



Supporting Information

Unprecedented Mushroom Polyketide Synthases Produce the Universal Anthraquinone Precursor

*N. A. Löhr, F. Eisen, W. Thiele, L. Platz, J. Motter, W. Hüttel, M. Gressler, M. Müller, D. Hoffmeister**

Table of Contents

Experimental procedures	2
Table S1. NMR spectroscopy data for atrochrysone	6
Table S2. Fungal strains	7
Table S3. Oligonucleotide primers	8
Table S4. PCR parameters	9
Table S5. Plasmids	9
Table S6. HPLC parameters	9
Table S7. Sequences of polyketide synthases	10
Figure S1. Comparison of three classes of fungal non-reducing polyketide synthases	11
Figure S2. Amino acid sequence alignments	12
Figure S3. Agarose gels to verify <i>C. odorifer</i> PKS gene integration in the <i>A. niger</i> host genome	15
Figure S4. UV/Vis spectra of monomeric polyketides	16
Figure S5. 1D and 2D NMR spectra of atrochrysone	17
Figure S6. LC-MS/MS analyses of monomeric polyketides	23
Figure S7. Analysis of dimeric polyketides	24
Figure S8. Analysis of the putative pannorin	25
Figure S9. Heptaketide synthase activity model	26
Figure S10. SDS-polyacrylamide gel electrophoresis with recombinant CoPKS4	27
Figure S11. Purity of commercial malonyl-CoA	28
Figure S12. Circular dichroism spectroscopy	29
 References	 30

Experimental Procedures

1. Microbiological methods

Strains and cultivation: *Cortinarius odorifer* SF013765 was routinely grown on solid Modified Melin-Norkrans (MMN)^[1] medium at 20 °C for 28 d. For extraction of chromosomal DNA, the fungus was grown in liquid Moser B medium,^[2] shaken at 120 rpm, at 20 °C. The mycelium was collected after 18 d, washed, dried, and ground to a powder in liquid nitrogen. Genomic DNA of *C. odorifer* was isolated following a described protocol.^[3]

Aspergillus niger expression strain ATNT16ΔpyrGx24^[4] served as heterologous expression platform. Genotypes of individual transformants are given in Table S2. All strains were routinely cultured at 30 °C on *Aspergillus* minimal medium (AMM)^[5] supplemented with 100 mM D-glucose and 20 mM L-glutamine (AMM-G100Gln20). Conidia suspensions were obtained from solid 2 % (w/v) agar slope cultures of AMM-G100Gln20. The slopes were overlaid with 5 mL of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) containing 0.01 % (v/v) Tween 20. Conidia were harvested by swabbing the culture surface with an inoculation loop. The suspensions were then filtered using a 40 µm Cell Strainer (Greiner Bio-One). For routine cultivation and secondary metabolite analysis, liquid cultures of AMM-G100Gln20 were inoculated with 1 × 10⁶ conidia mL⁻¹ and incubated at 30 °C and 150 rpm, for 48 h. Transformants were supplemented with 20 µg mL⁻¹ doxycycline to induce transgene expression. For negative controls with the parental strain *A. niger* ATNT16ΔpyrGx24 10 mM uridine was added.

For routine cloning and plasmid propagation, *Escherichia coli* XL1-Blue (Agilent) was grown on solid LB medium (per liter: 5 g yeast extract, 10 g tryptone, 10 g NaCl, 20 g agar) supplemented with 50 µg mL⁻¹ carbenicillin, if required.

2. Molecular biology methods

Genomic sequencing and sequence analysis: Genomic DNA of *C. odorifer* was sequenced using Illumina MiSeq technology. Gene models were predicted with Augustus,^[6] similar protein sequences were identified using BlastP.^[7] Sequence analyses and alignments were carried out with Geneious software (version 7.1.9, Biomatters, Ltd.).

Construction of expression plasmids for *C. odorifer* PKS genes: For heterologous production of native polyketide synthases in *A. niger* ATNT16ΔpyrGx24, the intron-disrupted *C. odorifer* genes *copks1* – 6 were amplified from genomic DNA, using oligonucleotides listed in Table S3. All reactions were performed in 20 µL total volume using 0.4 U Phusion DNA polymerase (NEB). The reaction mixture included 0.2 mM (each) deoxynucleoside triphosphate, 0.5 µM (each) oligonucleotide primer (Table S3) in HF buffer, supplied with the enzyme. PCR parameters (condition I) are given in Table S4.

The resulting amplicons were electrophoretically purified on agarose gels and ligated to *Nsi*I-linearized phis_SM-Xpress vectors^[4,8] using the NEBuilder HiFi DNA Assembly Master Mix (NEB). The constructed expression plasmids (Table S5) were pNAL004 (to produce CoPKS5), pNAL005 (CoPKS6), pNAL006 (CoPKS4), pNAL021 (CoPKS2), pNAL022 (CoPKS3). The expression plasmid pNAL020 (for CoPKS1) was constructed similarly, but using PCR-amplified phis_SM-Xpress vector (PCR condition II, oMG267/268, Tables S3 and S4).

To produce a CoPKS4 fusion protein that C-terminally comprises a Strep-tag II, the gene was PCR-amplified (condition I, Table S4) with primers oNAL218/248 (Table S3), gel purified, and ligated to *Nsi*I-linearized vector

pSM_StrepTag_X_URA (provided by M. Brock, University of Nottingham), to yield plasmid pNAL046. The pSM_StrepTag_X_URA vector is a derivative of plasmid phis_SM-Xpress^[4,8] in which the coding sequence for the His-tag is replaced by the sequence coding for the Strep-tag II minimal peptide sequence WSHPQFEK.^[9] The sequence is flanked on the 5'-end by an *NsiI* restriction site containing the ATG start codon and on the 3'-end by an *NcoI* restriction site allowing the addition of the Strep-tag II sequence either at the C- or the N-terminus of the protein of interest (M. Brock, personal communication). As a selection marker for fungal transformation, the plasmid additionally contains an URA-blaster cassette suitable for marker recycling in transformants.

For all expression plasmids (listed in Table S5), accurate amplification, and ligation of inserts with vectors was verified via analytical restriction digests and DNA sequencing.

Transformation of *A. niger* and genetic analysis of transformants: PEG-mediated transformation of *A. niger* protoplasts followed a described protocol.^[10] The transformants were selected for uracil prototrophy on AMM-G100Gln20 agar plates, supplemented with 1.2 M sorbitol. Genomic DNA of individual transformants was isolated as described.^[11] Full-length integration of the respective expression cassettes (*terA*-promoter/PKS gene/*trpC*-terminator) was then confirmed by diagnostic PCR using oligonucleotides oMG370/oNAL156 (condition I, Table S4). At least three PCR-confirmed transformants per construct were subsequently used as biological replicates for metabolic analyses. The transformants are shown in Table S2.

cDNA analysis: To compare the predicted with the actual splicing pattern, the cDNAs resulting from expression of gDNA-based *C. odorifer copks1* and *copks4* in *A. niger* tNAL024 and tNAL002 were analyzed. The mycelia were harvested and ground under liquid nitrogen. Total RNA was isolated from the respective expression strains using the SV Total RNA Isolation Kit (Promega) with an additional digestion of residual genomic DNA by Baseline-ZERO DNase (Biozym). Reverse transcription was performed with anchored oligo-(dT)₁₈ primers and RevertAid Reverse Transcriptase (ThermoFisher) at 42 °C for 60 min. The coding sequences were PCR-amplified from the first strand reaction, using oligonucleotides oNAL141/142 (Table S3) for *copks1* and oNAL070/071 in case of *copks4* using cycling condition III (Table S4). The gel-purified amplicons were ligated to cloning vector pJET1.2, to yield plasmids pNAL101 (*copks1*) and pNAL102 (*copks4*). Their inserts were sequenced which confirmed the *in silico* predicted exon/intron-junction pattern from the Augustus gene prediction tool for both genes.^[6]

3. Analytical and preparative methods

Liquid chromatography and mass spectrometry: To analyze natural products, the culture filtrates of *A. niger* transformants were extracted with ethyl acetate. If required, the culture filtrate was acidified to pH<4 with HCl prior to extraction. The organic phase was collected, dried over anhydrous sodium sulfate, and evaporated to dryness. The crude extracts were then dissolved in methanol and subjected to UHPLC-MS analysis using a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm particle size, thermostatted at 40 °C) on an Agilent 1290 Infinity II instrument, interfaced to an Agilent 6130 single quadrupole mass detector, operated in alternating positive/negative mode and applying gradient I (Table S6). UV/Vis spectra were recorded from λ=200 – 500 nm and chromatograms were extracted at λ = 395 nm. In addition, gradient II (Table S6) was used for rapid screening of extracts.

To purify atrochrysone from fungal cultures for NMR spectroscopy, the cultivation time was extended to up to 60 h and 30 µg mL⁻¹ doxycycline was added for induction. For initial compound purification, crude extracts from larger-scale fermentations (10 and 15 L total culture volume, respectively) of *A. niger* tNAL002 and tNAL024 were subjected to size exclusion chromatography on Sephadex LH-20 (60 × 4 cm) with methanol as eluent. Based on analytical UHPLC-MS runs (gradient II; Table S6), selected fractions were pooled and further purified via

reversed phase semi-preparative HPLC using a Zorbax Eclipse XDB-C18 column (9.4 × 250 mm, 5 µm, thermostatted to 40 °C) on an Agilent 1260 chromatograph, equipped with a diode-array detector. For final isolation of atrochrysone, gradient III (Table S6) was applied. The same gradient was used to pre-purify a putative heptaketide shunt product of CoPKS4 from *A. niger* tNAL002 (Figure S8). HR-ESIMS and tandem MS spectra were recorded on a Thermo Scientific Exactive Orbitrap instrument, using a reversed phase Accucore C18 column (100 × 2.1 mm, 2.6 µm) and applying gradient IV (Table S6).

Nuclear magnetic resonance spectroscopy: NMR spectra were recorded on a Bruker Advance DRX 400 spectrometer operating at 400 MHz (for ¹H NMR) and 100.6 MHz (for ¹³C NMR). The chemical shifts (δ) are given in ppm with a solvent resonance as internal standard (¹H NMR: CD₃OD 3.31; ¹³C NMR: CD₃OD 49.0).

Circular dichroism spectroscopy: Measurements were conducted with a JASCO J 810 spectrometer; the scan speed was 500 nm min⁻¹ in a range of 200 – 500 nm. Samples of atrochrysone were dissolved in MeOH (LC-MS grade) with an estimated final concentration of 1.5 mM. The measurement was performed in a 1 mm cuvette and repeated five times. The spectrum of background measurement with MeOH was subtracted from the sample spectrum.

4. Synthetic methods

Synthesis of 6-hydroxymusizin: The chemical synthesis of 6-hydroxymusizin was performed as previously demonstrated in a multistep synthesis with slight amendments.^[12] Naphthalene core structure was synthesized via MICHAEL-DIECKMANN condensation. Methyl 2,4-dimethoxy-6-methylbenzoate served as MICHAEL-donor which was synthesized by O-methylation of orsellinic acid with methyl iodide (3-fold excess). The MICHAEL-acceptor 4-methoxy-3-penten-2-one was synthesized according to Kraus *et al.* with subsequent distillation.^[13] For the tandem MICHAEL-DIECKMANN condensation, LDA was generated *in situ* by adding dropwise *n*-butyllithium (4 mmol, 2.5 M) to a solution of diisopropylamine (5 mmol) in THF (20 mL) at 0 °C. After 30 minutes, the solution was cooled down to -50 °C. Methyl 2,4-dimethoxy-6-methylbenzoate (1 mmol) was added quickly within 1 min and the mixture was stirred again for 30 min. At -30 °C, MICHAEL-acceptor (4 mmol) was added dropwise to the reaction mixture. After 2 h at -30 °C, the mixture was stirred overnight (20 h) without cooling. The reaction was stopped by adding 4 mL ethanol and 4 mL acetic acid. Subsequently, mixture was extracted by using 200 mL ammonium chloride and 3 × 20 mL ethyl acetate. Subsequently, the mixture was transferred into a separatory funnel with saturated ammonium chloride and extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. Torachrysone-8-O-methylether was isolated from crude extract automated flash chromatography (puriFlash XS520Plus, Interchim).^[12]

Demethylation was performed with boron tribromide (6-fold excess) in methylene chloride. After stirring at room temperature for 20 h, the reaction was quenched with semi-saturated sodium hydrogencarbonate and subsequent acidification with hydrochloric acid (3 M). The aqueous phase was extracted with ethyl acetate. After drying over anhydrous sodium sulfate the solvent was removed under reduced pressure. 6-hydroxymusizin was purified with automated flash chromatography on a silica gel column (12 g, 30 µm, Interchim) (methylene chloride and methanol, gradient 97:3 to 90:10 over 15 min).

