

Supporting Information for

Elucidation of Pyranonigrin Biosynthetic Pathway Reveals a Mode of Tetramic Acid, Fused γ -Pyrone and *exo*-Methylene Formation

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1. Supporting Methods

1.1 Reagents, strains and general techniques for DNA manipulation.

All of the chemicals were purchased from Tokyo Chemical Industry Co. Ltd., Sigma-Aldrich and Wako Pure Chemical Industries, Ltd. unless otherwise specified. Purchased chemicals were of reagent grade and used without further purification. *Aspergillus niger* A1179 and *A. nidulans* A1145 were obtained from the Fungal Genetics Stock Center in the USA. *A. oryzae* NBRC 30102 was obtained from National Institution of Technology and Evaluation Biological Resource Center in Japan. *Escherichia coli* XL1-Blue (Agilent Technologies) and *E. coli* TOP10 (Thermo Fisher Scientific Inc.) were used for plasmid propagation by standard procedures. Overproduction of recombinant proteins was carried out in *E. coli* BL21 (DE3) (Thermo Fisher Scientific Inc.). DNA restriction enzymes were used as recommended by the manufacturer (Thermo Fisher Scientific Inc.). PCR was carried out using PrimeSTAR GXL DNA polymerase (TAKARA Bio Inc.) as recommended by the manufacturer. Sequences of PCR products were confirmed through DNA sequencing (Macrogen Japan Corporation). *Saccharomyces cerevisiae* BY4741¹ and BY4705² were obtained from the Yeast Genetic Resource Center in Japan. *S. cerevisiae* BY4741 was used for homologous recombination-based molecular cloning of genes and plasmid assembly.

1.2 Biosynthetic gene cluster responsible for producing pyranonigrin F 1 in *A. niger* A1179.

Figure S1. The organization of the pyranonigrin biosynthetic gene cluster in *A. niger* A1179.

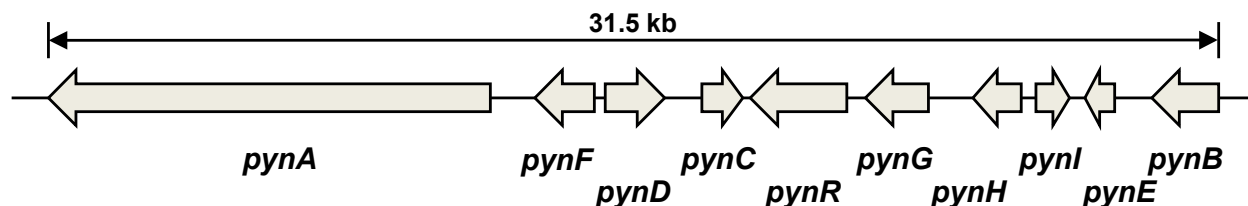


Table S1. Deduced functions of the open reading frames (ORFs) in the pyranonigrin biosynthetic gene cluster in *A. niger* A1179.

Gene name	Protein name	Proposed protein function (most informative protein ^a)
<i>pynA</i> (ANI_1_1528094)	PynA	PKS-NRPS hybrid
<i>pynF</i> (ANI_1_1530094)	PynF	MFS transporter
<i>pynD</i> (ANI_1_1532094)	PynD	Cytochrome P450
<i>pynC</i> (ANI_1_1534094)	PynC	Methyltransferase
<i>pynR</i> (ANI_1_1536094)	PynR	Transcription factor
<i>pynG</i> (ANI_1_1538094)	PynG	Flavin-dependent oxidase (FMO)
<i>pynH</i> (ANI_1_1540094)	PynH	Aspartyl protease
<i>pynI</i> (ANI_1_1542094)	PynI	Thioesterase
<i>pynE</i> (ANI_1_1544094)	PynE	NAD(P)-binding oxidoreductase
<i>pynB</i> (ANI_1_1546094)	PynB	Flavin-dependent oxidase (FMO)

^a Deduced functions of the ORFs identified within the biosynthetic gene cluster were based on the percentage sequence identity/similarity to known proteins as determined by Protein BLAST search against the NCBI non-redundant database.³

1.3 Construction of mutated strains of *A. niger*.

1.3.1 Preparation of the *pynR* promoter-replacement *A. niger* A1179 strain, AnKW2.

For the construction of AnKW2 (**Fig. S2A**), we followed our previously established method⁴ for initially integrating the *Afp_{pyrG}* gene (Afu2g08360 encoding for an *A. fumigatus* orotidine-5'-phosphate decarboxylase) and a *glaA* promoter⁵ into the genome of *A. niger* A1179 to place *pynR* under the control of the *glaA* promoter for activating transcription of the genes in the *pyn* cluster (**Fig. S1** and **Table S1**). *A. niger* A1179 is a mutant strain of deficient in *kusA* and *pyrG*.⁶ The resultant strain, AnKW1, possessed *Afp_{pyrG}* and was capable of activating the pyranonigrin biosynthetic gene cluster (**Figure S6**). Subsequently, in order to construct each *pyn* gene-deficient mutants more effectively, *Afp_{pyrG}* integrated in the AnKW1 genome was replaced with

a pyrithiamine resistance gene *ptrA*⁷ via targeted homologous recombination between the *ptrA*-carrying plasmid pKW20085⁸ and the genome of AnKW1, yielding AnKW2. We achieved efficient gene targeting by using this strain with uridine/uracil auxotrophic selection. For gene disruption, a disruption cassette was prepared on a plasmid as described below.

1.3.2 Construction of disruption cassette for preparing AnKW2.

The disruption cassette included a selection marker sandwiched in between a 5' side- and a 3' side-flanking fragment (Rec5' and Rec3', respectively) as shown in **Fig. S2A**. Rec5' and Rec3' are a 1,000-base pair fragment that is homologous to the site of recombination at or near the 5' and 3' end of the target gene in the *A. niger* A1179 genome. The primer sets given in **Tables S2–S4** were used to prepare the required flanking homologous regions for each of the target genes. The sequences of the primer sets are listed in **Table S9**. PCR amplification of Rec5', Rec3' and the selectable marker fragments were carried out using PrimeSTAR GXL DNA polymerase as recommended by the manufacturer (TAKARA Bio Inc.).

For plasmid construction, the four fragments (Rec5', *AfpYrG*, *glaA* promoter and *pynR*), each at 50 to 150 ng in a total volume of 45 µL, were mixed with 2 µg of a suitable pre-digested delivery vector. For AnKW1 preparation, pRS423⁹ was used as a delivery vector (**Table S2**). The mixture was transformed into *S. cerevisiae* BY4741 for constructing the plasmid possessing deletion cassette through *in vivo* homologous recombination. These five DNA fragments were joined *in situ* by the endogenous homologous recombination activity of *S. cerevisiae* through the 25-bp homologous sequences present at the ends of those DNA fragments. The desired transformants were selected for the presence of the selection marker. For AnKW1 preparation, transformants were selected on a histidine-deficient plate, because pRS423 carried a *HIS3* selection marker. The resulting plasmid pKW20155 (**Fig. S2A**) was recovered from the yeast transformant and transferred to *E. coli*. The plasmid was amplified in *E. coli* for subsequent characterization by restriction enzyme digestion and DNA sequencing to confirm its identity. For targeted homologous recombination, a PCR product was first amplified from the plasmid carrying a desired cassette using the corresponding Rec5' forward and Rec3' reverse primers (**Table S2, S9, Fig. S2A**). Then this PCR product was used to transform *A. niger* A1179.

1.3.3 Transformation of *A. niger*.

A mutant *A. niger* A1179 (or AnKW1 or AnKW2) strain was initially cultured on Czapek Dox (CD) agar plates¹⁰ containing 10 mM uridine and 5 mM uracil at 30 °C for 5 days. Approximately 1×10^6 to 4×10^6 of conidia collected from a single plate were used to inoculate 200 mL of CD medium containing 10 mM uridine and 5 mM uracil. This culture was shaken for 24 h at 30 °C. Grown cells were collected by filtration and washed with 0.8 M sodium chloride. The cells were incubated with 1 mL of 10 mM sodium phosphate buffer (pH 6.0) containing 0.8 M sodium chloride, 50 mg/mL lysing enzyme (Sigma-Aldrich) and 1,500 units of β -glucuronidase at room temperature for 3 h. The resulting protoplasts were filtered and subsequently centrifuged at $2,500 \times g$ for 5 min at room temperature. The collected protoplasts were washed with 0.8 M sodium chloride and centrifuged to remove the wash solution. The cells were suspended in 200 μ L of STC buffer at pH 8.0 (0.8 M sodium chloride, 10 mM calcium chloride and 10 mM Tris-HCl). Then 40 μ L of PEG solution at pH 8.0 (400 mg/mL polyethylene glycol 4,000, 50 mM calcium chloride and 50 mM Tris-HCl) was added to the protoplast suspension. The mixture was subsequently combined with 5 μ g of the DNA fragment with which the cells were to be transformed. The mixture was incubated on ice for 20 min to allow the transformation to proceed. After incubation on ice, 1 mL of the PEG solution was added to the reaction mixture, and the mixture was incubated at room temperature for additional 5 min. The cells were plated on the CD agar plates containing 0.8 M NaCl in order to select the desired mutants derived from *A. niger* A1179 or AnKW2, meanwhile, CD agar plates containing 0.8 M NaCl, 10 mM uridine 5 mM uracil and 1 mg/mL 5-fluoroorotic acid (5-FOA) and 0.1 μ g/mL pyrithiamine was used for generating AnKW2 strain from AnKW1. Replacement of the target region was confirmed by diagnostic PCR (**Table S3, S4, S6, Fig. S5, S8**).

Figure S2. (A) A schematic diagram showing the generation of AnKW2 *via* AnKW1. (B) A general scheme for preparing a deletion mutant (e.g., *ApynA*) from AnKW2. Construction of a disruption cassette-containing plasmid (e.g., pKW20244) (Fig. S3) was accomplished using a yeast homologous recombination method (see Section 1.4.1 for details). *AopyrG* (AOR_1_1464054) encodes an orotidine-5'-phosphate decarboxylase in the genome of *A. oryzae*.

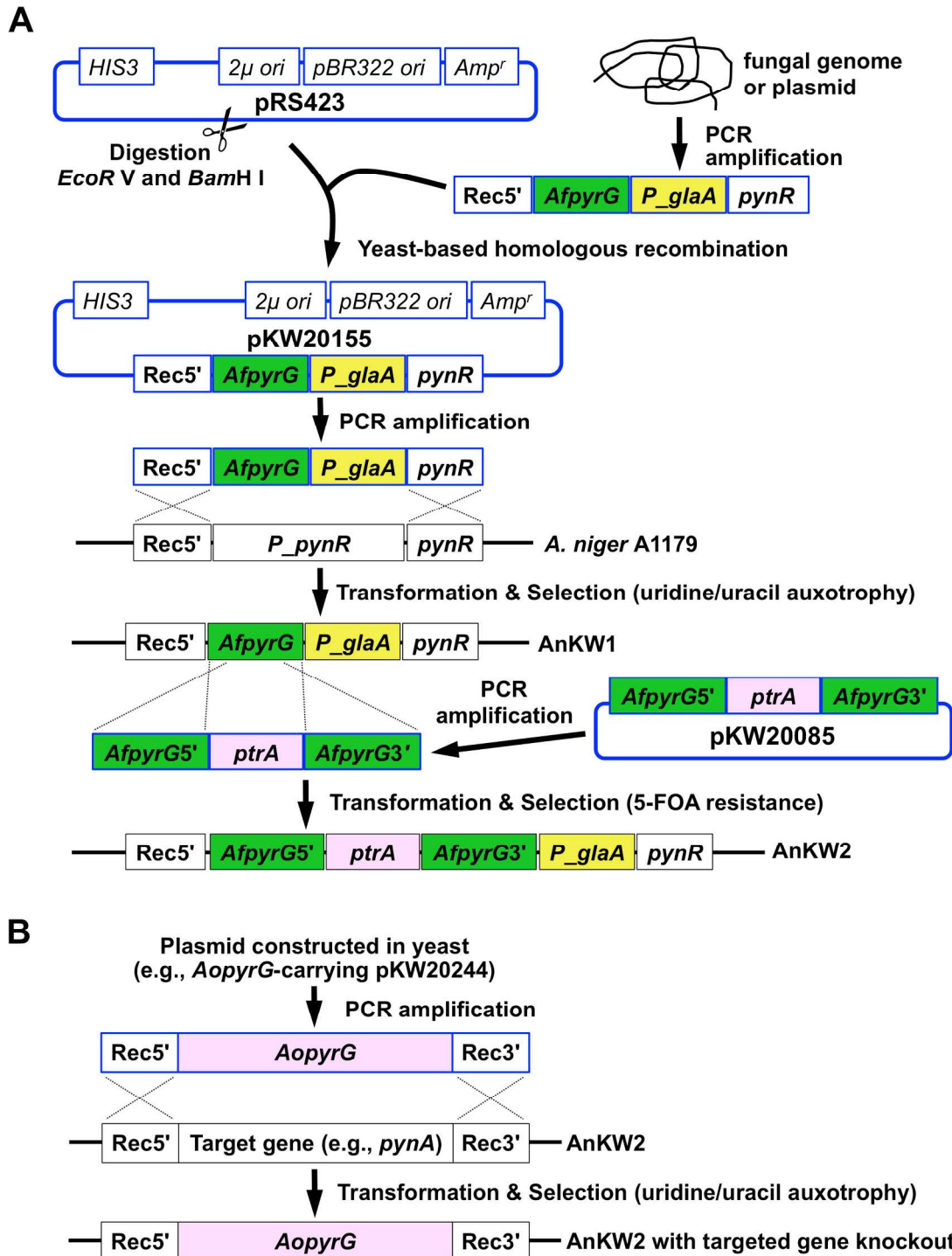


Figure S3. Map of plasmid pKW20244 carrying the disruption cassette for deletion of *pynA* is shown as a representative example of gene disruption plasmids prepared in this study. Rec5': 1,000-bp 5'-side-flanking fragment homologous to one side of the recombination site in the AnKW2 genome; Rec3': 1,000-bp 3'-side-flanking fragment homologous to the other side of the recombination site in the AnKW2 genome.

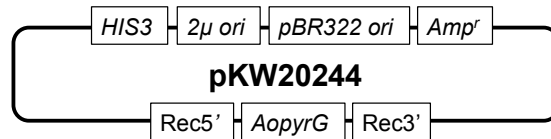


Table S2. The primer set used to create the plasmid for replacement of the promoter of *pynR*.

Plasmid name	Target gene	Marker	Primer set for Rec5'	Primer set for marker	Primer set for p_glaA	Primer set for Rec3'	Digested original vector
pKW20155	<i>P_pynR</i>	<i>AfpyrG</i>	pKW20155-F1 pKW20155-R1	pKW20155-F2 pKW20155-R2	pKW20155-F3 pKW20155-R3	pKW20155-F4 pKW20155-R4	pRS423/ <i>EcoRV</i> , <i>Bam</i> HI

Table S3. The list of primer sets used to confirm successful generation of AnKW1.

Plasmid name	Target gene	Marker	Primer set for positive PCR (left arm)	Primer set for positive PCR2 (right arm)	Primer set for negative PCR
pKW20155	<i>P_pynR</i>	<i>AfpyrG</i>	pKW20155-pos-LF pKW20155-pos-LR	pKW20155-pos-RF pKW20155-pos-RR	<i>P_pynR</i> -neg-F <i>P_pynR</i> -neg-R

Table S4. The list of primer sets used to confirm successful generation of AnKW2.

Plasmid name	Target gene	Marker	Primer set for positive PCR	Primer set for negative PCR
pKW20085	<i>AfpyrG</i>	<i>ptrA</i>	Afu8g00540::PtrA-RF pKW20155-pos-RR	Afu2g08360-neg-F Afu2g08360-neg-R

Figure S4. A schematic diagram showing the primer annealing sites for PCR-based confirmation of a mutated strain using the gDNA as a template. (A and B) Confirmation for AnKW1. (C and D) Confirmation for AnKW2.

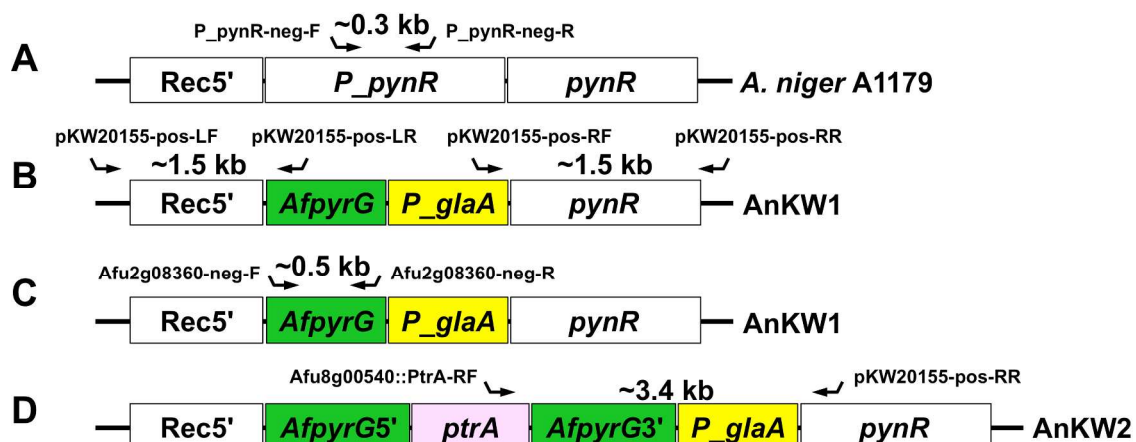
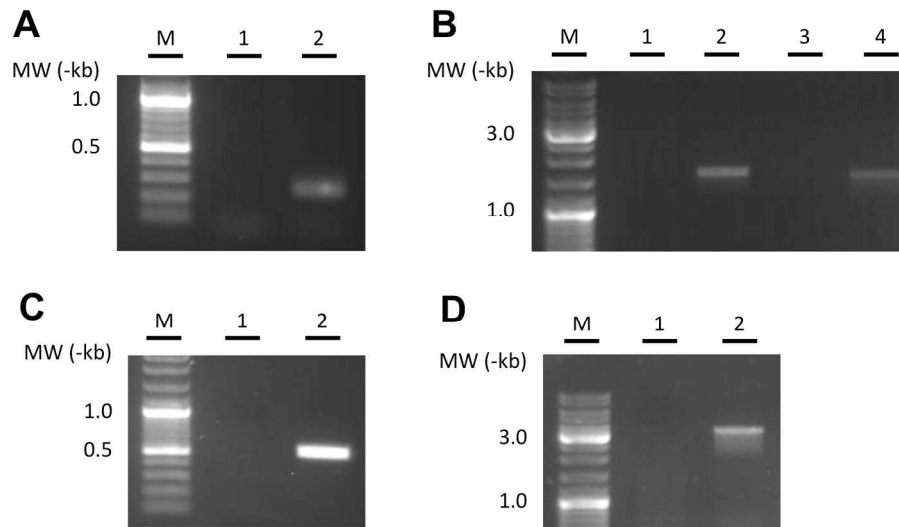


Figure S5. PCR analysis for confirming the generation of AnKW1 and AnKW2. Amplifications of PCR products from gDNA of AnKW1 and AnKW2 using the primer set described in **Table S3** and **S4**. The sequences of the primer sets are listed in **Table S9**. Lane M, molecular weight marker. (A) lane 1: template - AnKW1 gDNA, primers - negative PCR set in **Table S3** (see **Fig. S4B** for primer binding arrangement); lane 2: template - *A. niger* A1179 gDNA, primers - negative PCR set in **Table S3** (**Fig. S4A**). (B) lane 1 and 3: template - *A. niger* A1179 gDNA, primers - positive PCR set in **Table S3** (**Fig. S4A**); lane 2 and 4: template - AnKW1 gDNA, primers - positive PCR2 set in **Table S3** (**Fig. S4B**). (C) lane 1: template - AnKW2 gDNA, primers - negative PCR set in **Table S4** (**Fig. S4D**); lane 2: template - AnKW1 gDNA, primers - negative PCR set in **Table S4** (**Fig. S4C**). (D) lane 1: template - AnKW1 gDNA, primers - positive PCR set in **Table S4** (**Fig. S4C**); lane 2: template - AnKW2 gDNA, primers - positive PCR set in **Table S4** (**Fig. S4D**).

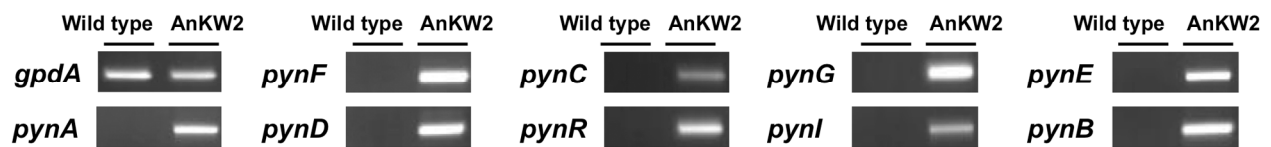


1.3.4 Reverse transcriptional analysis of expression of *pyn* genes.

A. niger A1179 and AnKW2 strain was cultured in a Czapek Dox Starch Tryptone (CD-ST) liquid medium^[4] (NaNO₃ 6 g/L, KCl 0.52 g/L, KH₂PO₄ 1.52 g/L, starch 20 g/L, MgSO₄•7H₂O 0.49 g/L and tryptone 20 g/L supplemented with a mixture comprised of FeSO₄•7H₂O 1 g/L, ZnSO₄•7H₂O 8.8 g/L, CuSO₄•7H₂O 0.4 g/L, Na₂B₄O₇•10H₂O 0.1 g/L, (NH₄)₆Mo₇O₂₄•4H₂O 0.05 g/L and adjusted to pH6.5 with 1 N KOH) containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. Total RNA was isolated from mycelium using the Ambion RNAqueous kit (Thermo Fisher Scientific Inc.). *A. niger* A1179 was used as a negative control.

First strand cDNA synthesis was performed using the SMARTer RACE cDNA Amplification kit (Clontech Laboratories, Inc.) with an oligo(dT)₂₀ primer. Primer sets used for amplifying the target genes are listed in **Table S6** except for the following: *pynR*-RT-PCR-F and *pynR*-RT-PCR-R were used to amplify *pynR*, and *gpdA*-RT-PCR-F and *gpdA*-RT-PCR-R were used to amplify *gpdA*. The sequences of the primers are listed in **Table S9**. Amplified products were visualized by agarose gel electrophoresis with ethidium bromide using UV (254 nm) transilluminator (**Fig. S6**).

