formula(e) evaluated with 1 results within limits (up to 25 closest results for each mass)
Elements Used:
C: 0-70 H: 0-100 O: 0-14

Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
257.1386	257.1389	-0.3	-1.2	3.5	4.5	C13 H21 O5

Figure S3.70 HRMS data for compound **23**.



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Single Mass Analysis (displaying only valid results)
Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
473 formula(e) evaluated with 6 results within limits (up to 80 closest results for each mass)
Elements Used:
C: 0-70 H: 0-110 N: 0-9 O: 0-10 Na: 0-1

Minimum:				-1.5		
Maximum:		5.0	20.0	50.0		

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
239.1282	239.1283	-0.1	-0.4	4.5	28.1	C13 H19 O4
	239.1273	0.9	3.8	6.5	29.9	C12 H16 N4 Na
	239.1297	-1.5	-6.3	9.5	30.9	C14 H15 N4
	239.1259	2.3	9.6	1.5	27.8	C11 H20 O4 Na
	239.1256	2.6	10.9	5.5	28.8	C9 H15 N6 O2
	239.1243	3.9	16.3	0.5	27.6	C8 H19 N2 O6

Figure S3.71 HRMS data for compound **16**.

Compound 20 (known from literature[32])

20

1-undecen-2,3-dicarboxylic acid
Chemical formula: $C_{13}H_{22}O_4$

Yellow oil; $[\alpha]_D^{25}$ – 9 (c = 0.27, MeOH); UV (λ_{max}): 211 nm. ^{13}C NMR data ($CDCl_3$, 150 MHz): δ_C 179.7 (C-1), 171.8 (C-4), 137.5 (C-3), 129.8 (C-13), 47.2 (C-2), 32.0 (C-10), 29.7 (C-5), 29.5 (C-7), 29.5 (C-8), 29.4 (C-9), 27.5 (C-6), 22.8 (C-11), 14.2 (C-12); ^{1}H NMR data ($CDCl_3$, 600 MHz): δ_H 6.53 (1H, s, H-13a), 5.83 (1H, s, H-13b), 3.41 (1H, t, J = 7.3 Hz, H-2), 1.93 (1H, m, H-5b), 1.73 (1H, m, H-5a), 1.33 (2H, m, H-6), 1.28 (2H, m, H-11), 1.24-1.34 (6H, m, H-7/H-8/H-9), 1.25 (2H, m, H-10), 0.88 (3H, t, J = 6.9 Hz, H-12); NMR data are consistent with those previously reported,[32] see table S3.12; ESI-MS m/z 241 $[M–H]^–$, 243 $[M+H]^+$. HR-ESI-MS m/z 241.1441 $[M–H]^–$ (calcd. for $C_{13}H_{21}O_4$, 241.1440).

pos.	δ_C / ppm	δ_H / ppm (J / Hz)	δ_C / ppm literature[32]	δ_H / ppm (J / Hz) literature[32]
1	179.7	-	177.9	-
2	47.2	3.41, 1H, t (7.3)	48.0	3.39, 1H, t (7.4)
3	137.5	-	138.3	-
4	171.8	-	171.4	-
5	29.7	1.73, 1H, m 1.93, 1H, m	30.3	1.75, 1H, m 1.93, 1H, m
6	27.5	1.33, 2H, m	27.5	1.24-1.40, 2H, m
7	29.5	1.24-1.34, 2H, m	29.4	1.24-1.40, 2H, m
8	29.5	1.24-1.34, 2H, m	29.3	1.24-1.40, 2H, m
9	29.4	1.24-1.34, 2H, m	29.2	1.24-1.40, 2H, m
10	32.0	1.25, 2H, m	31.8	1.24-1.40, 2H, m
11	22.8	1.28, 2H, m	22.6	1.24-1.40, 2H, m
12	14.2	0.88, 3H, t (6.9)	14.1	0.86, 3H, t (6.7)
13	129.8	6.53, 1H, s 5.83, 1H, s	128.4	6.54, 1H, s 5.82, 1H, s

Table S3.12 ^{1}H NMR (600 MHz) data and ^{13}C NMR (150 MHz) data for **20** in $CDCl_3$. Literature[32] data was measured at 500 MHz in $CDCl_3$.

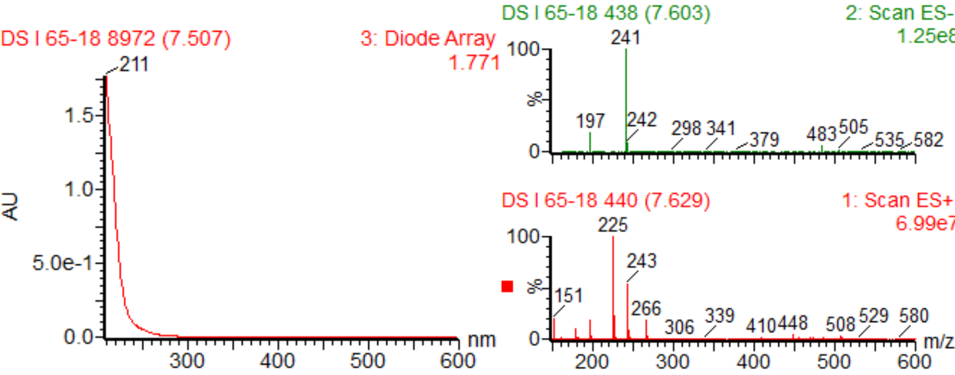


Figure S3.72 UV spectrum and ESI spectrum for **20**.

