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Cytochrome P450-Catalyzed Regio- and Stereoselective Phenol Coupling of Fungal Natural Products

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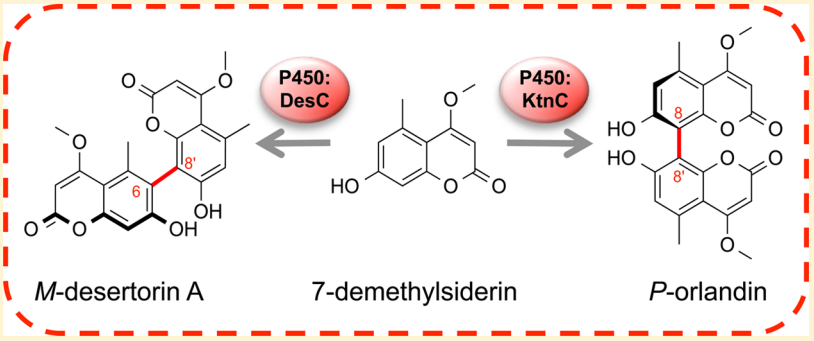
Supporting Information



ABSTRACT: For almost 100 years, phenoxy radical coupling has been known to proceed in nature. Because of the linkage of their molecular halves (regiochemistry) and the configuration of the biaryl axis (stereochemistry), biaryls are notoriously difficult to synthesize. Whereas the intramolecular enzymatic coupling has been elucidated in detail for several examples, the bimolecular intermolecular coupling could not be assigned to one single enzyme in the biosynthesis of axially chiral biaryls. As these transformations often take place regio- and stereoselectively, enzyme-catalyzed control is reasonable. We now report the identification and expression of fungal cytochrome P450 enzymes that catalyze regio- and stereoselective intermolecular phenol couplings. The cytochrome P450 enzyme KtnC from the kotanin biosynthetic pathway of *Aspergillus niger* was expressed in *Saccharomyces cerevisiae*. The recombinant cells catalyzed the coupling of the monomeric coumarin 7-demethylsiderin both regio- and stereoselectively to the 8,8′-dimer *P*-orlandin, a precursor of kotanin. The sequence information obtained from the kotanin biosynthetic gene cluster was used to identify in silico a similar gene cluster in the genome of *Emericella desertorum*, a producer of desertorin A, the 6,8′-regioisomer of orlandin. The cytochrome P450 enzyme DesC was also expressed in *S. cerevisiae* and was found to regio- and stereoselectively catalyze the coupling of 7-demethylsiderin to *M*-desertorin A. Our results show that fungi use highly specific cytochrome P450 enzymes for regio- and stereoselective phenol coupling. The enzymatic activities of KtnC and DesC are relevant for an understanding of the mechanism of this important biosynthetic step. These results suggest that bimolecular phenoxy radical couplings in nature can be catalyzed by phenol-coupling P450 heme enzymes, which might also apply to the plant kingdom.

INTRODUCTION

The biaryl axis is the central structural element in a broad variety of structurally interesting and biologically active natural products, ranging from small molecules to biopolymers.[1] A prominent example is lignin, a polymer of plant cell walls and the most abundant aromatic substance in nature. The biphenyl structure is included at approximately 10% in spruce lignin.[2] Although less abundant, melanins are widespread biaryl-containing natural products, synthesized by bacteria, animals, plants and fungi. Distinct microorganisms specifically produce low-molecular-weight biaryls such as vioxanthin,[3] julichrome Q_{3-3},[4] and the well-known antibiotic vancomycin.[5] The biaryl axis can be rotationally hindered and therefore stereogenic. A significant portion of such dimeric and oligomeric compounds display axial chirality, necessitating that they are synthesized in an atroposelective manner. Furthermore, nature's capacity to perform such syntheses is highlighted by the isolation of increasing numbers of chiral biaryls.[6,7]