Figure S6. RT-PCR analysis of the expression of *pyn* cluster genes upon activation of the transcriptional regulator *pynR* in AnKW2. Glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* was used as a positive control. *A. nidulans* A1179 was used as the wild-type strain.



1.4 Construction of pyranonigrin biosynthetic gene knockout strains.

1.4.1 Construction of plasmids for pyranonigrin biosynthetic genes.

The general procedure was the same as described above for the AnKW2 preparation. For the selection marker, *AopyrG* was used. For preparing the disruption mutants, the *pyrG* deletion mutant strain AnKW2 was used as a recipient for the transformation. For preparing the disruption cassette for creating the $\Delta pynA$ strain, *AopyrG* was included in the cassette. For preparing disruption cassettes for creating $\Delta pynB$, $\Delta pynC$, $\Delta pynD$, $\Delta pynE$, $\Delta pynF$, $\Delta pynG$, $\Delta pynH$ and $\Delta pynI$ mutant strains, *AopyrG* was included in each of the cassettes. The *AopyrG* gene was amplified from *A. oryzae* gDNA by PCR using the AopyrG-F/AopyrG-R primer set (**Table S5**, primer sequences are given in **Table S9**). Each segments of Rec5' and Rec3' region was amplified from A1179 or AnKW2 gDNA by PCR using corresponding primer sets listed in **Table S5** (primer sequences are given in **Table S9**).

1.4.2 Confirmation of targeted deletion for *pynA*, *B*, *C*, *D*, *E*, *F*, *G*, *H* and *I* by PCR.

To verify that the target gene was replaced with the cassette, the gDNA isolated from the transformants was analyzed by diagnostic PCR. Three sets of PCR primers were designed for this verification (**Fig. S7** and **Table S6**). For the first set, one primer that anneals to the selection marker and another primer that anneals at the 3' side of the Rec3' region were designed (**Fig. S7A**, "positive PCR2" set in **Table S6**). With this primer set, wild type gDNA will not produce any PCR product with this primer set. However, a PCR product around 1.5 kb in size will be formed from the gDNA of a strain containing desired gene deletion. For the second set, one primer that anneals to the selection marker and another primer that anneals at the 5' side of the Rec5' region were designed (**Fig. S7A**, "positive PCR" set in **Table S6**). With this primer set, wild type gDNA will not produce any PCR product with this primer set. However, a PCR product around 1.5 kb in size will be formed from the gDNA of a strain containing desired gene deletion. For the third primer set, one primer that anneals near the 3' end of the target gene and another primer that anneals approximately 500 bp inside of the target gene (**Fig. S7B**, "negative PCR" set in **Table S6**). With this primer set, PCR on the gDNA of unaltered wild type strain, such as AnKW2, will yield a PCR product approximately 0.8 kb in size. PCR on the gDNA of strain having the target gene replaced by a selection marker will not yield the 0.8 kb PCR product. Combination of the results from those two separate PCR reactions ensured us that we had the targeted gene replaced by our desired selection marker, *AopyrG*. Results of the PCR analyses are given in **Fig. S8**.

Figure S7. A schematic diagram showing the primer design for the confirmation of a knockout strain by PCR using the gDNA as a template. (A) Confirmation for the generation of knockout strains. (B) Identification for the presence of AnKW2.

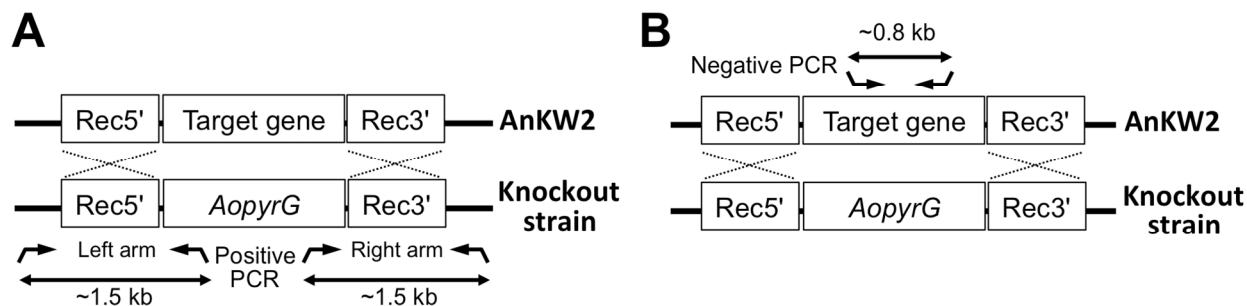


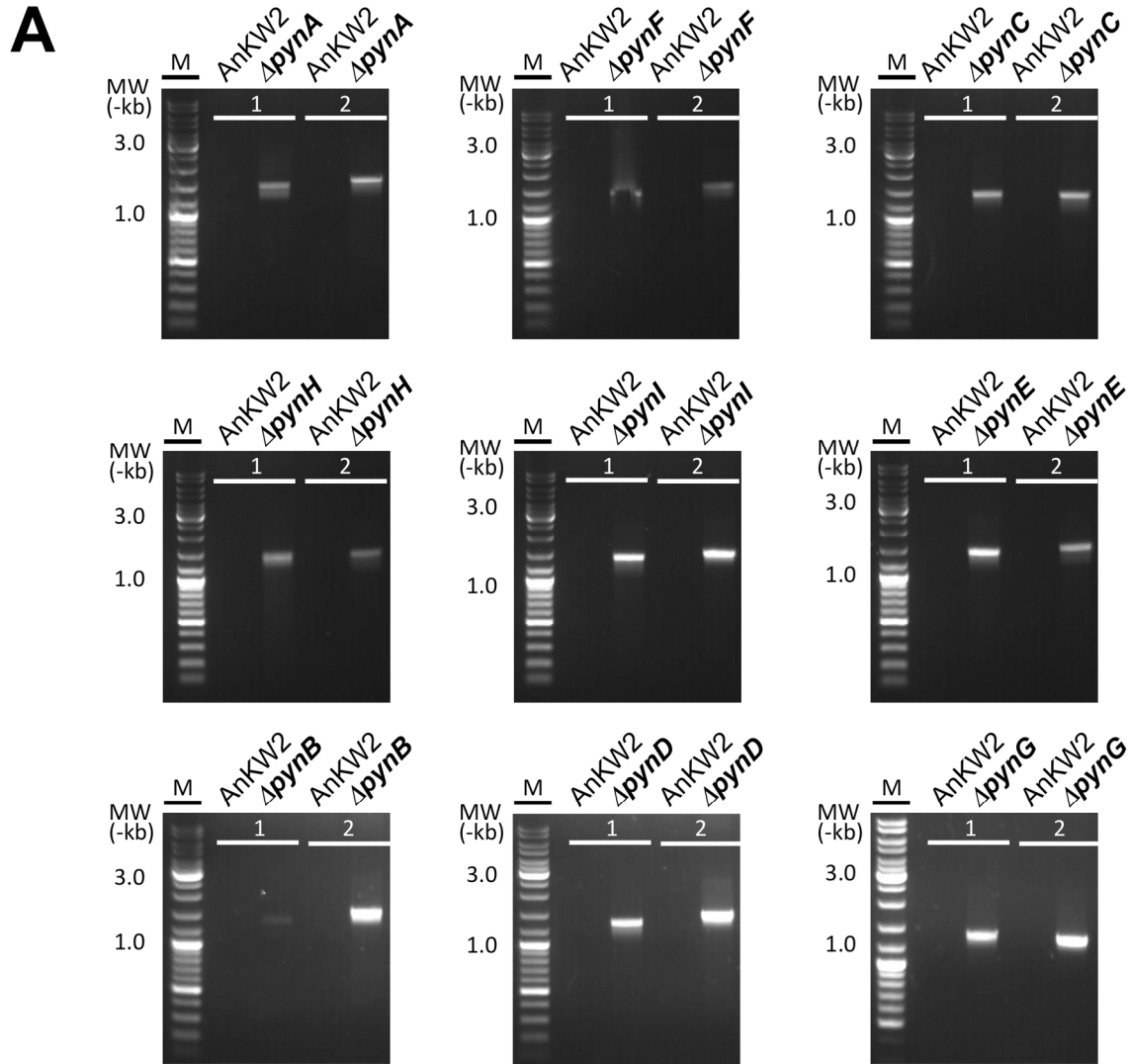
Table S5. The list of primer sets used to create the knockout cassettes for disrupting pyranonigrin biosynthetic genes.

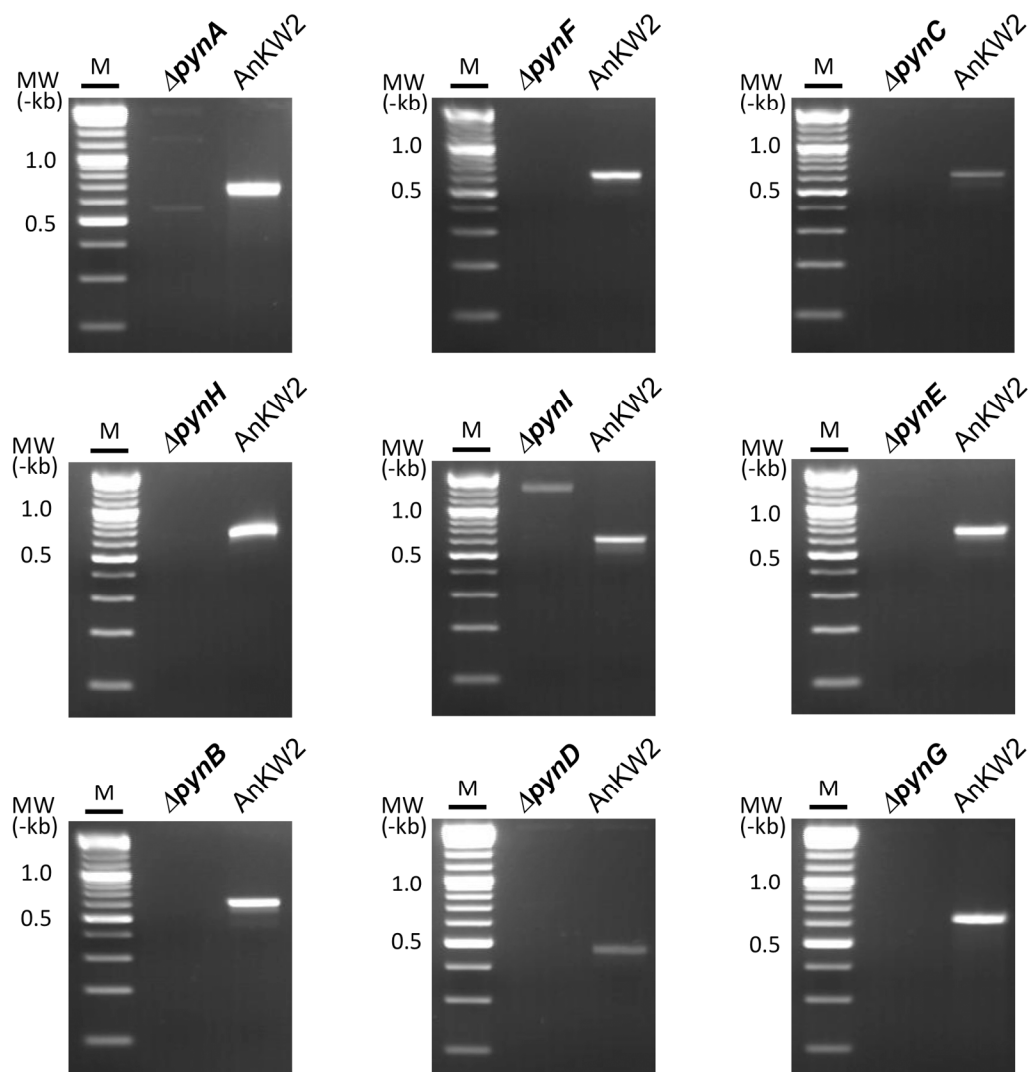
Plasmid name	Target gene	Marker	Primer set for Rec5'	Primer set for marker	Primer set for Rec3'	Digested original vector
pKW20244	<i>pynA</i> (<i>An11g00250</i>)	<i>AopyrG</i>	pKW20244-F1 pKW20244-R1	AopyrG-F AopyrG-R	pKW20244-F3 pKW20244-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20245	<i>pynF</i> (<i>An11g00260</i>)	<i>AopyrG</i>	pKW20245-F1 pKW20245-R1	AopyrG-F AopyrG-R	pKW20245-F3 pKW20245-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20246	<i>pynC</i> (<i>An11g00280</i>)	<i>AopyrG</i>	pKW20246-F1 pKW20246-R1	AopyrG-F AopyrG-R	pKW20246-F3 pKW20246-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20247	<i>pynH</i> (<i>An11g00310</i>)	<i>AopyrG</i>	pKW20247-F1 pKW20247-R1	AopyrG-F AopyrG-R	pKW20247-F3 pKW20247-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20248	<i>pynI</i> (<i>An11g00320</i>)	<i>AopyrG</i>	pKW20248-F1 pKW20248-R1	AopyrG-F AopyrG-R	pKW20248-F3 pKW20248-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20249	<i>pynE</i> (<i>An11g00330</i>)	<i>AopyrG</i>	pKW20249-F1 pKW20249-R1	AopyrG-F AopyrG-R	pKW20249-F3 pKW20249-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20250	<i>pynB</i> (<i>An11g00350</i>)	<i>AopyrG</i>	pKW20250-F1 pKW20250-R1	AopyrG-F AopyrG-R	pKW20250-F3 pKW20250-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20252	<i>pynD</i> (<i>An11g00270</i>)	<i>AopyrG</i>	pKW20252-F1 pKW20252-R1	AopyrG-F AopyrG-R	pKW20252-F3 pKW20252-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>
pKW20253	<i>pynG</i> (<i>An11g00300</i>)	<i>AopyrG</i>	pKW20253-F1 pKW20253-R1	AopyrG-F AopyrG-R	pKW20253-F3 pKW20253-R3	pRS423/ <i>EcoRV</i> , <i>BamHI</i>

Table S6. The list of primer sets used to confirm successful preparation of AnKW2 strains with disruption of the pyranonigrin biosynthetic genes.

Plasmid name	Target gene	Marker	Primer set for positive PCR (left arm)	Primer set for positive PCR2 (right arm)	Primer set for negative PCR
pKW20244	<i>pynA</i> (<i>An11g00250</i>)	<i>AopyrG</i>	pKW20244-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20244-pos-RR	pynA-RTPCR-F pynA-RTPCR-R
pKW20245	<i>pynF</i> (<i>An11g00260</i>)	<i>AopyrG</i>	pKW20245-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20245-pos-RR	pynF-RTPCR-F pynF-RTPCR-R
pKW20246	<i>pynC</i> (<i>An11g00280</i>)	<i>AopyrG</i>	pKW20246-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20246-pos-RR	pynC-RTPCR-F pynC-RTPCR-R
pKW20247	<i>pynH</i> (<i>An11g00310</i>)	<i>AopyrG</i>	pKW20247-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20247-pos-RR	pynH-RTPCR-F pynH-RTPCR-R
pKW20248	<i>pynI</i> (<i>An11g00320</i>)	<i>AopyrG</i>	pKW20248-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20248-pos-RR	pynI-RTPCR-F pynI-RTPCR-R
pKW20249	<i>pynE</i> (<i>An11g00330</i>)	<i>AopyrG</i>	pKW20249-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20249-pos-RR	pynE-RTPCR-F pynE-RTPCR-R
pKW20250	<i>pynB</i> (<i>An11g00350</i>)	<i>AopyrG</i>	pKW20250-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20250-pos-RR	pynB-RTPCR-F pynB-RTPCR-R
pKW20252	<i>pynD</i> (<i>An11g00270</i>)	<i>AopyrG</i>	pKW20252-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20252-pos-RR	pynD-RTPCR-F pynD-RTPCR-R
pKW20253	<i>pynG</i> (<i>An11g00300</i>)	<i>AopyrG</i>	pKW20253-pos-LF AopyrG-pos-LR	AopyrG-pos-RF pKW20253-pos-RR	pynG-RTPCR-F pynG-RTPCR-R

Figure S8. PCR analysis for confirming the deletion of *pynA*, *B*, *C*, *D*, *E*, *F*, *G*, *H* and *I* using the gDNA of $\Delta pynA$ /AnKW2, $\Delta pynB$ /AnKW2, $\Delta pynC$ /AnKW2, $\Delta pynD$ /AnKW2, $\Delta pynE$ /AnKW2, $\Delta pynF$ /AnKW2, $\Delta pynG$ /AnKW2, $\Delta pynH$ /AnKW2 and $\Delta pynI$ /AnKW2 as templates, respectively. Amplifications of PCR products from AnKW2 and knockout strains were performed using the primer sets described in **Table S6**. The sequences of the primer sets are listed in **Table S9**. Lane M, molecular weight marker. (A) Positive PCR for left arm (lanes 1) and right arm (lanes 2) with knockout and AnKW2 strain gDNAs. See **Fig. S7A** for primer-binding arrangement. (B) Negative PCR with knockout and AnKW2 strain gDNAs. See **Fig. S7B** for primer-binding arrangement.



B

1.5 Cloning of the pyranonigrin biosynthetic genes.

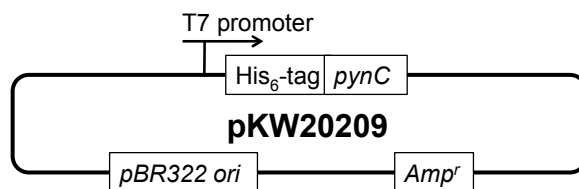
The open reading frame (ORF) of the pyranonigrin biosynthetic gene was predicted based on the *A. niger* genome sequence information available from NCBI database, and their predicted function was determined by comparison to known proteins using the BLAST peptide sequence database search program.³ To construct vectors for expression of the gene in *E. coli* or yeast, we isolated total RNA from AnKW2 using the Ambion RNAqueous kit (Thermo Fisher Scientific Inc.). The SMARTer RACE cDNA Amplification kit (Clontech Laboratories, Inc.) was used for synthesizing cDNA according to the protocol supplied by the manufacturer. *A. niger* AnKW2 strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. Total RNA was isolated from the mycelium using the Ambion RNAqueous kit (Thermo Fisher Scientific Inc.). The mycelia weighing 100 mg were suspended in 100 µL of the elution buffer and 1 mL of the lysis/binding solution provided by the kit and subsequently flash-frozen in liquid nitrogen. The frozen mixture was ground with a refrigerated mortar and pestle for 2 min. The resulting cell powder was then allowed to thaw into a lysate solution. This solution was centrifuged with $13,000 \times g$ at 4 °C for 2 min. The supernatant was mixed with 700 µL of 64% (v/v) ethanol to a final ethanol concentration of 32% and allowed to stand for 30 sec on ice. The resulting supernatant was loaded onto a spin column, and the RNA was eluted from the column with the elution buffer provided by the kit. To examine the quantity and quality of the isolated total RNA, the RNA solution was checked by agarose gel electrophoresis. Subsequently, DNase (3.0 units) was added to the isolated RNA to digest the genomic DNA at 37 °C for 30 min.

Using the SMARTer RACE cDNA Amplification kit (Clontech Laboratories, Inc.), a full-length cDNA of target gene was synthesized by a reverse transcriptase with an adaptor sequence—oligo(dT)₂₀ primer using the mRNA isolated as described above as a template. Then, using 5' and 3' RACE methods, we successfully determined the correct 5' and 3' ends of the ORF, which differed from the database annotations. The ORF was expressed to obtain a purified enzyme in its active form.

1.6 Construction of pKW20209 for expression of *pynC*.

For molecular cloning of *pynC* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *pynC* was amplified by PCR with two primers pKW20209-F1/pKW20209-R1 (**Tables S7 and S9**) and ligated with the pET21c(+) expression vector that was digested with *Nde* I (10 units) and *Xho* I (10 units) to generate pKW20209 (**Fig. S9**) using GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific Inc.). The identity of the resulting vector pKW20209 was confirmed by DNA sequencing (Macrogen Japan Corporation). This plasmid was used for expression of *pynC* for purification of PynC for *in vitro* reactions (See Section 1.13, 1.16 and **Figs. S16 and S18**).

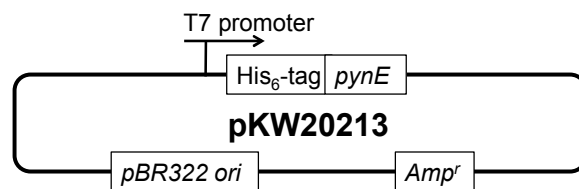
Figure S9. Map of plasmid pKW20209 for the production of PynC in *E. coli*.



1.7 Construction of pKW20213 for expression of *pynE*.

For molecular cloning of *pynE* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *pynE* was amplified by PCR with two primers pKW20213-F1/pKW20213-R1 (**Tables S7 and S9**) and ligated with pET21c(+) expression vector that was digested with *Nde* I (10 units) and *Xho* I (10 units) to generate pKW20213 (**Fig. S10**) using GeneArt Seamless Cloning and Assembly kit. The identity of the resulting vector pKW20213 was confirmed by DNA sequencing. This plasmid was used for expression of *pynE* for purification of PynE for *in vitro* reactions (See Section 1.13, 1.18 and **Figs. 4 ii and S16**).

Figure S10. Map of plasmid pKW20213 for expression of *pynE* in *E. coli*.



1.8 Construction of pKW20298 for expression of *pynG*.

For molecular cloning of *pynG* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *pynG* was amplified by PCR with two primers pKW20298-F1/pKW20298-R1 (Tables S7 and S9) and ligated with pET41c(+) expression vector that was digested with *Bam*H I (10 units) and *Xho* I (10 units) to generate pKW20298 (Fig. S11) using GeneArt Seamless Cloning and Assembly kit. The identity of the resulting vector pKW20298 was confirmed by DNA sequencing. This plasmid was used for expression of *pynG* for purification of PynG for *in vitro* reactions (See Section 1.13, 1.14, 1.15 and Figs. 2 ii, S16 and S17).

Figure S11. Map of plasmid pKW20298 for expression of *pynG* in *E. coli*.

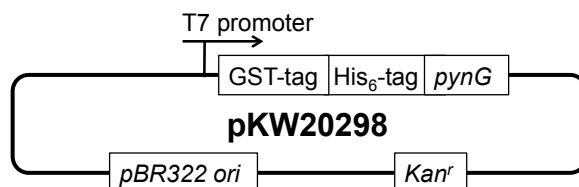


Table S7. The primer set used to create the plasmid for expression of *pynC*, *E* and *G* in *E. coli*.

