

## VIP Very Important Paper

## The Ketosynthase Domain Controls Chain Length in Mushroom Oligocyclic Polyketide Synthases

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The nonreducing iterative type I polyketide synthases (NR-PKSs) CoPKS1 and CoPKS4 of the webcap mushroom *Cortinarius odorifer* share 88% identical amino acids. CoPKS1 almost exclusively produces a tricyclic octaketide product, atrochrysonic carboxylic acid, whereas CoPKS4 shows simultaneous hepta- and octaketide synthase activity and also produces the bicyclic heptaketide 6-hydroxymusizin. To identify the region(s) controlling chain length, four chimeric enzyme variants were

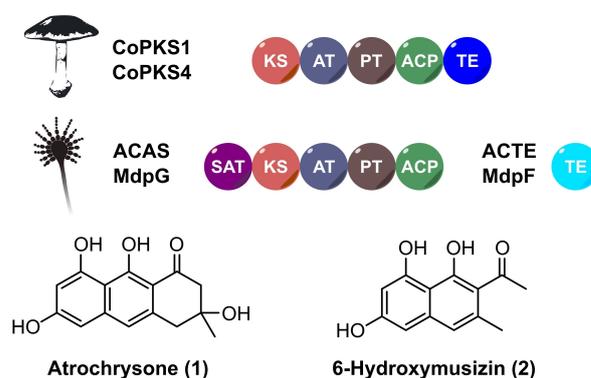
constructed and assayed for activity in *Aspergillus niger* as heterologous expression platform. We provide evidence that the  $\beta$ -ketoacyl synthase (KS) domain determines chain length in these mushroom NR-PKSs, even though their KS domains differ in only ten amino acids. A unique proline-rich linker connecting the acyl carrier protein with the thioesterase domain varies most between these two enzymes but is not involved in chain length control.

## Introduction

Polyketide synthases (PKSs) account for a major share of the natural product repertoire of filamentous fungi.<sup>[1]</sup> The amazing structural diversity of polyketides results from i) the selection of starter and extender units, ii) the extent of reductive processing during each extension cycle, and iii) post-PKS modifications, for example, by oxidative coupling into dimers, prenylation, methylation or other group transfer reactions.<sup>[2]</sup> The genetic and biochemical foundation of these processes is well understood.<sup>[3]</sup> Another fundamental factor that contributes to metabolic diversity is the number of condensation steps that lead to a given chain length. Fungi rely on monomodular iterative type I PKSs that comprise a single set of catalytic domains, that is, the  $\beta$ -ketoacyl synthase (KS) domain catalyzes multiple elongation rounds and thus requires chain-length regulation. However, the regulative program behind chain-length control is probably the least well understood parameter of fungal PKSs and contrasts bacterial modular type I PKSs in which each condensation step is catalyzed by a dedicated module within the megasynthase.<sup>[4]</sup>

The Basidiomycota, which include most mushroom-forming filamentous fungi, are known to produce polyketides of various lengths. In the case of aromatic compounds produced by

nonreducing (NR-)PKSs, only enzymes for the tetraketide orsellinic acid were known, among them ArmB and others, which all belong to the same evolutionary category of PKSs.<sup>[5]</sup> Recently, CoPKS1 and CoPKS4 (Figure 1) from the webcap mushroom *Cortinarius odorifer*,<sup>[6]</sup> were described as the first representatives of a previously elusive evolutionary clade of basidiomycete NR-PKSs. CoPKS1 and CoPKS4 are involved in the biosynthesis of oligocyclic aromatic polyketides, among them atrochrysonic acid (1), the general intermediate of numerous mono- and dimeric fungal (and plant) natural products.<sup>[7]</sup> Remarkably, these enzyme twins vary in their product spectrum although they share 88.1% identical amino acids (Figures S1 and S2 in the Supporting Information). CoPKS1 has a strong preference for octaketides and produces the universal anthraquinone precursor atrochrysonic carboxylic acid (Scheme 1). However, the near-identical CoPKS4 exhibits dual hepta-/octaketide activity and simultaneously produces 6-hydroxymusizin (2, Figure 1).