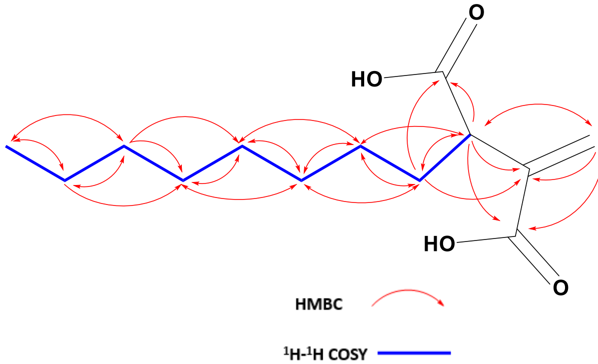


Figure S3.73 Key HMBC and ^{1}H-^{1}H COSY correlations of **20**.

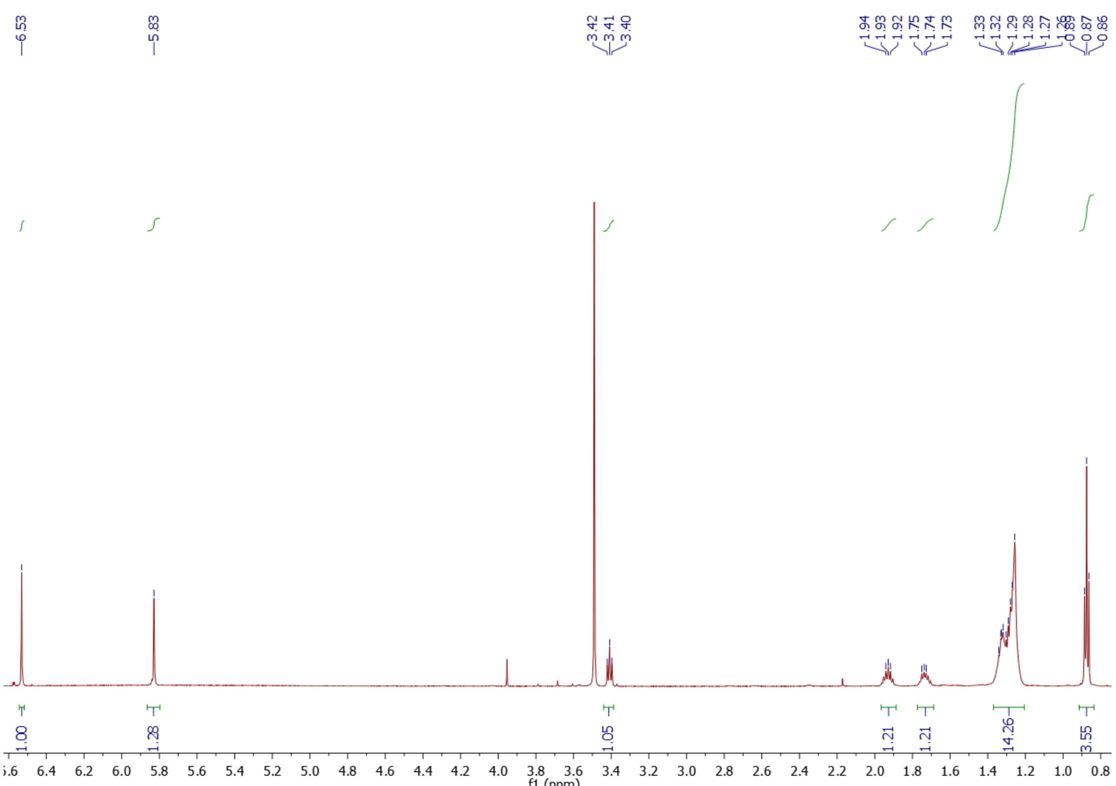


Figure S3.74 ^{1}H NMR of compound **20**.