5. Biochemical methods

Heterologous production and purification of CoPKS4: To purify the CoPKS4-Strep-tag II fusion protein from *A. niger* tNAL048 the Strep-Tactin Superflow resin and recommended buffers (iba life sciences) were used. A 400 mL culture of Yeast Peptone Dextrose medium, supplemented with 30 µg mL⁻¹ doxycycline, was inoculated with 1 × 10⁶ conidia mL⁻¹ and incubated for 36 h at 30 °C and 150 rpm. The mycelium was then separated from

the culture filtrate, washed with tap water, ground under liquid nitrogen, resuspended in 15 mL 1 × buffer W and further disrupted by sonication. Cell debris was removed by centrifugation (4 °C, 4500 × g, 20 min), and the supernatant was cleared via a 0.45 µm GD/X syringe filter device (Whatman). The cell-free protein extract was then subjected to affinity chromatography, using a gravity flow column loaded with Strep-Tactin Superflow resin (column volume, CV = 2 mL). The column was washed with 8 CV 1 × buffer W, before eluting six times with 0.5 CV 2 × buffer E. Protein purification was verified by polyacrylamide gel electrophoresis (12 % Laemmli gel, Figure S10). The eluate was repeatedly desalted on an Amicon Ultra-15 centrifugal filter unit (Merck, 100 kDa cutoff) and equilibrated in reaction buffer (see below).

***In vitro* product formation assay:** Product formation enzyme assays followed a previously described protocol^[14] with minor modifications. Product formation assays were carried out in triplicate in 250 µL reactions containing 2.5 mM acetyl-CoA, 5 mM malonyl-CoA, and approximately 7 µg of purified protein in reaction buffer (PBS). An identical reaction with heat-denatured protein served as negative control. An assay with intact protein but in the absence of acetyl-CoA and malonyl-CoA was run for additional control. The reaction mixtures were incubated at 22 °C in the dark, for 12 h. After lyophilization, the residue was dissolved in 50 µL methanol and subjected to chromatography using gradient I (Table S6).

6. Bioinformatic methods

Phylogenetic tree construction: Evolutionary analyses were conducted using MEGA X.^[15] The analyses were based on the sequences of β-ketoacyl synthase domains of characterized basidiomycete or ascomycete PKSs (Table S7). The extracted protein sequences were aligned using the Muscle algorithm^[16] performing 10,000 iterations. Phylogenetic tree construction was performed using the Maximum Likelihood method and Le Gascuel 2008 model.^[17]

Table S1. ^1H (400 MHz) and ^{13}C (100.6 MHz) NMR spectroscopy data for atrochrysone (**1**) in CD_3OD .*

Pos.	δ_{H} (mult. J [Hz])	δ_{C}	HMBC	COSY
1		203.9	-	-
2	2.75 (dd, 17.3, 1.66, 1H); 2.86 (d, 17.3, 1H)	51.8	1, 3, 4, 9a, ^[a] 11	2, 4, 11 ^[a]
3	4.61 (s, br, -OH)	71.4	-	-
4	2.99 (d, 16.0, 1 H); 3.05 (d, 16.0, 1 H)	43.8	2, 3, 4a, 9a, 10, 11 ^[a]	2, 4, 10, 11 ^[a]
4a		137.7	-	-
5	6.48 (d, 2.04, 1 H)	103.1	6 ^[a] , 7, 8a, 10	7, 10 ^[a]
6	-	163.1	-	-
7	6.32 (d, 2.04, 1 H)	102.2	5, 6, 8, 8a,	5
8	-	161.1	-	-
8a	-	108.0	-	-
9	-	163.1 ^[b]	-	-
9a	-	109.2	-	-
10	6.82 (s, 1 H)	118.0	4, 5, 8a ^[a] , 9a, 10a	4, 5 ^[a]
10a	-	142.9	-	-
11	1.38 (s, 3 H)	29.0	1 ^[a] , 2, 3, 4, 4a ^[a]	2 ^[a] , 4 ^[a]

* Spectra were evaluated according to their phenotype.

[a] Weak signal.

[b] Tentative assignment.

Table S2. Fungal strains and their genotypes.

Strain	Genotype	Reference
<i>Cortinarius odorifer</i> SF013765	wild type	
<i>Aspergillus niger</i> ATNT16 Δ pyrGx24	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i>	[4]
<i>Aspergillus niger</i> tNAL000	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:His₆_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL002	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:copks4_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL003	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:copks5_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL004	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:copks6_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL024	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:copks1_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL025	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:copks2_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL026	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA:copks3_pyrG</i>	This study
<i>Aspergillus niger</i> tNAL048	TetOn: <i>terR_ble</i> ; Δ pyrG:: <i>ptrA</i> ; <i>PterA: copks4-streptagII_pyrG</i>	This study

Table S3. Oligonucleotides used in this study.

oCB041	AACTTCTCATCACAGCACCATGCCACCAAATACTGTTAAC	<i>copks4</i>	Cloning of pNAL006
oCB042	CCATGGTGATGGTGATGATGTCACCCCATCTTGTTCAACC	<i>copks4</i>	Cloning of pNAL006
oCB044	AACTTCTCATCACAGCACCATGTCACCAGATATTAACAAAGCTG	<i>copks5</i>	Cloning of pNAL004
oCB045	CCATGGTGATGGTGATGATGTCATCCCATCTTGTTTAACCAAC	<i>copks5</i>	Cloning of pNAL004
oCB047	AACTTCTCATCACAGCACCATGCCACCAAATACCGCTAAAAAAGC	<i>copks6</i>	Cloning of pNAL005
oCB048	CCATGGTGATGGTGATGATGTCATCCCATCTTGTTCAACCGG	<i>copks6</i>	Cloning of pNAL005
oMG267	CATGGTGCTGTGATGAGAAG	phis_SM-Xpress	Cloning of pNAL020
oMG268	CAGCAGTGATTCAATCTGAACC	phis_SM-Xpress	Cloning of pNAL020
oMG370	GATCCTCTCTCTGATATTGTCG	phis_SM-Xpress	Proof of transgene integration
oNAL070	GCCACCAAATACTGTTAACAAAGC	<i>copks4</i>	Cloning of pNAL034
oNAL071	CACCCCATCTTGTTCAACCGG	<i>copks4</i>	Cloning of pNAL034
oNAL098	TAACAACTTCTCATCACAGCACCATGCCACCAAACACTGCTAAC	<i>copks1</i>	Cloning of pNAL020
oNAL099	CGGTTCAAGATTGAAATCACTGCTGTCATCCCGCCTTGTTCAACCAACC	<i>copks1</i>	Cloning of pNAL020
oNAL113	TTAACAACTTCTCATCACAGCACCATGCATTTACCAAATTCTGC	<i>copks2</i>	Cloning of pNAL021
oNAL114	GCTGTTATCCATGGTGATGGTGATGATGTCACGCCGATTGCTCAACC	<i>copks2</i>	Cloning of pNAL021
oNAL115	TAACAACTTCTCATCACAGCACCATGCATTTACGAAATTCTGCAAACC	<i>copks3</i>	Cloning of pNAL022
oNAL116	GCTGTTATCCATGGTGATGGTGATGATGTCACACCTTGTTCAAGGAGC	<i>copks3</i>	Cloning of pNAL022
oNAL141	ATGCCACCAAACACTGCTAAC	<i>copks1</i>	Cloning of pNAL033
oNAL142	TCATCCCGCCTTGTTCAACC	<i>copks1</i>	Cloning of pNAL033
oNAL156	GTGAGGGTTGAGTACGAGATT	phis_SM-Xpress	Proof of transgene integration
oNAL218	CATTTAACAACTTCTCATCACAGCACCATGCATCCACCAAATACTGTTAACAAAGC	<i>copks4</i>	Cloning of pNAL046
oNAL248	CAGATTGAAATCACTGCTGTTACCATGGCTTCTCGAACTGGGGGTGGGACCACCCCATCTTGTTCAACCGG	<i>copks4</i>	Cloning of pNAL046

Table S4. PCR parameters.

Condition	Thermal cycling	Final elongation
I	35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 3 min	72 °C for 7 min
II	34 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 4 min	72 °C for 7 min
III	40 cycles of 98 °C for 10 s, 58 °C for 15 s, 72 °C for 3 min	72 °C for 7 min

Table S5. Plasmids used in this study.

Plasmid name	Vector backbone	Gene	Reference
phis_SM-Xpress	pUC19	-	[4,8]
pSM_StrepTag_X_URA	pUC19	-	This study
pNAL004	phis_SM-Xpress	<i>copks5</i> (gDNA)	This study
pNAL005	phis_SM-Xpress	<i>copks6</i> (gDNA)	This study
pNAL006	phis_SM-Xpress	<i>copks4</i> (gDNA)	This study
pNAL020	phis_SM-Xpress	<i>copks1</i> (gDNA)	This study
pNAL021	phis_SM-Xpress	<i>copks2</i> (gDNA)	This study
pNAL022	phis_SM-Xpress	<i>copks3</i> (gDNA)	This study
pNAL046	pSM_StrepTag_X_URA	<i>copks4-streptagII</i> (gDNA)	This study
pNAL101	pJET	<i>copks1</i> (cDNA)	This study
pNAL102	pJET	<i>copks4</i> (cDNA)	This study

Table S6. HPLC parameters. Eluents were: 0.1 % formic acid in water (eluent A) and acetonitrile (eluent B). In case of gradient IV, eluent B was 0.1 % formic acid in acetonitrile.

Gradient	Flow [mL min ⁻¹]	Time [min]	Eluent B [%]	Column
I	1	0.00	5	Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm particle size)
		1.00	5	
		1.50	25	
	1.1	3.50	25	
		6.00	30	
		6.50	60	
	1	7.50	100	
II	1	0.00	5	Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm particle size)
		0.50	25	
		1.50	25	
		3.00	30	
		3.50	100	
III	2.5	0.00	40	Zorbax Eclipse XDB-C18 column (9.4 × 250 mm, 5 µm particle size)
		5.00	40	
		15.00	48	
		19.00	100	
IV	0.2	0.00	5	Accucore C18 column (100 × 2.1 mm, 2.6 µm particle size)
		10	98	
		22	99	

Table S7. Sequences of polyketide synthases used to reconstruct a phylogenetic tree.

Species	PKS	NCBI Accession/ JGI protein ID	Type	Reference
<i>Armillaria mellea</i>	ArmB	JQ801748.1	NR	[18]
<i>Aspergillus fumigatus</i>	Alb1	AAC39471.1	NR	[19]
<i>Aspergillus nidulans</i>	AptA	Q5B0D0.1	NR	[20]
	wA	CAA46695.2	NR	[21]
	MdpG	Q5BH30.1	NR	[22]
	PksSt	AAA81586.1	NR	[23]
<i>Aspergillus parasiticus</i>	AflC	AAS66004.1	NR	[24]
<i>Aspergillus terreus</i>	LovB	AAD39830.1	HR	[25]
	LovF	AAD34559.1	HR	[26]
	ACAS	Q0CCY3.1	NR	[27]
BY1 ^[a]	PPS1	KX819293.1	HR	[28]
	PKS1	APH07629.1	NR	[29]
<i>Cladosporium fulvum</i>	ClaG	P0CU67.1	NR	[30]
<i>Cortinarius odorifer</i>	CoPKS1	OL512945	NR	this study
	CoPKS4	OL512946	NR	this study
<i>Laetiporus sulphureus</i>	LpaA	QNJ99675.1	HR	[31]

^[a] BY1 is a taxonomically unidentified stereaceous basidiomycete.

