

Biosynthesis

Unprecedented Mushroom Polyketide Synthases Produce the Universal Anthraquinone Precursor

Nikolai A. Löhr, Frederic Eisen, Wiebke Thiele, Lukas Platz, Jonas Motter, Wolfgang Hüttel, Markus Gressler, Michael Müller, and Dirk Hoffmeister*

Dedicated to Professor Dr. Wolfgang Steglich

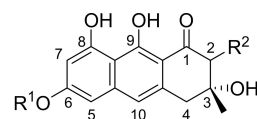
Abstract: (Pre-)anthraquinones are widely distributed natural compounds and occur in plants, fungi, microorganisms, and animals, with atrochrysone (**1**) as the key biosynthetic precursor. Chemical analyses established mushrooms of the genus *Cortinarius*—the webcaps—as producers of atrochrysone-derived octaketide pigments. However, more recent genomic data did not provide any evidence for known atrochrysone carboxylic acid (**4**) synthases nor any other polyketide synthase (PKS) producing oligocyclic metabolites. Here, we describe an unprecedented class of non-reducing (NR-)PKS. In vitro assays with recombinant enzyme in combination with in vivo product formation in the heterologous host *Aspergillus niger* established CoPKS1 and CoPKS4 of *C. odorifer* as members of a new class of atrochrysone carboxylic acid synthases. CoPKS4 catalyzed both hepta- and octaketide synthesis and yielded 6-hydroxymusizin (**6**), along with **4**. These first mushroom PKSs for oligocyclic products illustrate how the biosynthesis of bioactive natural metabolites evolved independently in various groups of life.

biosynthesize and diversify the key intermediate atrochrysone (**1**, Figure 1). Compound names such as torosachrysone (**2**), named after the tropical shrub *Cassia torosa*, or phlegmacin (**3**), after the mushroom subgenus *Phlegmacium*, reflect their diverse origins.^[2] The chiral octaketide atrochrysone carboxylic acid (**4**, Figure 1), a derivative of 3,4-dihydroanthracene-1(2*H*)-one, is the common and first tricyclic biosynthetic intermediate to **1**. The multitude of these tricyclic, polyfunctional compounds originates from subsequent post-PKS modifications of **1** that include, e.g., *O*-methylation, glycosylation, hydroxylation, oxidation, as well as regio- and atropselective dimerization via oxidative phenol coupling.^[3]

The first fungal gene encoding an atrochrysone carboxylic acid synthase (AptA) was identified, and shown to be involved in asperthecin biosynthesis in the mold *Aspergillus nidulans*.^[4a] Other enzyme examples include MdpG for monodictyphenone assembly, GedC for geodin biosynthesis, ClaG for cladofulvin, and ACAS, which is involved in the biosynthesis of endocrocin (**8**) and emodin (**9**).^[4b–e] These examples consistently support the idea that fungal atrochrysone carboxylic acid synthases are monomodular iterative enzymes that lack an intrinsic thioesterase (TE)-domain.

Introduction

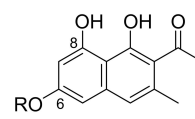
Anthraquinone and pre-anthraquinone polyketides represent one of the largest classes of natural products.^[1] Remarkably, vascular plants, fungi, bacteria, and some invertebrates, have developed the metabolic capacity to



R¹=R²=H: (3R)-Atrochrysone (**1**)

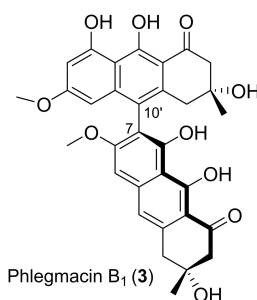
R¹=CH₃, R²=H: (3R)-Torosachrysone (**2**)

R¹=H, R²=COOH: (3R)-Atrochrysone carboxylic acid (**4**)

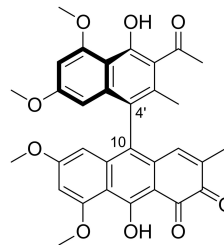


R=H: 6-Hydroxymusizin (**6**)

R=CH₃: Torachrysone (**7**)



Phlegmacin B₁ (**3**)



Rufoolivacin A (**5**)

Figure 1. Chemical structures of fungal monomeric and dimeric hepta- and octaketides.