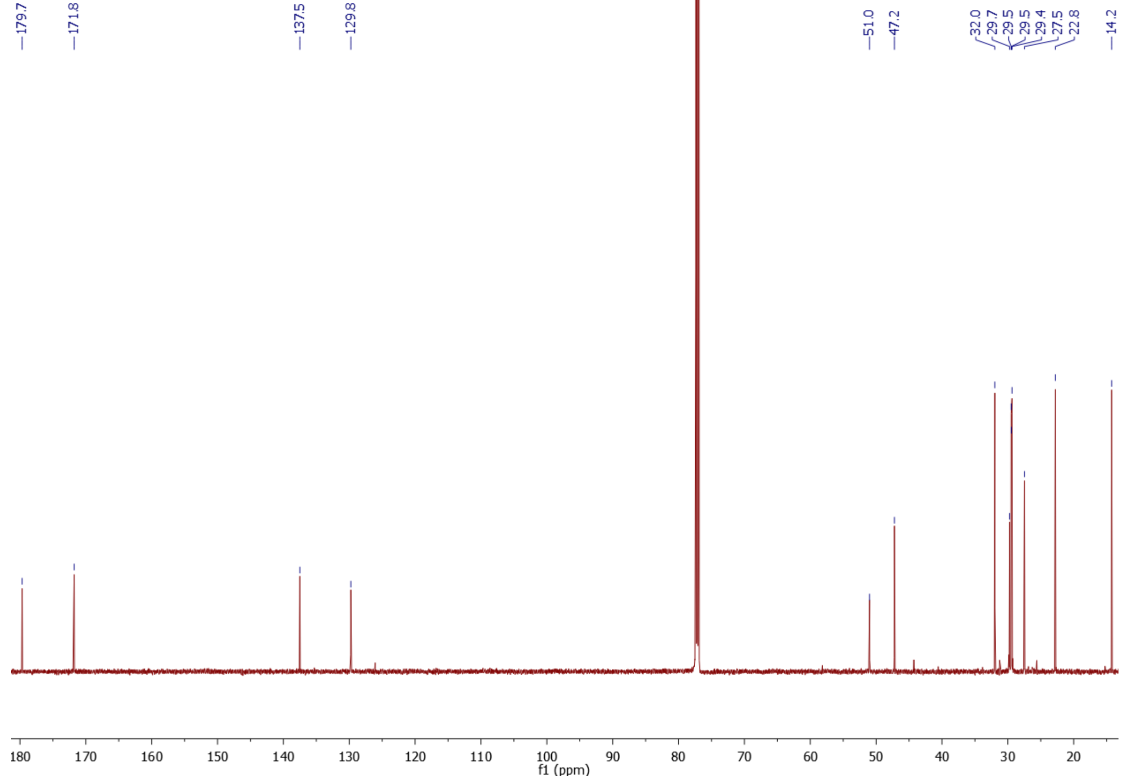


Figure S3.75 ^{13}C NMR of compound **20**.

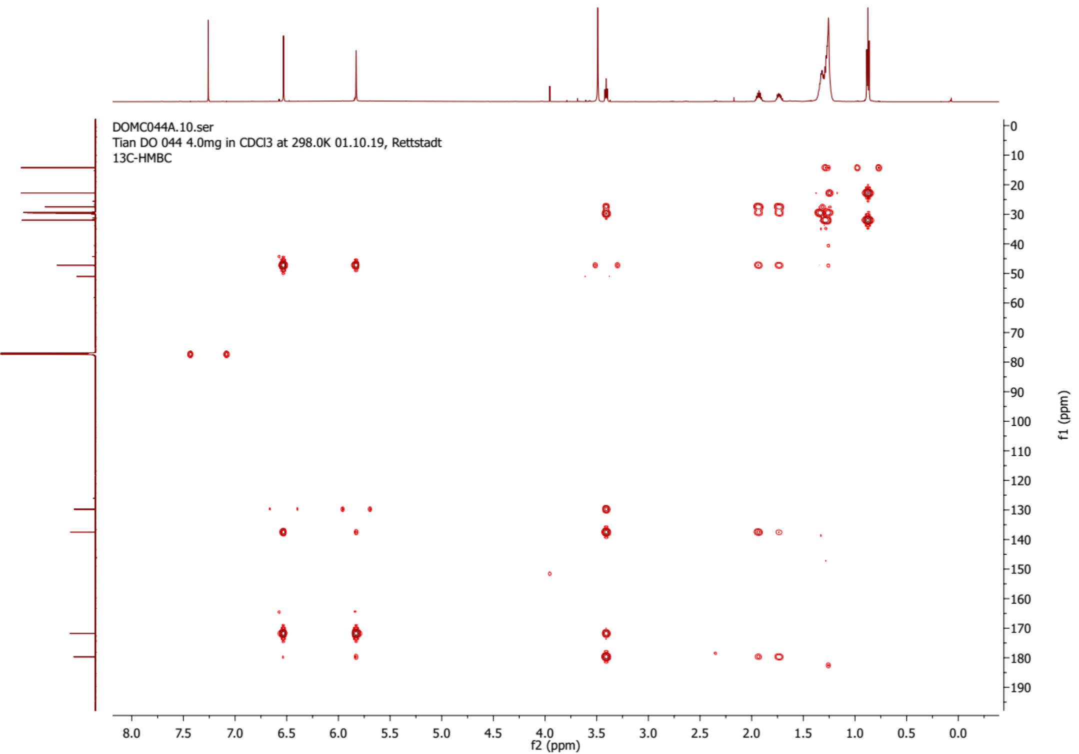


Figure S3.76 HMBC of compound **20**.