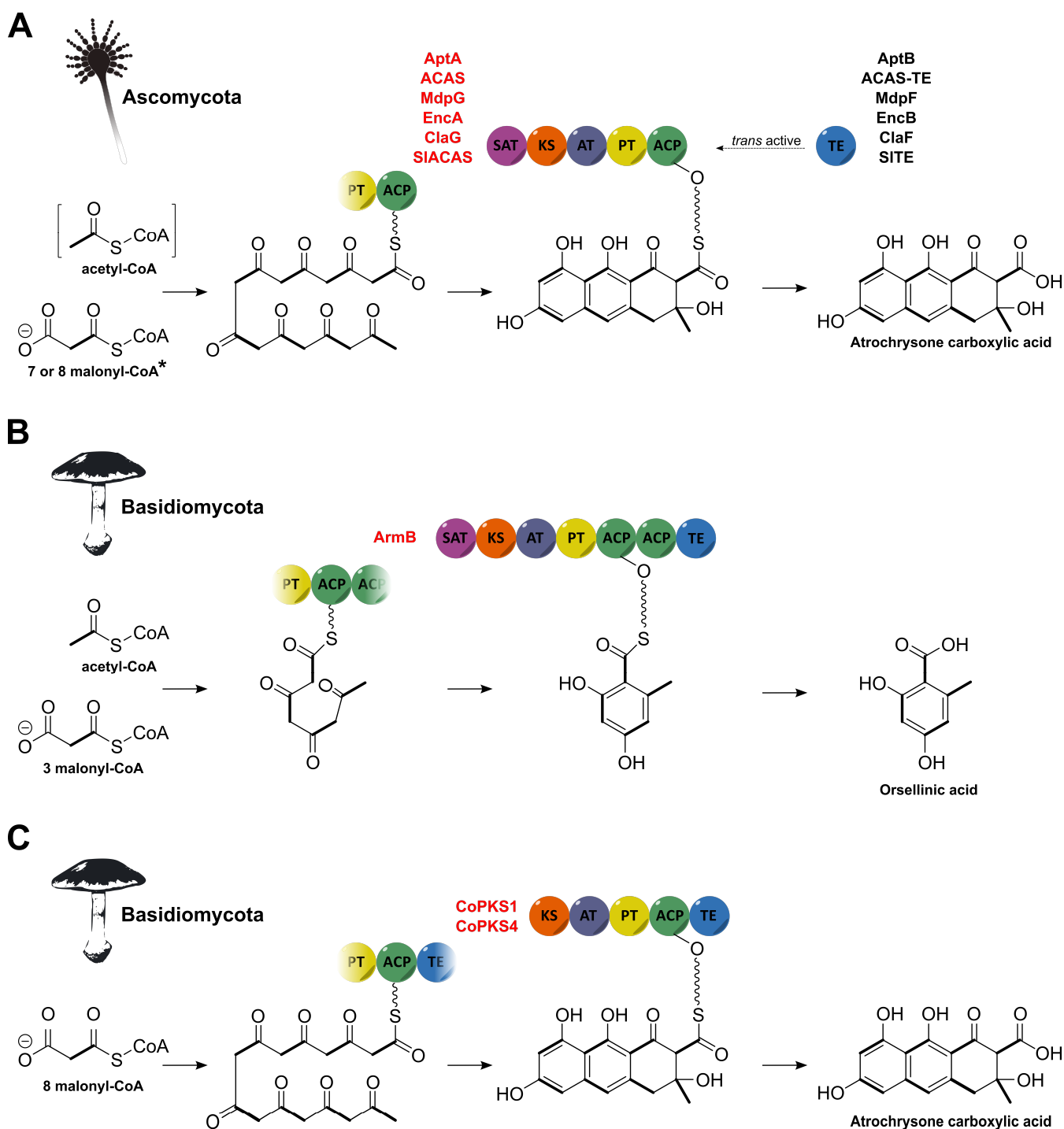
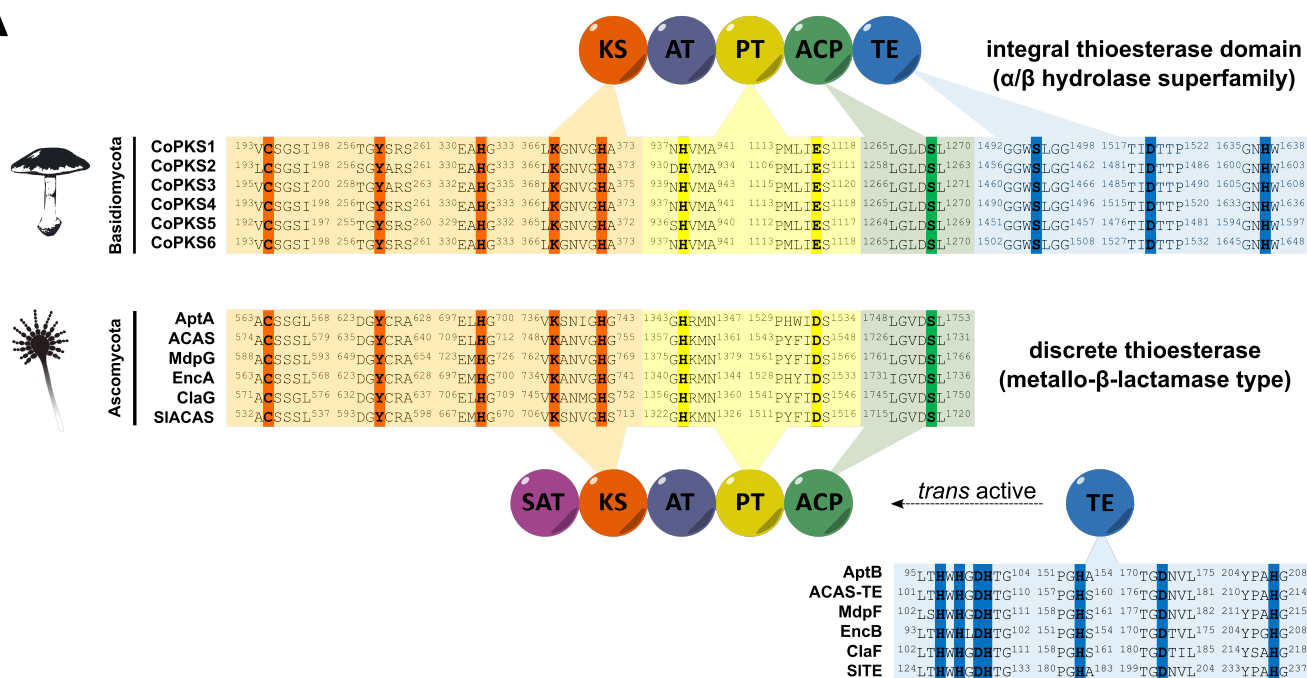


Figure S1. Comparison of three classes of fungal iterative non-reducing polyketide synthases (PKSs). A) Ascomycete atrochrysone carboxylic acid synthases. Functional PKS/thioesterase (TE) pairs are: AptA/AptB^[20] (*Aspergillus nidulans*), ACAS/ACAS-TE^[27] (*A. terreus*), MdpG/MdpF^[22] (*A. nidulans*), EncA/EncB^[32] (*A. fumigatus*), ClaG/ClaF^[30] (*Cladosporium fulvum*), SIACAS/SITE^[33] (*Stemphylium lycopersici*). The asterisk denotes inconsistent literature data^[27,30,32,33] on the requirement of acetyl-CoA as starter and, consequently, the number of extender units. B) Basidiomycete orsellinic acid synthases, e.g., represented by *Armillaria mellea* ArmB.^[18] C) *Cortinarius* atrochrysone carboxylic acid synthases CoPKS1 and CoPKS4.

A



B

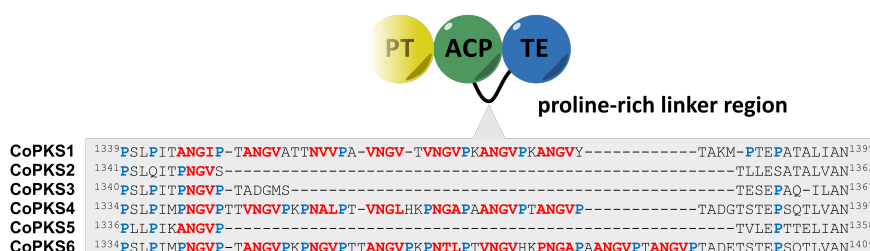
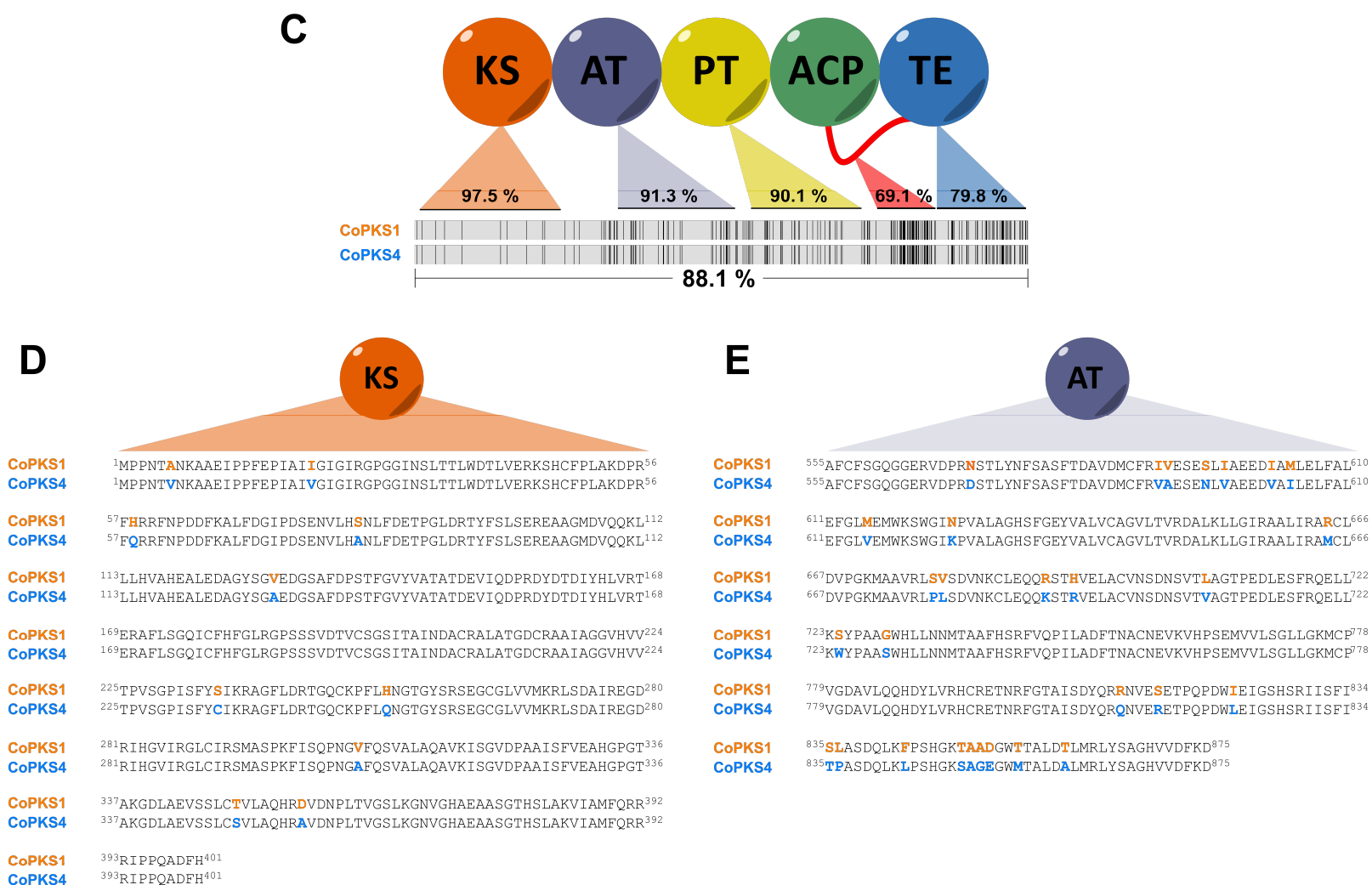


Figure S2. Alignment of amino acid sequences of fungal polyketide synthases.

A) Alignments of active site regions and catalytic residues in the β-ketoacyl synthase (KS), acyltransferase (AT), product template (PT), acyl carrier protein (ACP) and thioesterase (TE) domains in *Cortinarius* PKSs and comparison to ascomycete PKSs. An alignment of active site residues of the discrete ascomycete TE is presented as well. Functional ascomycete PKS/TE pairs are: AptA/AptB^[20] (*Aspergillus nidulans*), ACAS/ACAS-TE^[27] (*A. terreus*), MdpG/MdpF^[22] (*A. nidulans*), EncA/EncB^[32] (*A. fumigatus*), ClaG/ClaF^[30] (*Cladosporium fulvum*), SIACAS/SITE^[33] (*Stemphylium lycopersici*).

B) Proline-rich linker region between the ACP and TE domains region of CoPKSs. Proline residues are highlighted in blue, the repeated motif is shown in red.