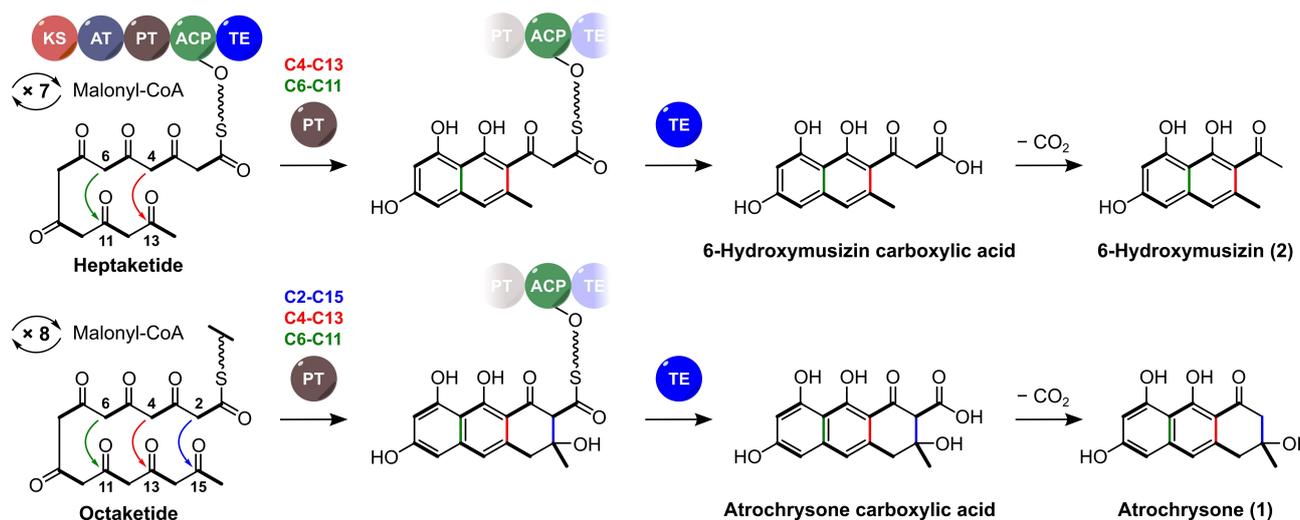
**Figure 1.** Comparison of the domain arrangements of nonreducing polyketide synthases from Basidiomycota and Ascomycota. Domain acronyms: SAT-starter unit; ACP-transacylase, KS- $\beta$ -ketoacyl synthase, AT-acyltransferase, PT-product template, ACP-acyl carrier protein, TE-thioesterase. The ascomycete enzymes (ACAS/MdpG) depend on a discrete, and hence *trans* acting, TE domain of the metallo- $\beta$ -lactamase-type (ACTE/MdpF).<sup>[8]</sup>

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**Scheme 1.** Proposed biosynthetic model of *Cortinarius* PKS-produced hepta- and octaketides. Acetate units are highlighted in bold, C–C bonds made by PT-catalyzed cyclizations are highlighted in different colors. The third octaketide ring might be cyclized spontaneously by C2–C15 aldol addition.<sup>[8]</sup> Decarboxylation to the final products 1 and 2 might occur spontaneously or by host enzymes.<sup>[7,8]</sup>

Therefore, this pair of enzymes represents an ideal system to explore how chain length is determined in this newly discovered class of mushroom NR-PKSs. Another advantageous feature that supports this research is the minimalistic domain architecture (KS-AT-PT-ACP-TE, Figure 1) of these remarkably small NR-PKSs (CoPKS4: 1666 aa, CoPKS1: 1668 aa). Furthermore, the SAT-domainless architecture of CoPKSs eliminates the possibility of a promiscuous starter unit selection to explain products of different lengths.<sup>[9]</sup> Notably, these mushroom enzymes are evolutionarily unrelated to atrochryson carboxylic acid synthases (ACASs) found in aspergilli and other ascomycetes.<sup>[8]</sup> This is, for example, reflected by their dissimilar domain setup, as the latter enzymes feature a SAT domain but rely on discrete TE domains of the metallo- $\beta$ -lactamase-type for product release.