The chemical synthesis of chiral biaryls from two achiral monomers, hence the stereoselective intermolecular coupling, is particularly challenging. A myriad of synthetic approaches in organic chemistry has been developed to construct axially chiral compounds.[8] In contrast, our mechanistic understanding of enzyme-catalyzed oxidative coupling reactions is limited largely to some laccases, peroxidases and cytochrome P450 enzymes.[6,7,9] Laccases and peroxidases efficiently catalyze the formation of free radicals, which in turn can couple to yield biaryls.[10,11] These enzymes, though, were reported to be unselective with respect to the biaryl axis, and an intermolecular atroposelective biaryl coupling could not be achieved.[12] For the

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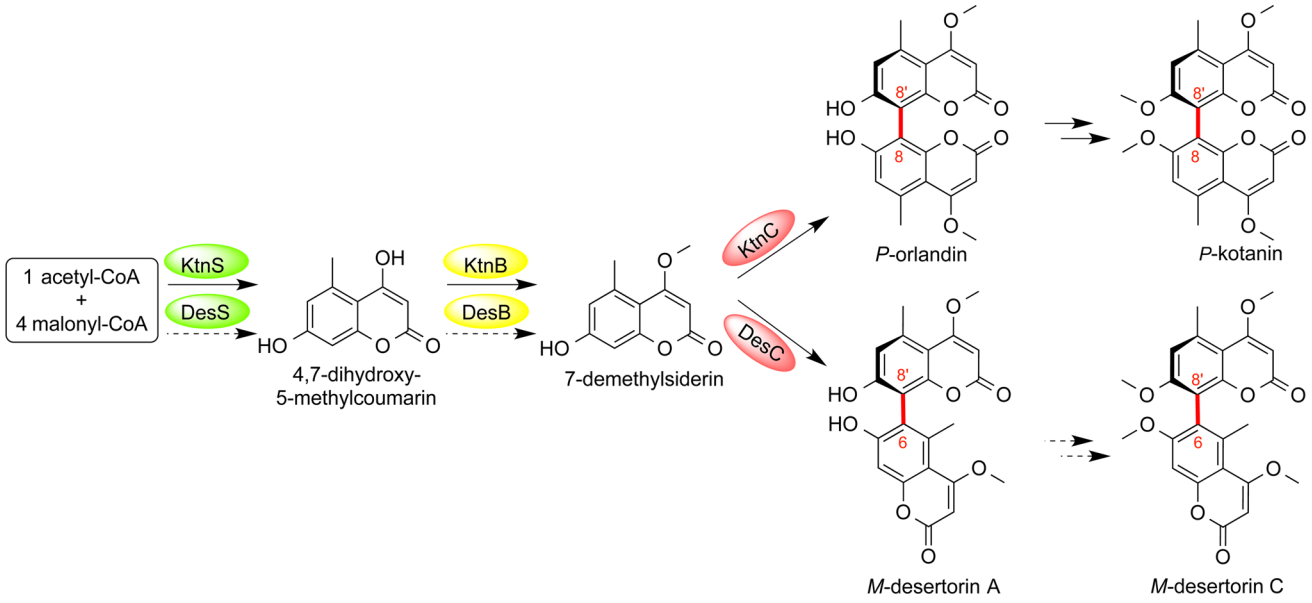
Scheme 1. Biosynthetic Pathway to Kotanin According to Stothers and Stoessl,[21] Hüttel and Müller,[22] Gil Girol and Co-Workers,[20] and Our Results, and to Desertorin C Elucidated in This Work[a]



[a]The full arrows correspond to experimentally proven steps, and the dashed arrows to steps identified by in silico analysis of genomic information and analogy to kotanin biosynthesis.

biosynthesis of (+)-pinoresinol in the plant *Forsythia suspensa*, it has been shown that the monomeric precursor *E*-coniferyl alcohol is oxidized by a laccase, but the product is obtained selectively only in the presence of "dirigent proteins". Aside from that, dirigent proteins are catalytically not active.[12−14]