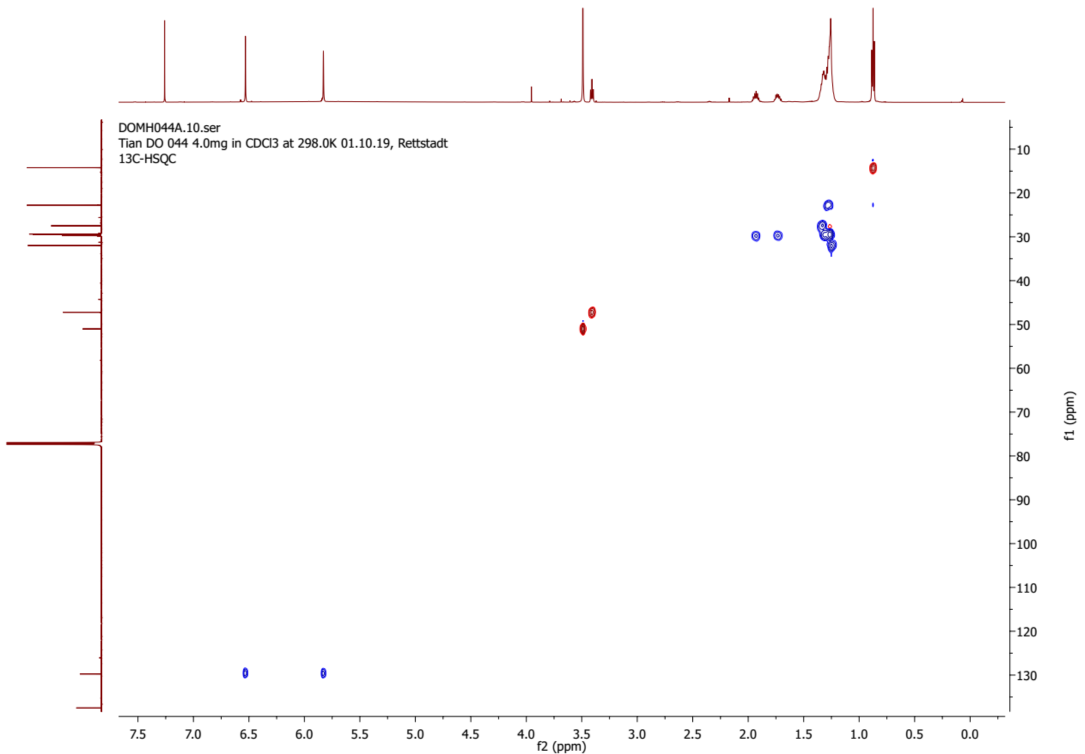


Figure S3.77 HSQC of compound **20**.