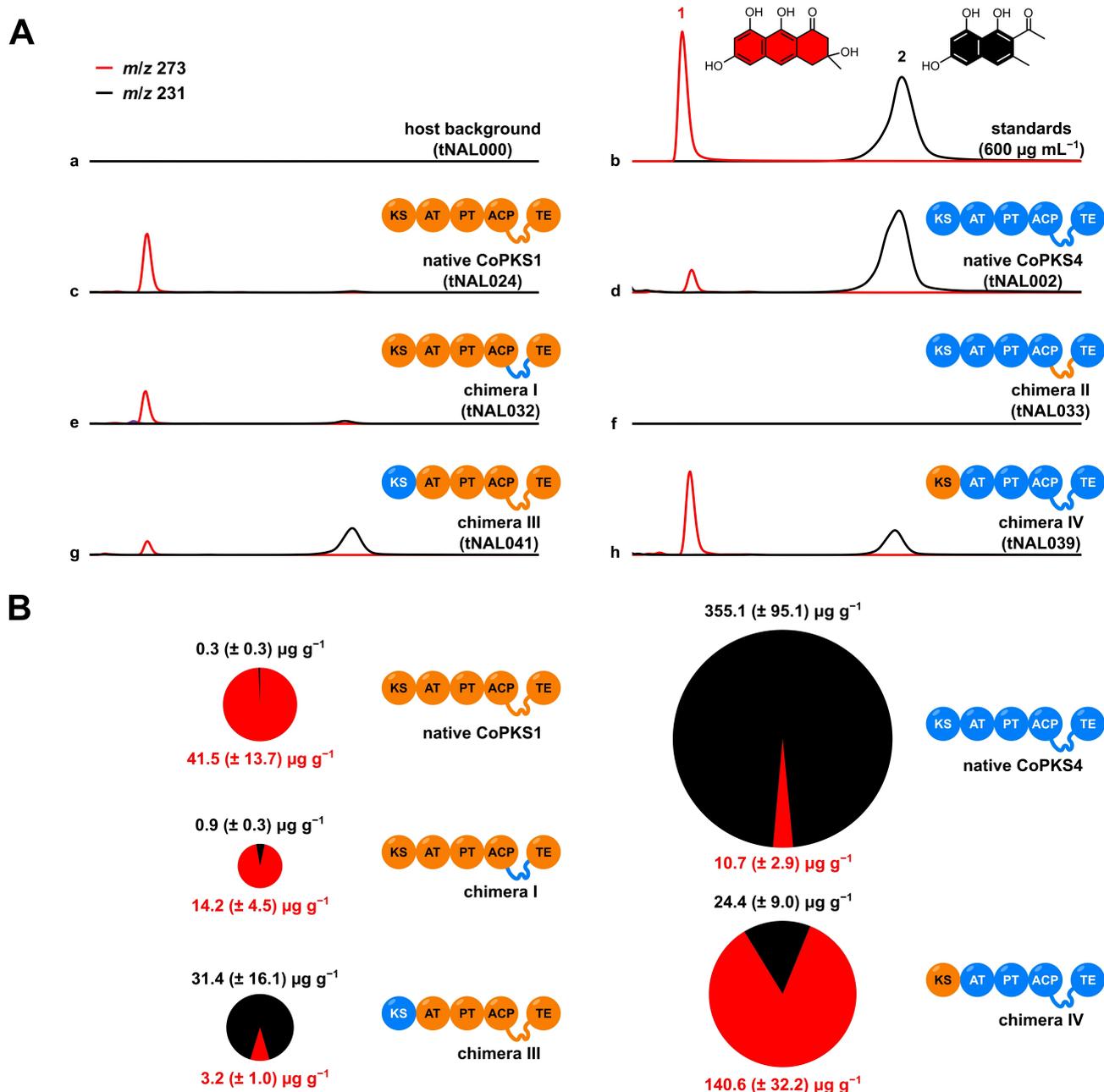
According to our previously published model of biosynthetic events, the primary enzymatic products of *Cortinarius* PKS are  $\beta$ -keto carboxylic acids, which can undergo spontaneous decarboxylation (Scheme 1).<sup>[6,8]</sup> The detection of the decarboxylated follow-up products atrochryson (1), and 6-hydroxymusizin (2), established CoPKS4 as a dual hepta-/octaketide synthase, whereas the CoPKS1 product spectrum is dominated by octaketide 1, and only traces of heptaketide 2 were detected.<sup>[6]</sup> In this report, we address how chain length is regulated in the twin enzyme pair CoPKS1/4 which were, for the first time for mushroom PKSs, subjected to enzyme engineering. Four chimeric enzymes were generated by swapping the respective parts of their genes. This approach maintains the integrity of the multidomain setup and the covalent linkages of the various domains and comes closest to the native situation which should help minimize artifact formation.<sup>[10]</sup>

## Results and Discussion

First, we considered a yet elusive proline-rich linker region<sup>[6]</sup> between the ACP and TE domains a strong candidate for chain length regulation, as it represents the most variable portion (Figure S2C; 58.4% aa identity) of these otherwise near-identical enzymes. Therefore, hybrid genes to produce two chimeric enzymes were constructed in which a 108 aa section containing the linker and adjacent sequences was mutually swapped. Chimera I consists of CoPKS1 but harbors the proline-rich linker of CoPKS4, while chimera II represents the inverse enzyme.

We introduced the genes for chimeras I and II individually in *Aspergillus niger* (Figure S3A) as heterologous host for subsequent doxycycline-induced expression using the ATNT method, which is based on the *Aspergillus terreus* transcriptional activator TerR and its target promoter *PterA*.<sup>[11]</sup> To infer the functions of the chimeric enzymes, the transformed aspergilli were cultivated and the metabolic products, extracted from the mycelium with ethyl acetate, were analyzed chromatographically. The production of 1 and 2 was quantified to assess the octa- and heptaketide synthase activity, respectively (Figures 2 and S4). The *A. niger* transformants tNAL024 and tNAL002 producing the native PKSs CoPKS1 and CoPKS4 were included for positive control, and a transformant (tNAL000)<sup>[6]</sup> carrying the insertless expression vector pSMX2-URA served as negative control.