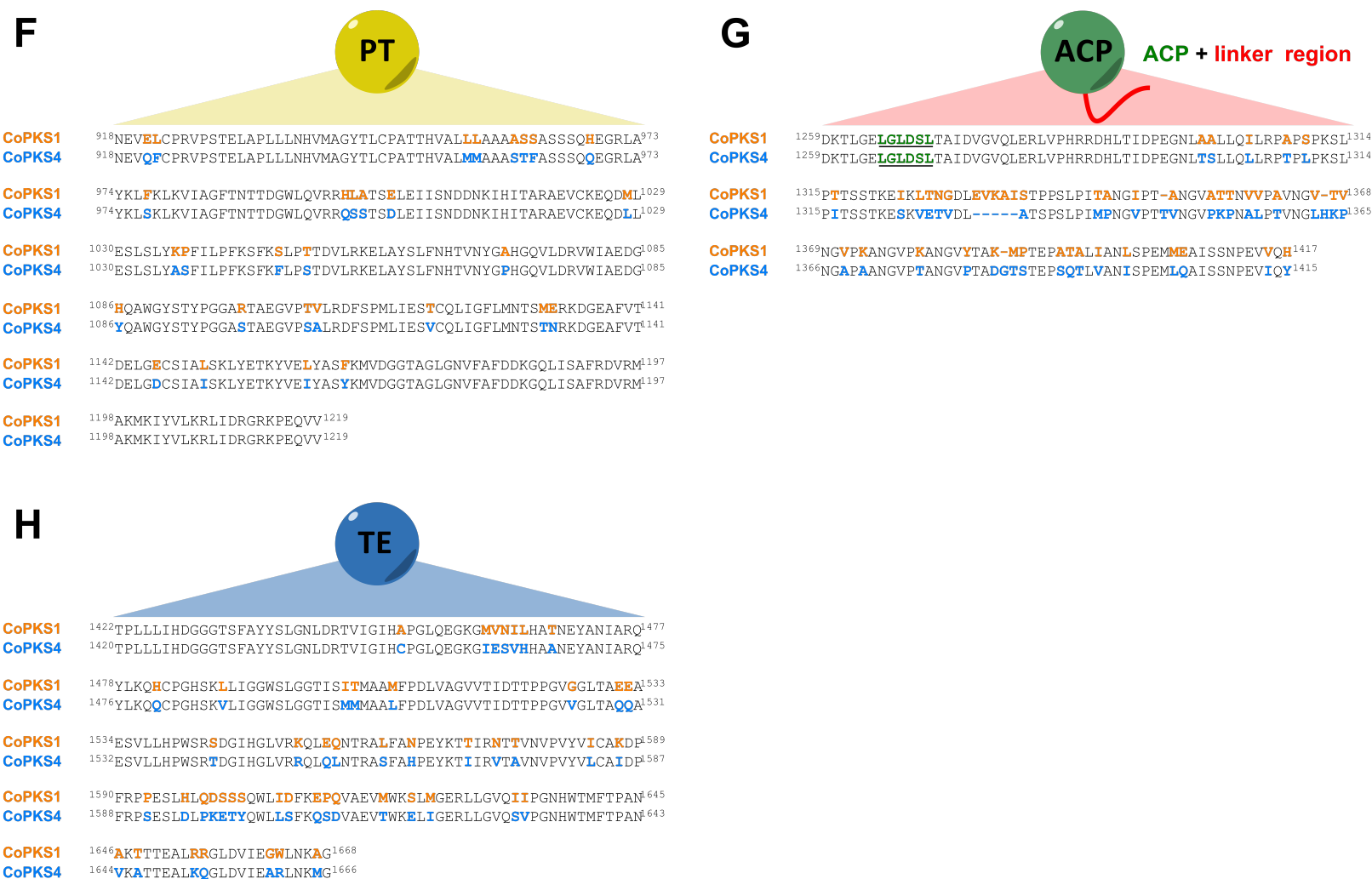


Figure S2 (continued). Alignment of CoPKS1 and CoPKS4. C) Overall sequence similarities; D) Alignment of β -ketoacyl synthase (KS) domains; E) Alignment of acyltransferase (AT) domains; F) Alignment of product template (PT) domains; G) Alignment of acyl carrier proteins (ACP) and linker regions; H) Alignment of thioesterase (TE) domains.

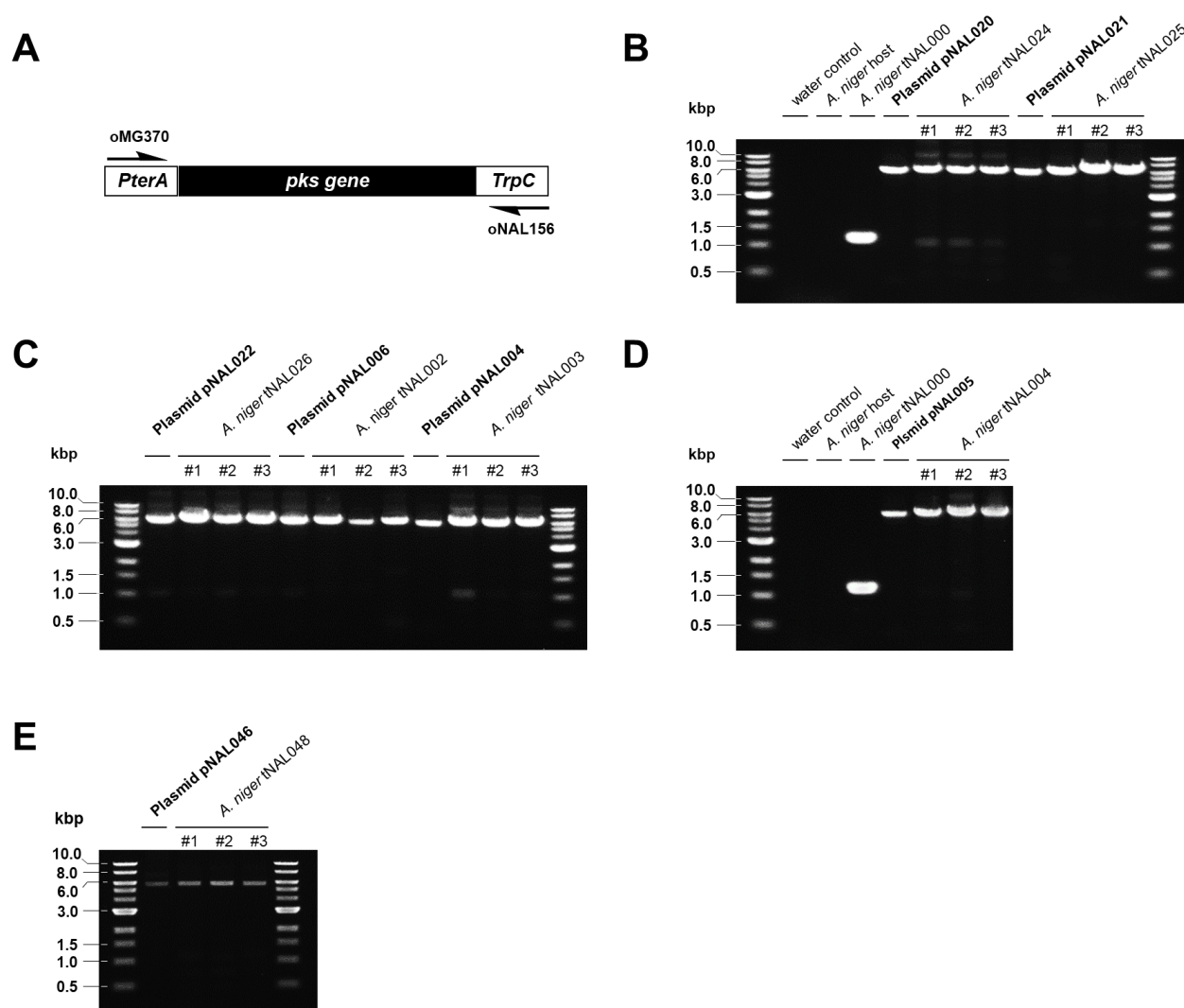


Figure S3. Agarose gel electrophoresis to verify integration of *C. odorifer* PKS genes in the genome of *A. niger* ATNT16ΔpyrGx24.

A) PCR strategy to amplify the entire cassette of *PterA* promoter/PKS gene/*trpC* terminator. Expected amplicon lengths in case of a full-length accurate integration range from 6.1 to 6.2 kilobase pairs (kbp).

Sizes DNA marker bands in kbp are indicated. Water control: water instead of chromosomal DNA was added; *A. niger* host: DNA of untransformed *A. niger* host strain ATNT16ΔpyrGx24 was added for negative control; *A. niger* tNAL000: DNA of the *A. niger* host, transformed with insert-less expression vector phis_SM-Xpress was added for negative control. For positive control, the PCR product obtained with the respective pNAL expression plasmid as template DNA is shown. Three independent transformants are shown per transformed PKS gene.

B) tNAL024 and tNAL025: *A. niger* harboring the genes for CoPKS1 and 2, respectively.

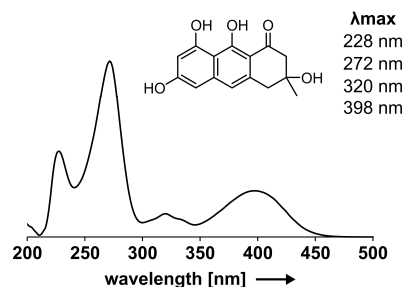
C) tNAL026, tNAL002, and tNAL003: *A. niger* harboring the genes for CoPKS3, 4, and 5.

D) tNAL004: *A. niger* harboring the genes for CoPKS6.

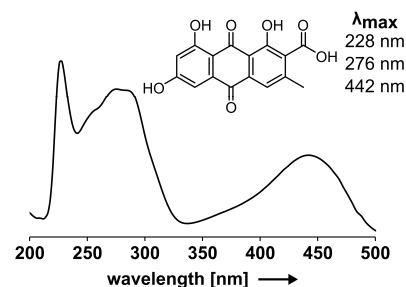
E) tNAL048: *A. niger* harboring the genes for CoPKS4-StrepTagII.

A**Standards**

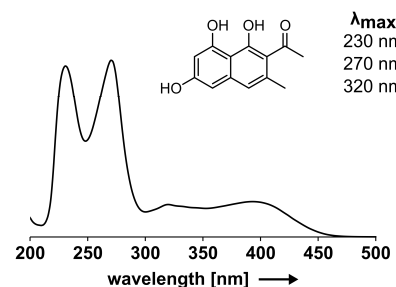
Atrochrysone (1)



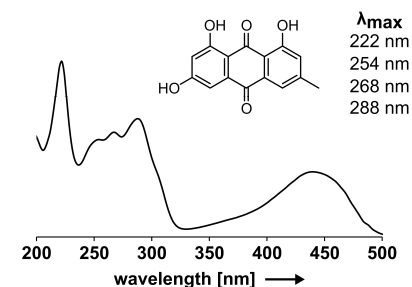
Endocrocin (8)



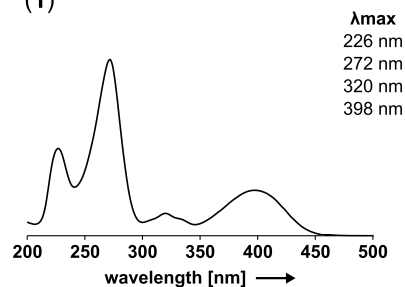
6-Hydroxymusizin (6)



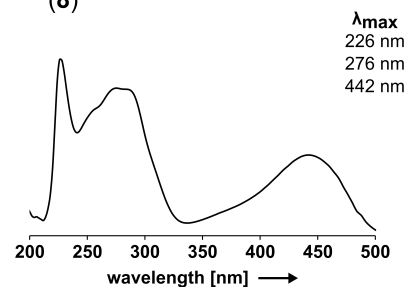
Emodin (9)

**B****Compounds from recombinant Aspergilli**

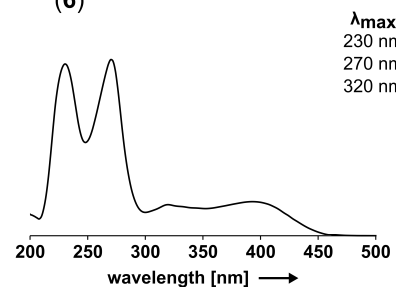
(1)



(8)



(6)



(9)

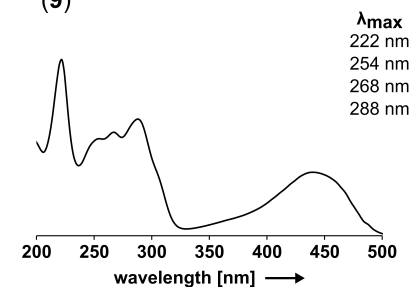


Figure S4. UV/Vis spectra of monomeric polyketides, isolated from *A. niger* tNAL002 or tNAL024. A) Standards of atrochrysone (1), endocrocin (8), 6-hydroxymusizin (6) and emodin (9). B) Representative UV/Vis spectra of compounds of the transformants tNAL002 (CoPKS4) and tNAL024 (CoPKS1).