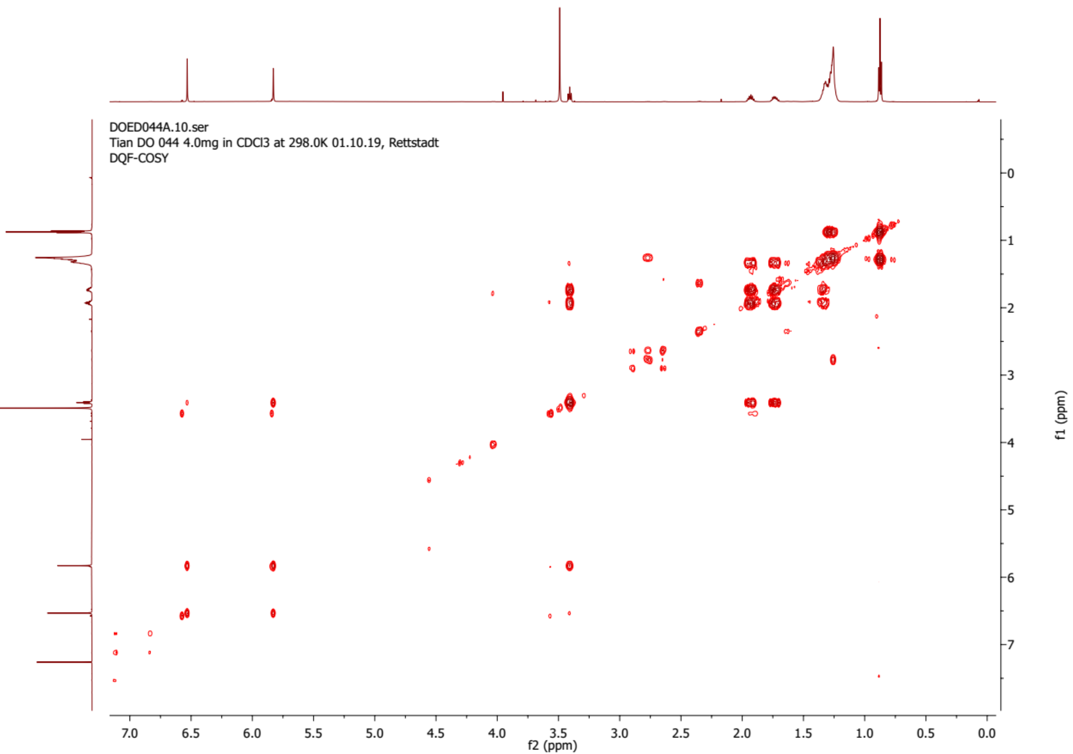
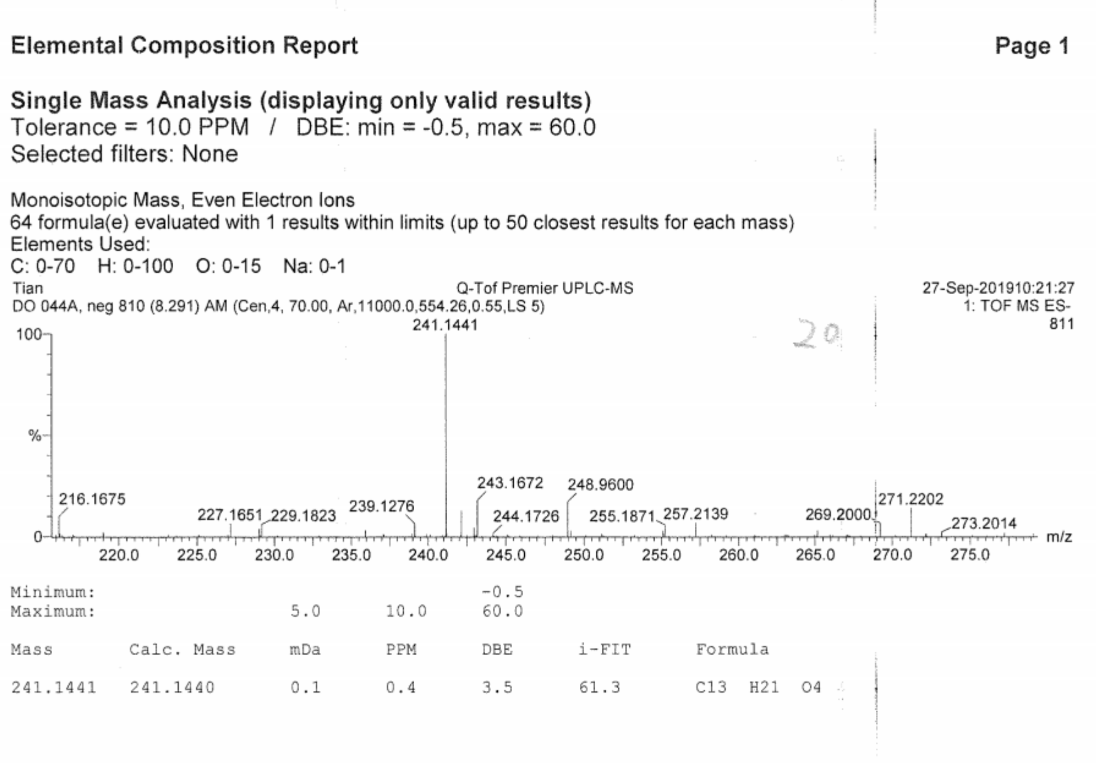