[*] N. A. Löhr, J. Motter, Dr. M. Gressler, Prof. Dr. D. Hoffmeister
Department Pharmaceutical Microbiology at the Hans-Knöll-Institute, Friedrich-Schiller-Universität
Beutenbergstrasse 11a, 07745 Jena (Germany)
E-mail: dirk.hoffmeister@leibniz-hki.de

F. Eisen, Dr. W. Thiele, L. Platz, Dr. W. Hüttel, Prof. Dr. M. Müller
Institute of Pharmaceutical Sciences
Albert-Ludwigs-Universität Freiburg
Albertstrasse 25, 79104 Freiburg (Germany)

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This activity is supplied in *trans* by a separately encoded metallo- β -lactamase-like TE (Figure 2A, Figure S1).^[4e]

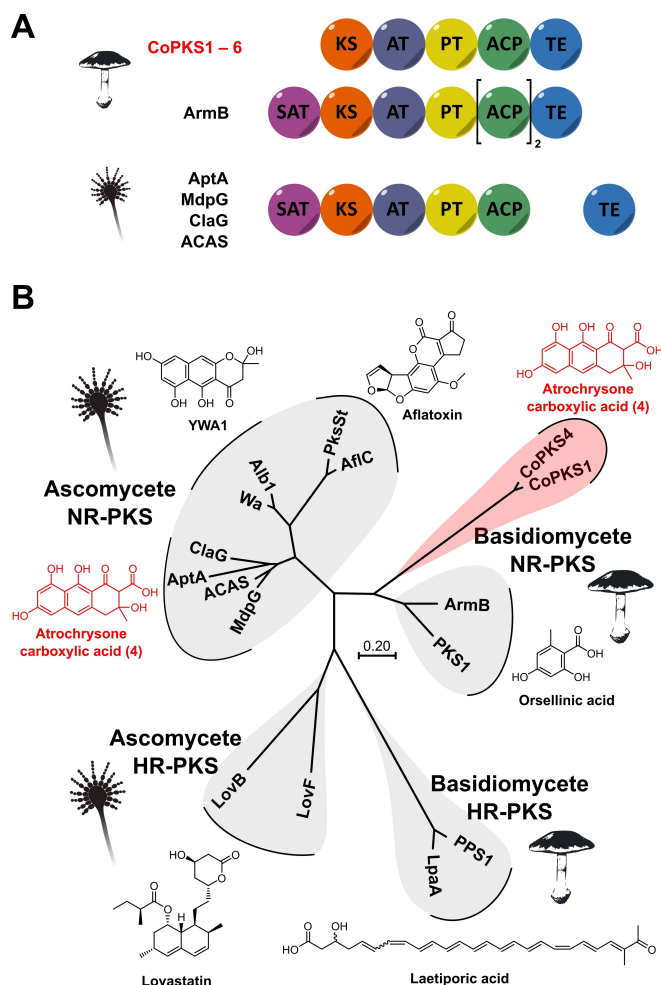


Figure 2. A) Comparison of domain arrangements of fungal non-reducing polyketide synthases. Domain acronyms: SAT: starter unit; ACP: acyl carrier protein; KS: β -ketoacyl synthase; AT: acyltransferase; PT: product template; ACP: acyl carrier protein; TE: thioesterase. B) Phylogenetic tree reconstructing the relationship of fungal PKSs. Representative products are shown for the respective PKS clades. The bar indicates the uncorrected pairwise distance. The branch lengths are proportional to the number of substitutions per site. Accession numbers of amino acid sequences are provided in the Supporting Information.

With 2000+ species globally, the basidiomycete genus *Cortinarius* is one of the most species-rich mushroom genera.^[5] These fungi are prolific producers of atrochrysone-derived mono- or dimeric dihydroanthracenones and corresponding anthrones or anthraquinones.^[2,6] However, no precedent exists for how mushrooms biosynthesize oligocyclic polyketides, although synthases for monocyclic aromatic tetraketides (e.g., the orsellinic acid synthase ArmB, Figure S1) have been described.^[7] Given seminal stable-isotope feeding experiments with *Cortinarius* pre-anthraquinones that unequivocally proved their polyketidic origin,^[8] we hypothesized that biosynthesis of **4** was catalyzed in mushrooms by AptA/ACAS-type synthases as well.