Expression of the gene for chimera II (*A. niger* transformant tNAL033) did not lead to any product formation (Figure 2). In mono- or multimodular PKSs, linker regions are critical for interdomain<sup>[10]</sup> and intermodule communication.<sup>[12]</sup> Therefore, the swapped linker may have resulted in impaired domain communication and substrate shuttling in chimera II. In contrast, the linker region of the dual hepta-/octaketide synthase CoPKS4 in active chimera I (*A. niger* tNAL032) generated a fully active enzyme. Chimera I produced 14.2 ( $\pm$  4.5)  $\mu$ g of 1 per gram lyophilized mycelium and 0.9 ( $\pm$



**Figure 2.** In vivo activity assays with *Cortinarius* chimeric PKSs. A) Extracted ion chromatograms (EICs) of ethyl acetate extracts are shown for  $m/z$  273  $[M-H]^-$  to detect the octaketide-derived atrochryson (1) and  $m/z$  231  $[M-H]^-$  for the heptaketide-derived 6-hydroxymusizin (2). Domains and linker regions of CoPKS1 are shown in orange; those of CoPKS4 are in blue. Trace a represents an overlay of individual EICs for the ethyl acetate extract of *A. niger* tNAL000 harboring the insertless expression vector to monitor the metabolic background of the host (negative control). Trace b: standards of 1 and 2 ( $600 \mu\text{g mL}^{-1}$ ). Lower traces: *A. niger* expressing the genes for c) native CoPKS1 (*A. niger* tNAL024), d) native CoPKS4 (*A. niger* tNAL002), e) chimera I (*A. niger* tNAL032), f) chimera II (*A. niger* tNAL033), g) chimera III (*A. niger* tNAL041), and h) chimera IV (*A. niger* tNAL039). B) Pie charts displaying the concentrations of 1 (red) and 2 (black) in  $\mu\text{g per g}$  lyophilized mycelium. The standard deviation is based on three biological replicates, each technically triplicated.

$0.3 \mu\text{g g}^{-1}$  of 2. Both the ratio and the yields are in the same range, compared to that of tNAL024 producing the native CoPKS1 which yielded  $41.5 (\pm 13.7) \mu\text{g}$  of 1 and  $0.3 (\pm 0.3) \mu\text{g g}^{-1}$  of 2.

These results demonstrate that chimera I still showed a strong preference for octaketide biosynthesis. We therefore conclude that the proline-rich region, that is, the most variable portion between CoPKSs, does not impact chain length and

rather fulfills a yet unknown, but essential function in *C. odorifer* PKSs. Subsequently, we focused on the KS domain. Outside the arena of basidiomycete polyketide metabolism, previous work had demonstrated a key role of the KS domain to control chain length in polyketide biosynthesis.<sup>[9,13]</sup> Structural data from closely related mammalian fatty acid synthases indicate that KS-mediated chain length regulation occurs through spatial restrictions in the binding channel.<sup>[14]</sup> The SAT-domainless setup

of the CoPKS1/CoPKS4 twins eliminates the search for a proper interdomain junction between the SAT and KS domains. Thus, only minimally invasive protein manipulations were necessary to create KS-swapped chimeras III and IV. KS domains were annotated using the PFAM database.<sup>[15]</sup> To identify suitable sites for interdomain dissection, linker preference profiles were generated (Figure S5) using DomCUT.<sup>[16]</sup> Based on these predictions, the first 401 amino acids were selected for the swapping experiments.

Chimera III consists of the KS of CoPKS4 fused to the CoPKS1 AT–PT–ACP–TE tetradomain, while chimera IV represents the inverse enzyme. Intriguingly, the expression of the gene for chimera III (*A. niger* tNAL041, Figure 2) dramatically changed the product titer when compared to the native CoPKS1 expression strain. With  $31.4 (\pm 16.1) \mu\text{g g}^{-1}$  of **2**, this compound was now nearly ten times more abundant than octaketide **1** ( $3.2 (\pm 1.0) \mu\text{g g}^{-1}$ ), although its tetradomain AT–PT–ACP–TE derives from the octaketide synthase CoPKS1. While the ratio was clearly shifted in favor of heptaketide **2**, chimera III did not reach the ratio of native CoPKS4 that produces a 33-fold excess of **2** with  $355.1 (\pm 95.1) \mu\text{g g}^{-1}$  versus  $10.7 (\pm 2.9) \mu\text{g g}^{-1}$  of **1**.