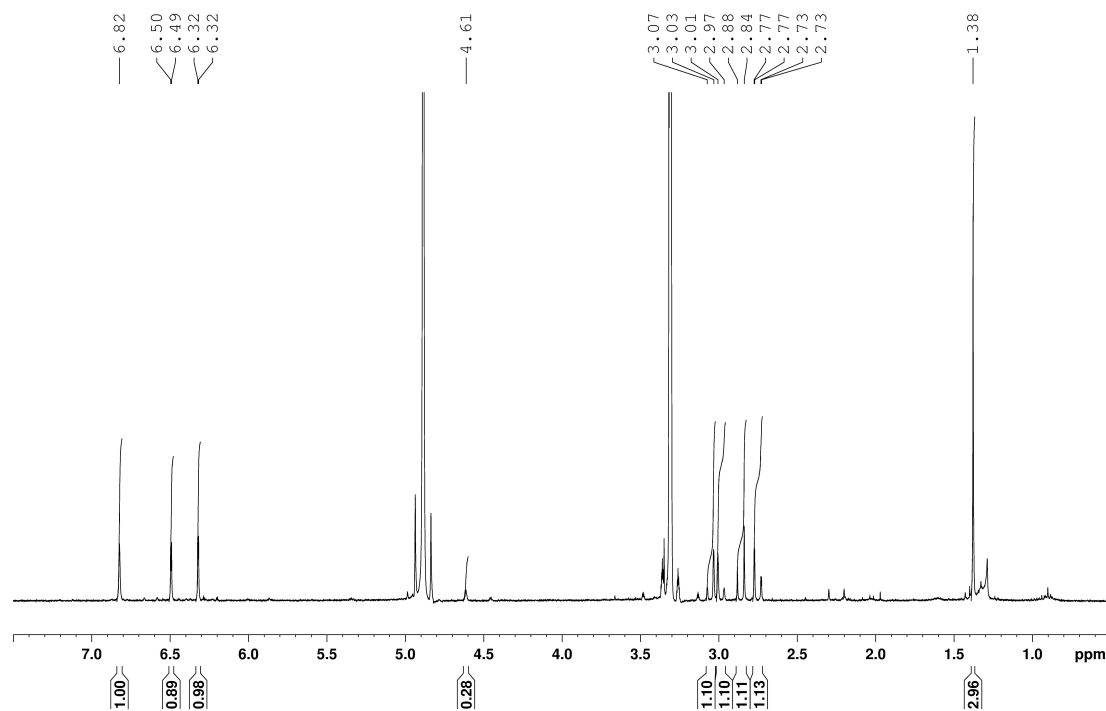


Figure S5A. ^1H NMR spectrum of atrochryson, produced by CoPKS1 (400 MHz, CD_3OD).

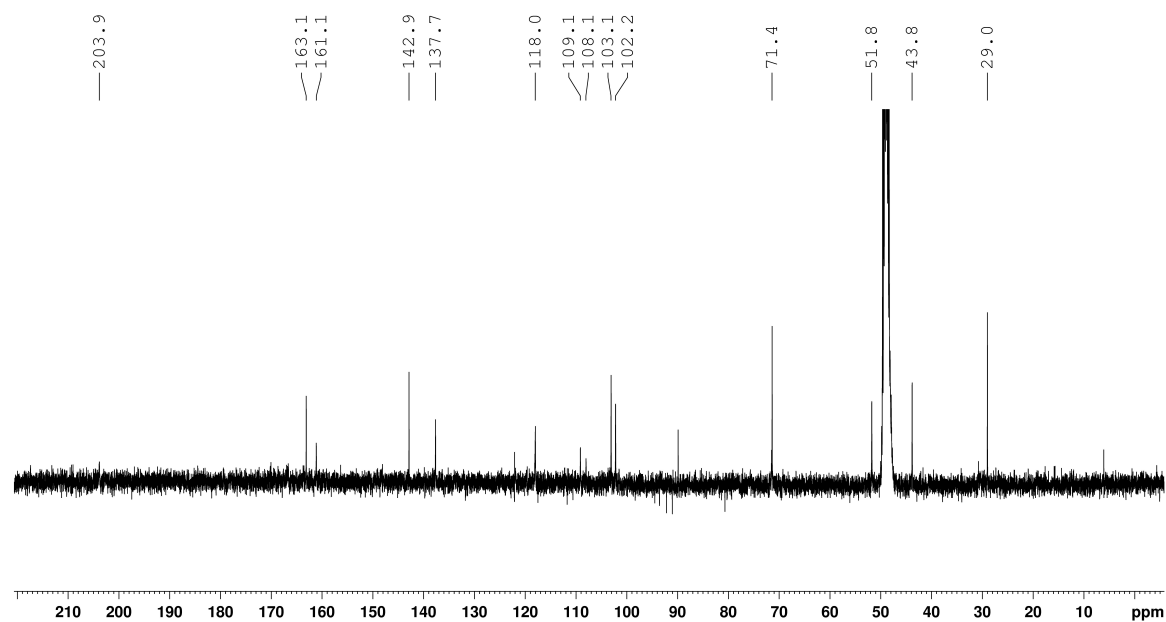


Figure S5B. ^{13}C NMR spectrum of atrochrysone, produced by CoPKS1 (100.6 MHz, CD_3OD).

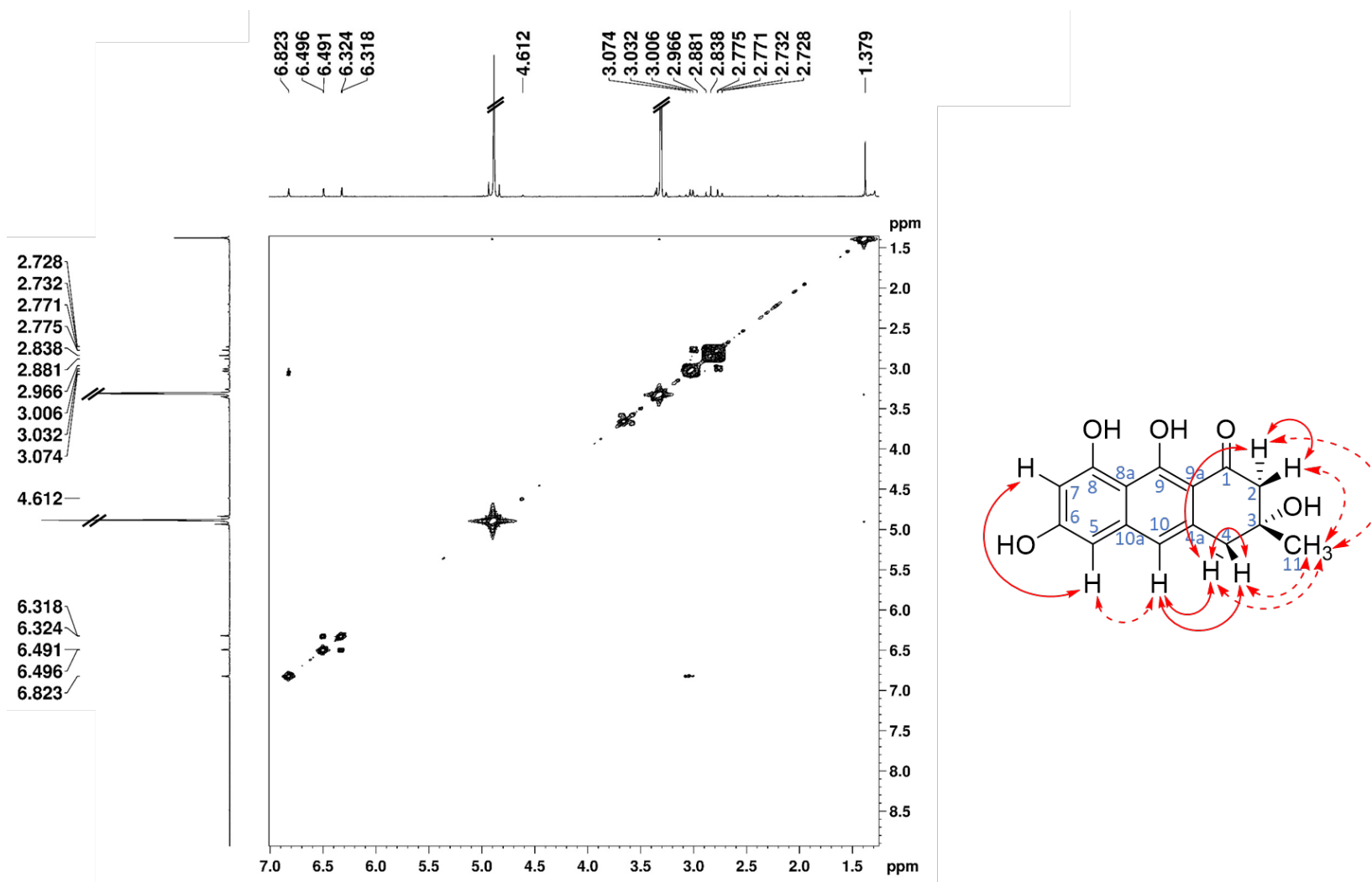


Figure S5C. ^1H - ^1H COSY spectrum of atrochrysone, produced by CoPKS1.

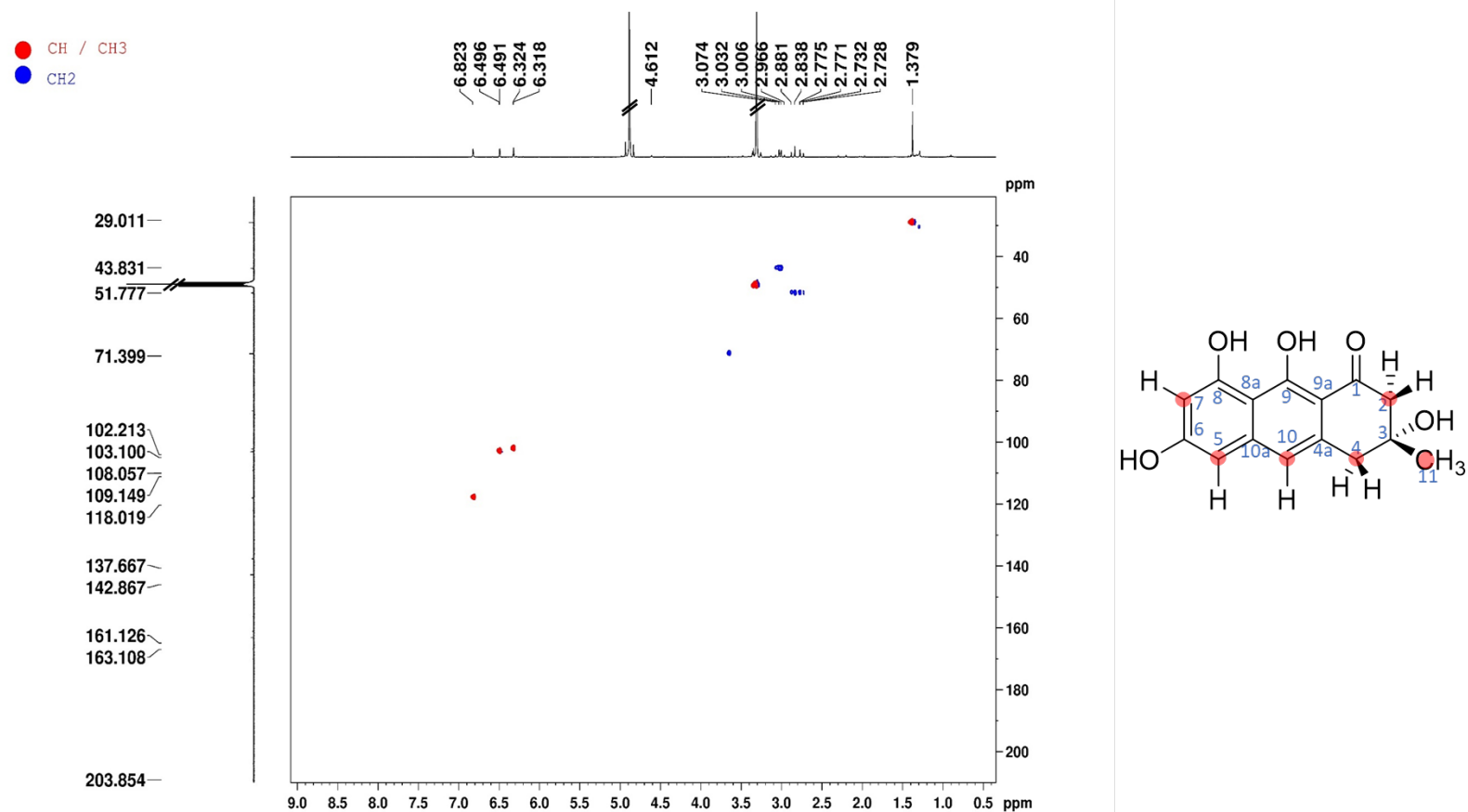


Figure S5D. HSQC spectrum of atrochryson, produced by CoPKS1.