Results and Discussion

To test this hypothesis, we sequenced the genomic DNA of *Cortinarius odorifer*, a Northern hemispheric symbiotic mushroom producing **3** and the hepta-/octaketide dimer rufoolivacin (**5**).^[9] Contrary to our hypothesis, the genome does not encode any AptA/ACAS-type synthase. However, six genes for an unusual type of non-reducing (NR-)PKSs were detected, hereafter referred to as CoPKS1–6. The predicted enzymes are 1627–1678 aa in length (Table 1) and composed of canonical NR-PKSs domains: β -ketoacyl synthase (KS), acyltransferase (AT), product template (PT), and acyl carrier protein (ACP) that show typical active site motifs (Figures 2A and S2A).^[10] Unlike typical fungal NR-PKSs, none of the *C. odorifer* genes encodes a starter unit: ACP transacylase (SAT) domain. However, they include an integral α/β hydrolase superfamily-type TE domain, which contrasts with AptA/ACAS-type synthases. Building upon a previously published classification of fungal PKSs,^[11] a phylogenetic placement, based on their KS domains, revealed that CoPKS1–6 belong to a distinct evolutionary clade i) which split early from other basidiomycete NR-PKSs; and ii) for which neither a genetically nor biochemically characterized representative exists yet (Figure 2B).

Another extraordinary feature of CoPKS1–6 is a repetitive proline-rich region (9–20 % proline residues) between the ACP and TE domains, present in one to nine copies (Table 1, Figure S2B). Given that *C. odorifer* encoded only one other NR-PKS whose phylogenetic placement is with ArmB-type orsellinic acid synthases (Figure 2B), we consid-

Table 1: Features of *Cortinarius odorifer* SAT-domainless polyketide synthases.

Name	Length (aa)	Product ^[a,b]	Repeats ^[c]	Identity [%] ^[d]	<i>A. niger</i> strain
CoPKS1	1668	1 (<i>R</i> -excess)	7	88.1	tNAL024
CoPKS2	1633	inactive	1	76.4	tNAL025
CoPKS3	1637	inactive	1	84.3	tNAL026
CoPKS4	1666	1 (<i>S</i> -excess), 6	7	–	tNAL002
CoPKS5	1627	inactive	1	82.5	tNAL003
CoPKS6	1678	inactive	9	91.8	tNAL004

[a] Verified experimentally by product formation in the heterologous host *Aspergillus niger*. [b] The actual enzymatic product is atrochrysone carboxylic acid (**4**). Due to its inherent instability, the chromatographically detected compound is its decarboxylated follow-up product atrochrysone (**1**). [c] Refers to the number of repetitive sequences at the ACP/TE domain interfaces. [d] Referenced to CoPKS4.

ered CoPKS1–6 strong candidates to catalyze the synthesis of **4**, even though their evolutionary origin is different of that of AptA/ACAS-type PKSs.

Cortinarius species and most other mushroom-forming fungi are not amenable to genetic manipulation and reverse genetics strategies. Therefore, we introduced the genes for CoPKS1–6 individually in *A. niger* as heterologous host for subsequent doxycycline-induced expression using the ATNT method, which is based on the *A. terreus* transcriptional activator TerR and its target promoter *PterA*.^[12] For all *C. odorifer* PKS genes, gDNA was used to transform *A. niger* (Table 1, Figure S3). All PKSs were produced in their native, tag-less form.

To infer their function, we cultivated the transformed *Aspergilli* and chromatographically analyzed the metabolic products extracted with ethyl acetate from the culture broth.