Plasmid name	Gene	primer set	Digested original vector
pKW20209	<i>pynC</i>	pKW20209-F1 pKW20209-R1	pET21c/ <i>Nde</i> I, <i>Xho</i> I
pKW20213	<i>pynE</i>	pKW20213-F1 pKW20213-R1	pET21c/ <i>Nde</i> I, <i>Xho</i> I
pKW20298	<i>pynG</i>	pKW20298-F1 pKW20298-R1	pET41b/ <i>Bam</i> HI, <i>Xho</i> I

1.9 Construction of pKW10007 for expression of *pynH*.

For molecular cloning of *pynH* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. Genomic DNA extraction were performed as described above. The *pynH* and *P_{coxA}* were amplified by PCR with two sets of primers pKW10007-F1/pKW10007-R1 and pKW20124-F1/pKW20124-R1 (Tables S8 and S9) and ligated with pKW20093⁴ expression vector that was digested with *Mlu* I (10 units) and *Sma* I (10 units) to generate pKW10007 (Fig. S12) using yeast-based homologous

recombination. The identity of the resulting vector pKW10007 was confirmed by DNA sequencing. This plasmid was used for expression of *pynH* for *in vivo* reactions (See Section 1.20 and **Fig. 3 ii**).

Figure S12. Map of plasmid pKW10007 for expression of *pynH* in *A. nidulans* A1145.¹¹

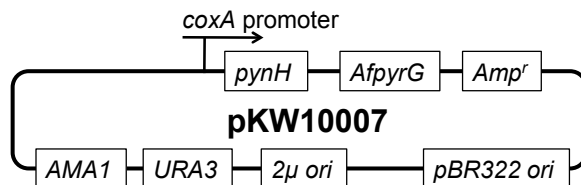


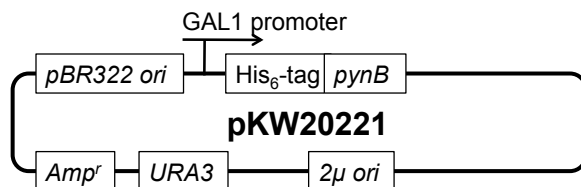
Table S8. The primer set used to create the plasmid for expression of *pynH* in *A. nidulans* A1145.

Plasmid name	Gene	primer set	Digested original vector
pKW10007	<i>pynH</i>	pKW10007-F1 pKW10007-R1	pKW20093/ <i>Mlu</i> I, <i>Sma</i> I
	<i>A. niger P_coxA</i>	pKW20124-F1 pKW20124-R1	

1.10 Construction of pKW20221 for expression of *pynB*.

For molecular cloning of *pynB* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *pynB* was amplified by PCR with two primers pKW20221-F1/pKW20221-R1 (**Table S9**) and ligated with pKW1250⁴ expression vector that was digested with *Sma* I (10 units) and *Hind* III (10 units) to generate pKW20221 (**Fig. S13**) using yeast-based homologous recombination. The identity of the resulting vector pKW20221 was confirmed by DNA sequencing. This plasmid was used for expression of *pynB* for purification of PynB for *in vivo* reactions (See Section 1.21 and **Fig. 5 ii, iii, and iv**).

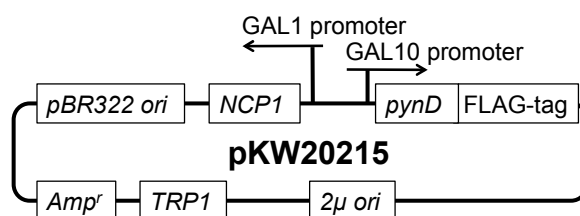
Figure S13. Map of plasmid pKW20221 for expression of *pynB* in *S. cerevisiae*.



1.11 Construction of pKW20215 for expression of *pynD* in *S. cerevisiae*.

For molecular cloning of *pynD* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *pynD* was amplified by PCR with two primers pKW20215-F1/pKW20215-R1 (**Table S9**) and ligated with pKW5050⁴ expression vector that was digested with *Xho* I (10 units) to generate pKW20215 (**Fig. S14**) using yeast-based homologous recombination. The identity of the resulting vector pKW20215 was confirmed by DNA sequencing. This plasmid was used for expression of *pynD* for *in vivo* reactions (See Section 1.19 and **Fig. 2 v**).

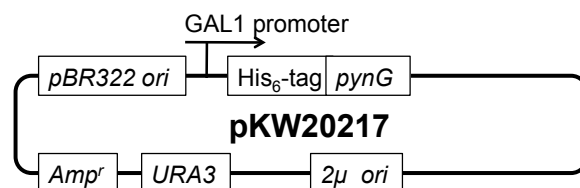
Figure S14. Map of plasmid pKW20215 for expression of *pynD* in *S. cerevisiae*.



1.12 Construction of pKW20217 for expression of *pynG* in *S. cerevisiae*.

For molecular cloning of *pynG* from *A. niger* AnKW2, this strain was cultured in a CD-ST liquid medium containing 10 mM uridine and 5 mM uracil at 30 °C for 3 days with 180 r.p.m. RNA extraction and cDNA synthesis were performed as described above. The *pynG* was amplified by PCR with two primers pKW20217-F1/pKW20217-R1 (**Table S9**) and ligated with pKW1250⁴ expression vector that was digested with *Sma* I (10 units) and *Hind* III (10 units) to generate pKW20217 (**Fig. S15**) using yeast-based homologous recombination. The identity of the resulting vector pKW20217 was confirmed by DNA sequencing. This plasmid was used for expression of *pynG* for *in vivo* bioconversion of **7** (See Section 1.19 and **Fig. 2 iv** and **v**).

Figure S15. Map of plasmid pKW20217 for expression of *pynG* in *S. cerevisiae*.



1.13 Production and purification of PynC, E and G.

Overexpression and subsequent protein purification of PynC was performed as follows: BL21 (DE3) harboring the desired plasmid was grown overnight in 10 mL of LB medium with 100 µg/mL carbenicillin at 37 °C. Each liter of fresh LB medium with 100 µg/mL carbenicillin was inoculated with 5 mL of the overnight culture and incubated at 37 °C until the optical density at 600 nm (OD₆₀₀) reached 0.6. Then expression of each gene was induced with 100 µM isopropylthio-β-D-galactoside (IPTG) at 15 °C. Incubation was continued for another 24 h, after which cells were harvested by centrifugation at 10,000 × g for 10 min. All subsequent procedures were performed at 4 °C or on ice. Harvested cells were resuspended in disruption buffer [0.1 M Tris-HCl (pH 7.4), 0.1 M NaCl, and 20 mM imidazole]. Cells were disrupted by French Press, and the lysate was clarified by centrifugation at 10,000 × g for 10 min. The supernatant and precipitate were recovered as the soluble and insoluble fraction, respectively. The soluble fraction containing protein was applied onto a Ni-NTA (QIAGEN) column. After washing the column with 10 mM imidazole and 0.1 M NaCl in 0.1 M Tris-HCl (pH 7.4), the target protein was eluted with 0.1 M imidazole and 0.1 M NaCl in 0.1 M Tris-HCl (pH 7.4). The purified proteins were pooled and dialyzed against 0.1 M Tris-HCl (pH 7.4), 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M NaCl and 25% (v/v) glycerol. Protein concentration was estimated using the Bio-Rad protein assay kit with bovine immunoglobulin G as a standard. Purified protein samples were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) using Tris-HCl 10% of polyacrylamide gel stained with Coomassie Brilliant Blue R-250 stain solution (CBB; nacalai tesque) (**Fig. S16**). The purified protein was pooled and further concentrated with Amicon Ultra centrifugal concentrator (Millipore). For production and purification of PynE and PynG, the experimental procedure was performed with essentially the same procedure used for purifying PynC as described above.

1.14 Western blotting analyses for *pynG* expression in *E. coli*.

Western blotting analysis was performed to confirm the expression of *pynG* from the plasmid pKW20298 constructed as described earlier for the production of N-terminal hexahistidine-tagged PynG. The nickel affinity chromatography fraction was applied to SDS–PAGE to separate the proteins in the sample. Subsequently, a standard western blotting analysis was carried out on

the gel with the chemiluminescent system (Thermo Fisher Scientific Inc.) using an alkaline phosphatase-based detection method to identify the presence of our target enzymes (**Fig. S17**). Anti-poly-histidine mouse monoclonal antibody HIS-1 (Sigma-Aldrich) was used for visualization of the target protein.

Figure S16. SDS–PAGE analysis of the nickel affinity chromatography fractions of PynC, E and G stained with CBB. Lane M: molecular weight marker; 1: PynC (42 kDa); 2: PynE (33 kDa); and 3: PynG (82 kDa).

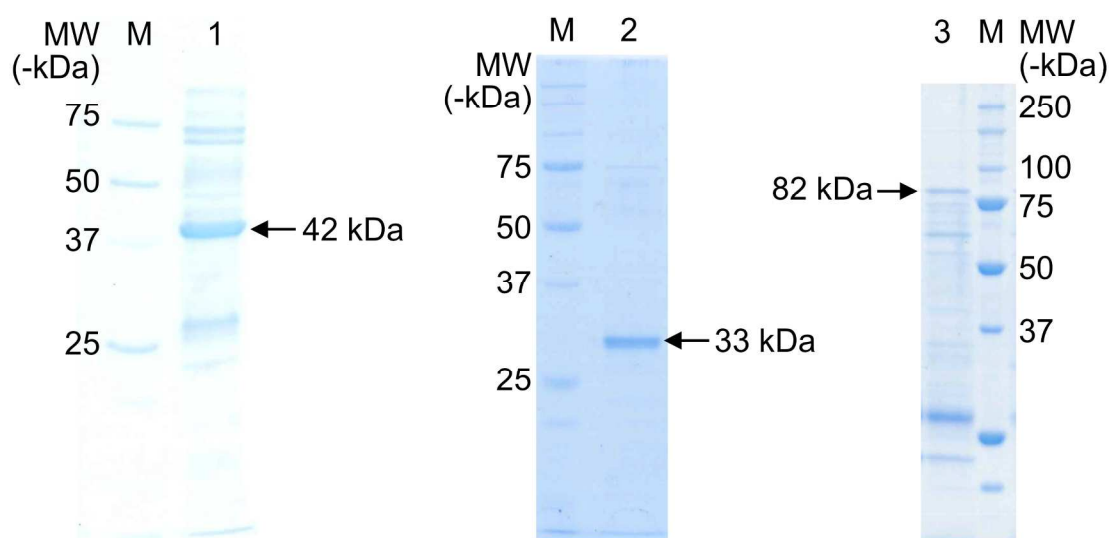
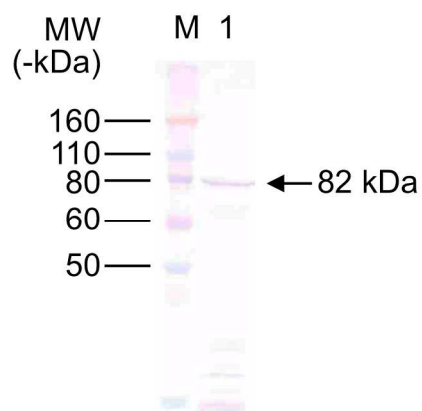


Figure S17. Western blotting analyses for the nickel affinity chromatography fraction of PynG. M: molecular weight marker; 1: PynG (82 kDa).



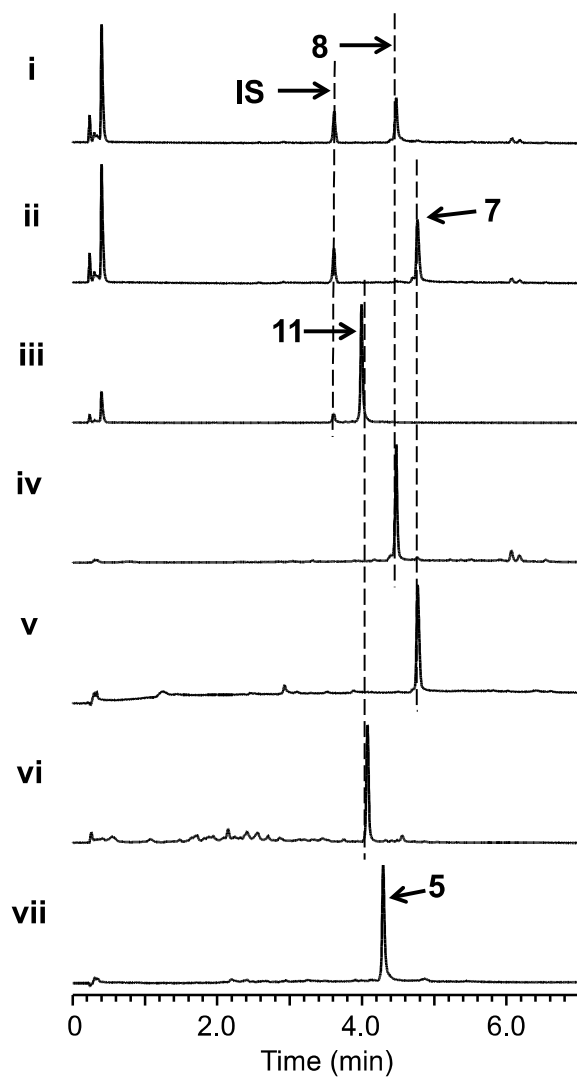
1.15 *In vitro* transformation of **7** with PynG.

A solution with a total volume of 500 μ L comprised of 100 μ L of nickel affinity chromatography fraction containing PynG (see **Fig. S16** and **S17** for the result of the SDS–PAGE and the Western analysis), 100 μ M of **7**, 1 mM NADPH, 2 mM FAD, 0.1 M Tris-HCl (pH 7.4) and 0.1 M NaCl was incubated at 30 °C for 1 h. Heat-inactivated (100 °C for 5 min) PynG was used in reaction as a negative control. After a 1-h incubation, the reaction mixture was quenched by addition of 500 μ L of ethyl acetate including 3.3 μ g/mL of *N*-Boc-L-tryptophan methyl ester as an internal standard. The organic layer was concentrated *in vacuo*. The dried material was dissolved in 100 μ L of *N,N*-dimethylformamide (DMF) and subjected to liquid chromatography–mass spectrometry (LC–MS) analysis. The LC–MS analysis was performed with a Thermo SCIENTIFIC Exactive liquid chromatography mass spectrometer using both positive and negative electrospray ionization. Samples were analyzed using an ACQUITY UPLC 1.8 μ m, 2.1 \times 50 mm C18 reversed-phase column (Waters) and separated on a linear gradient of 5–50% (v/v) CH₃CN in H₂O supplemented with 0.05% (v/v) formic acid at a flow rate of 500 μ L/min. The results of the assay are given in **Figure 2** in the main text.

1.16 Methylation activity by *in vitro* assay of PynC.

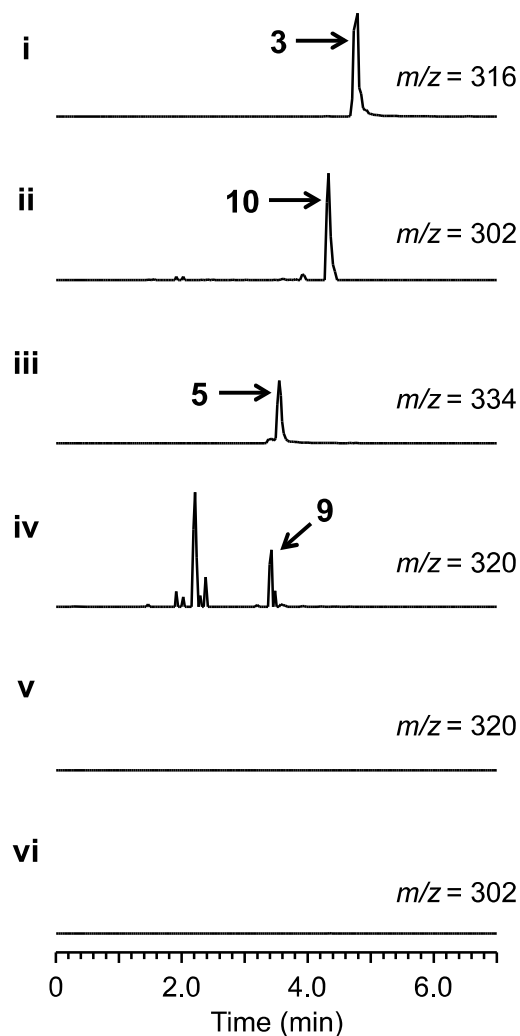
A solution with a total volume of 500 μ L comprised of 100 μ L of nickel affinity chromatography fraction containing PynC (see **Fig. S16** for the result of the SDS–PAGE analysis), 50 μ M of **8** or **11**, 1 mM *S*-adenosyl-L-methionine (SAM), 0.1 M NaCl and 0.1 M Tris-HCl at pH 7.4 was incubated at 30 °C for 1 h. The reaction (250 μ L) was terminated by extraction with ethyl acetate (500 μ L). The extract was dried *in vacuo*. The dried residue was dissolved in DMF (100 μ L), and the resulting solution was subjected to LC–MS analysis (**Fig. S18**). Heat-inactivated (100 °C for 5 min) PynC was used in reaction as a negative control. The results of the assay are given in **Figure S18**.

Figure S18. *In vitro* analysis of the methylation activity of partially purified PynC against **8** and **11**. LC traces of the reaction mixture containing (i) **8** and heat-inactivated PynC, (ii) **8** and PynC, (iii) **11** and PynC are shown. Authentic references are given in (iv) **8**, (v) **7**, (vi) **11** and (vii) **5**.



1.17 Metabolic profile from the cultures of AnKW2-based *pynC* deletion strain.

Figure S19. HPLC traces of metabolic extracts from the culture of $\Delta pynC$ /AnKW2 comparing to the retention time of metabolites from AnKW2. (i) LC trace corresponding to the m/z for **3** ($m/z = 316$) of metabolic extracts from AnKW2 and (ii) LC trace corresponding to the m/z for **10** ($m/z = 302$) of metabolic extracts from $\Delta pynC$ /AnKW2. (iii) LC trace corresponding to the m/z for **5** ($m/z = 334$) of metabolic extracts from AnKW2 and (iv) LC trace corresponding to the m/z for **9** ($m/z = 320$) from $\Delta pynC$ /AnKW2. (v) Extracted mass of 320 of metabolic extracts from AnKW2 and (vi) extracted mass of 302 of metabolic extracts from AnKW2.



1.18 *In vitro* assay for the activity of PynE against **3** and **10**.

The assay mixture (500 μ L) containing 12 μ M of the purified PynE (see **Fig. S16** for the production and purification procedure), 100 μ M of **3** or **10**, 1 mM NADPH, 0.1 M Tris-HCl (pH 7.4) and 0.1 M NaCl was incubated at 30 °C for 1 h. The reaction (250 μ L) was terminated by extraction with ethyl acetate (500 μ L). The extract was dried *in vacuo*. The dried residue was dissolved in DMF (100 μ L), and the resulting solution was subjected to LC–MS analysis. Heat-inactivated (100 °C for 5 min) PynC was used in reaction as a negative control. The results of the assay are given in **Figure 4** in the main text.

1.19 *In vivo* transformation of **7** to **4/6** by expressing *pynG* and **7** to **5** by co-expressing *pynG* and *pynD* in *S. cerevisiae*.

SCKW12 generated from *S. cerevisiae* BY4705¹² was transformed with pKW20217 (**Fig. S15**) for the production of **4/6** by feeding **7** to the strain. Similarly, SCKW12 was transformed with pKW20217 and pKW20215 (**Fig. S14**) for the production of **5** by feeding **7** to the strain. Selected cells were grown overnight in SC medium (2 mL) without uracil (for transformants with pKW20217) or L-tryptophan and uracil (for co-transformants with pKW20217 and pKW20215) at 30 °C for 40 h with 180 r.p.m. The overnight culture (100 μ L) was transferred into 2 mL of the fresh SC medium without L-leucine, and the culture was incubated at 30 °C for 48 h. Fresh YPD medium (1 mL) was inoculated with each of the culture (100 μ L) and incubated at 30 °C for 15 h. Then, expression of each gene was induced with 2% D-galactose at 30 °C, and incubation was continued for another 9 h. Substrate **7** was added to the medium to a final concentration of 200 μ M, and the culture was incubated for an additional 4 h at 30 °C. The resultant culture (500 μ L) was terminated by extraction with ethyl acetate (500 μ L). The extract was dried *in vacuo*. The dried residue was dissolved in DMF (100 μ L), and the resulting solution was subjected to LC–MS analysis. The LC–MS analysis was performed with essentially the same procedure described above. SCKW12 harboring the expression vectors without *pynG* and *pynD* (pKW1250 and pKW5050) was used as a negative control. The results of the assay are given in **Fig. 2** in the main text.

1.20 Bioconversion of **5** to **3** by expression of *pynH* in *A. nidulans* A1145.

Bioconversion of **5** to **3** was performed by using engineered *A. nidulans* A1145. *A. nidulans* A1145 was transformed with pKW10007 (**Fig. S12**) that contained an overexpression cassette of *pynH* for the bioconversion experiments in which **5** was fed to the strain. Selected cells were grown overnight in CD-ST medium (20 mL) with 0.5 µg/mL pyridoxine HCl and 0.125 µg/mL riboflavin at 30 °C for 48 hours with 180 r.p.m. The culture was transferred into 0.5 mL of the fresh CD-ST medium with 0.5 µg/mL pyridoxine HCl and 0.125 µg/mL riboflavin. Subsequently, substrate **5** was added to the medium to a final concentration of 400 µM, and the culture was incubated for an additional 3 hours at 18 °C. The resultant culture was filtered to separate the liquid medium and the cells. One milliliter of the liquid medium was extracted with ethyl acetate. The ethyl acetate extract was combined and concentrated *in vacuo* and the dried residue was dissolved in DMF (50 µL). The resulting solution was subjected to LC–MS analysis. LC–MS analysis was performed with a Thermo Scientific Exactive liquid chromatography mass spectrometer using both positive and negative electrospray ionization. Samples were analyzed using an ACQUITY UPLC 1.8 µm, 2.1 × 50 mm C18 reversed-phase column (Waters) and separated on a linear gradient of 10–80% (v/v) CH₃CN in H₂O supplemented with 0.05% (v/v) formic acid at a flow rate of 500 µL/min. The results of the assay are given in **Fig. 3** in the main text.