Cytochrome P450 enzymes display various grades of selectivity. The synthase CYP158A2 from *Streptomyces coelicolor* A3(2) can couple flaviolin to at least two different dimers and a trimer.[15] The enzyme P450mel from *Streptomyces griseus*, which is involved in the biosynthesis of hexahydroxyperylenequinone melanin,[16] was found to regioselectively catalyze the aryl coupling of 1,3,6,8-tetrahydroxynaphthalene (THN) to a corresponding dimer. The stereochemistry of this reaction is still unknown. Our group recently described the first bacterial biocatalytic system that performs a regio- and stereoselective intermolecular oxidative C−C phenol coupling of the preanthraquinone julichrome in *Streptomyces aurantiacus*.[4]

The only reported eukaryotic cytochrome P450 enzyme that catalyzes a regio- and stereospecific—yet not stereoselective—intermolecular phenol coupling is the berbamunine synthase from the plant *Berberis stolonifera*.[17] In vivo studies have shown that this synthase catalyzes a selective C−O coupling in the synthesis of the dimeric alkaloid (*R*,*S*)-berbamunine.[17] Nevertheless, when the enzyme was purified from plant cell cultures or recombinantly expressed in insect cells, two or more dimeric products were identified.[18,19] Moreover, the ratio of the products could be altered by changing the ratio of the substrates and the source of the cytochrome P450 reductase (CPR).[19]

With a focus on oxidative phenol coupling in eukaryotes, we have identified the polyketide synthase (PKS) gene cluster for the biosynthesis of the bicoumarin *P*-kotanin in the fungus *Aspergillus niger* FGSC A1180 by deletion experiments (Scheme 1). The cytochrome P450 enzyme KtnC was shown to be essential for the dimerization of the monomeric coumarin 7-demethylsiderin to orlandin, which is further O-methylated to yield kotanin.[20]

Herein, we show that the heterologously expressed cytochrome P450 enzymes KtnC from *A. niger* and its homologue DesC from *Emericella desertorum* catalyze the regio- and stereoselective biaryl coupling of the same monomeric precursor to different isomers. This clearly shows that the selectivity is independent of other proteins. Taking as reference other enzymes that catalyze similar reactions (EC 1.14.21.*), we have named the cytochrome P450 enzymes "bicoumarin synthases".

MATERIALS AND METHODS

Chemicals. Chemical reagents and solvents were obtained from Sigma-Aldrich, Fluka, AppliChem, Roth or Acros. The coumarins 7-demethylsiderin, [2-^{13}C]-7-demethylsiderin, 4-demethylsiderin, siderin, 4,7-dihydroxy-5-methylcoumarin, kotanin, and orlandin were synthesized as described previously.[22]

Strains and Cultivation. For full experimental details, see the Supporting Information (SI) text. The fungal strains used in this work were *A. niger* FGSC A1180, *E. desertorum* CBS 653.73, and *Aspergillus alliaceus* DSM 813. For RNA extraction, *A. niger* and *E. desertorum* were cultivated with potato dextrose medium at 28 °C without shaking. Mycelium was collected aseptically after 6 and 18 days, respectively, and stored at −80 °C until further processing. For genome sequencing, *E. desertorum* CBS 653.73 was grown in potato dextrose agar plates at 25 °C for 12 days, and *A. alliaceus* DSM 813 was grown in 100 mL cultures with potato dextrose medium at 22 °C (120 rpm) for 4 days.

Genome Sequencing and Analysis. The genome sequencing of *E. desertorum* and *A. alliaceus* and the assembly were performed by the de novo sequencing service of BaseClear (Netherlands). The sequence was analyzed using the following servers and programs: Augustus,[23] antiSMASH,[24] Galaxy,[25] Clustal Omega,[26] BLAST,[27] and Geneious (Biomatters Ltd.). The phylogenetic tree and the alignments of cytochrome P450 enzymes were performed with Geneious using the neighbor-joining method and ClustalW, respectively. Sequence data have been deposited at the National Center for Biotechnology Information (NCBI) (accession numbers KT583602 and KT583603).