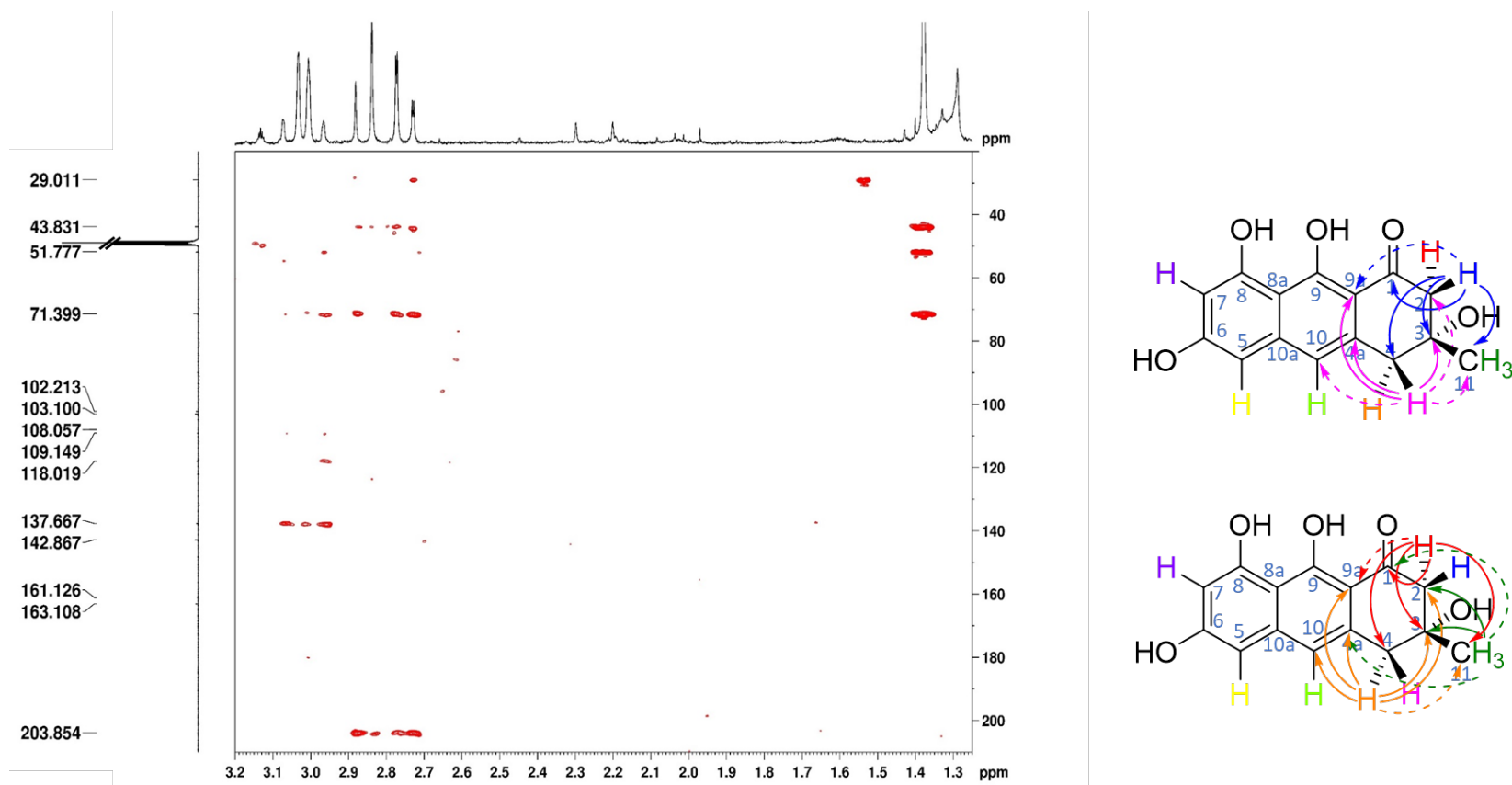


Figure S5E. HMBC spectrum (aliphatic protons) of atrochrysone, produced by CoPKS1.

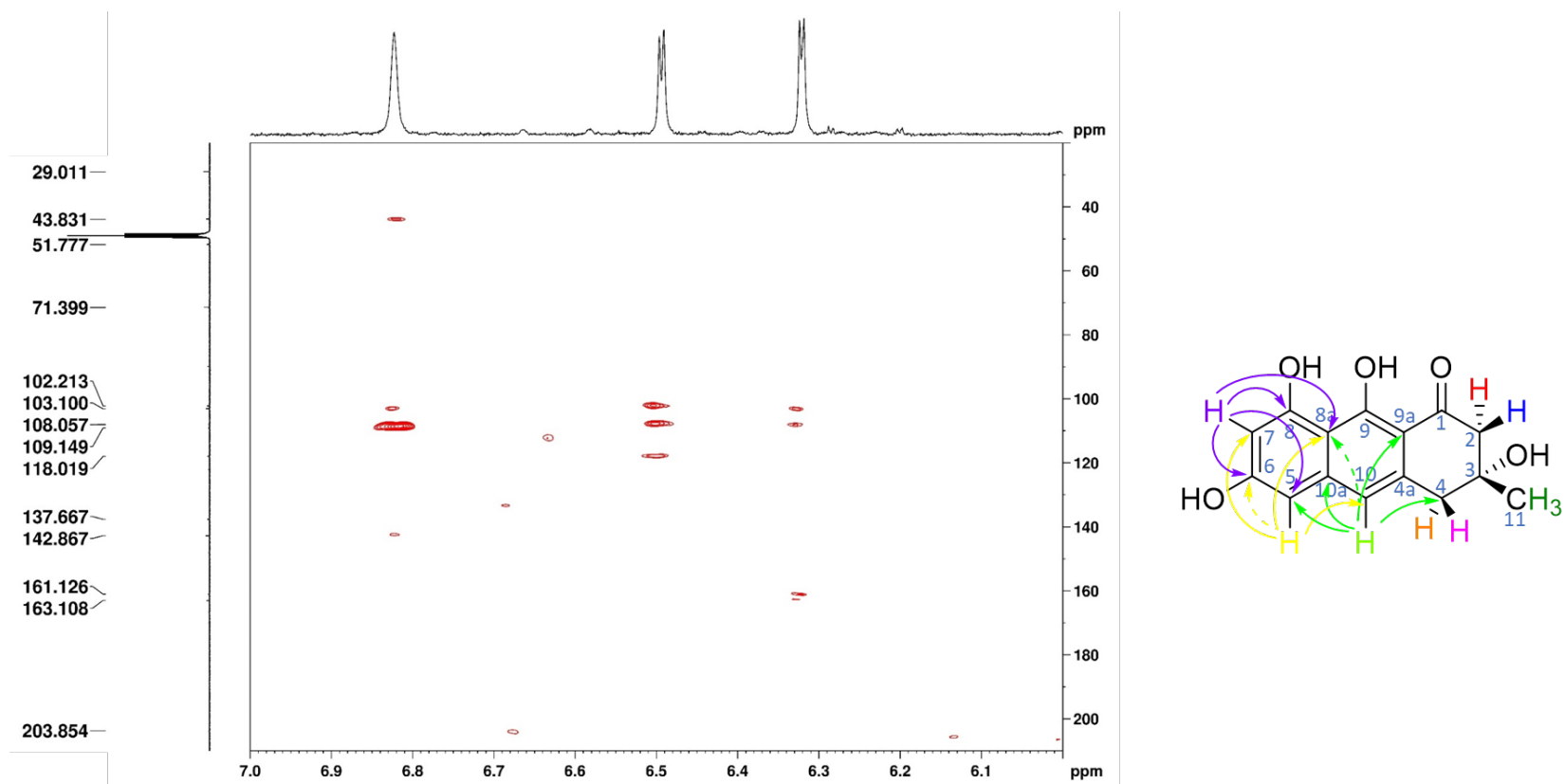
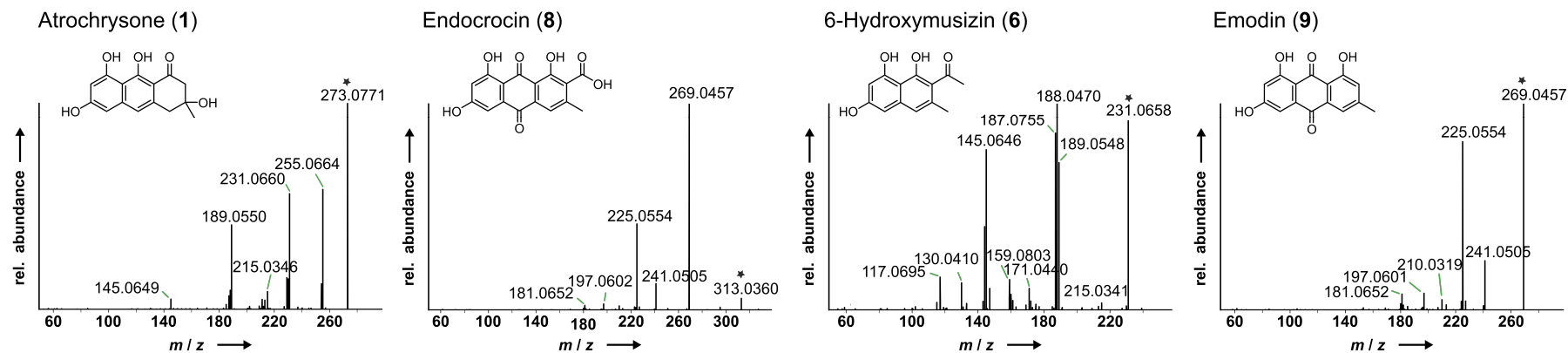


Figure S5F. HMBC spectrum (aromatic protons) of atrochrysone, produced by CoPKS1.

A

Standards



B

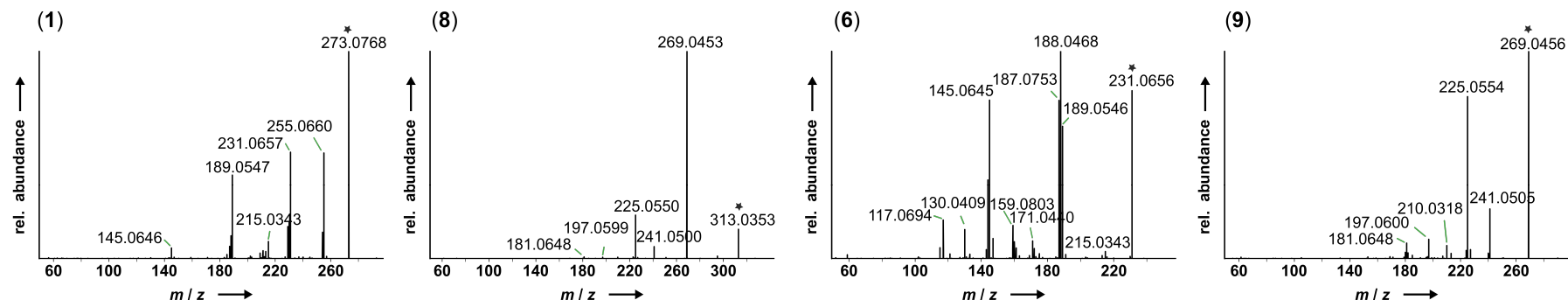
Compounds from recombinant *Aspergilli*

Figure S6. LC-MS/MS spectra of monomeric polyketides. A) LC-MS/MS spectra of standards of atrochrysone (1), endocrocin (8), 6-hydroxymusizin (6) and emodin (9). B) Representative LC-MS/MS spectra of compounds of the transformants *A. niger* tNAL002 (CoPKS4) and tNAL024 (CoPKS1). All spectra were recorded in negative ionization mode $[M-H]^-$. Asterisks denote parental ions. Calculated masses ($[M-H]^-$) are: m/z 273.0769 (1), m/z 313.0354 (8), m/z 231.0663 (6), and m/z 269.0456 (9).