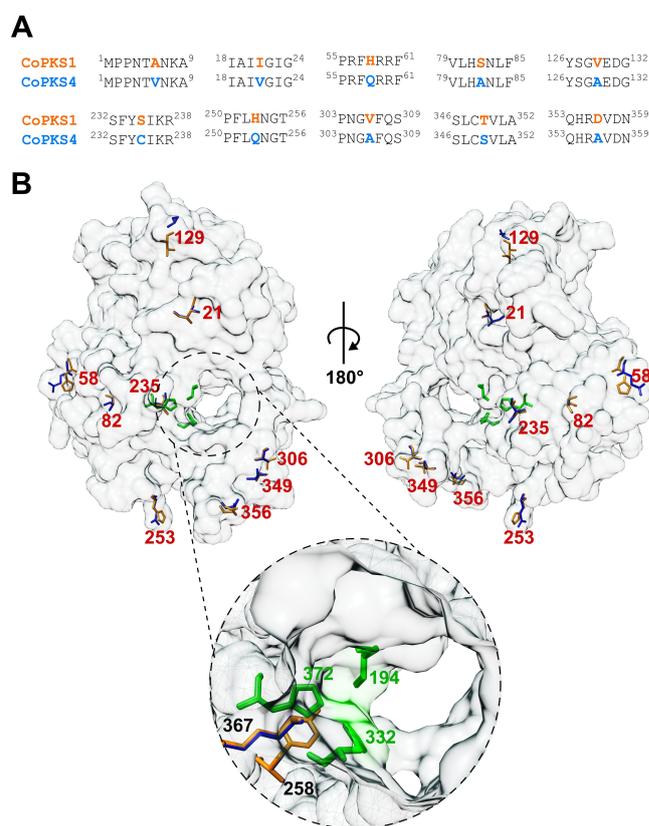
Likewise, the reciprocal exchange also led to a shifted product profile as chimera IV in *A. niger* tNAL039 showed a clear preference for octaketide synthesis. With  $140.6 (\pm 32.2) \mu\text{g g}^{-1}$ , **1** was now nearly six times more abundant than **2** ( $24.4 (\pm 9.0) \mu\text{g g}^{-1}$ ). Interestingly, the highest titer of **1** was thus produced by this non-native PKS.

Mechanistic studies on chain length control in fungal PKSs were previously performed with PksA<sup>[10a]</sup> of the aflatoxin pathway and with AfoE involved in asperfuranone biosynthesis.<sup>[17]</sup> As these enzymes accepted starter units of various lengths, NR-PKSs appear intrinsically programmed for a given final chain length and not to perform a fixed number of elongation rounds.<sup>[9,10a]</sup> Our work on *Cortinarius* PKSs, as well as previous results on the terrein synthase TerA<sup>[18]</sup> and the mushroom polyene synthases PPS1 and LpaA<sup>[19]</sup> seem to contrast this view. In these cases, product populations of variable lengths were observed: tri- through pentaketides for TerA,<sup>[18]</sup> C<sub>20</sub>/C<sub>22</sub> for PPS1,<sup>[19a]</sup> and C<sub>26</sub>/C<sub>28</sub>/C<sub>30</sub>/C<sub>32</sub> for LpaA.<sup>[19b]</sup>

The results on CoPKS1/4 advance the previous findings from a mechanistic angle as the variable lengths are associated with the KS domain, according to our results. This is particularly intriguing given the repeatedly observed phenomenon of duplicated natural product biosynthesis genes in Basidiomycota, leading to near-identical isoenzymes. This phenomenon may root in an evolutionary need to redundantly secure a particular pathway.<sup>[2b,20]</sup> However, the overwhelming sequence identity of the respective KS domains of CoPKSs 1 and 4 (97%; with only ten amino acid substitutions in total; Figure S2B) may also point to a sophisticated evolutionary mechanism to create and control structural diversity of the product at the PKS level. It includes the principal enzymatic capacity to produce various chain lengths, complemented by minute amino acid sequence changes within the isoenzymes that fine-tune selectivity and lead to highly selective (CoPKS1) or less selective dual-product enzymes (CoPKS4).

To develop a hypothesis how this fine-tuning occurs at the enzymatic level, we performed an in silico<sup>[21]</sup> structural comparison of the modeled CoPKS1 and CoPKS4 KS domains (Figures 3 and S6). The 15 N-terminal residues, including one deviating between CoPKS1 and 4 (Ala6 vs. Val6) were excluded as no obvious similarity to any known PKS was found and a structure for this portion was not reliably predicted. The modeled KS domains adopt the familiar thiolase fold with highly similar structures and the canonical catalytic triad (Cys194-His332-His372) in a plausible spatial arrangement.<sup>[22]</sup>