Expression of KtnC and DesC in *Saccharomyces cerevisiae*. For full experimental details, see the SI text. The RNA extraction, and

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first-strand and second-strand cDNA synthesis were done according to Nazir and co-workers,[28,29] with slight modifications. The plasmid pESC-HIS (Agilent Technologies) was used as the expression vector in the strain *S. cerevisiae* MH272-3fα (ura3 leu2 his3 trp1 ade2).[30] The whole-cell biotransformation was performed by inoculating *S. cerevisiae* in synthetic galactose medium (composition, g/L: 20 galactose, 6.8 yeast nitrogen base without amino acids, 0.10 L-leucine, 0.02 L-tryptophan, 0.02 uracil, 0.02 adenine) containing 0.05 mM [2-^{13}C]-7-demethylsiderin or 0.5 mM 7-demethylsiderin. The cultures (5−50 mL) were grown for 2−7 days (200 rpm) at 30 °C.

Analytical Methods. For full experimental details, see the SI text. (i) For HPLC-DAD-MS analysis, *S. cerevisiae* culture (1 mL) was extracted with 1 volume of chloroform. The chloroform phase was concentrated, redissolved in methanol (300 μL), and purified by solid-phase extraction. The analysis was performed with a LC Series 1100 system (Agilent) coupled to an API 2000 mass spectrometer with an ion source (AB Sciex) using a ZORBAX Eclipse XDB-C8 column (5 μm, 4.6 × 150 mm, Agilent). As mobile phase, a linear gradient of acetonitrile and 0.1% (v/v) formic acid was applied. (ii) For the structural analysis of the coupling products, the products were extracted with chloroform and purified by thin-layer chromatography or semipreparative liquid chromatography. Circular dichroism (CD) was carried out on a Jasco J-810 spectrophotometer (Jasco International Co.). The molar circular dichroism ($\Delta\varepsilon$) of the dimeric coumarins was calculated from the raw data (θ, mdeg) using the respective molar concentration.

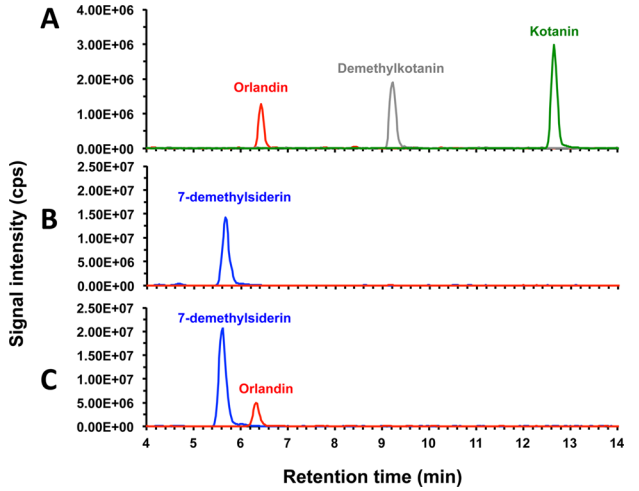

Figure 1. HPLC-MS analysis of the KtnC enzymatic activity: extracts from (A) *A. niger*, (B) *S. cerevisiae*::pESC-HIS negative control, and (C) *S. cerevisiae*::pESC-HIS::*ktnC*. The coumarins were detected by single-ion monitoring at *m*/*z* 207 for the substrate 7-demethylsiderin (blue), 411 for orlandin (red), 425 for demethylkotanin (gray), and 439 for kotanin (green).