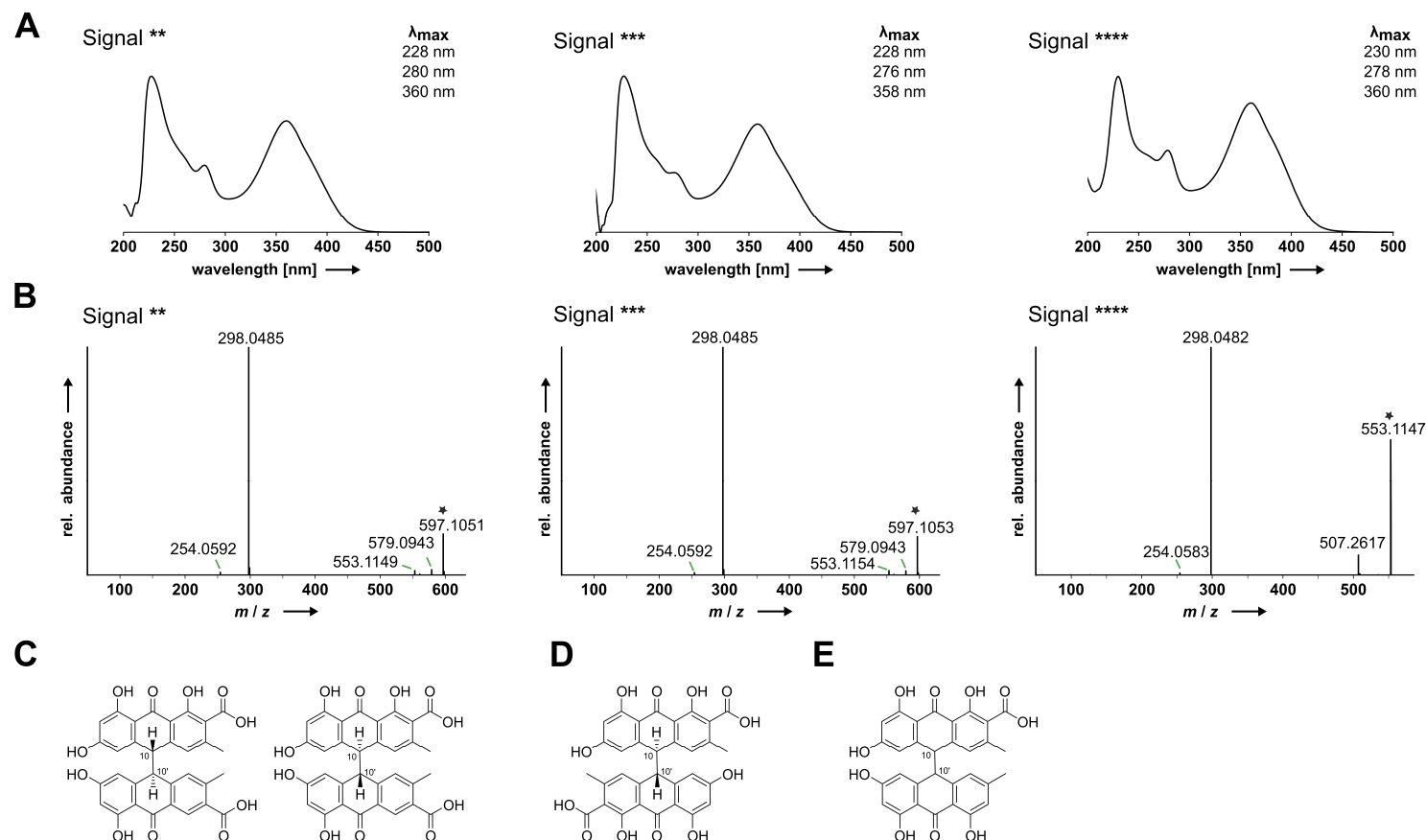


Figure S7. Analysis of dimeric polyketides. A) Representative UV/Vis spectra of dimeric compounds of the transformants *A. niger* tNAL002 (CoPKS4) or tNAL024 (CoPKS1). B) Representative LC-MS/MS spectra of dimeric compounds. All spectra were recorded in negative ionization mode $[M-H]^-$. The single asterisk in mass spectra denotes parental ions. Calculated masses ($[M-H]^-$) are: m/z 597.1039 (**), m/z 597.1039 (***), m/z 553.1140 (****). C) Proposed structures for signals ** or ***: mixture of enantiomers ($10S$, $10'S$; left) and ($10R$, $10'R$; right) of endocrocin anthrone homodimers. D) Proposed structure for signals ** or ***: *meso*-form of ($10R$, $10'S$) of endocrocin anthrone homodimer. E) Proposed structure for signal ****: heterodimeric endocrocin anthrone - emodin anthrone. Awakawa *et al.* speculated that the dimerization reactions are catalyzed by an endogenous *Aspergillus* host enzyme.^[27]

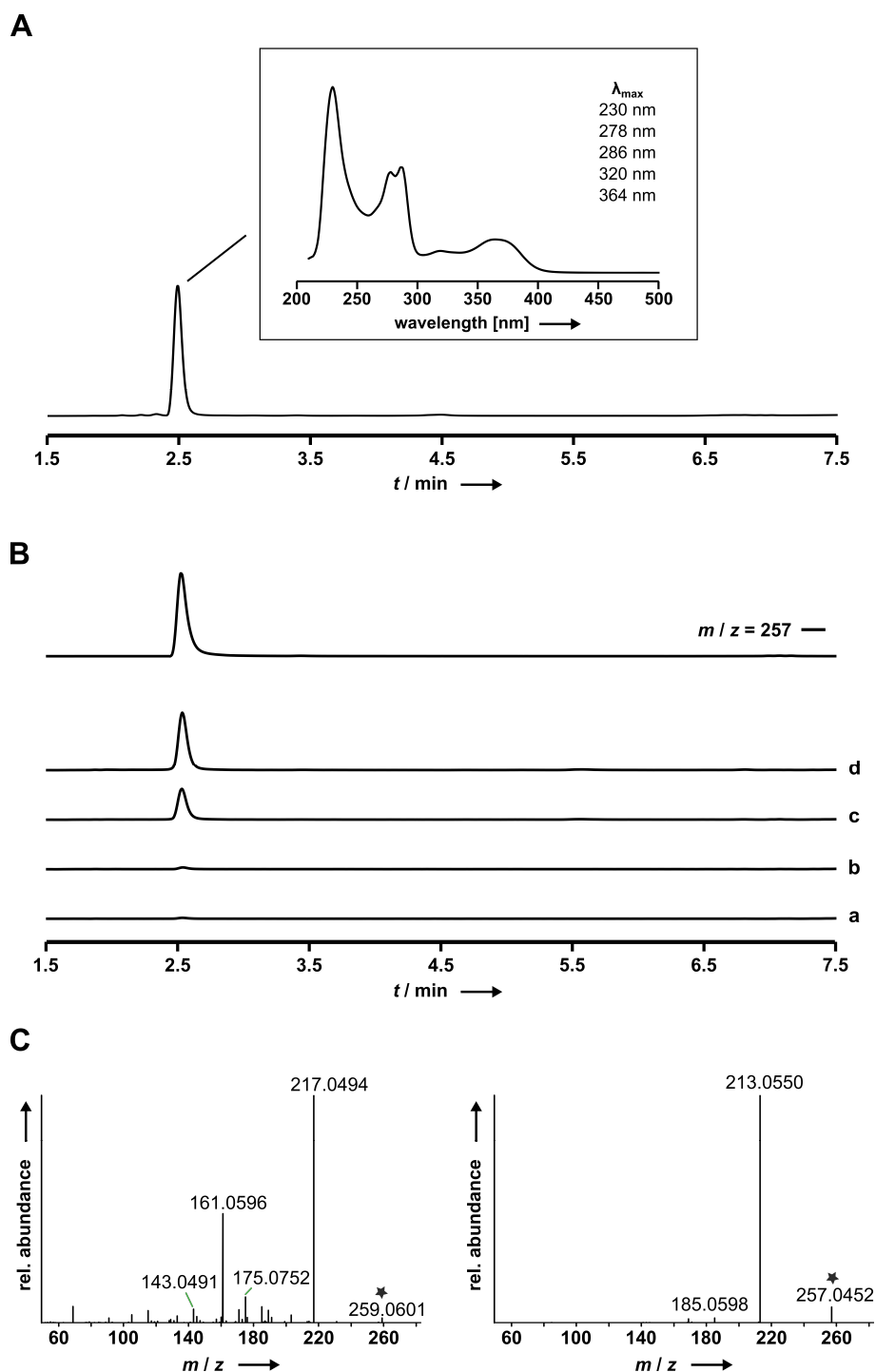


Figure S8. A) Chromatogram of pre-purified putative pannorin, recorded at $\lambda = 395$ nm. The inset shows the respective UV/Vis spectrum. The local maxima are in agreement with literature data.^[34] B) *In vitro* product formation by CoPKS4. Extracted ion chromatograms (EICs) of lyophilized reactions are shown for m/z 257 $[M-H]^-$ to detect the putative pannorin. The top trace represents a pre-purified standard. Traces a: negative control with intact CoPKS4 but without any substrates; traces b: negative control with heat-treated CoPKS4, acetyl-CoA and malonyl-CoA; traces c: reaction with CoPKS4, acetyl-CoA and malonyl-CoA; traces d: reaction with CoPKS4 and malonyl-CoA only. C) LC-MS/MS spectra of putative pannorin. The spectrum was recorded in both, positive (left; $[M+H]^+$) and negative (right; $[M-H]^-$) ionization mode. The asterisk denotes the parental ion. Calculated masses are: m/z 259.0601 ($[M+H]^+$) and m/z 257.0456 ($[M-H]^-$).

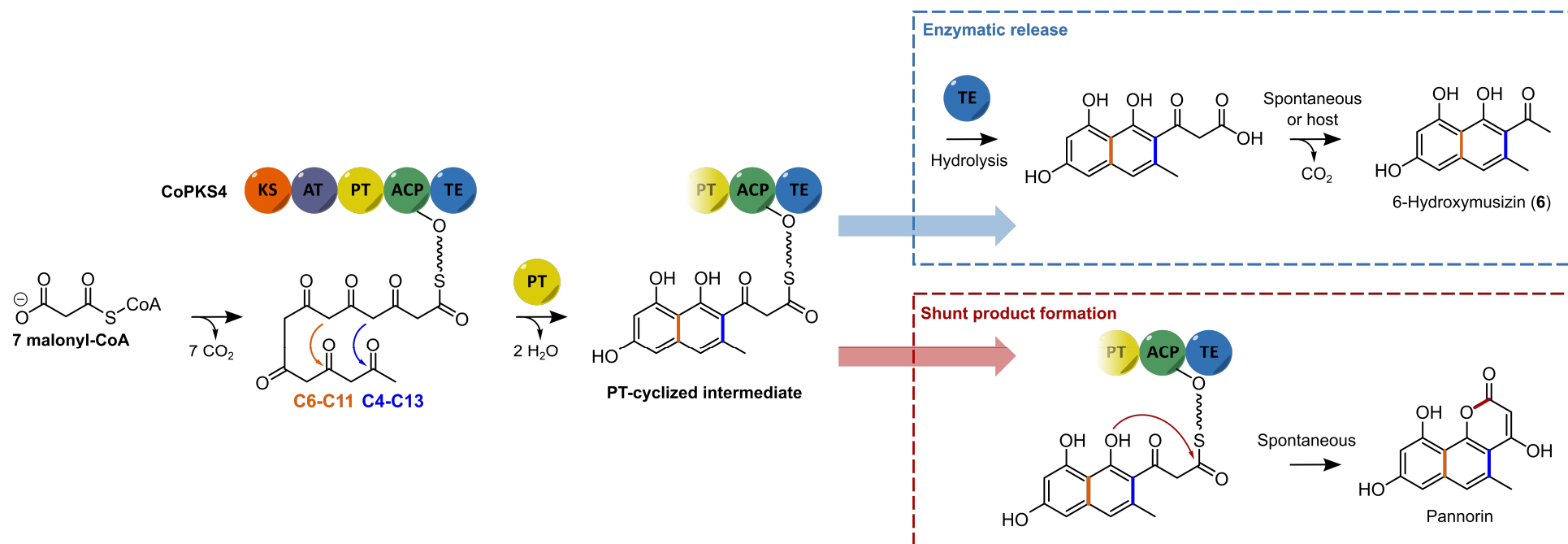


Figure S9. Heptaketide synthase activity of CoPKS4. Proposed biosynthetic actions of CoPKS4 in *A. niger*. Accordingly, the cyclization mode, catalyzed by the PT domain, is C6-C11, C4-13, which leads to a bicyclic ACP-linked intermediate. Offloading, followed by decarboxylation, yields 6-hydroxymusizin (**6**). Lactone formation, yielding pannorin, occurs non-enzymatically.