Both the untransformed parental *A. niger* host and a transformant (tNAL000) carrying the insert-less expression vector phis_SM-Xpress were included for comparison as negative controls. Expression of the *C. odorifer copks1* gene (*A. niger* tNAL024) led to active enzyme, as evident from LC-MS analysis of the culture supernatants. Six additional peaks were found (Figure 3), which appear at t_R =2.6 min, at t_R =2.9 min, at t_R =3.9 min (signal **), at t_R =4.4 min (signal ***), at t_R =6.7 min (signal ****), and at t_R =6.9 min. Importantly, the major signal at t_R =2.6 min corresponded to a mass of m/z 273.0766 $[M-H]^-$ and therefore pointed to compound **1**. This was supported by the UV/Vis spectrum with local maxima at λ =226, 272, 320, and 398 nm (Figure S4), which is in agreement with literature data.^[13] We also observed traces of another compound (t_R =1.9 min) whose mass m/z 317.0667 $[M-H]^-$ (Figure 3) was in accordance with that of **4**, the notoriously instable direct precursor of **1**.^[4e]

For final evidence, we purified **1** to homogeneity. From a total of 15 L tNAL024 culture broth, approximately 5 mg pure compound were isolated, and the structure was identified as **1** by 1D and 2D NMR spectroscopy (Figure 4, Figure S5, Table S1).^[13]

Thus, CoPKS1 has atrochrysone carboxylic acid synthase activity and represents both the first mushroom octaketide PKS and first characterized member of the entire evolutionary clade to which it belongs. The compounds were characterized by i) HR-ESIMS (Figure 3), ii) UV/Vis spectra (Figure S4), and iii) LC-MS/MS analyses (Figure S6, S7). The signal at t_R =2.9 min was identified as endocrocin (**8**). Signals ** and *** probably represent isomeric endocrocin anthrone homodimers,^[4e] **** the heterodimer of emodin anthrone and endocrocin anthrone,^[4d-e] and the peak at t_R =6.9 min was assigned to emodin (**9**). The identity of **8** and **9** was further confirmed by comparison with standards. Surprisingly, this set of products is congruent with the compounds found for in vivo activity of the phylogenetically unrelated ascomycete PKSs ACAS and ClaG which led to the published model of biosynthetic events (Scheme 1).^[4de] Awakawa et al. speculated that the observed dimers (represented by signals marked **, ***, and **** in Figure 3) were formed due to enzymatic activity intrinsic to the host, which was *A. oryzae*.^[4e] Our observed dimeriza-