Figure S3.78 ^1H-^1H COSY of compound **20**.



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Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 PPM / DBE: min = -0.5, max = 60.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
64 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-70 H: 0-100 O: 0-15 Na: 0-1

Minimum:				-0.5		
Maximum:		5.0	10.0	60.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
241.1441	241.1440	0.1	0.4	3.5	61.3	C13 H21 O4

Figure S3.79 HRMS data for compound **20**.

Compound 21 (proposed structure)

OH CO$_2$H CO$_2$H

21

Chemical formula: $C_{13}H_{22}O_5$

UV (λ_{max}): 211 nm. ESI-MS *m/z* 257 [M–H]$^-$, 515 [2M–H]$^-$, 241 [M–H$_2$O+H]$^+$, 259 [M+H]$^+$, 281 [M+Na]$^+$, 539 [2M+Na]$^+$. HR-ESI-MS *m/z* 257.1387 [M–H]$^-$ (calcd. for $C_{13}H_{21}O_5$, 257.1389).

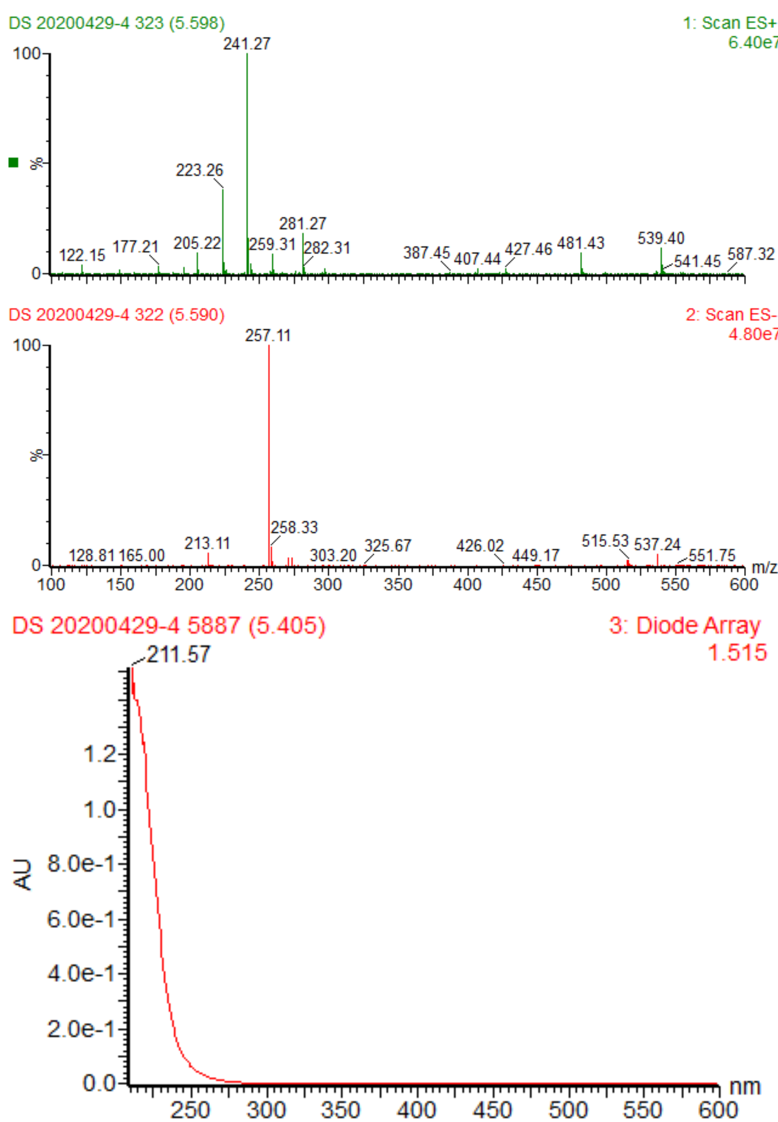
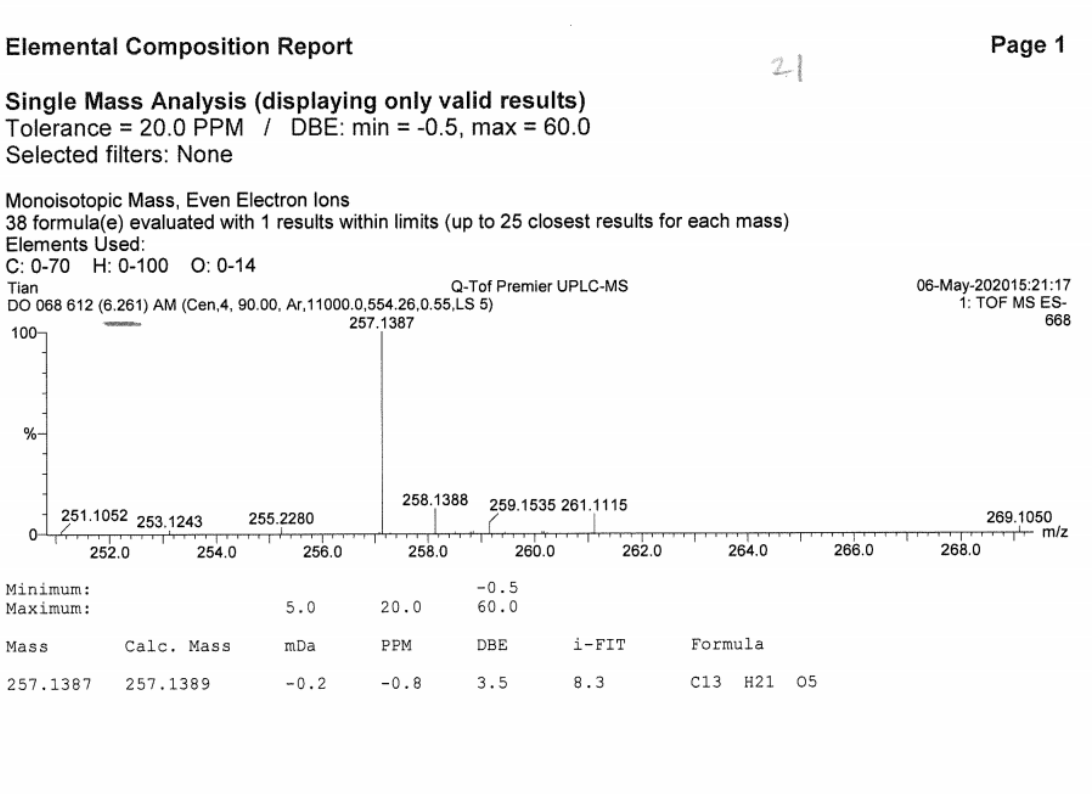