1.21 *In vivo* transformation of **2** or **11** to **3** or **10** by expression of *pynB* in *S. cerevisiae*.

SCKW12 was transformed with pKW20221 (**Fig. S12**) for the production of **3** or **10** by feeding **2** or **11** to the strain. Selected cells were grown overnight in SC medium (2 mL) without uracil at 30 °C for 40 h with 180 r.p.m. The overnight culture (100 µL) was transferred into 2 mL of the fresh SC medium without L-leucine and the culture was incubated at 30 °C for 48 h. Fresh YPD medium (1 mL) was inoculated with each of the culture (100 µL) and incubated at 30 °C for 15 h. Then, expression of each gene was induced with 2% D-galactose at 30 °C, and incubation was continued for another 9 h. Substrate **2** or **11** was added to the medium to a final concentration of 200 µM, and the culture was incubated for an additional 4 h at 30 °C. The resultant culture (500 µL) was terminated by extraction with ethyl acetate (500 µL). The extract was dried *in vacuo*. The dried residue was dissolved in DMF (100 µL), and the resulting solution was subjected to

LC–MS analysis. The LC–MS analysis was performed with essentially the same procedure described above. SCKW12 harboring the expression vectors without *pynB* (pKW1250) was used as a negative control. The results of the assay are given in **Fig. 5** in the main text.

1.22 Engineered biosynthesis of **1** in AnKW1.

For isolation of the biosynthetic product **1**, the engineered strain AnKW1 was incubated in 150 mL of CD-ST medium for 2 days with 180 r.p.m. Five liters of the same medium was inoculated with 150 mL of the culture and incubated at 30 °C for 6 days using the BioFlo/CelliGen 115 fermentor system (Eppendorf, Inc.). The resultant culture was filtrated. The acetone extract of mycelia was concentrated *in vacuo* and extracted with ethyl acetate to give an oily residue. This residue was fractionated by ODS gel flash column chromatography with 25–75% H₂O (20 mM NaH₂PO₄ and 1 mM EDTA)/CH₃CN gradient elution. The fractions containing the desired material were pooled and dried. The residues were purified by reversed-phase HPLC (Wako Pure Chemical Industries, Ltd., Wakosil-II 5C18 5 µm, 20 × 250 mm) using a linear gradient of 60–100% (v/v) CH₃OH in H₂O over 30 min at a flow rate of 8.0 mL/min. The fractions containing the desired material were pooled and dried. A part of the residue were purified by reversed-phase HPLC (Nacalai Tesque Inc., Cosmosil 5C18 MS-II 5 µm, 20 × 250 mm) on an isocratic of 70% CH₃CN in H₂O (20 mM NaH₂PO₄ and 1 mM EDTA) over 30 min at a flow rate of 8.0 mL/min. The fractions containing **1** were collected, concentrated and subsequently extracted with ethyl acetate. This procedure afforded 110 mg of **1**.

NMR spectra were obtained with a JEOL JNM-ECA 500 MHz spectrometer (¹H 500 MHz, ¹³C 125 MHz). ¹H NMR chemical shifts are reported in parts per million (ppm) using the proton resonance of residual solvent as reference: CDCl₃ δ 7.26, DMSO-*d*₆ δ 2.50 and CD₃OD δ 3.31. ¹³C NMR chemical shifts are reported relative to CDCl₃ δ 77.16, DMSO-*d*₆ δ 39.5 and CD₃OD δ 49.0.¹³ Mass spectra were recorded with a Thermo SCIENTIFIC Exactive liquid chromatography mass spectrometer by using both positive and negative ESI. LC–MS was conducted with a Thermo SCIENTIFIC Exactive liquid chromatography mass spectrometer by using positive electrospray ionization. Samples were separated for analysis on an AQUITY UPLC 1.8 µm, 2.1 × 50 mm C18 reversed-phase column (Waters) using a linear gradient of 5–80% (v/v) CH₃CN in H₂O supplemented with 0.05% (v/v) formic acid at a flow rate of 500 µL/min.

1.23 Engineered biosynthesis of **11** in the AnKW2 *ΔpynC* mutant strain.

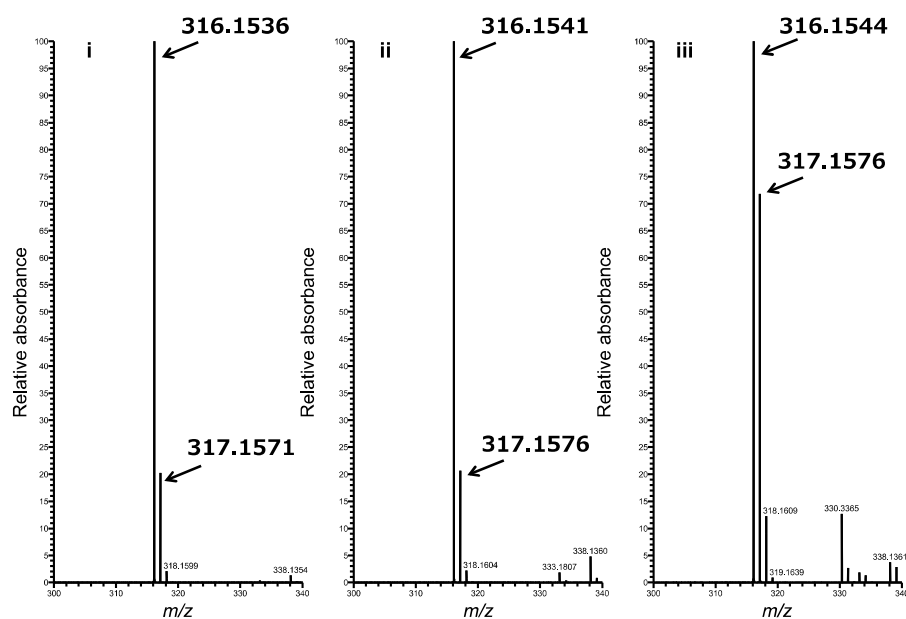
For isolation of the biosynthetic product **11**, the AnKW2 *ΔpynC* mutant strain was incubated in 150 mL of CD-ST medium for 39 hrs with 180 r.p.m. Five liters of fresh CD-ST liquid medium was inoculated with 20 mL of the culture and incubated at 30 °C for 3 days using the BioFlo/CelliGen 115 fermentor system (Eppendorf, Inc.). The resultant culture was directly extracted with MeOH (5 L). The extract was filtrated, concentrated and subsequently extracted with ethyl acetate (5 L). The ethyl acetate extract was concentrated *in vacuo* to give an oily residue, which was fractionated by silica gel flash column chromatography with 0–100% CH₃OH/CHCl₃ gradient elution. The fractions containing the desired material were pooled and dried. The residue were purified by reversed-phase HPLC (Nacalai Tesque Inc., Cosmosil 5C18 MS-II 5 μm, 20 × 250 mm) on an isocratic of 50% CH₃CN in H₂O over 80 min at a flow rate of 8.0 mL/min. The fractions containing **11** were collected, and this procedure afforded 93.9 mg of **11**.

1.24 Bioconversion experiments for producing **3** from fed [1-¹³C]-L-serine in AnKW2.

Bioconversion experiments using fed [1-¹³C]-L-serine were performed by using AnKW2. AnKW2 was grown overnight in CD medium (20 mL) containing 10 mM uridine and 5 mM uracil at 30 °C for 48 hours with 180 r.p.m. The culture (200 μL) was transferred into 2 mL of the fresh CD-ST medium supplemented with 10 mM uridine and 5 mM uracil. Subsequently, 20 μL of sterilized [1-¹³C]-L-serine (500 mM) was added to the medium to a final concentration of 5 mM, and the culture was incubated for an additional 24 hours at 30 °C. The resultant culture was filtered to separate the liquid medium and the cells. 250 μL of the liquid medium was extracted with ethyl acetate (250 μL). The ethyl acetate extracts were combined and concentrated *in vacuo*, and the dried residue was dissolved in DMF (50 μL). The resulting solution was subjected to LC–MS analysis (**Fig. S20**). LC–MS analysis was performed with a Thermo Scientific Exactive liquid chromatography mass spectrometer using both positive and negative electrospray ionization. Samples were analyzed using an ACQUITY UPLC 1.8 μm, 2.1 × 50 mm C18

reversed-phase column (Waters) and separated on a linear gradient of 10–80% (v/v) CH₃CN in H₂O supplemented with 0.05% (v/v) formic acid at a flow rate of 500 μ L/min.

Figure S20. LC traces corresponding to the m/z for **3**. (i) $m/z = 316$ of $[M+H]^+$ as a control isolated from AnKW2 without feeding L-serine. (ii) $m/z = 316$ of $[M+H]^+$ as a control isolated from regular L-serine fed AnKW2. (iii) $m/z = 317$ of $[M+H+1]^+$ as a control isolated from [¹³C]-L-serine fed AnKW2.



2. Supporting Results

Table S9. Oligonucleotide primer sequences. DNA primers were designed on the basis of sequence data obtained from the *A. niger* sequence database.

Primer name	Sequence, 5'-3'
pKW20155-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCTCACTTCCGTGACGGTGAGGACATT
pKW20155-R1	TTCAACACAGTGGAGGACATACCCGTATCCGCCCCACTGCTTATTCCTT
pKW20155-F2	CGGGTATGTCCTCCACTGTGTGAA
pKW20155-R2	AGCAACCAGCGATAAGCAGGCGATA
pKW20155-F3	TATCGCCTGCTATCGTGTGTTGCTGACCTTCCACGGAAGTGACCTGCTG
pKW20155-R3	CGCAAGAGCCATCGCCATTGCTGAGGTGTAATGATGCTG
pKW20155-F4	CTTCATCCCCAGCATCATTACACCTCAGCAATGGCCAAGAAAGCTGAGTTAC
pKW20155-R4	GGTGGCGGCCGCTCTAGAACTAGTGGACCAGTCAGAGGCGATTTCATGCAG
pKW20155-pos-LF	ACACTACCCCACTCGATCACCTTGG
pKW20155-pos-LR	ACTTGCAATTGCTCAACGGTCGCATA
pKW20155-pos-RF	CTCGTCCGTTTACAAGCTGAAGAGC
pKW20155-pos-RR	ATAGATTATCCGTCGCGCATCCAG
P_pynR-neg-F	GTCAAGGTTCTTGCTGTGCCAGTG
P_pynR-neg-R	CAGTTGAACACGGGGCTCCAAAATC
Afu2g08360::PtrA-RR	GGTAATAGACTCGCCCTCCA
Afu8g00540::PtrA-RF	TCAGTTTCGTCCGAGGACTT
Afu2g08360-neg-F	ACATCCTCACCGATTTCAGC
Afu2g08360-neg-R	CCGATGCAGGAGTCTGGTAT
pKW20244-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCCCTAGCACTGGATCTCCTCCCCACA
pKW20244-R1	TTCAACACAGTGGAGGACATACCCGGGACGACGTTGAAGGGTCGAATAGG
AopyrG-F	CGGGTATGTCCTCCACTGTGTGAAGGTTCTCCACTTGC GGAGACAAAG
AopyrG-R	TTACATGGCGGAACATGGCTTGAACCCGGCTGATGCATTAAAAACAACG
pKW20244-F3	GTTCAAGCCATGTTCCGCCATGTAATGCCGGCTACTTCAGCATGGCTATC
pKW20244-R3	GGTGGCGGCCGCTCTAGAACTAGTGGAGTGGAACGACGCTCTCTCGTTCCG
pKW20245-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCAATCGAGCGAGTCTTGCCACTTTTC
pKW20245-R1	TTCAACACAGTGGAGGACATACCCGGCGTTTACAGGGTGGAAGGTCTGTG
pKW20245-F3	GTTCAAGCCATGTTCCGCCATGTAATACTACCCAGCGCAGCTAGGTGTCAT
pKW20245-R3	GGTGGCGGCCGCTCTAGAACTAGTGTCGCGAAGAGCAATCCTCCCTAATG
pKW20246-F1	GTATCGATAAGCTTGATGCGGCCGCGGAGGGTGACTGTGAAGCGAGATA
pKW20246-R1	TTCAACACAGTGGAGGACATACCCGTTGTGTGGGACAGGACATTGTGTGA
pKW20246-F3	GTTCAAGCCATGTTCCGCCATGTAATTTGACCCTCAAGCAACGTCAGACC
pKW20246-R3	GGTGGCGGCCGCTCTAGAACTAGTGACAGGTTTACGCCGATTGCATTTTT
pKW20247-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCTGGAGAGAGTGCTGGCATGAATGTG
pKW20247-R1	TTCAACACAGTGGAGGACATACCCGCTGGTCCTTGTTCCTGCGTTTGCT
pKW20247-F3	GTTCAAGCCATGTTCCGCCATGTAAGACGGAGAATCCAAGACCGTAGATCA
pKW20247-R3	GGTGGCGGCCGCTCTAGAACTAGTGTAAGAGCCGATGTCGAAATGAGGA
pKW20248-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCCCATCCTTGGTAGAAGGACGCTTGC
pKW20248-R1	TTCAACACAGTGGAGGACATACCCGTGAAGGAGGCTTTTGGCTTTCTTCC

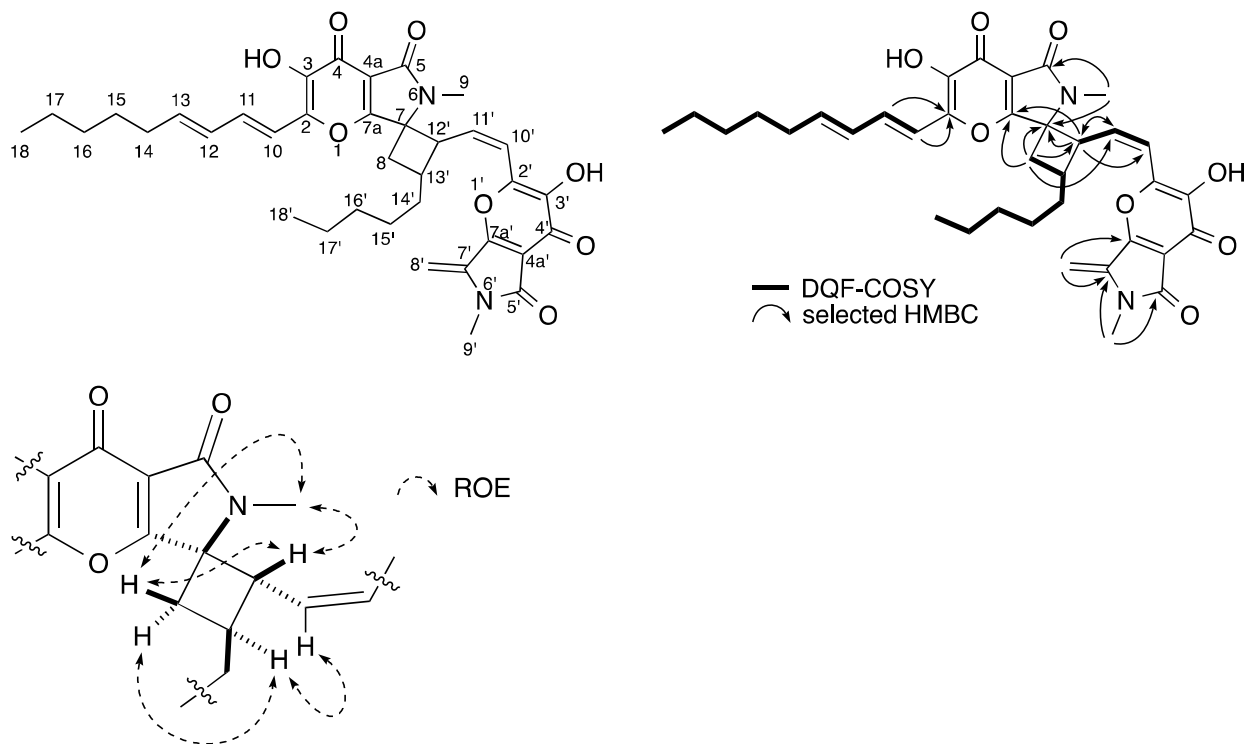
Primer name	Sequence, 5'-3'
pKW20248-F3	GTTC AAGCCATGTTCCGCCATGTAAATTTGCGAATGCTGTTCAACGATGG
pKW20248-R3	GGTGGCGGCCGCTCTAGAACTAGTGGCACCCAGAACATCGACCAAGAGAC
pKW20249-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCCGCATCGCTCGGCTGTGGAAGACTGTA
pKW20249-R1	TTCAACACAGTGGAGGACATACCCGTGTTGTGTCTGAAGGATGAAGGATTATGGA
pKW20249-F3	GTTCAAGCCATGTTCCGCCATGTAAGGCGAAGTGAGTGAGTTTTGGGTTA
pKW20249-R3	GGTGGCGGCCGCTCTAGAACTAGTGGATTACAGTCGGAGCTCCAGCCACA
pKW20250-F1	TCGACGGTATCGATAAGCTTGATGCGGCCCGCGAAAACCATACGCGACTAGCAGTGTCA
pKW20250-R1	TTCAACACAGTGGAGGACATACCCGTGCAGATGCTGTGATTTTGATCCTCTTG
pKW20250-F3	GTTCAAGCCATGTTCCGCCATGTAAGATCGCTCGGCTGTGGAAGACTGTA
pKW20250-R3	TCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGAGGAAGGCAATGGTTGGTTTGGACA
pKW20252-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCCACAGCTTGGAACAAGGAAAGCCAGA
pKW20252-R1	TTCAACACAGTGGAGGACATACCCGCAAGAAATGAAGTGGGCGTGATTGG
pKW20252-F3	GTTCAAGCCATGTTCCGCCATGTAAGGCTCACGTCTGCTTTGCAGTATGA
pKW20252-R3	GTGGCGGCCGCTCTAGAACTAGTGTCAATTATGCTCATTTTCGACCATGGAA
pKW20253-F1	GGTCGACGGTATCGATAAGCTTGATGCGGCCCGCAGCGCTGTTCCACATTGAGACAACC
pKW20253-R1	TTCAACACAGTGGAGGACATACCCGCCAATCCTTCGACACATGGCTGCTA
pKW20253-F3	GTTCAAGCCATGTTCCGCCATGTAATCACTTCCGTGACGGTGAGGACATT
pKW20253-R3	GGTGGCGGCCGCTCTAGAACTAGTGTATCCCGCCCCACTGCTTATTCCTT
pKW20244-pos-LF	ATCCTCTCTCGCTACGTGGCAGCTC
AopyrG-pos-LR	TGGGGTACTCTTCATTGGGACGTTG
AopyrG-pos-RF	CGGGTATCCGCTCCAAAGCTGTCTA
pKW20244-pos-RR	GGACATGAAGGGAGAATCAGCGACA
pKW20245-pos-LF	TCCAGAATACCGTCGGCTCGTCTCT
pKW20245-pos-RR	GACACGACAAGCAAGCCCCACAATA
pKW20246-pos-LF	CCCCGAAAACATGCATTGTGTGCGATT
pKW20246-pos-RR	ATGCTACATCGGCTCAACCGTCTCC
pKW20247-pos-LF	TCACCTCCTCCACGTGTTCTCATC
pKW20247-pos-RR	CCGTAGGGCATTAGGAGCCAGTGAA
pKW20248-pos-LF	GCGGCTTGCTTTCCATCCAGAGTAG
pKW20248-pos-RR	CCCCAGACGGAGACTTTCTCATCCTC
pKW20249-pos-LF	TATCTGCGGCCAAAGATTGGGTGAG
pKW20249-pos-RR	TCGTCGTTAACCCCGTTGATCTTCA
pKW20250-pos-LF	TCATTTTGGAACAGGGGTTCCCGTA
pKW20250-pos-RR	GGGATACCAGGGTTGGTGTGGTGA
pKW20252-pos-LF	CGCGATCAAGAATAATGATGCAAGGA
pKW20252-pos-RR	CGGGGACCAGGAAGAAATCGAAGAT
pKW20253-pos-LF	GGGATTGCAGACTTCGCGTGGTAAT
pKW20253-pos-RR	CGACGGAAC TGGCATT TTAATGAGC
pynA-RTPCR-F	TTGGCCGGTATCATAAAAGC
pynA-RTPCR-R	AATAGGCATACCGTGGCAAG
pynF-RTPCR-F	CTCTTTAGCGTGGGAATGCT
pynF-RTPCR-R	TCGAACCACAAAAGTCGATG
pynC-RTPCR-F	GCTGTACTGGGTACGGAAGC
pynC-RTPCR-R	GCAGGAAGTGAAAAGGATGA

pynH-RTPCR-F	TCGCACACATCTTCGATTTC
pynH-RTPCR-R	TGGAGAATCTGCTCCGAGAT
pynI-RTPCR-F	CAGCGATGTCTTCCATCAGA
pynI-RTPCR-R	ACAAATGGACCCCAGCTTC
pynE-RTPCR-F	GCCACCTACGATTGTCTCAA
pynE-RTPCR-R	TGTTGAGCTCTTGCTCTTCG
pynB-RTPCR-F	TATCTCCTAGGGGGCTGGTC
pynB-RTPCR-R	CTCCTCCACGTGTTCTCAT
pynD-RTPCR-F	AAACCAACCATTGCCTTCCT
pynD-RTPCR-R	ATATGATCCCCGGTCAACAA
pynG-RTPCR-F	CTCCGCTAGAGGCTTCGTTT
pynG-RTPCR-R	GGAAATGTGACGTTGTGCTC
pynR-RTPCR-F	ACGCTCTGTATCCCCAACAC
pynR-RTPCR-R	CGAAGGACAGGTCCAGAGAG
gpdA-RTPCR-F	GTCCTCTCCAACGCTTCTTG
gpdA-RTPCR-R	TCGTACCAGGAGACGAGCTT
pKW20209-F1	TAAGAAGGAGATATACAATGTCCCCGTCCACCATCAACC
pKW20209-R1	TGGTGGTGGTGGTGCCCTGCAACCACCAACTCCACAACCC
pKW20213-F1	TAAGAAGGAGATATACAATGTCCAAACCAACCATTGCCTTCC
pKW20213-R1	TGGTGGTGGTGGTGCCCCCAACAAGTCAATGTCACCTTCTCATCCA
pKW20298-F	CCCATGGATATCGGGATGAACCCGAACCAACCACACG
pKW20298-R	GTGGTGGTGGTGGTGCTAGGCAGTCTTCTTCGCGGCCTC
pKW10007-F1	TCATTGCAACCCACCCACCAGGACAATGTTCCCTGCTCTCGTATTTGGTCTCTG
pKW10007-R1	GGACATACCCGTAATTTTCTGGGCATTAAATTCTGGGGGCGGGATTAGTCACATAC
pKW20124-F1	CGGGTGTTCTTGACGATGGCATCCTGCGGCCGCTGTGGAATCTCCTCCTTGC
pKW20124-R1	CGCCTACCGCAAGAGCCATCGCCATTGTCCTGGTGGGTGGGT
pKW20221-F1	TCACCATCACCATCATCATCATATGAGACTCTCCGCTAGAGGCTTCGTTTG
pKW20221-R1	TTCGCTTATTTAGAAGTGCGCGCCTTAGCAACTAATCCCCTCCGGCACG
pKW20215-F1	AAGAATTTTGAATAATCAATATAAATGTGGCGTATTCCTGTTATCGTG
pKW20215-R1	ACTTGTCATCGTCATCTTTATAATCTGCTTCCTCCATTCCCTGCTTATCT
pKW20217-F1	TCACCATCACCATCATCATCATATGAACCCGAACCAACCACACG
pKW20217-R1	TTCGCTTATTTAGAAGTGCGCGCCTTAGGCAGTCTTCTTCGCGGCCTC

2.1 Chemical characterization of **1**.

Table S10. NMR data of compound **1** in CDCl₃. Isolated amount: 110 mg from 5 L of a culture as a yellow powder. The molecular formula of **1** was established by mass data [ESI-MS: m/z 631 (M+H)⁺; HRESIMS: m/z 631.3015 (M+H)⁺, calcd. for C₃₆H₄₃N₂O₈⁺, 631.3014, Δ = 0.1 mmu].