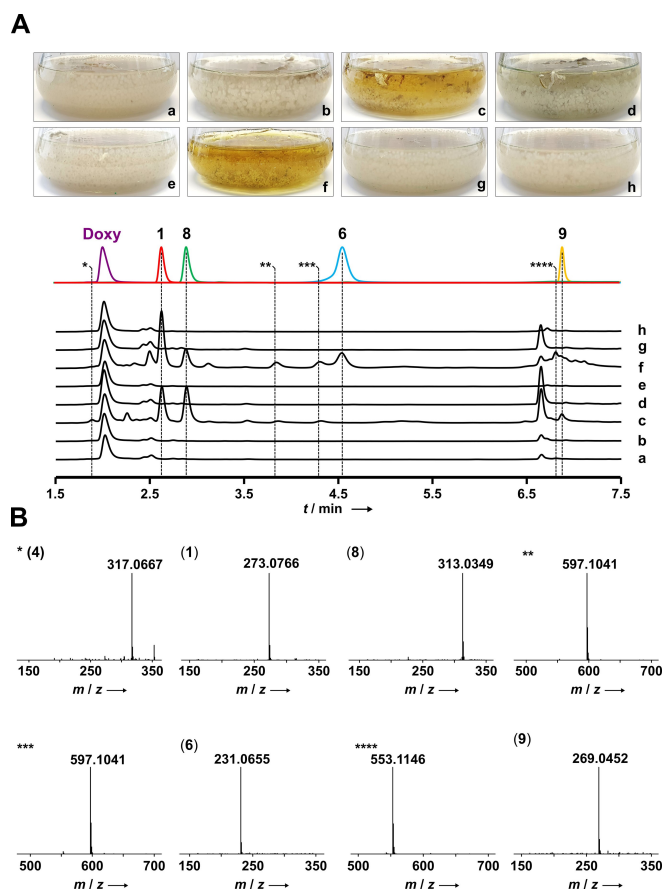


Figure 3. In vivo activity assays with *Cortinarius* PKSs. A) Liquid cultures and chromatograms of ethyl acetate extracts of recombinant *A. niger* strains expressing *C. odorifer* PKS genes. The yellow color of some cultures indicates polyketide formation by recombinant strains. Chromatograms were recorded at λ =395 nm. The top trace in panel A represents an overlay of individual chromatograms for standards of doxycycline (Doxy), atrochrysone (**1**), endocrocin (**8**), 6-hydroxymusizine (**6**), and emodin (**9**). Trace a: Untransformed *A. niger* host; b: *A. niger* tNAL000; traces c–h: *A. niger* harboring the genes for CoPKS1 (c, *A. niger* tNAL024), CoPKS2 (d, *A. niger* tNAL025), CoPKS3 (e, *A. niger* tNAL026), CoPKS4 (f, *A. niger* tNAL002), CoPKS5 (g, *A. niger* tNAL003), and CoPKS6 (h, *A. niger* tNAL004), respectively. B) Representative HR-ESIMS spectra (negative mode) of signals, extracted from the CoPKS4 producing *A. niger* tNAL002. In addition to the major signals (identified as compounds **1**, **6**, **8**, **9**, and putative dimers, marked **, ***, and ****), the assumed primary PKS product atrochrysone carboxylic acid (**4**) was detected in traces, as indicated by an asterisk. Calculated masses ($[M-H]^-$) are: m/z 317.0667 (**4**), 273.0769 (**1**), 313.0354 (**8**), 597.1039 (**), 597.1039 (***), 231.0663 (**6**), 553.1140 (****) and 269.0456 (**9**).

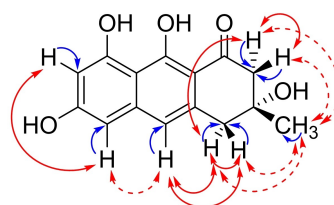
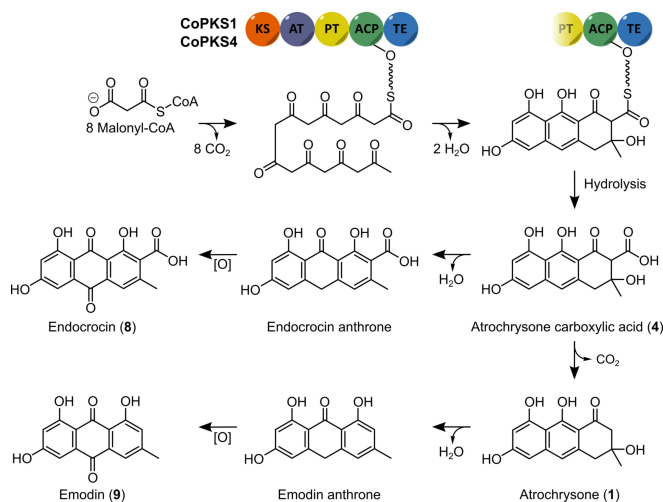


Figure 4. Structure of atrochrysone (**1**). 1H - 1H COSY correlations are shown in red, HSQC correlation in blue.



Scheme 1. Sequence of oligocyclic octaketide biosynthesis and modification after heterologous production of *C. odorifer* PKSs in *A. niger*. Please see Figure S9 for a model of the CoPKS4-catalyzed heptaketide biosynthesis.

tions with *A. niger* as host argue that *Aspergilli* generally may be capable of such enzymatic dimerizations.

Next, we investigated CoPKSs 2–6. Expression of the genes for CoPKSs 2, 3, 5, and 6 (*A. niger* transformants tNAL025, 026, 003, and 004, respectively) did not lead to an altered profile (Figure 3), compared to negative controls, although the genes were properly integrated (Figure S3). Expression of the gene encoding CoPKS4 in *A. niger* tNAL002 led to a metabolic change: the extracted broth showed chromatographic signals for **1**, **8**, **9**, and the above dimers. As was the case with CoPKS1, trace amounts of putative **4** were present as well. Compound **1** was purified from tNAL002. Subsequent 1D and 2D NMR spectroscopy led to spectra virtually identical to those recorded for the CoPKS1 product. Hence, we concluded that CoPKS4 possesses atrochrysone carboxylic acid synthase activity as well. Still, the overall product profile of CoPKS4 was different from that of CoPKS1 as an additional compound was detected ($t_R = 4.5$ min, m/z 231.0655 [$M-H$][−], compound **6** in Figure 3). Its deduced sum formula of C₁₃H₁₂O₄ appeared incompatible with an octaketide, but was consistent with that of the naphthalene-type heptaketide 6-hydroxymusizin (**6**), a natural product described as a glycoside from the shrub *Cassia angustifolia*.^[14] Final evidence came from a chromatographic comparison with a synthetic standard.^[15]