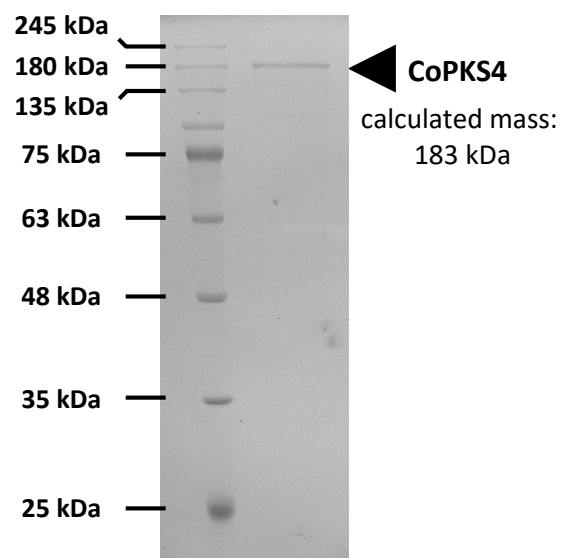


Figure S10. SDS-polyacrylamide gel electrophoresis. Left lane: molecular weight marker. Right lane: CoPKS4, recombinantly produced in *A. niger* tNAL048.

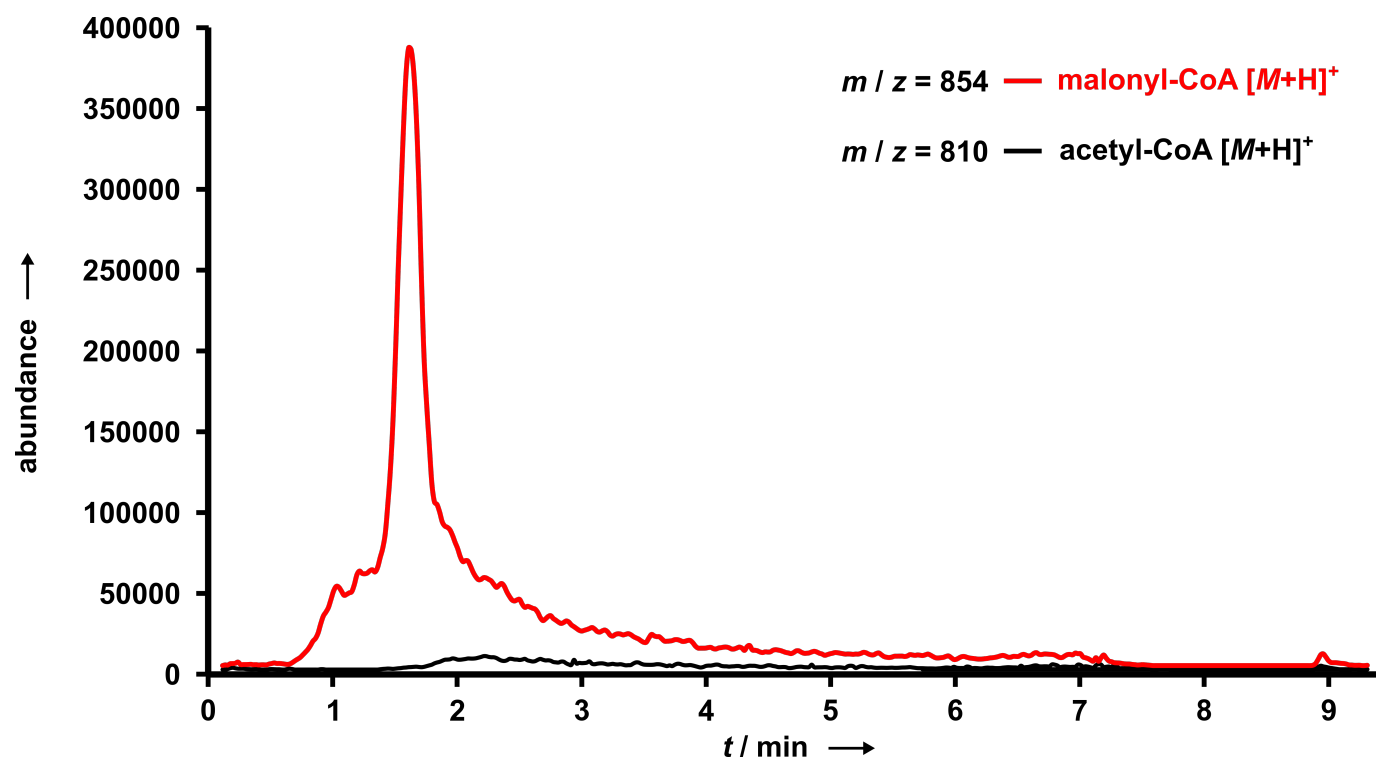


Figure S11. Chromatographic and mass spectrometric analysis of the purity of commercial malonyl-CoA. Extracted ion chromatograms are shown for m/z 854 $[M+H]^+$ to detect malonyl-CoA (red trace) and m/z 810 $[M+H]^+$ to detect acetyl-CoA (black trace).

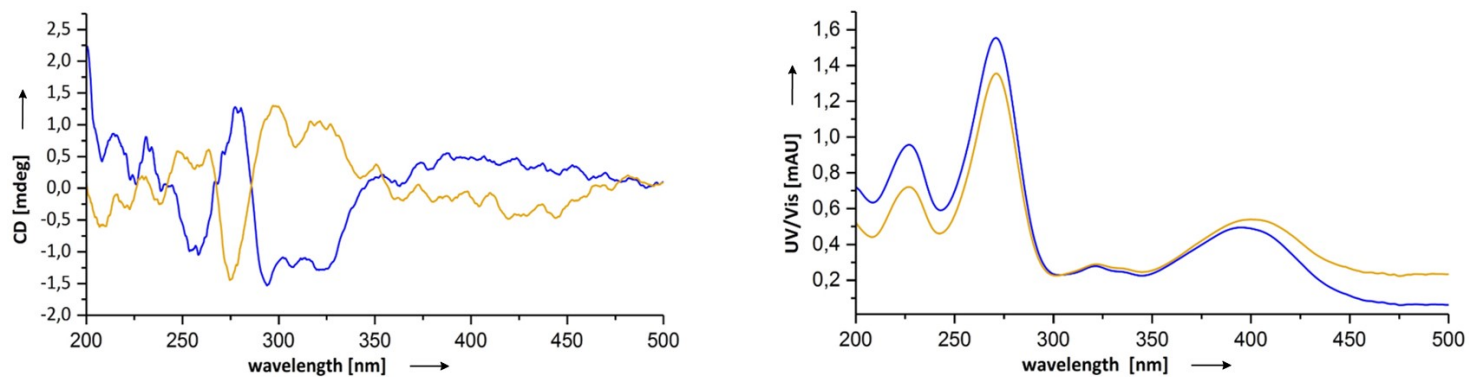


Figure S12. CD and UV/Vis spectra of atrochryson produced by *Cortinarius* PKSs: CoPKS1 (ochre), CoPKS4 (blue). Spectra were recorded in MeOH.

References

- [1] D. H. Marx, *Phytopathol.* **1969**, *59*, 152–163.
- [2] M. Moser, *Die Pilze Mitteleuropas*, 4th ed., Klinkhardt, Bad Heilbrunn, **1960**, pp. 58–62.
- [3] K. Shimizu, N. P. Keller, *Genetics* **2001**, *157*, 591–600.
- [4] E. Geib, M. Brock, *Fungal Biol. Biotechnol.* **2017**, *4*, 13.
- [5] G. Pontecorvo, J. A. Roper, L. M. Hemmons, K. D. Macdonald, A. W. J. Bufton in *Advances in Genetics* 5th ed (Ed.: M. Demerec), Academic Press, New York, NY, **1953**, pp. 141–238.
- [6] M. Stanke, R. Steinkamp, S. Waack, B. Morgenstern, *Nucleic Acids Res.* **2004**, *32*, 309–312.
- [7] S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **1997**, *25*, 3389–3402.
- [8] E. Geib, F. Baldeweg, M. Doerfer, M. Nett, M. Brock, *Cell Chem. Biol.* **2019**, *26*, 223–234.
- [9] T. G. M. Schmidt, A. Skerra, *Nat. Protoc.* **2007**, *2*, 1528–1535.
- [10] E. Geib, M. Gressler, I. Viedernikova, F. Hillmann, I. D. Jacobsen, S. Nietzsche, C. Hertweck, M. Brock, *Cell Chem. Biol.* **2016**, *23*, 587–597.
- [11] H. Girardin, J.-P. Latgé, T. Srikantha, B. Morrow, D. R. Soll, *J. Clin. Microbiol.* **1993**, *31*, 1547–1554.
- [12] W. Thiele, S. Obermaier, M. Müller, *ACS Chem. Biol.* **2020**, *15*, 844–848.
- [13] G. A. Kraus, M. E. Krolski, J. Sy, *Org. Synth.* **1989**, *67*, 202–203.
- [14] S.-H. Kang, R. P. Pandey, C.-M. Lee, J.-S. Sim, J.-T. Jeong, B.-S. Choi, M. Jung, D. Ginzburg, K. Zhao, S. Y. Won, T.-J. Oh, Y. Yu, N.-H. Kim, O. R. Lee, T.-H. Lee, P. Bashyal, T.-S. Kim, W.-H. Lee, C. Hawkins, C.-K. Kim, J. S. Kim, B. O. Ahn, S. Y. Rhee, J. K. Sohng, *Nat. Commun.* **2020**, *11*: 5875.
- [15] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, *Mol. Biol. Evol.* **2018**, *35*, 1547–1549.
- [16] R. C. Edgar, *BMC Bioinformatics* **2004**, *5*:113.
- [17] S. Q. Le, O. Gascuel, *Mol. Biol. Evol.* **2008**, *25*, 1307–1320.
- [18] G. Lackner, M. Bohnert, J. Wick, D. Hoffmeister, *Chem. Biol.* **2013**, *20*, 1101–1106.
- [19] H.-F. Tsai, Y. C. Chang, R. G. Washburn, M. H. Wheeler, K. J. Kwon-Chung, *J. Bacteriol.* **1998**, *180*, 3031–3038.
- [20] E. Szewczyk, Y.-M. Chiang, C. E. Oakley, A. D. Davidson, C. C. C. Wang, B. R. Oakley, *Appl. Environ. Microbiol.* **2008**, *74*, 7607–7612.
- [21] M. E. Mayorga, W. E. Timberlake, *Mol. Gen. Genet.* **1992**, *235*, 205–212.
- [22] Y.-M. Chiang, E. Szewczyk, A. D. Davidson, R. Entwistle, N. P. Keller, C. C. C. Wang, B. R. Oakley, *Appl. Environ. Microbiol.* **2010**, *76*, 2067–2074.
- [23] J.-H. Yu, T. J. Leonard, *J. Bacteriol.* **1995**, *177*, 4792–4800.
- [24] J. Yu, P.-K. Chang, K. C. Ehrlich, J. W. Cary, D. Bhatnager, T. E. Cleveland, G. A. Payne, J. E. Linz, C. P. Woloshuk, J. W. Bennett, *Appl. Environ. Microbiol.* **2004**, *70*, 1253–1262.
- [25] L. Hendrickson, C. R. Davis, C. Roach, D. K. Nguyen, T. Aldrich, P. C. McAda, C. D. Reeves, *Chem. Biol.* **1999**, *6*, 429–439.
- [26] J. Kennedy, K. Auclair, S. G. Kendrew, C. Park, J. C. Vederas, C. R. Hutchinson, *Science* **1999**, *284*, 1368–1372.
- [27] T. Awakawa, K. Yokota, N. Funa, F. Doi, N. Mori, H. Watanabe, S. Horinouchi, *Chem. Biol.* **2009**, *16*, 613–623.
- [28] P. Brandt, M. García-Altares, M. Nett, C. Hertweck, D. Hoffmeister, *Angew. Chem. Int. Ed.* **2017**, *56*, 5937–5941.
- [29] J. Braesel, J. Fricke, D. Schwenk, D. Hoffmeister, *Fungal Genet. Biol.* **2017**, *98*, 12–19.
- [30] S. Griffiths, C. H. Mesarich, B. Saccomanno, A. Vaisberg, P. J. G. M. De Wit, R. Cox, J. Collemare, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 6851–6856.
- [31] P. S. Seibold, C. Lenz, M. Gressler, D. Hoffmeister, *J. Antibiot.* **2020**, *73*, 711–720.
- [32] F. Y. Lim, Y. Huo, Y. Chen, J.-H. Oh, I. Lee, T. S. Bugni, N. P. Keller, *Appl. Environ. Microbiol.* **2012**, *78*, 4117–4125.
- [33] L. Sun, G. Liu, Y. Li, D. Jiang, W. Guo, H. Xu, R. Zhan, *Metab. Eng.* **2019**, *54*, 212–221.
- [34] A. G. Newman, A. L. Vagstad, P. A. Storm, C. A. Townsend, *J. Am. Chem. Soc.* **2014**, *136*, 7348–7362.