$[\alpha]_D^{23}$ +2.7 (c 0.1, CHCl₃), IR (ATR) 1198, 1227, 1380, 1468, 1632, 1709, 2855, 2927, 2953, 3302 cm⁻¹.



Position	δ_{H}		mult. (J in Hz)	HMBC	δ_{C}
2					145.8
3					145.4 or 142.1
4					168.1 or 167.4
4a					112.0 or 110.4
5					163.3
7					64.1
7a					176.0
8 α	1.82	1H	dd (14.2, 5.7)	7, 7a, 11', 12'	35.1
8 β	2.43	1H	dd (14.2, 7.9)	7, 7a, 11', 12'	
9	3.07	3H	s	5, 7	25.5 or 25.4
10	6.46	1H	d (15.3)	2, 11, 12	116.1
11	6.63	1H	dd (15.3, 10.8)	2, 10, 12, 13	134.3
12	6.19	1H	dd (15.3, 10.8)	10, 14	129.8
13	5.87	1H	dt (15.3, 7.4)	11, 14, 15	141.8
14	2.15	2H	ddd (7.4, 7.4, 7.4)	12, 13, 15	33.2
15	1.43	2H	m	13, 14, 17	28.7
16	1.31	2H	m	14, 18	31.9
17	1.31	2H	m	15, 16	22.7
18	0.88	3H	t (6.8)	16, 17	14.1
2'					145.9
3'					145.4 or 142.1
4'					168.1 or 167.4
4a'					112.0 or 110.4
5'					161.6
7'					137.3
7a'					163.8
8'	5.04	1H	d (2.8)	7a'	94.4
	4.91	1H	d (2.8)	7', 7a'	
9'	3.08	3H	s	5', 7'	25.5 or 25.4
10'	5.88	1H	d (10.2)		121.9
11'	6.24	1H	ddd (10.2, 2.6, 2.5)	12'	134.6
12'	4.31	1H	dd (5.4, 2.6)	7, 7a, 10', 11'	40.6
13'	2.66	1H	m		34.1
14'	1.62	1H	m		36.1
	1.44	1H	m		
15'	1.44	2H	m		27.3
16'	1.31	2H	m	17'	31.6
17'	1.31	2H	m	16'	22.6
18'	0.88	3H	t (6.8)	16', 18'	14.1
3-OH	n.d.				
3'-OH	n.d.				

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Figure S21. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **1** in CDCl_3 .

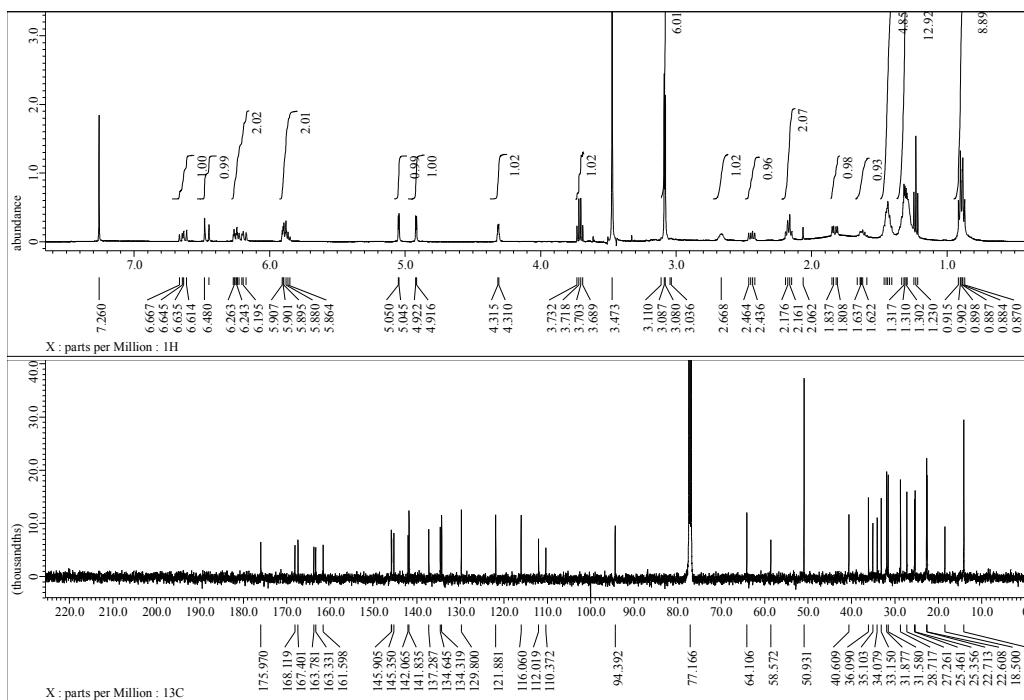


Figure S22. DQF-COSY spectrum of **1** in CDCl_3 (500 MHz). DQF-COSY, double quantum filtered-correlated spectroscopy.

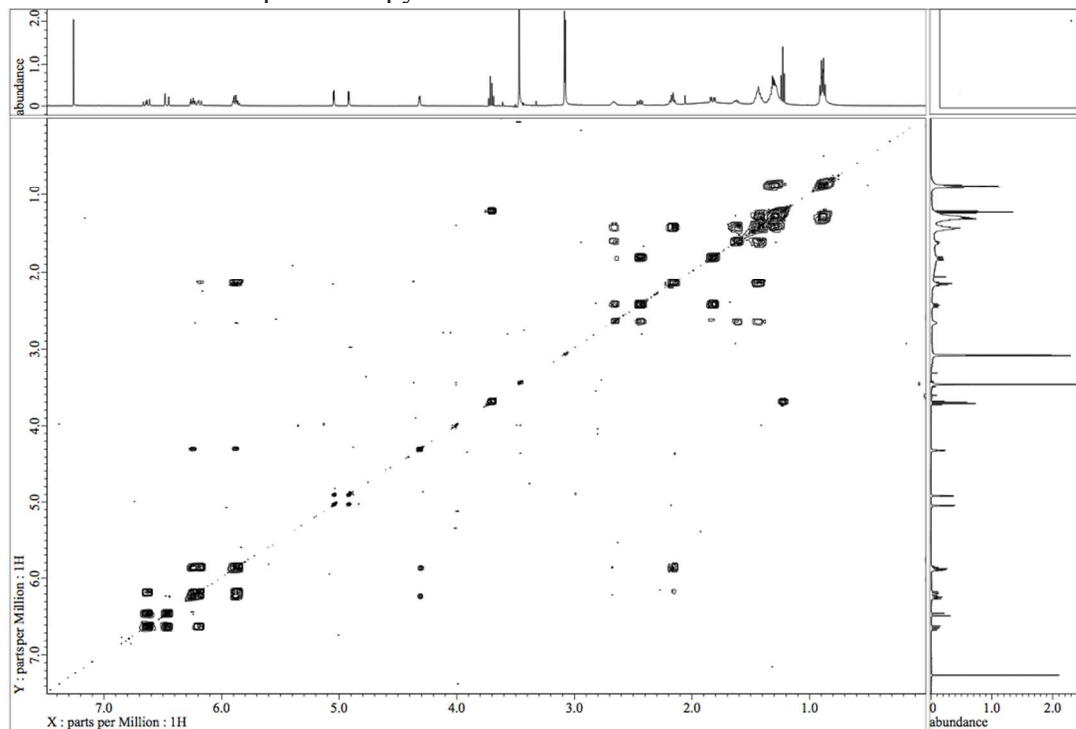


Figure S23. HMQC spectrum of **1** in CDCl₃ (500 MHz). HMQC, heteronuclear multiple quantum coherence.

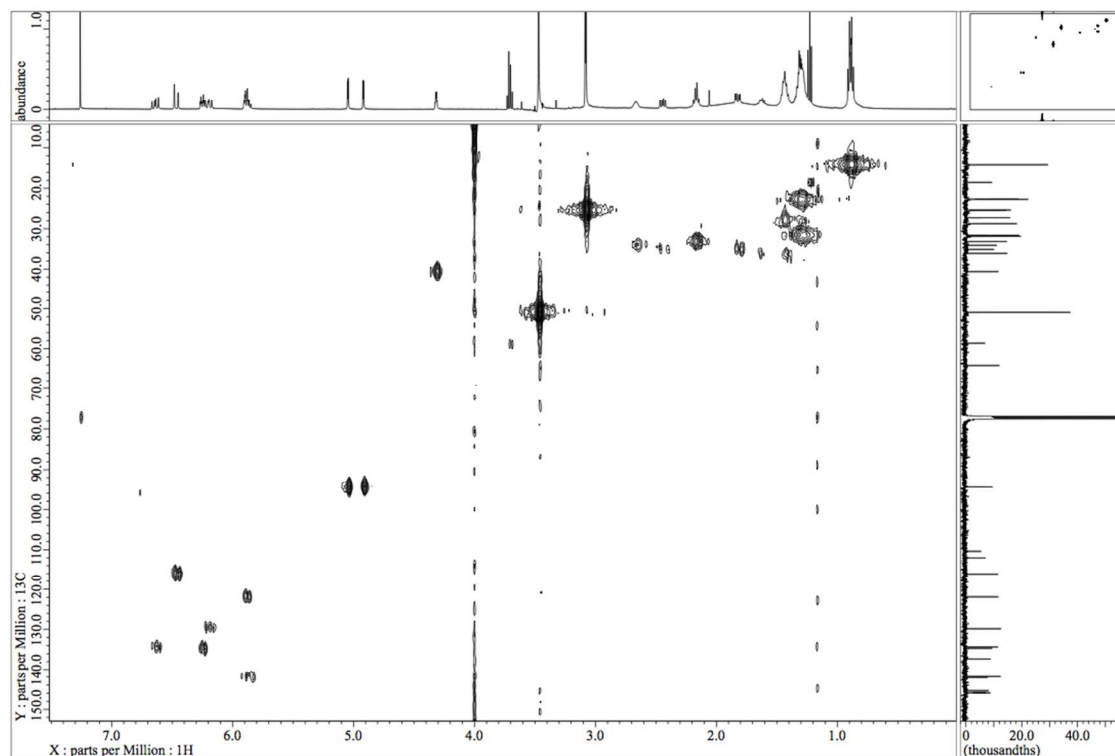


Figure S24. HMBC spectrum of **1** in CDCl₃ (500 MHz). HMBC, heteronuclear multiple bond correlation.

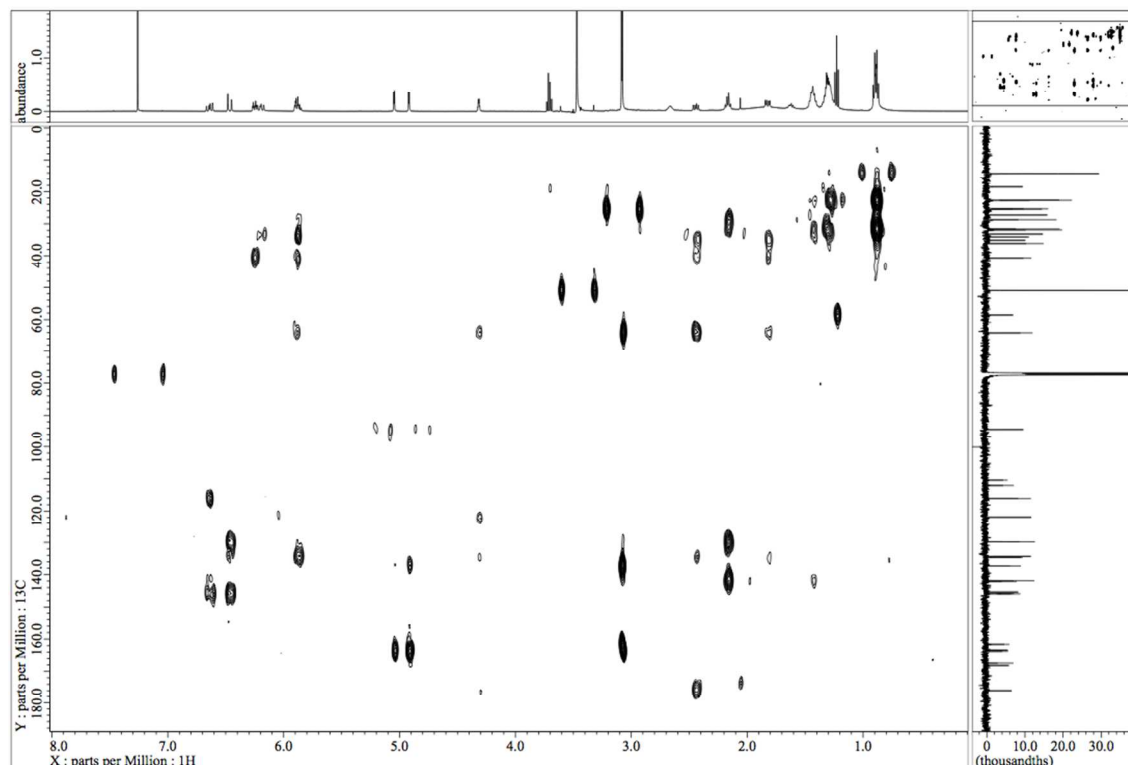
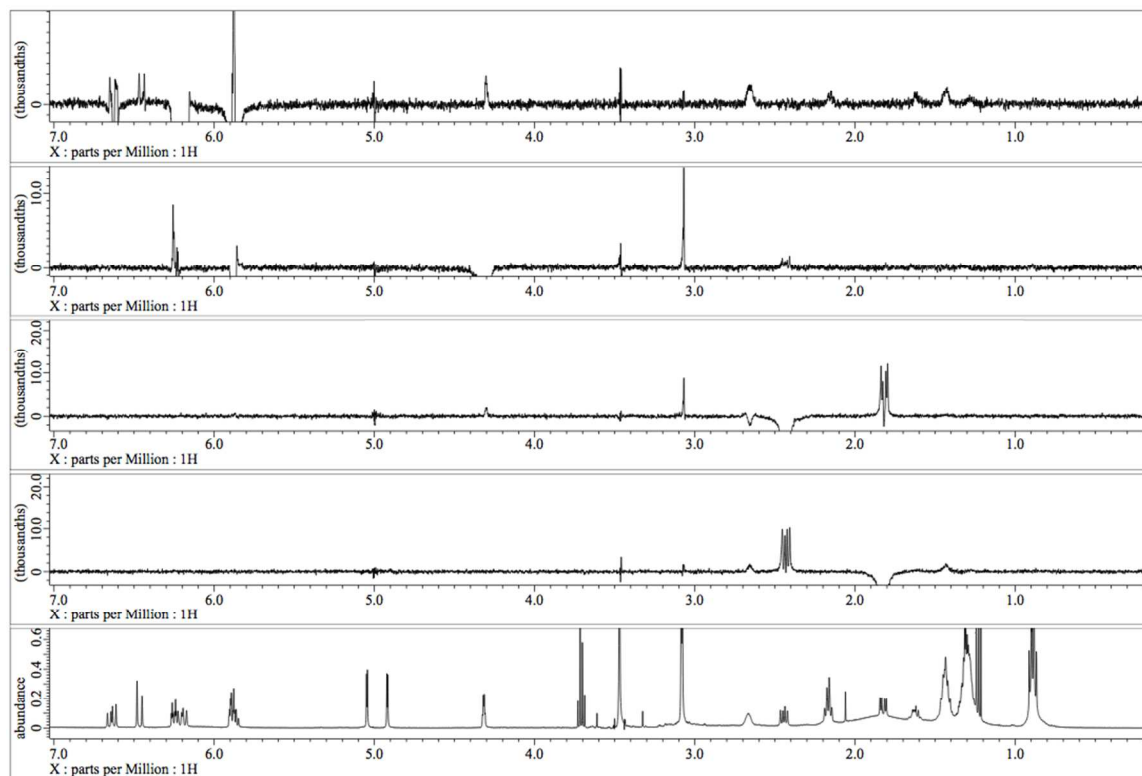


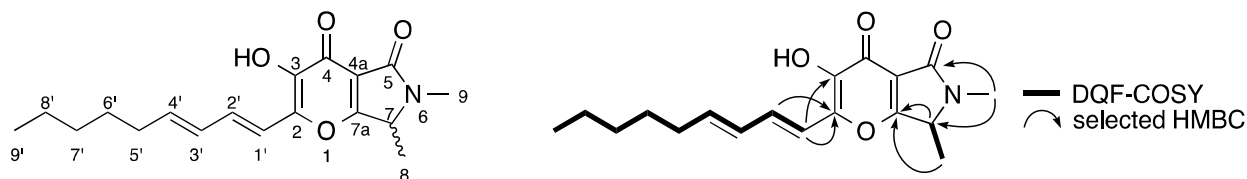
Figure S25. 1D-ROESY spectrum of **1** in CDCl₃ (500 MHz). ROESY: Rotating-frame nuclear Overhauser effect spectroscopy.



2.2 Chemical characterization of 2.

Table S11. NMR data of compound **2** in CDCl₃. Isolated amount: 3.5 mg from 5 L of a culture as a pale yellow solid. The molecular formula of **2** was established by mass data [ESI-MS: m/z 318 (M+H)⁺; HRESIMS: m/z 318.1700 (M+H)⁺, calcd. for C₁₈H₂₄NO₄⁺, 318.1700, Δ = 0.0 mmu].

[α]_D²³ +7.4 (c 0.1, CHCl₃), IR (ATR) 1020, 1396, 1469, 1650, 1700, 2828, 2917, 2952, 3394, 3730 cm⁻¹.



Position	δ_H	mult. (J in Hz)		HMBC	δ_C
2					146.1
3					141.9
4					168.3
4a					112.1
5					163.8
7	4.32	1H	q (7.0)	7a, 8	55.8
7a					175.5
8	1.55	3H	d (7.0)	7, 7a	15.7
9	3.01	3H	s	5, 7	26.5
1'	6.53	1H	d (16.0)	2, 3, 2', 3'	116.2
2'	6.94	1H	dd (16.0, 11.5)	2, 4'	134.6
3'	6.25	1H	dd (15.5, 11.5)	1', 2', 4', 5'	129.9
4'	6.06	1H	dd (15.5, 7.5)	2', 5', 6'	141.9
5'	2.19	2H	dd (7.5, 7.0)	3', 4', 6', 7'	33.2
6'	1.45	2H	tt (7.0, 7.0)	4', 5', 7', 8'	28.8
7'	1.32	2H	m	5', 8'	31.5
8'	1.32	2H	m	6', 7'	22.6
9'	0.92	3H	t (6.8)	7', 8'	14.2
3-OH	n.d.				

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Figure S26. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **2** in CDCl_3 .

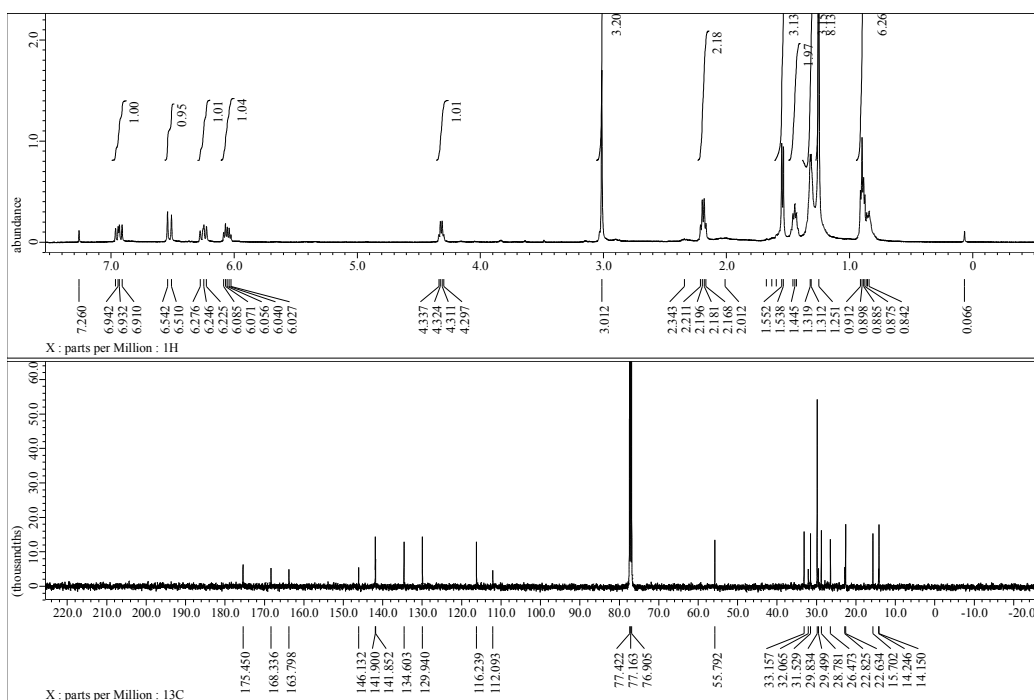


Figure S27. DQF-COSY spectrum of **2** in CDCl_3 (500 MHz).