Curiously, heptaketides with a naphthalene scaffold have been isolated before from *C. odorifer*, e.g., torachrysone (**7**) (Figure 1), which is the 6-*O*-methylether of **6**, as typical representative. It may undergo heterodimerization with a tricyclic octaketide by oxidative phenol coupling and further 8-*O*-methylation, to yield **5** that, in fact, was isolated from *C. odorifer*, among other species.^[2b,9a] Based on our in vivo data, we therefore conclude that CoPKS4 provides dual hepta- and octaketide synthase activity. In addition to **6**, we detected yet another compound ($t_R = 2.5$ min) whose mass

m/z 257.0452 [$M-H$][−] and UV/Vis spectrum (Figure S8) are compatible with that of pannorin, which may be viewed as a non-enzymatically formed heptaketide shunt product (Figure S9).

Hence, CoPKS4 differs from the closely related CoPKS1 (sequence alignment in Figure S2) and the ascomycete AptA/ACAS-type PKSs. In the fungal realm, precedents exist, however, for multi-product PKSs that synthesize compounds of dissimilar chain lengths, e.g., the highly reducing polyene PKSs PPS1 and LpaA,^[16] PKS1 of *Colletotrichum lagenarium* that produces tetra- to hexaketides,^[17] and the terrein synthase TerA of *A. terreus*, which makes tri- to pentaketides.^[18]

We could not rule out that the different chain lengths reflect a host effect, caused by the *A. niger* background. We sought to unequivocally confirm the chain length variation as an intrinsic PKS feature. Therefore, we ran in vitro product formation assays with CoPKS4 Strep-tagged fusion protein, which was heterologously produced in *A. niger* tNAL048 and purified by affinity chromatography (Figure S10). Interestingly, the reaction with malonyl-CoA as sole substrate led to **1** formation, as shown by LC-MS analysis (Figure 5). In addition to minor amounts of a heptaketide shunt product, presumably pannorin (Figure S8), we also detected **6**, which proves simultaneous heptaketide synthase activity of CoPKS4. Neither molecule was detected in control reactions with heat-inactivated enzyme or in the absence of malonyl-CoA substrate. As no auxiliary enzyme, i.e., a discrete TE was required for the synthesis of **1** and **6**, we unequivocally confirmed *Cortinarius* CoPKS4 alone is sufficient to provide hepta- and octaketide synthase activity to these fungi.

The SAT-domainless design of CoPKS4 implies that all building blocks to initiate and extend polyketide biosyn-

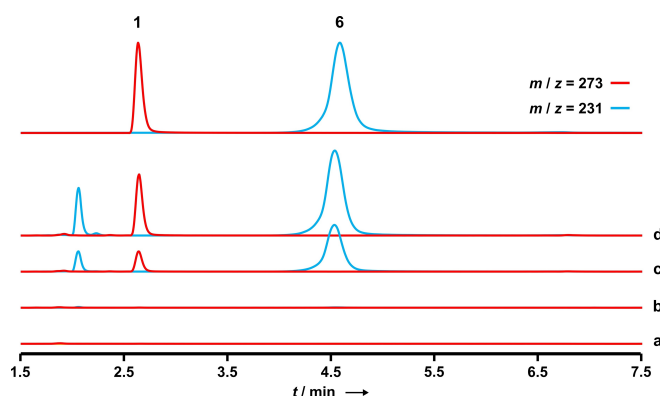


Figure 5. In vitro product formation by CoPKS4. Extracted ion chromatograms (EICs) of lyophilized reactions are shown for m/z 273 [$M-H$][−] (red traces) and m/z 231 [$M-H$][−] (blue traces) to detect the octaketide atrochrysone (**1**) and the heptaketide 6-hydroxymusizin (**6**), respectively. The top trace represents an overlay of individual EICs of standards. Trace a: negative control with intact CoPKS4 but without any substrates; trace b: negative control with heat-treated CoPKS4, acetyl-CoA and malonyl-CoA; trace c: reaction with CoPKS4, acetyl-CoA and malonyl-CoA; trace d: reaction with CoPKS4 and malonyl-CoA only. The enzymatic reactions in c and d showed a second product at m/z 231 ($t_R = 2.1$ min), which remained unidentified due to low quantity.