RESULTS

Functional Expression of *A. niger* KtnC. The biosynthesis of kotanin in *A. niger* FGSC A1180 includes a coupling step from 7-demethylsiderin to the *P*-atropisomer of orlandin.[22] This step is putatively catalyzed by the cytochrome P450 enzyme KtnC.[20] To confirm the origin of the selectivity, we functionally expressed KtnC in *S. cerevisiae*. As *S. cerevisiae* CPR is able to transfer electrons to numerous heterologously expressed eukaryotic cytochrome P450 enzymes,[29] the coexpression of *A. niger* cytochrome P450 reductase CprA was deemed to be unnecessary. The *ktnC* gene was cloned into the shuttle plasmid pESC-HIS. *S. cerevisiae* cells harboring the construct pESC-HIS::*ktnC* and the parental plasmid pESC-HIS (negative control) were cultivated in induction medium, and the orlandin precursor 7-demethylsiderin was fed. After 4 days, the formation of a product was detected by liquid chromatography (HPLC-DAD) only in the cells harboring *ktnC*. The retention time and UV spectrum were identical with those of the authentic orlandin standard. Further analysis by mass spectrometry (MS) revealed the *m*/*z* peak expected for the $[M + H]^+$ ion of the dimeric coumarin (*m*/*z* 411) (Figure 1).

The orlandin product was purified by preparative thin-layer chromatography and analyzed by CD spectroscopy. The compound was optically active, and the absolute configuration was assigned as *P* by comparison with *P*-orlandin isolated from *A. niger* (Figure 2A).

In order to verify the regioselectivity of the bioconversion, [^{13}C-2]-labeled 7-demethylsiderin was fed to *S. cerevisiae*::pESC-HIS::*ktnC*. As expected, a peak of *m*/*z* 413 for the $[M + H]^+$ ion of the dimer orlandin was detected. This product was also purified by preparative thin-layer chromatography, and then analyzed by ^{13}C NMR spectroscopy. The signal due to the carboxylic carbons (^{13}C-2,2′: 162.38 ppm; Figure S1) agreed well with the data reported for the 8,8′-dimer orlandin.[22] The regioselectivity of the KtnC catalysis was hence confirmed, as no traces of regioisomers were detected by HPLC-DAD-MS analysis or NMR spectroscopy. This is in agreement with the results of feeding experiments performed with *A. niger*, which resulted in the selective formation of 8,8′-dimeric coumarins.[22]

The substrate specificity of KtnC was assayed with different monomeric coumarins, namely, 4-demethylsiderin, siderin, 4,7-dihydroxy-5-methylcoumarin, 4,7-dihydroxycoumarin, 4-methylumbelliferone, and umbelliferone (Figure S3). KtnC did not show activity with these compounds, which indicates a stringent substrate specificity.

Our experiments clearly show that KtnC catalyzes the intermolecular coupling of 7-demethylsiderin, regioselectively to the 8,8′-dimer and stereoselectively to the *P*-atropisomer. As the expression of KtnC in *S. cerevisiae* was undertaken without the coexpression of CprA or other *A. niger* proteins, it can be concluded that the regio- and stereoselectivity of the coupling depends exclusively on the cytochrome P450 enzyme.

We postulated that other bisiderin-producing fungi would have homologous biosynthetic gene clusters, whose cytochrome P450 enzymes also determine the selectivity of the intermolecular coupling. In particular, *E. desertorum* is a producer of desertorin A, a 6,8′-regioisomer of orlandin,[31,32] and for this reason this fungus was further investigated. In this case, we expected to identify a cytochrome P450 enzyme with 6,8′-regioselectivity.

Identification of the Desertorin Biosynthetic Gene Cluster in *E. desertorum*. The fungus *E. desertorum* CBS 653.73 was cultivated in Czapek Dox medium with corn steep liquor, and the culture was analyzed by HPLC-DAD-MS in order to confirm the production of desertorins. Three major peaks displayed the characteristic UV spectrum of coumarins, and the mass spectrometric analysis revealed signals corresponding to the $[M + H]^+$ ions of desertorin A (*m*/*z* 411), B (*m*/*z* 425), and C (*m*/*z* 439). The identity of the latter was confirmed by comparison with a desertorin C standard.[33]