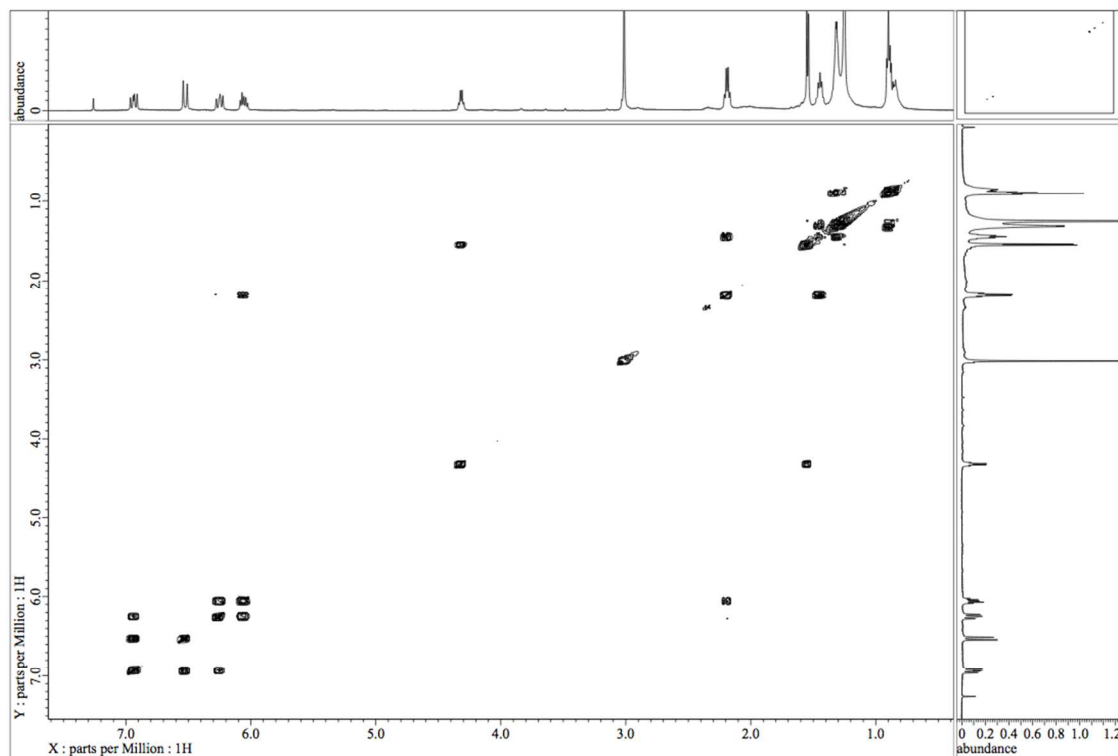


Figure S28. HMQC spectrum of **2** in CDCl₃ (500 MHz).

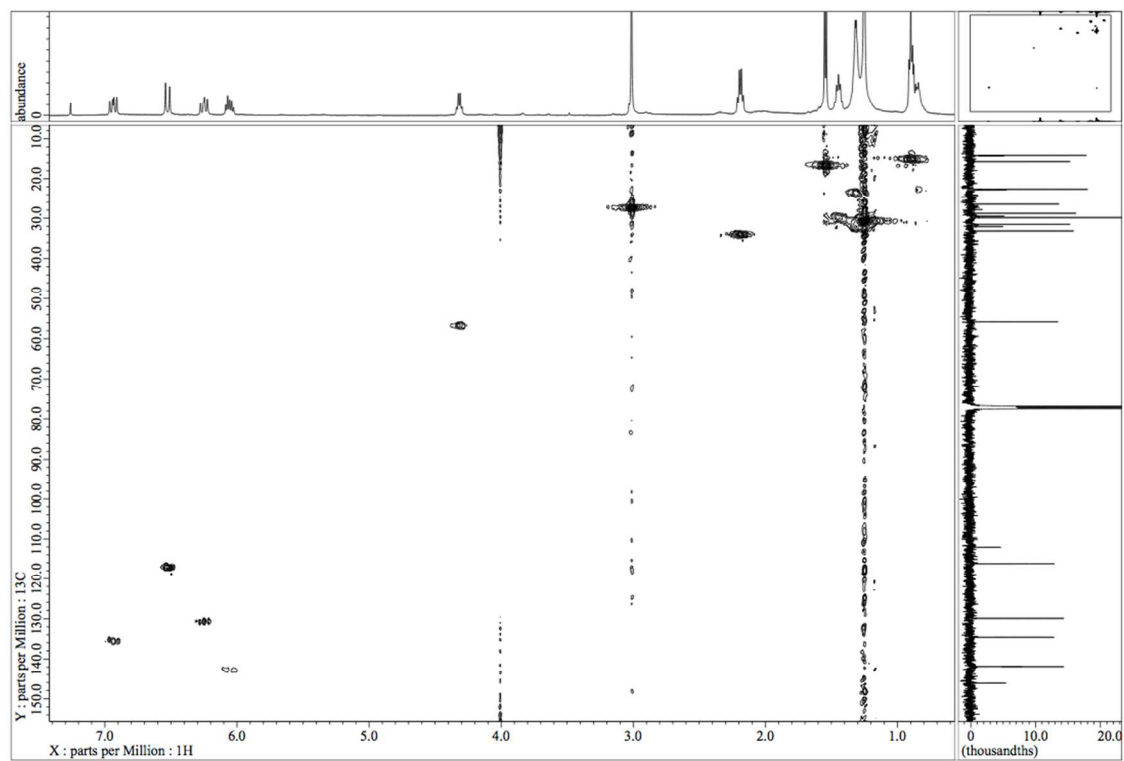
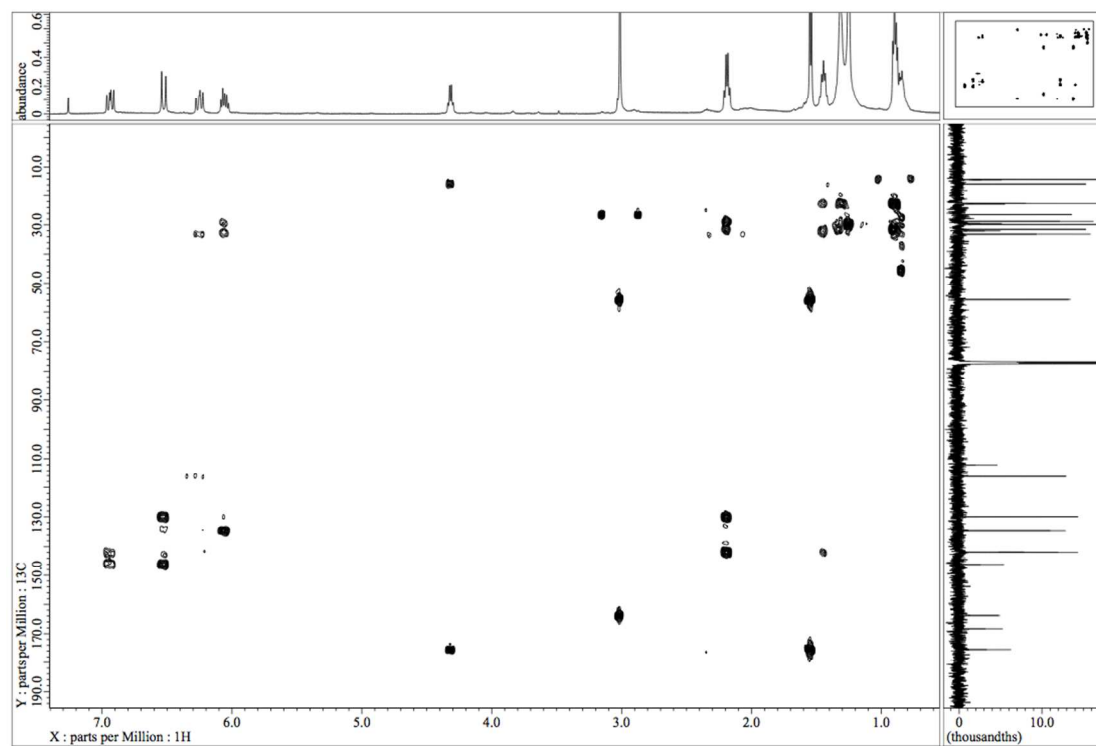
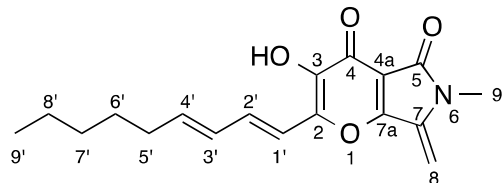


Figure S29. HMBC spectrum of **2** in CDCl₃ (500 MHz).



2.3 Chemical characterization of compound 3.

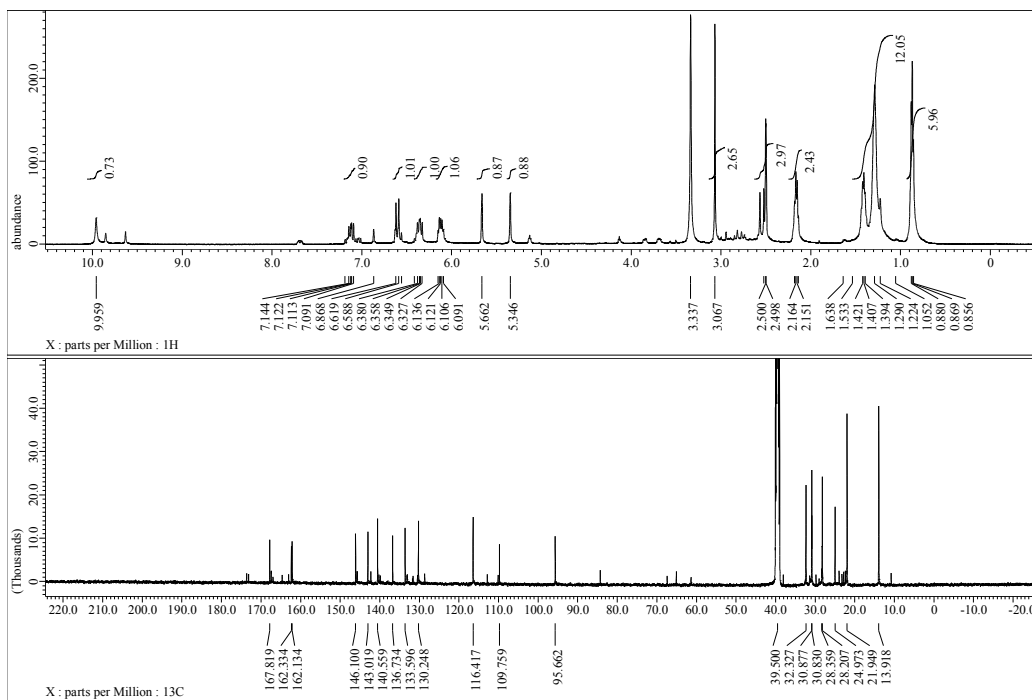
Table S12. NMR data of compound **3** in DMSO-*d*₆. Isolated amount: 15 mg from 5 L of a culture as a yellow powder. The molecular formula of **3** was established by mass data [ESI-MS: *m/z* 316 (M+H)⁺; HRESIMS: *m/z* 316.1541 (M+H)⁺, calcd. for C₁₈H₂₂NO₄⁺, 316.1543, Δ = 0.2 mmu].



Position	Isolated		Reported ¹⁴		Isolated	Reported ¹⁴
	δ _H	mult. (<i>J</i> in Hz)	δ _H	mult. (<i>J</i> in Hz)	δ _C	δ _C
2					146.1	146.7
3					143.0	143.6
4					167.8	168.4
4a					109.8	110.3
5					162.1	162.7
7					136.7	137.3
7a					162.3	162.9
8	5.66	1H br s	5.65	1H br s	95.7	96.2
	5.35	1H br s	5.33	1H br s		
9	3.07	3H	3.05	3H s	25.0	25.5
1'	6.61	1H d (15.5)	6.59	1H d (15.5)	116.4	117.0
2'	7.12	1H dd (15.5, 10.9)	7.10	1H dd (15.5, 11.5)	133.6	134.2
3'	6.35	1H dd (15.5, 10.9)	6.34	1H dd (15.0, 11.5)	130.2	130.8
4'	6.12	1H ddd (15.5, 6.9, 6.9)	6.11	1H m	140.6	141.1
5'	2.16	2H m	2.14	2H m	32.3	32.9
6'	1.41	2H m	1.39	2H m	28.2	28.8
7'	1.29	2H m	1.27	2H m	30.9	31.4
8'	1.29	2H m	1.27	2H m	21.9	22.5
9'	0.87	3H t (6.8)	0.87	3H t (6.5)	13.9	14.5
3-OH	9.96	1H s	9.94	s		

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

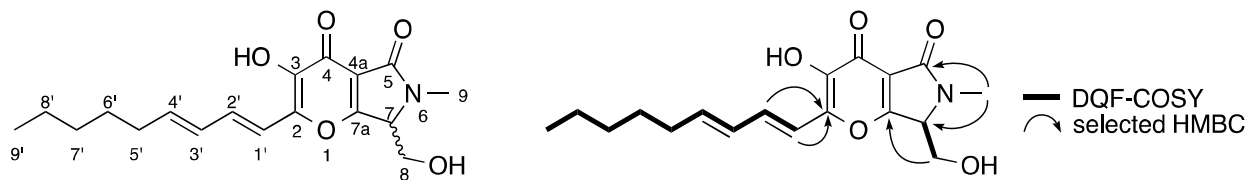
Figure S30. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **3** in $\text{DMSO-}d_6$.



2.4 Chemical characterization of 5.

Table S13. NMR data of compound **5** in CD₃OD. Isolated amount: 30.6 mg from 5 L of a culture as a yellow powder. The molecular formula of **5** was established by mass data [ESI-MS: m/z 334 (M+H)⁺; HRESIMS: m/z 334.1648 (M+H)⁺, calcd. for C₁₈H₂₄NO₅⁺, 334.1649, Δ = 0.1 mmu].

$[\alpha]_D^{23}$ -0.2 (c 0.1, CHCl₃), IR (ATR) 989, 1224, 1391, 1481, 1631, 1693, 2855, 2928, 2954, 3356 cm⁻¹.



Position	δ_H	mult. (J in Hz)		HMBC	δ_C
2					148.8
3					143.7
4					170.1
4a					115.0
5					167.4
7	4.51	1H	br s	7a	63.6
7a					174.8
8	4.11	1H	br d (12.5)	7a	58.6
	4.07	1H	br d (12.5)		
9	3.02	3H	s	5, 7	26.8
1'	6.59	1H	d (15.3)	2, 3, 3'	117.5
2'	6.99	1H	dd (14.7, 10.8)	2, 4'	135.2
3'	6.28	1H	dd (14.7, 10.8)	1', 2', 5'	131.4
4'	6.06	1H	dt (14.5, 7.1)	2', 5', 6'	141.9
5'	2.19	2H	dt (7.1, 6.8)	3', 4', 7'	34.0
6'	1.46	2H	tt (6.8, 6.8)	4', 5', 7', 8'	29.9
7'	1.34	2H	m	5', 9'	32.6
8'	1.34	2H	m	6', 7', 9'	23.6
9'	0.92	3H	t (6.8)	7', 8'	14.4
3-OH	n.d.				
8-OH	n.d.				

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Figure S31. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **5** in CD_3OD .

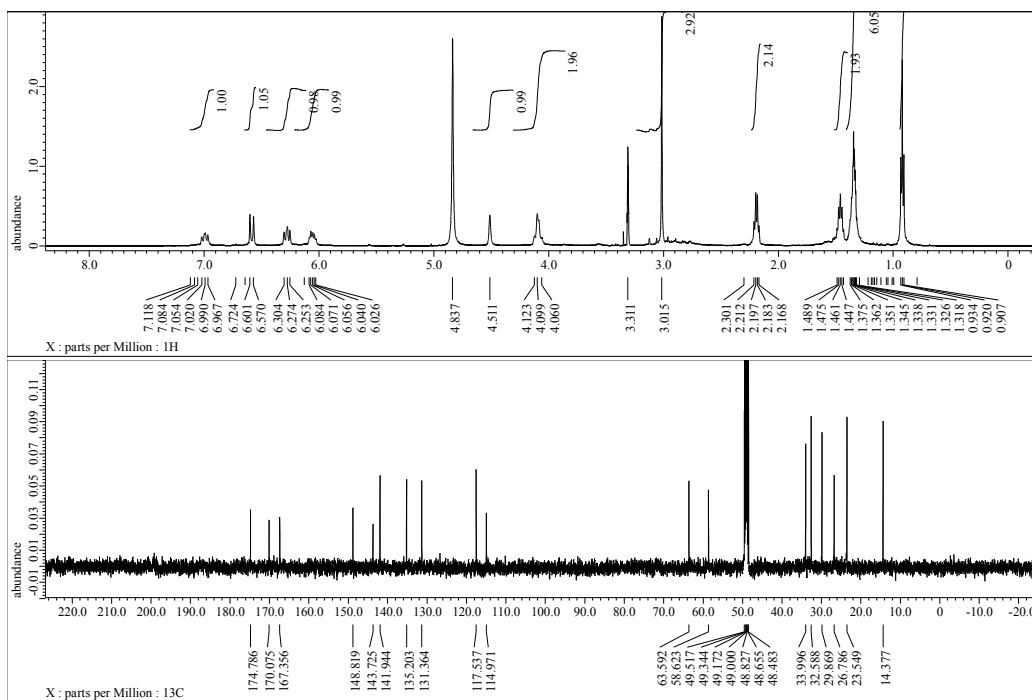


Figure S32. DQF-COSY spectrum of **5** in CD_3OD (500 MHz).

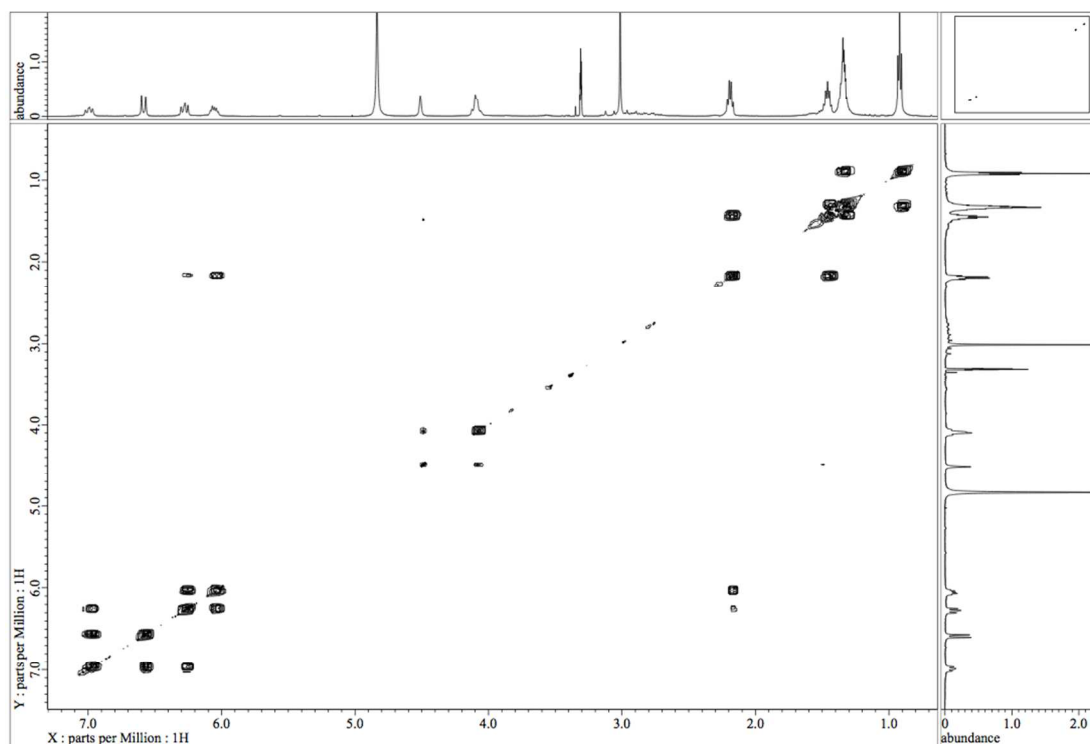


Figure S33. HMQC spectrum of **5** in CD₃OD (500 MHz).