thesis enter via the AT domain, confirming an earlier hypothesis.^[10c] However, for most PKSs, the origin of the formal acetate for chain initiation is not yet clear. For bacterial type II PKSs, Leadlay and co-workers demonstrated a non-condensing KS-catalyzed decarboxylation of malonyl-CoA for the actinorhodin PKS, which was also found for a PKS from *Photorhabdus*.^[19] Similar observations were made for the 6-deoxyerythronolide B synthase, a multimodular type I system.^[20] Furthermore, a radiotracer experiment with [1-¹⁴C]acetyl-CoA and the fungal ACAS showed that no radioactivity was incorporated and, thus, acetyl-CoA does not serve as a starter.^[4c] In the past, due to impurities of commercial CoA thioesters, in vitro assays of PKSs were misinterpreted in that decarboxylation had occurred enzymatically.^[21] Therefore, malonyl-CoA as substrate for our experiment was analyzed in-house for its purity. However, it did not contain acetyl-CoA (Figure S11). Therefore, we assume a comparable decarboxylating activity is also inherent to the KS domains of the mushroom CoPKSs.

From the known *Cortinarius* octaketides, we hypothesized that the polyketide synthesis intrinsic to the mushrooms predominantly yields one enantiomer. To determine which enantiomer was produced, **1** was subjected to circular dichroism spectroscopy (Figure S12). Interestingly, both PKSs (CoPKS1/4) produced a mixture of enantiomers (3R and 3S) in different ratios, respectively.

Conclusion

The wide distribution of (pre-)anthraquinones in nature indicates a privileged structure and points to an important but still elusive evolutionary advantage. From the chemical ecology perspective, the selective pressure to synthesize these oligocyclic compounds must have been immense in the various forms of life—from bacteria to fungi, plants, and invertebrates—and several enzymatic solutions have independently evolved to confer this biosynthetic capacity. In addition to the shikimate-based biosynthesis of the anthraquinone alizarin,^[22] chalcone synthase-like plant enzymes,^[23] bacterial type II PKSs,^[24] and ascomycete AptA/ACAS-type synthases,^[4] our work proves the existence of a fourth independent polyketide pathway.

The structural diversity accessible via only a few post-PKS tailoring steps may plausibly explain the privileged nature of atrochrysone-derived products. These modifications include regioselective methylations, hydroxylations, glycosylations, dehydrations (e.g., anthrones), oxidations (e.g., quinones and xanthenes) and prenylations (e.g., vismione group of anthranoids) and yield hundreds of different compounds, which all use **1** as intermediate and many of which are associated with distinct bioactivity.^[4,25] Regio- and stereoselective enzyme-mediated oxidative phenol coupling further massively increases the structural diversity.^[3] In return, (near-)identical atrochrysone derivatives emerged independently, regardless of the diversity of producing organisms, the habitat they live in, or the class of enzyme that biosynthesizes **1**. This phenomenon is exempli-

fied well by the phlegmacins, which were isolated both from fungi and plants.^[9,26] Our work on the chemistry and biosynthesis of polyketides in the mushroom *Cortinarius odorifer* revealed a new class of polyketide synthases which either selectively produce an octaketide or are intrinsically flexible to simultaneously biosynthesize hepta- and octaketides. Previous chemical analyses identified numerous mushrooms, within and outside the genus *Cortinarius*, as producers of bi- and tricyclic hepta/octaketides.^[2b,27] We therefore expect our work unveiled a fundamental and widely applicable principle of fungal natural product chemistry.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov/>, reference number 512945.

Keywords: Anthraquinones • Biosynthesis • Fungi • Octaketides • Polyketide Synthase

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