Figure S3.80 UV spectrum and ESI spectrum for **21**.



		mDa	PPM	DBE	i-FIT	Formula
Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		
Mass	**Calc. Mass**	**mDa**	**PPM**	**DBE**	**i-FIT**	**Formula**
257.1387	257.1389	-0.2	-0.8	3.5	8.3	C13 H21 O5

Figure S3.81 HRMS data for compound **21**.

Compound 22 (proposed structure)

22

Chemical formula: $C_{13}H_{22}O_6$

UV (λ_{max}): 200 nm. ESI-MS *m/z* 273 $[M–H]^-$, 547 $[2M–H]^-$, 257 $[M–H_2O+H]^+$, 275 $[M+H]^+$, 292 $[M+H_2O]^+$, 549 $[2M+H]^+$. HR-ESI-MS *m/z* 273.1339 $[M–H]^-$ (calcd. for $C_{13}H_{21}O_6$, 277.1338).

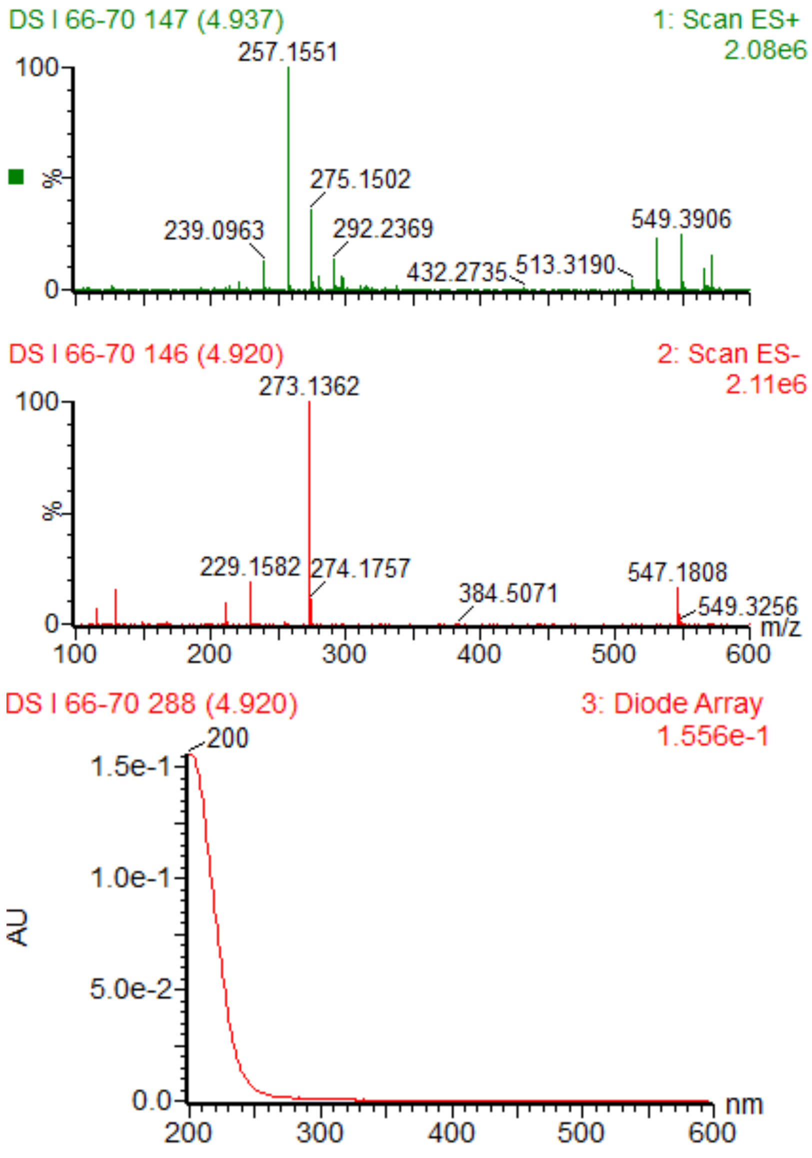
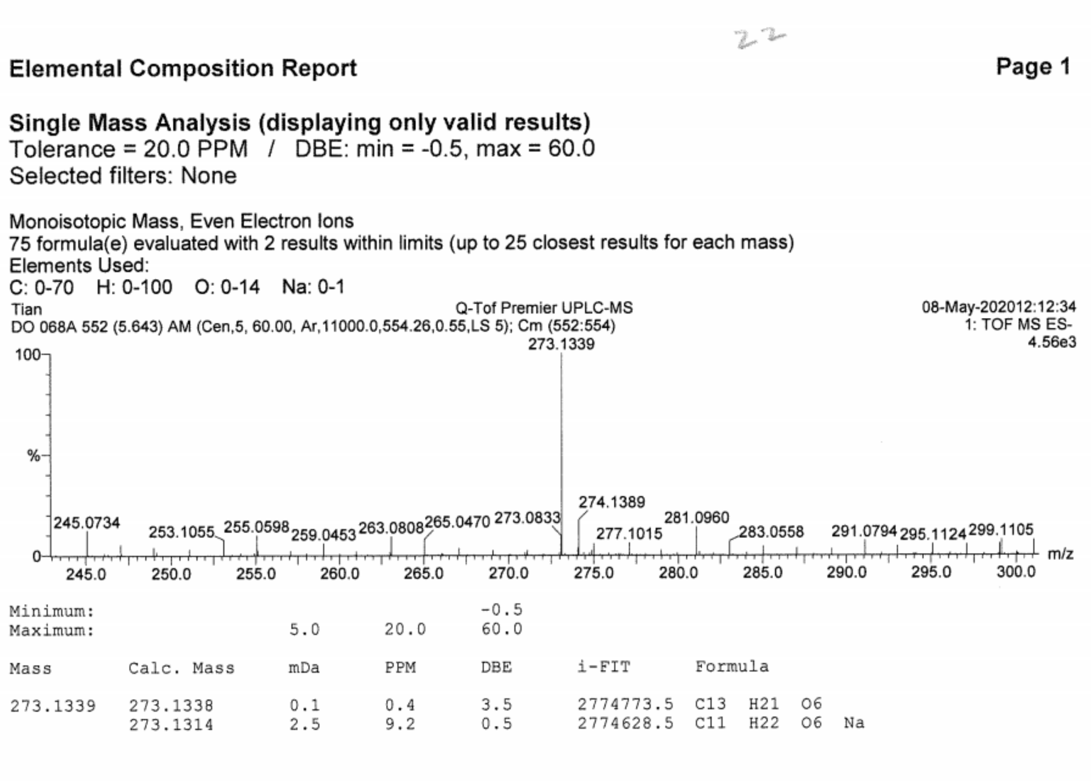


Figure S3.82 UV spectrum and ESI spectrum for **22**.



22

Elemental Composition Report Page 1

Single Mass Analysis (displaying only valid results)
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 60.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
75 formula(e) evaluated with 2 results within limits (up to 25 closest results for each mass)
Elements Used:
C: 0-70 H: 0-100 O: 0-14 Na: 0-1

		mDa	PPM	DBE	i-FIT	Formula
Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
273.1339	273.1338	0.1	0.4	3.5	2774773.5	C13 H21 O6
	273.1314	2.5	9.2	0.5	2774628.5	C11 H22 O6 Na

Figure S3.83 HRMS data for compound **22**.

Compound 24 (proposed structure)

24

Chemical formula: $C_{13}H_{20}O_5$

UV (λ_{max}): 214 nm. ESI-MS *m/z* 255 [M–H]$^-$, 511 [2M–H]$^-$, 239 [M–H_2O+H]$^+$, 257 [M+H]$^+$, 274 [M+H_2O]$^+$, 535 [2M+Na]$^+$. HR-ESI-MS *m/z* 255.1217 [M–H]$^-$ (calcd. for $C_{13}H_{19}O_5$, 255.1232).