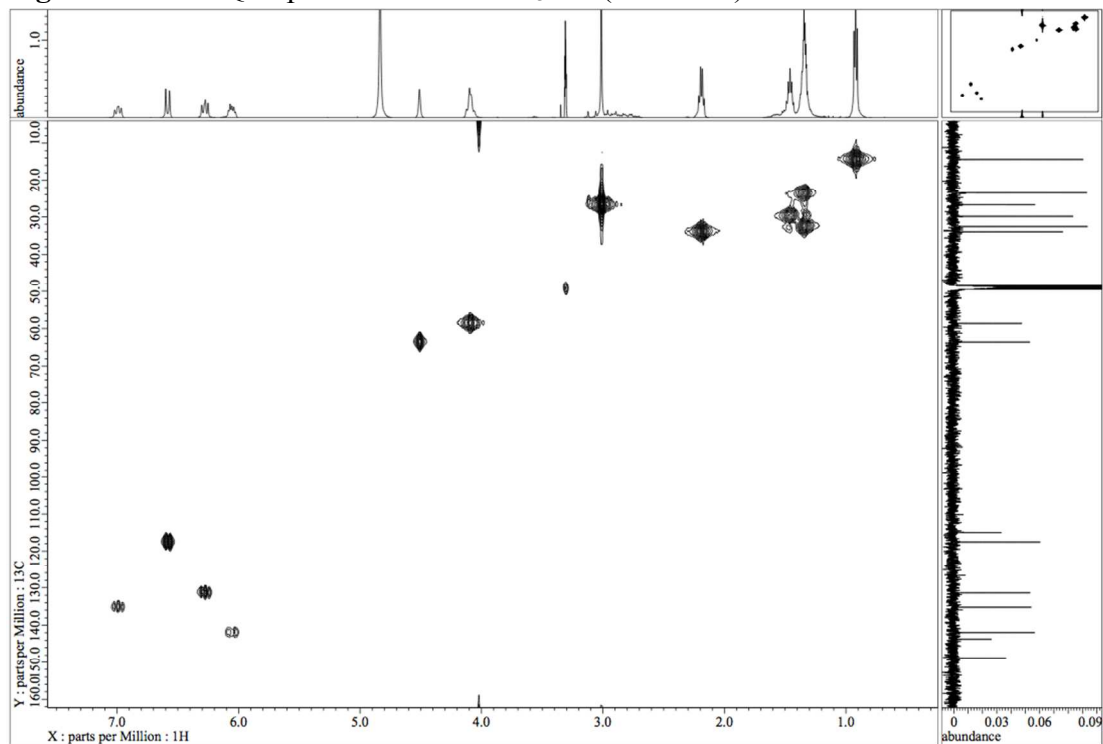
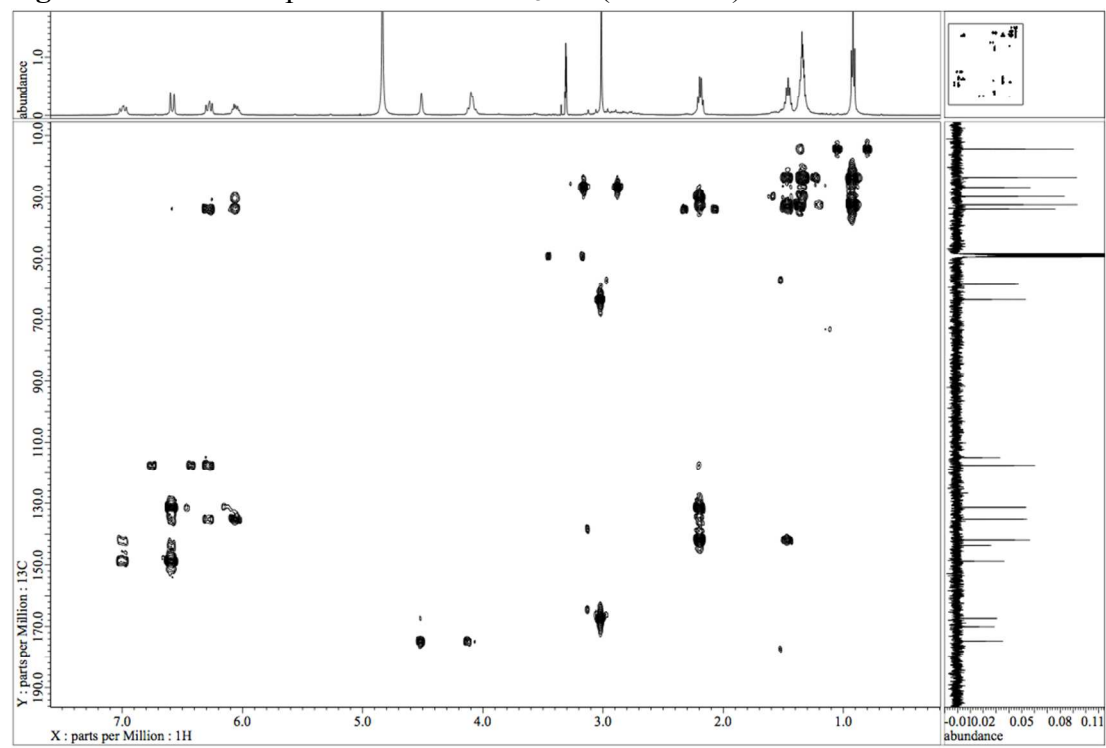
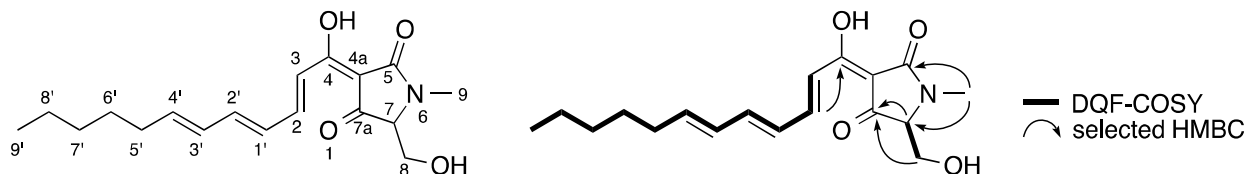


Figure S34. HMBC spectrum of **5** in CD₃OD (500 MHz).



2.5 Chemical characterization of 7.

Table S14. NMR data of compound **7** in CDCl₃. Isolated amount: 80 mg from 5 L of a culture as a yellow powder. The molecular formula of **7** was established by mass data [ESI-MS: m/z 320 (M+H)⁺; HRESIMS: m/z 320.1855 (M+H)⁺, calcd. for C₁₈H₂₆NO₄⁺, 320.1856, Δ = 0.1 mmu]. $[\alpha]_D^{17}$ –112.9 (c 0.3, CH₃OH), IR (ATR) 1616, 1686, 2859, 2925, 2954, 3373 cm^{–1}.



Position	δ_H		mult. (J in Hz)	HMBC	δ_C
2	7.46	1H	dd (15.0, 11.0)	4, 2'	145.5
3	7.06	1H	d (15.0)		120.4
4					173.4
4a					100.4
5					174.2
7	3.68	1H	dd (4.0, 4.0)		68.0
7a					193.5
8	4.02	1H	dd (11.5, 3.1)	7a	59.9
	3.88	1H	dd (11.5, 3.1)		
9	3.01	3H	s	5, 7	26.9
1'	6.34	1H	dd (15.0, 11.0)	3, 3'	129.0
2'	6.62	1H	dd (15.0, 11.5)	2, 4'	144.1
3'	6.18	1H	dd (15.0, 11.5)	1', 4', 5'	130.3
4'	6.00	1H	dt (15.0, 7.0)	2', 3', 5', 6'	142.9
5'	2.15	2H	dt (7.0, 7.0)	3', 4', 6', 7'	33.2
6'	1.41	2H	m	4', 5', 7', 8'	28.7
7'	1.29	2H	m	5', 9'	31.5
8'	1.29	2H	m	6', 7', 9'	22.6
9'	0.88	3H	t (6.5)	7', 8'	14.1
NH	n.d.				
4-OH	n.d.				
8-OH	n.d.				

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Figure S35. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **7** in CDCl_3 .

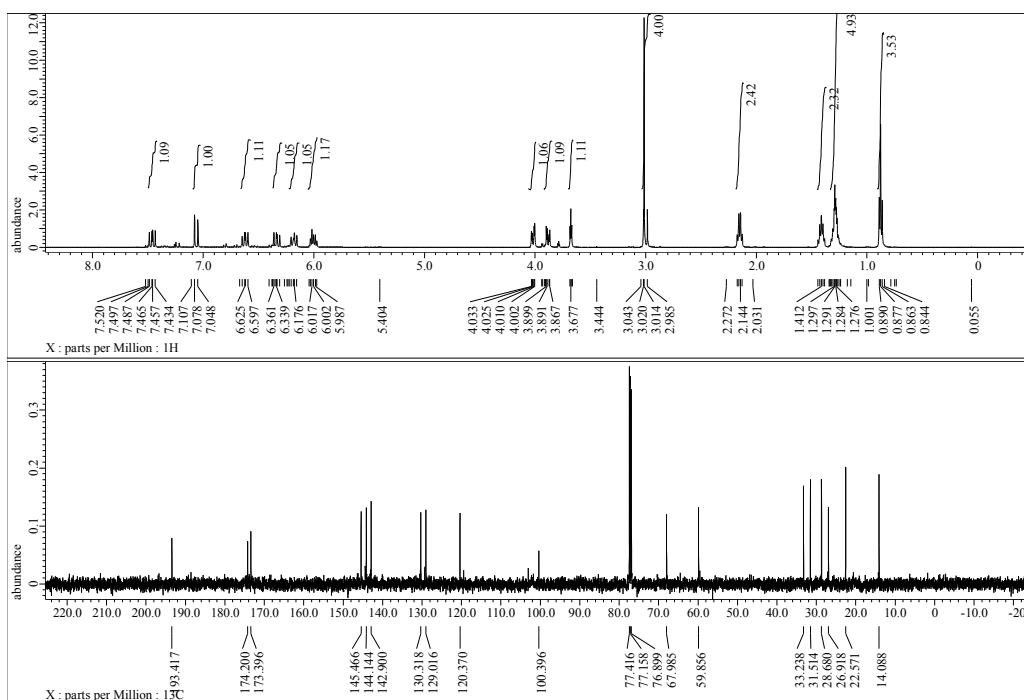


Figure S36. DQF-COSY spectrum of **7** in CDCl_3 (500 MHz).

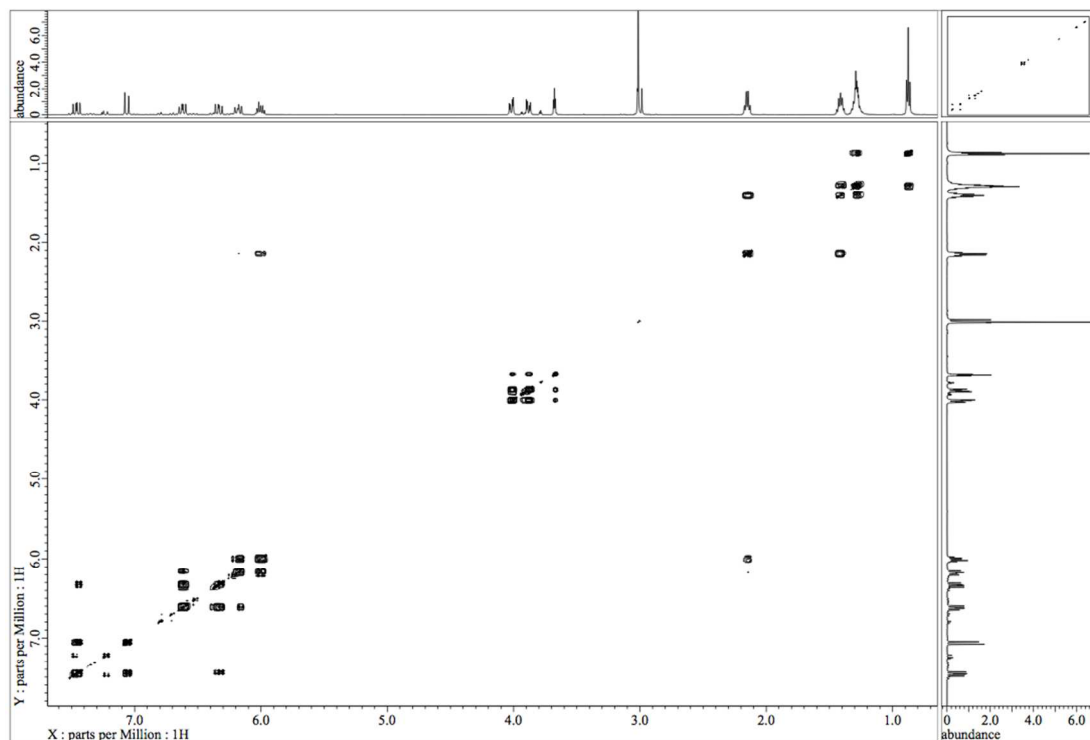


Figure S37. HMQC spectrum of **7** in CDCl₃ (500 MHz).

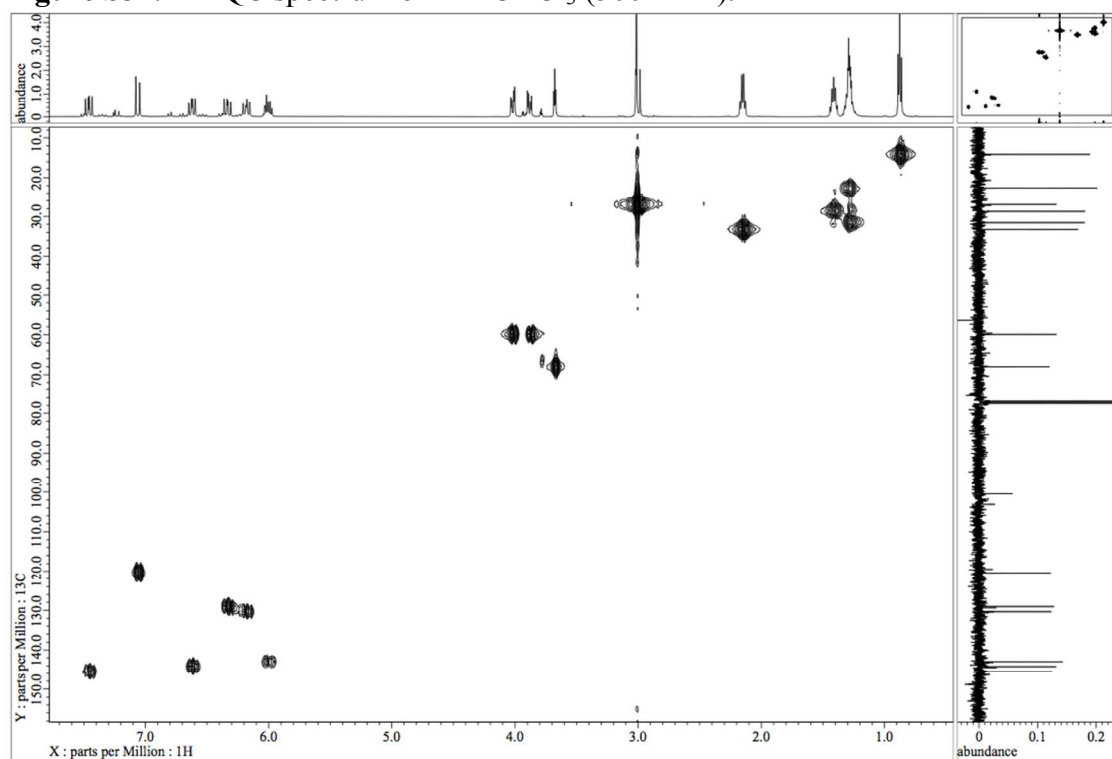
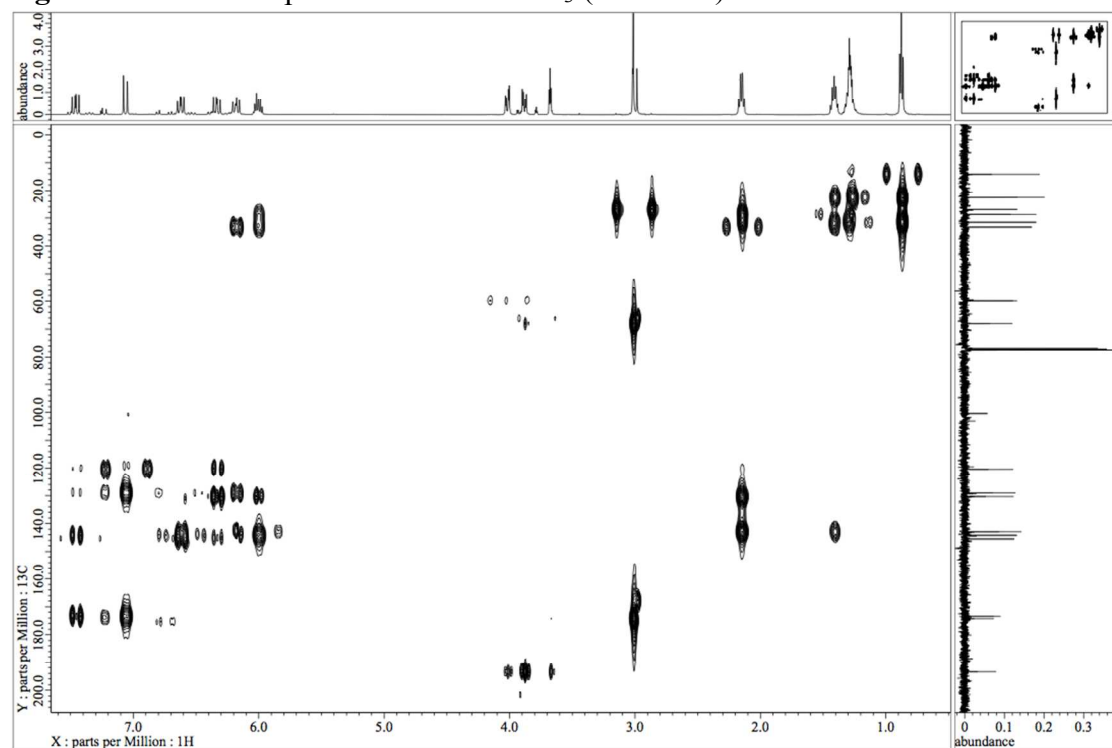
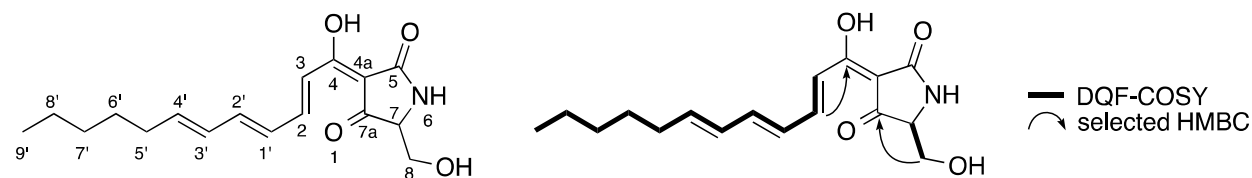


Figure S38. HMBC spectrum of **7** in CDCl₃ (500 MHz).



2.6 Chemical characterization of 8.

Table S15. NMR data of compound **8** in DMSO-*d*₆. Isolated amount: 6.9 mg from 5 L of a culture as a yellow powder. The molecular formula of **8** was established by mass data [ESI-MS: *m/z* 306 (M+H)⁺; HRESIMS: *m/z* 306.1698 (M+H)⁺, calcd. for C₁₇H₂₄NO₄⁺, 306.1700, Δ = 0.2 mmu]. [α]_D¹⁷ -210.6 (*c* 0.23, CH₃OH), IR (ATR) 1429, 1553, 1596, 2855, 2923, 2955, 3217 cm⁻¹.



The chemical structure of compound **8** is shown with proton positions labeled 1' through 9' and 1 through 8. The structure features a long chain of protons (1'-9') connected by double bonds, and a fused ring system containing a carbonyl group and a hydroxyl group. The DQF-COSY selected HMBC correlations are indicated by curved arrows between the protons and the carbons they correlate with.

Position	δ _H		mult. (<i>J</i> in Hz)	HMBC	δ _C
2	7.45	1H	dd (14.5, 11.5)	4, 2'	144.1
3	7.02	1H	d (14.5)		119.9
4					172.0
4a					100.7
5					175.4
7	3.85	1H	br s		64.1
7a					194.2
8	3.64	1H	dd (14.5, 3.1)		60.4
	3.59	1H	dd (14.5, 3.1)	7a	
1'	6.49	1H	dd (14.5, 11.5)	2, 3, 2', 3'	129.0
2'	6.83	1H	dd (14.5, 11.5)	2, 4'	143.9
3'	6.26	1H	dd (14.5, 11.5)	1', 2', 4', 5'	130.4
4'	6.06	1H	dt (14.5, 7.1)	2', 3', 5', 6'	142.2
5'	2.14	2H	dt (7.1, 6.9)	3', 4', 6', 7'	32.5
6'	1.38	2H	tt (6.9, 6.9)	4', 5', 7', 8'	28.1
7'	1.26	2H	m	5', 6', 8', 9'	30.9
8'	1.26	2H	m	6', 7', 9'	22.0
9'	0.85	3H	t (6.9)	7', 8'	13.9
NH	8.79				
4-OH	n.d.				

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Figure S39. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **8** in $\text{DMSO}-d_6$.

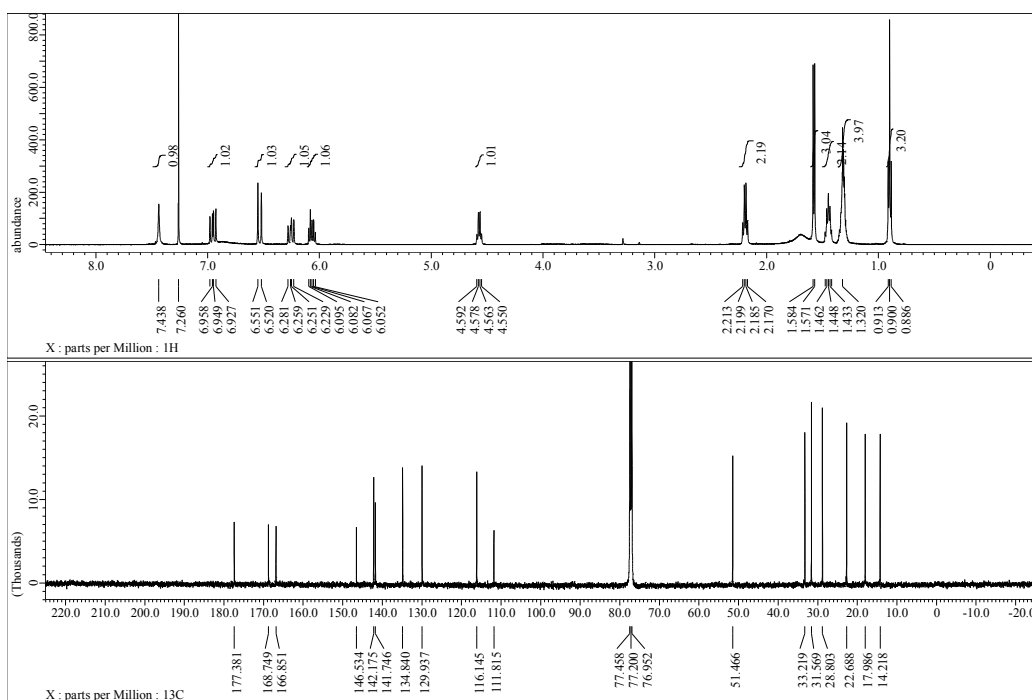


Figure S40. DQF-COSY spectrum of **8** in $\text{DMSO}-d_6$ (500 MHz).