The two superimposed KS models unambiguously showed that the nine positions with dissimilar residues are scattered across the KS domain and are located between 15.8 Å (Ser235-Cys194) and 34.9 Å (Ala129-Cys194) in CoPKS1 and between 15.8 Å (Cys235-Cys194) and 31.4 Å (Val129-Cys194) away from the active site region and binding pocket (distances of the C $\alpha$  atoms, Figure 3B). Therefore, the in silico data did not support a rational identification of particular amino acid residues that would make CoPKS1 a highly selective enzyme and that would confer the dual functionality on CoPKS4. Considering the dimeric structure of PKSs,<sup>[3,22a,23]</sup> that leads to connected



**Figure 3.** In silico models of the CoPKS1 and 4  $\beta$ -ketoacyl synthase (KS) domains. A) Alignment depicting sequence differences. B) Superimposed models of the respective KS domains highlighting deviating amino acid residues (CoPKS1: orange; CoPKS4: blue). The close-up of the active site pocket shows the perfectly superimposed catalytic triad (Cys194-His332-His372; green) within the domains. Also shown are two key cavity-lining residues (Tyr258 and Lys367). The 15 N-terminal residues were excluded as no obvious similarity to known PKSs was found and no structure could be reliably predicted.

substrate binding tunnels, protein-protein interactions could account for conformational changes that might explain the functional differences. Eventually, analysis of crystal structures as well as more biochemically characterized members of this PKS clade may become necessary to solve this question conclusively.

Another finding of the swapping experiments is that the TE domain of CoPKS1, even though its KS domain is programmed selectively for octaketide synthesis, is also capable of releasing heptaketides, as evident from chimera III. This is reminiscent of previous work on a chimeric NR-PKS, called SW-B, composed of *Aspergillus nidulans* WA and *Colletotrichum lagenarium* PKS1. In this example, the C-terminal Claisen-type cyclization domain (CLC) cyclizes a linear hexaketide intermediate as efficiently as the native heptaketide.<sup>[24]</sup> Interestingly, in fungal HR-PKSs, chain length is controlled by the substrate selectivity of the product-releasing TE.<sup>[25]</sup>

Ascomycete NR-PKSs of the same phylogenetic origin produce structurally similar compounds that share the same number of formal acetate units.<sup>[26]</sup> One notable exception pertains to a particular clade (clade V) of NR-PKSs, as the members of this clade produce multicyclic polyketides of various chain lengths and lack an intrinsic C-terminal TE/CLC domain.<sup>[26,27]</sup> Instead, they depend on a discrete metallo- $\beta$ -lactamase-type thioesterase for product release. The clade V includes synthases that produce hepta- (SpoP,<sup>[28]</sup> GsfA<sup>[29]</sup>), octa- (ACAS,<sup>[8a]</sup> MdpG<sup>[8b]</sup>), nona- (AptA,<sup>[30,31]</sup> VrtA<sup>[29]</sup>), and decaketides (AdaA<sup>[31]</sup>) with different modes of cyclization of the backbones. As a result of convergent evolution, the mushroom synthases CoPKS1 and 4 are the functional equivalent of the ascomycete clade V NR-PKSs.

The product variation within clade V points to flexible programming of the enzymes.<sup>[26]</sup> Motivated by our findings with the mushroom CoPKS1 and 4, ACAS<sup>[8a]</sup> from *A. terreus*, and MdpG<sup>[8b]</sup> from *A. nidulans* were analyzed. We particularly investigated if they have the capacity to produce the heptaketide **2**, although both enzymes are octaketide synthases belonging to NR-PKS clade V. The gDNAs encoding MdpG or ACAS and their respective corresponding discrete TE (MdpF or ACTE) were introduced into the double auxotrophy strain *A. niger* ATNT16\_2\_No. 17.1 to yield tNAL052 and tNAL059. After verifying the respective transgenes had fully integrated into the genome (Figure S3B), both strains were cultured and the metabolites were chromatographically analyzed, as described above. Indeed, traces of **2** were detected using single ion monitoring, which was further confirmed by MS/MS-analyses (Figure S7). The clade V heptaketide NR-PKS PkgA of *A. nidulans*, which is associated with alternariol biosynthesis, was previously shown to also produce minor amounts of hexaketide isocoumarins.<sup>[26]</sup> Likewise, our results demonstrated that ACAS and MdpG, even though being highly selective, are not exclusive with respect to the final product length as well. We thus hypothesize that the variability regarding the number of acetate building blocks known from clade V NR-PKS reflects an intrinsic flexibility which might be also true for other members.

Intriguingly, the sequence differences in KS domains among hepta-, octa-, nona-, and decaketide synthases from NR-PKS clade V are rather subtle (Figure S8). As the chain length is most likely determined by the volume of the KS active site, we do not expect apparent sequence motifs or single positions within the primary sequence at a KS domain level that would determine a specific chain length.<sup>[3b]</sup> Consequently, slight sequence variations during evolution seem to have shifted the preferred product length of a given enzyme.