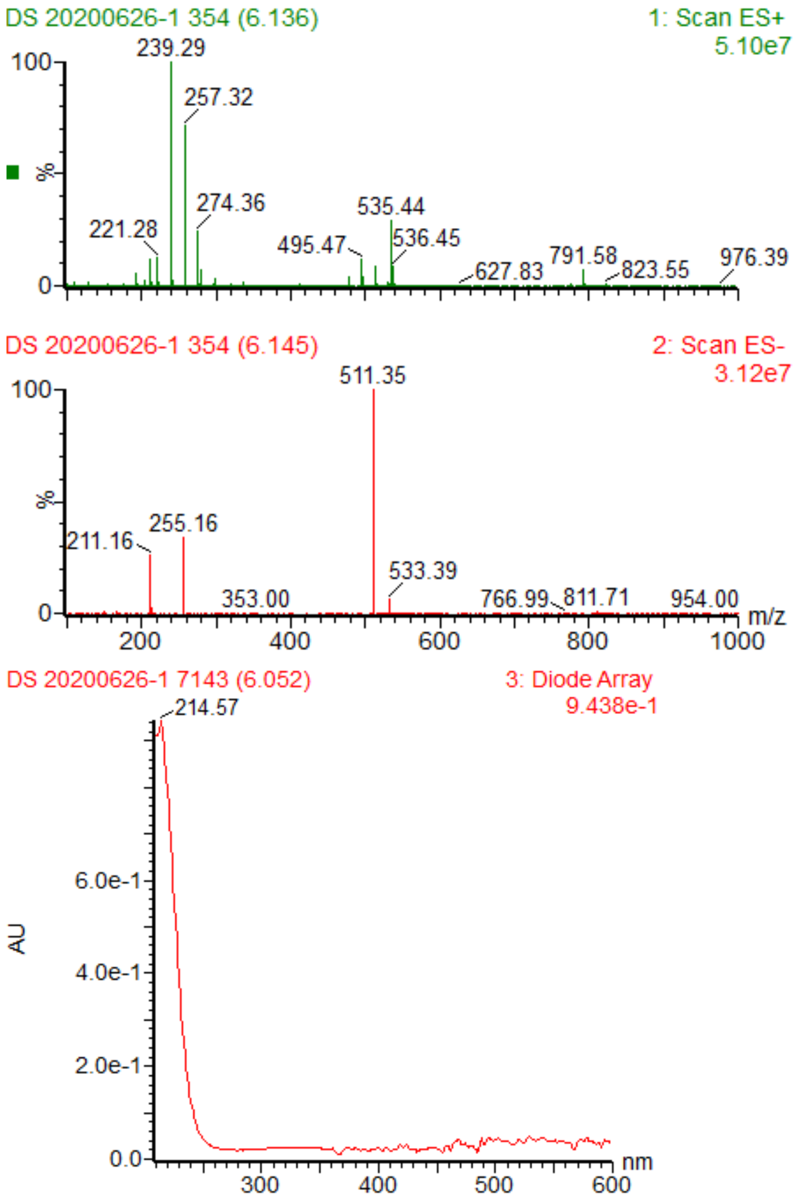
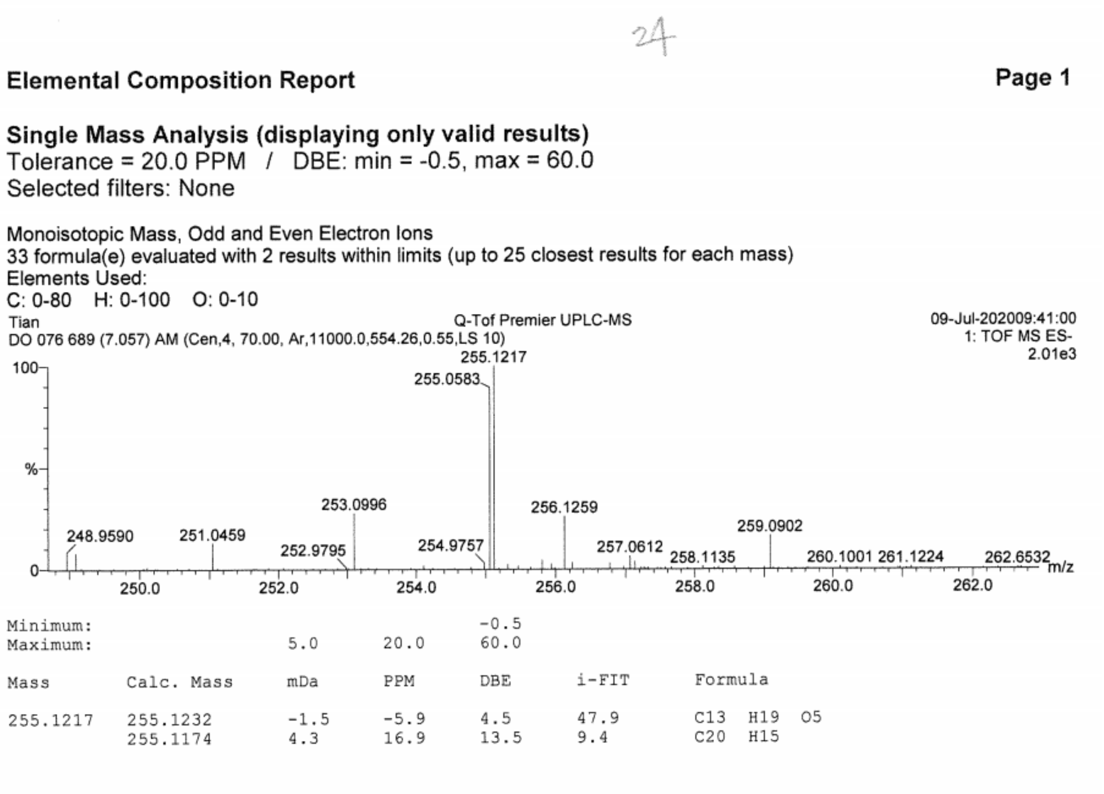


Figure S3.84 UV spectrum and ESI spectrum for **24**.



Minimum:				-0.5		
Maximum:		5.0	20.0	60.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
255.1217	255.1232	-1.5	-5.9	4.5	47.9	C13 H19 O5
	255.1174	4.3	16.9	13.5	9.4	C20 H15

Figure S3.85 HRMS data for compound **24**.

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