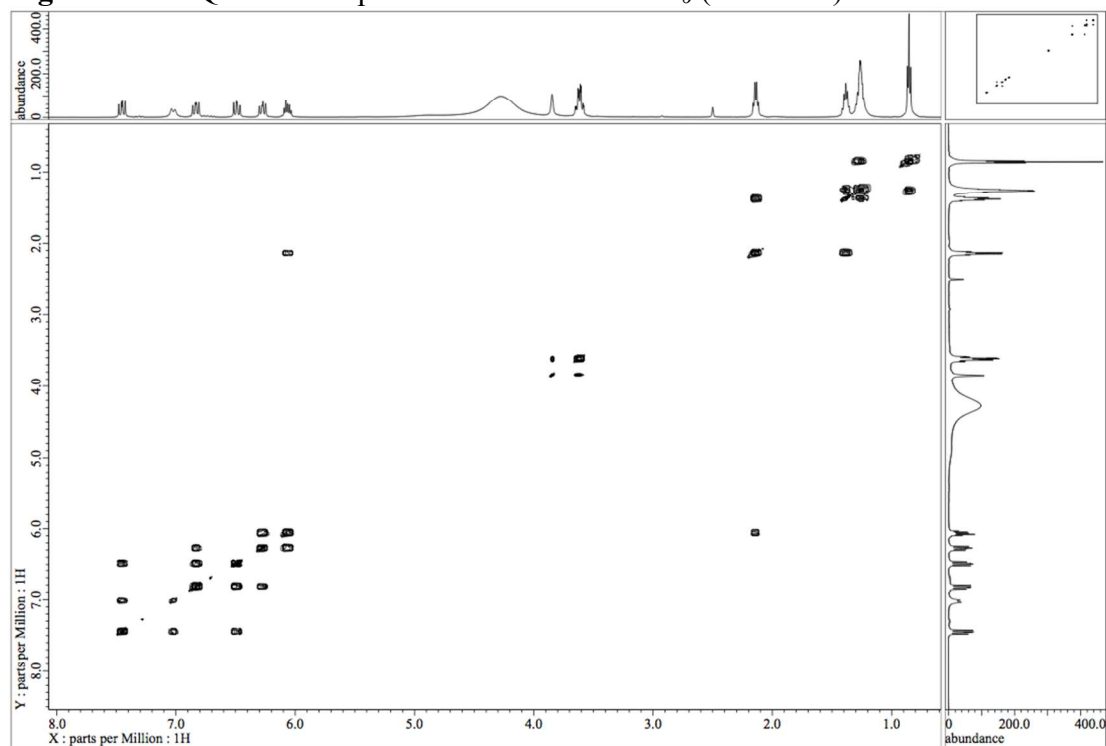


Figure S41. HMQC spectrum of **8** in DMSO- d_6 (500 MHz).

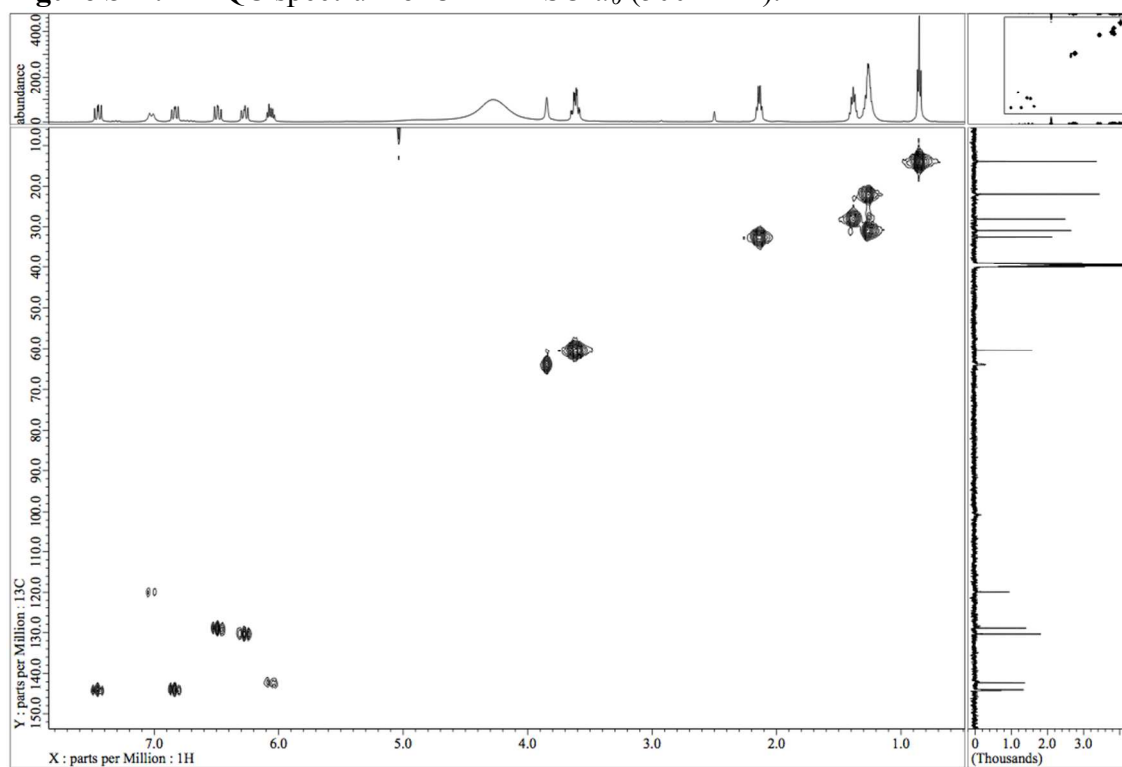
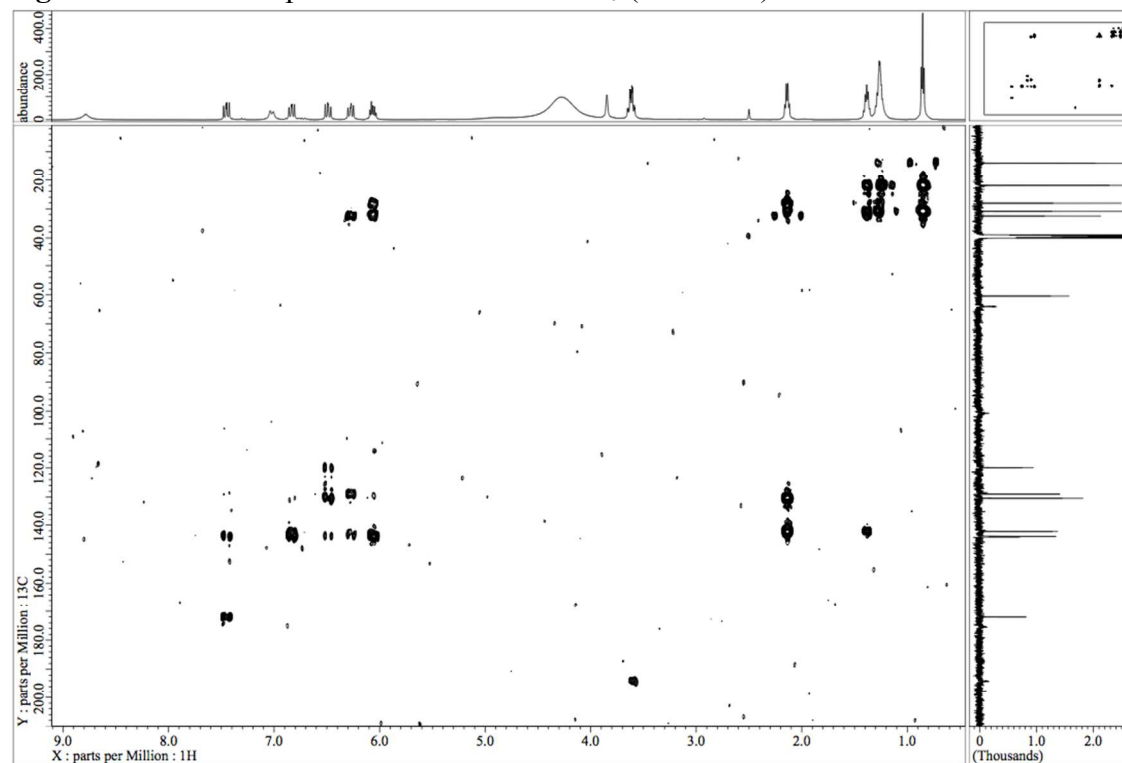
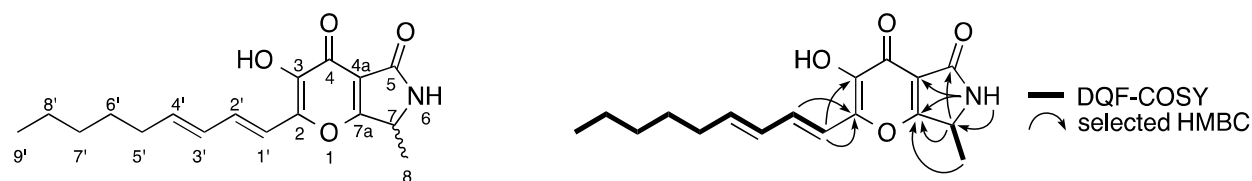


Figure S42. HMBC spectrum of **8** in DMSO- d_6 (500 MHz).



2.7 Chemical characterization of 11.

Table S16. NMR data of compound **11** in CDCl₃. Isolated amount: 94 mg from 5 L of a culture as a yellow powder. The molecular formula of **11** was established by mass data [ESI-MS: m/z 304 (M+H)⁺; HRESIMS: m/z 304.1542 (M+H)⁺, calcd. for C₁₇H₂₂NO₄⁺, 304.1543, Δ = 0.1 mmu]. [α]_D²⁴ +0.5 (*c* 0.1, CHCl₃), IR (ATR) 1212, 1397, 1485, 1634, 1732, 2875, 2919, 2956, 3337, 3508 cm⁻¹.



Position	δ_H	mult. (<i>J</i> in Hz)		HMBC	δ_C
2					146.5
3					141.7
4					168.7
4a					111.8
5					166.9
7	4.57	1H	q (7.5)	5, 7a, 8	51.5
7a					177.4
8	1.58	3H	d (6.5)	7, 7a	18.0
1'	6.54	1H	d (15.2)	2, 3, 2', 3'	116.1
2'	6.95	1H	dd (15.2, 10.5)	2, 4'	134.8
3'	6.26	1H	dd (15.0, 10.5)	1', 2', 4'	129.9
4'	6.07	1H	dt (15.0, 7.0)	2', 5', 6'	142.2
5'	2.19	2H	dd (7.0, 7.0)	3', 4', 6', 7'	33.2
6'	1.45	2H	tt (7.0, 7.0)	4', 8'	28.8
7'	1.32	2H	m	5', 9'	31.6
8'	1.32	2H	m	6', 7'	22.7
9'	0.90	3H	t (6.5)	7', 8'	14.2
NH	7.44	1H	s	7, 4a, 7a	
3-OH	n.d.				

¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

Figure S43. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra of **11** in CDCl_3 .

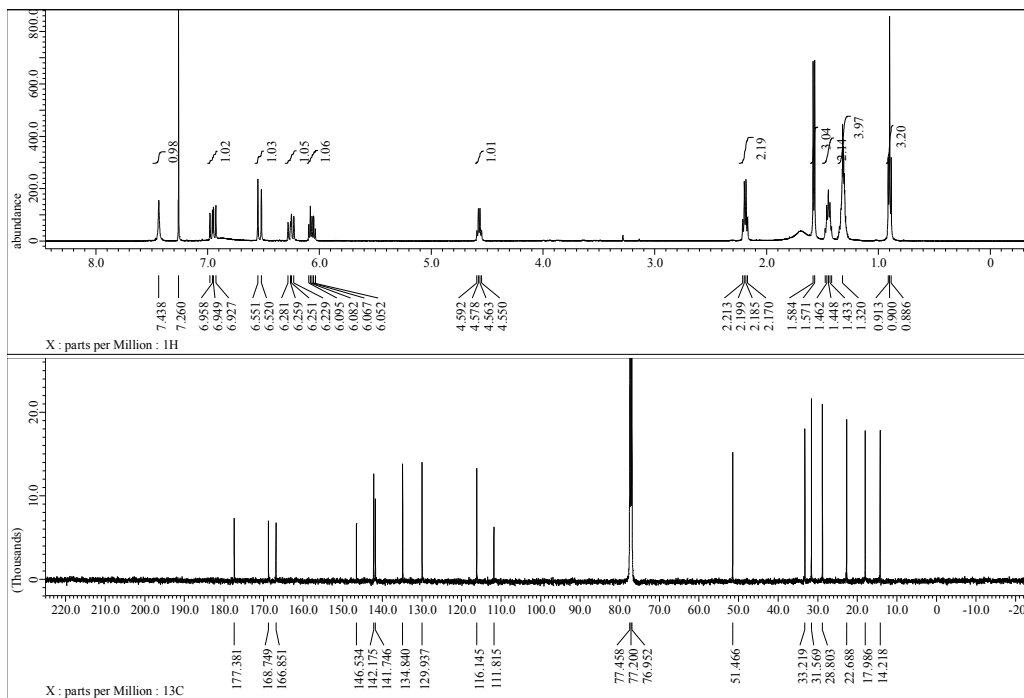


Figure S44. DQF-COSY spectrum of **11** in CDCl_3 (500 MHz).

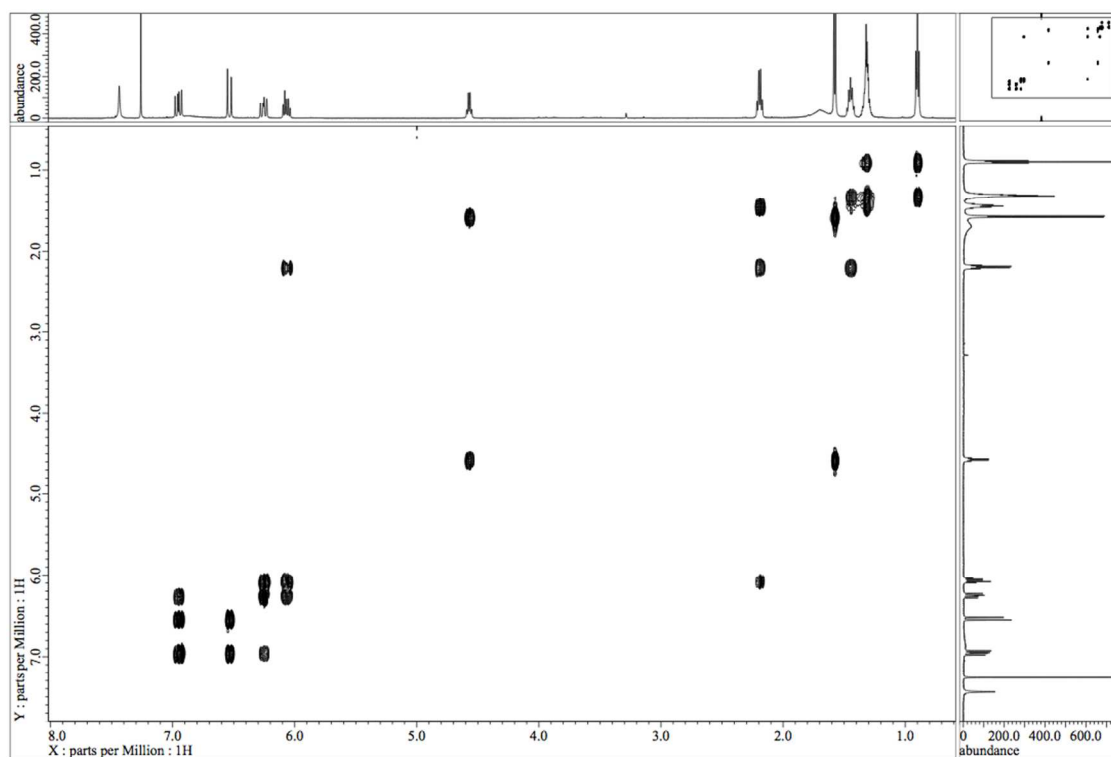


Figure S45. HMQC spectrum of **11** in CDCl₃ (500 MHz).

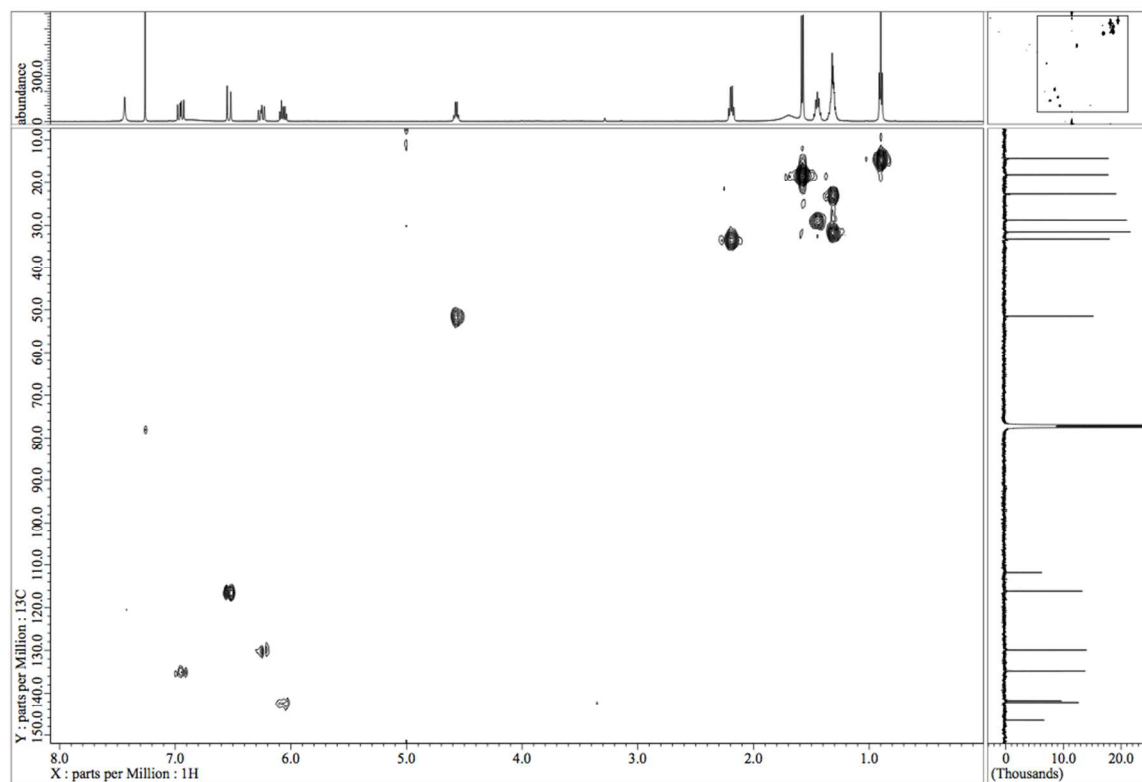
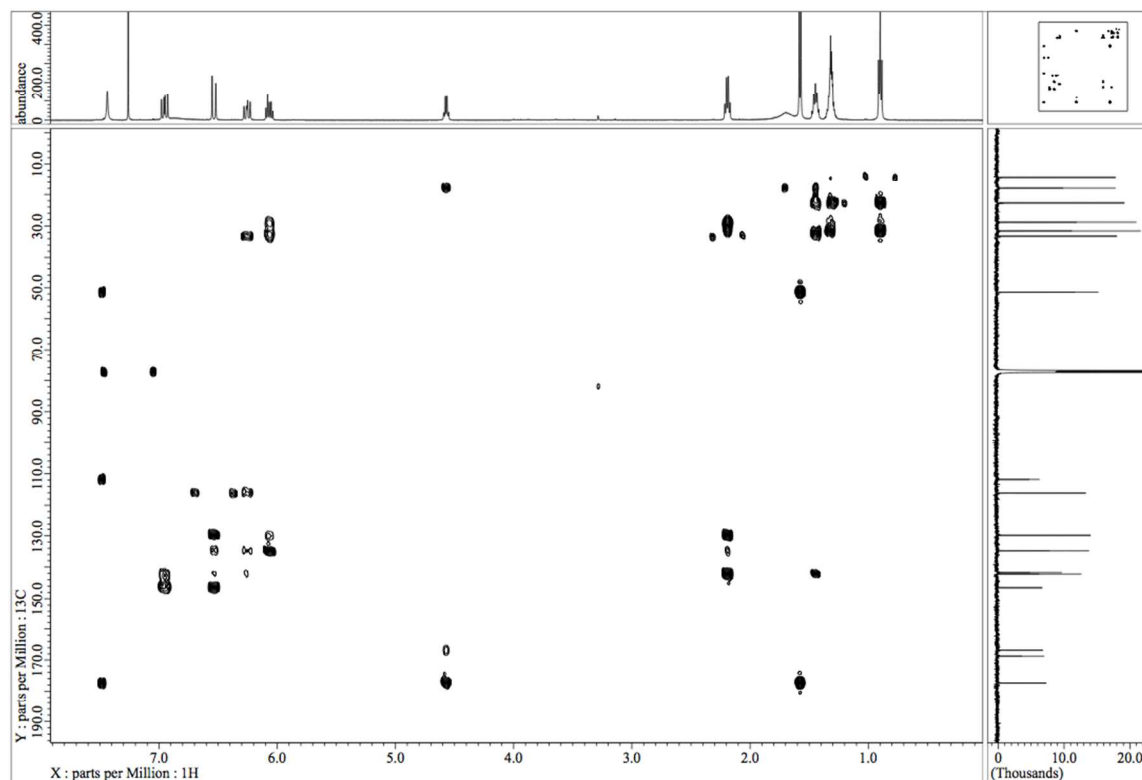
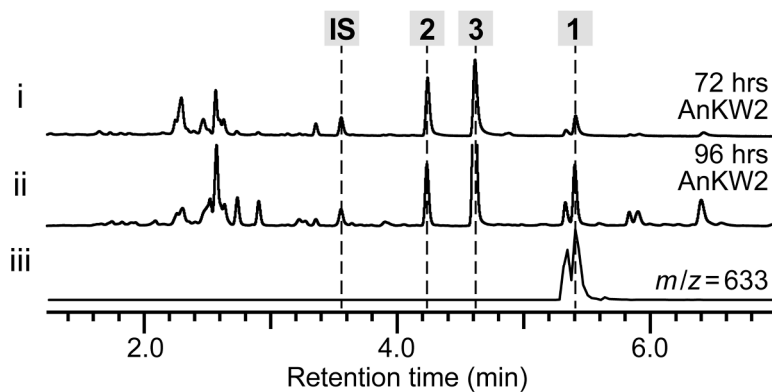


Figure S46. HMBC spectrum of **11** in CDCl₃ (500 MHz).



2.8 Metabolic profile from the cultures of AnKW2.

Figure S47. HPLC traces of metabolic extracts from the culture of AnKW2, showing accumulation of **1** over time. Traces (i) and (ii) were monitored at 280 nm. (i) Extract from the culture incubated for 72 h. (ii) Extract from the culture incubated for 96 h. (iii) Trace corresponding to the m/z of **1** ($m/z = 633$) extracted from (ii).



3. Supporting References

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