Beyond fungal systems, chain length control has been thoroughly investigated for polyketide synthases of bacteria and plants. The *Streptomyces coelicolor* type II actinorhodin PKS unequivocally supports a measuring mechanism, that is, the enzyme follows a fixed length, not a set number of condensation steps.<sup>[32]</sup> This notion is supported by plant and other type III PKSs of the chalcone synthase superfamily, where the volume of the active site pocket also determines product length.<sup>[33]</sup>

In addition to the PKS-intrinsic programming, terminal polyketide shortening was demonstrated during anthraquinone biosynthesis with the AntI lyase from *Photobacterium luminescens*. In this rare example, AntI tailors an octaketide into a heptaketide-anthraquinone through a retro-Claisen and Dieckmann reaction.<sup>[34]</sup>

## Conclusion

Controlling chain length during polyketide biosynthesis is a fundamental problem in natural product chemistry. This work addressed this question by using two closely related PKSs (CoPKS1 and CoPKS4) of the mushroom *C. odorifer*. Either enzyme produces both hepta- and octaketides, albeit at a strongly dissimilar ratio. The creation of chimeric mushroom PKSs proved adequate to identify the  $\beta$ -ketoacyl synthase domain as an important element that determines whether a hepta- or an octaketide is the predominant product. CoPKS1 and 4 each possess a unique proline-rich linker region between the acyl carrier protein and the thioesterase domain. Although this linker does not impact selectivity for a given product length, our chimeras show that it is critical for the overall activity.

From the PKSs investigated here and from other examples,<sup>[5,18,19]</sup> we conclude that *Cortinarius* and some other mushroom PKSs have evolved to intrinsically enable the biosynthesis of a metabolic corridor, rather than a single product, yet they adjust selectivity within this corridor by minor sequence variations between isoenzymes.

## Experimental Section

**Strains and cultivation:** The inducible *A. niger* ATNT system<sup>[11]</sup> was used for heterologous gene expression. Genotypes of each transformant are listed in Table S1. All strains were routinely cultured at 30 °C on *Aspergillus* minimal medium (AMM),<sup>[35]</sup> supplemented with 100 mM D-glucose and 20 mM L-glutamine (AMM-G100Gln20). Conidia suspensions were obtained and quantified as described.<sup>[6]</sup> For natural product analysis, liquid cultures of AMM-G100Gln20

(150 mL medium in 500 mL nonbaffled stand flasks) were inoculated with  $1.5 \times 10^8$  conidia  $\text{mL}^{-1}$  and incubated at 30 °C and 160 rpm, for 48 h. All transformants were supplemented with  $30 \mu\text{g mL}^{-1}$  doxycycline to induce transgene expression.<sup>[11]</sup> To express the two transgenes in tNAL052 and tNAL059 (Table S1), the double auxotrophic strain ATNT16\_2\_No. 17.1 was used, which was kindly provided by Dr. M. Brock (University of Nottingham, UK). The strain contains a deletion of the *pyrG* gene essential for uracil/uridine biosynthesis and a deletion of the *pabA* gene essential for *p*-aminobenzoic acid biosynthesis. To complement the *pabA*-negative phenotype in fungal cultivations, 1 L of AMM was supplemented with 1 mL of a 0.1% (w/v) *p*-aminobenzoic acid solution. For use of the *pabA* deletion as auxotrophic marker, the URA-blaster in the pSM\_StrepTag\_X\_URA plasmid was replaced by a functional copy of the *pabA* gene of *A. nidulans* resulting in plasmid pSM\_StrepTag\_X\_PABA as previously described.<sup>[36]</sup> For cloning and plasmid propagation, *Escherichia coli* XL1-Blue (Agilent) was routinely cultured on solid LB medium (per liter: 5 g yeast extract, 10 g tryptone, 10 g NaCl, 20 g agar), supplemented with  $100 \mu\text{g mL}^{-1}$  carbenicillin, when needed.

**Construction of expression plasmids for chimeric PKS genes:** To heterologously produce chimeric *Cortinarius* polyketide synthases in *A. niger* ATNT16 $\Delta$ pyrGx24, fragments of the intron-disrupted *C. odorifer* genes were amplified from plasmids pNAL020 (*copks1*) and pNAL006 (*copks4*), using oligonucleotides listed in Table S2A.<sup>[6]</sup> All reactions were performed in a total volume of 20  $\mu\text{L}$  using 0.4 U Phusion DNA polymerase (NEB). The reaction mixtures contained 0.2 mM (each) deoxynucleoside triphosphate and 0.5  $\mu\text{M}$  (each) oligonucleotide primer (Table S2A) in HF buffer supplied with the enzyme. PCR parameters are listed in Table S3. The resulting PCR products were electrophoretically purified in agarose gels and ligated to the linearized pSMX2-URA vector suitable<sup>[11,37]</sup> for the *A. niger* ATNT expression system, using the NEBuilder HiFi DNA Assembly Master Mix (NEB). The expression plasmids (Table S4) were pNAL030 to produce chimera I, pNAL031 (chimera II), pNAL039 (chimera III), and pNAL037 (chimera IV). Further details are given in the Supporting Information.

**Transformation of *A. niger*:** Polyethylene glycol-mediated transformation of *A. niger* protoplasts was performed as described.<sup>[38]</sup> Transformants were selected for prototrophy on AMM-G100Gln20 agar plates, supplemented with 1.2 M sorbitol. The genomic DNA of individual transformants was isolated as described.<sup>[39]</sup> Details on the genetic analysis of transformants are provided in the Supporting Information.

**Liquid chromatography and mass spectrometry:** Metabolite analysis and quantification of **1** and **2** was performed after 48 h (see first section for cultivation conditions) with mycelia of the *A. niger* strains. Three independent transformants per construct (three biological replicates) were cultured and analyzed independently three times each (three technical replicates). Mycelium was separated from the culture broth using Miracloth and rinsed thoroughly with HPLC-grade water. Subsequently, the mycelia were lyophilized. The samples were then ground to a fine powder and extracted with ethyl acetate (5 mL per 100 mg of dried mycelium). Extraction was carried out for 2 h on a shaker at 160 rpm and room temperature. The organic phases were filtered, dried over 20 g of anhydrous sodium sulfate, and evaporated to dryness in a rotary evaporator. The dried crude extracts were then dissolved in 500  $\mu\text{L}$  methanol and subjected to UHPLC-MS analysis using a Zorbax Eclipse Plus C<sub>18</sub> column (2.1  $\times$  50 mm, 1.8  $\mu\text{m}$  particle size, thermostatted at 40 °C) on an Agilent 1290 Infinity II instrument, interfaced to an Agilent 6130 single quadrupole mass detector (electrospray ionization (ESI)), operated in negative mode and applying gradient I (Table S5). Extracted ion chromatograms (EICs) based on mass-to-charge (*m/z*) ratios were recorded to detect **1** (*m/z* 273) and **2** (*m/z*

231), respectively. Authentic standards served as reference.<sup>[6]</sup> Quantification was then performed by manual determination of the area under the curve (AUC values) of the respective EICs using standards of **1** and **2** for calibration. All data were then referenced to lyophilized mycelial dry weight. In addition, the production of **1** and **2** was selectively monitored in tNAL052 and tNAL059 by single ion monitoring in the negative mode and applying gradient I (Table S5). HR-ESIMS and tandem MS spectra were recorded on a Thermo Scientific Exactive Orbitrap instrument, using a reversed phase Accucore C<sub>18</sub> column (100  $\times$  2.1 mm, 2.6  $\mu\text{m}$ ) and applying gradient II (Table S5).

**Bioinformatic and statistical methods:** Domain predictions and annotations were performed using the PFAM database.<sup>[15]</sup> To support the predictions and to identify suitable interfaces for domain swaps, linker preference profiles were generated (Figure S5) using DomCUT.<sup>[16]</sup> Error bars were generated based on three biological and three technical replicates using the Wilcoxon-Mann-Whitney test.<sup>[40]</sup> 3D structural models of the KS domain of CoPKS1 or CoPKS4 were generated using AlphaFold Colab,<sup>[21b]</sup> which is a slightly simplified version of AlphaFold v2.1.0.<sup>[21a]</sup> The models were superimposed, analyzed and visualized by using UCSF Chimera (version 1.13.1).<sup>[41]</sup>

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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 available in the supplementary material of this article.

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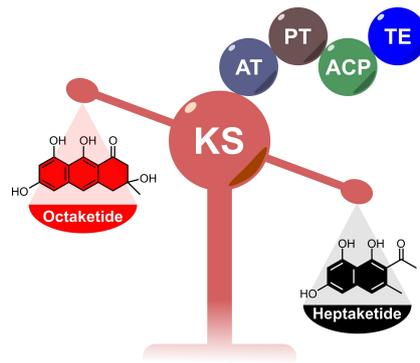
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## RESEARCH ARTICLE

**Unequal twins:** The mushroom polyketide synthase twins CoPKS1 and CoPKS4 differ strongly in their preference for hepta- or octaketide production. A series of chimeric CoPKS enzymes identified ten residues in the  $\beta$ -ketoacyl synthase domain as critical for chain length control, while a unique proline-rich linker is critical for the overall activity.



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1 – 8

**The Ketosynthase Domain Controls Chain Length in Mushroom Oligocyclic Polyketide Synthases**