The genome of *E. desertorum* was sequenced using the Illumina technique and assembled to 883 scaffolds (≥0.3 kb; N_{50}, 128 kb) with a total size of ≈29 Mb. The search for gene clusters homologous to the kotanin biosynthetic gene cluster was performed using complementary approaches: (i) The genome was searched for type I PKS gene clusters using the

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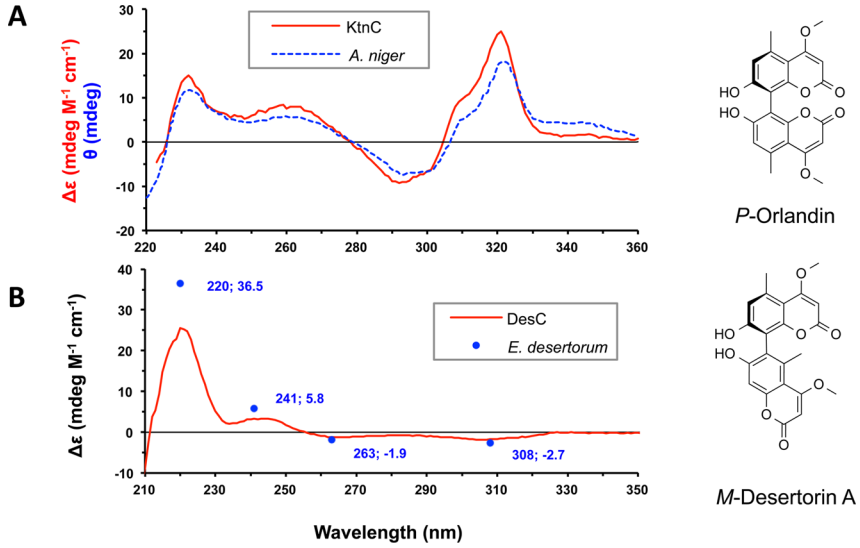

Figure 2. CD spectra of (A) *P*-orlandin and (B) *M*-desertorin A synthesized by feeding experiments in *S. cerevisiae* (solid red line) compared with material isolated from *A. niger* (dashed blue line)[22] and *E. desertorum* (solid blue circle).[31]

Table 1. Fungal Strains and Their Bicoumarins[a]

Bicoumarin synthases from different strains	Biosynthetic gene cluster	Fungal bicoumarins with different biaryl axes			
		8,8'	6,8'	3,8'	6,6'
	The same color indicates the same predicted function: PKS; cytochrome P450 enzyme; *O*-methyltransferase; methyltransferase	$R^1 = R^2$ = H: orlandin (only *A. niger*); R^1 = CH_3, R^2 = H: demethylkotanin; $R^1 = R^2 = CH_3$: kotanin	$R^1 = R^2$ = H: desertorin A; R^1 = CH_3, R^2 = H desertorin B; $R^1 = R^2 = CH_3$: desertorin C	R^1 = CH_3, R^3 = OH: aflavarin	$R^1 = R^2$ = H: isokotanin C; R^1 = CH_3, R^2 = H: isokotanin B; $R^1 = R^2 = CH_3$: isokotanin A
A. niger	*ktnS* *ktnC*	✓ **20, 22**	---	---	---
A. clavatus	*ACLA_043310*	✓ 34, 35, **36**	---	---	---
E. desertorum	*desS* *desC*	---	✓ **32**	---	---
A. flavus	*AFLA_108550*	✓ 37	---	✓ 37	---
A. oryzae	*AOR_1_1958144*	---	---	---	---
A. alliaceus	*bicS* *bicC*	✓ **38**	---	---	✓ **38**

0.08

[a]The function of the bicoumarin synthase was verified at protein level for *A. niger* (KtnC) and *E. desertorum* (DesC). The references in bold letters refer to the strains for which both the cytochrome P450 gene sequence is available and the bicoumarin production in the wild type strain was demonstrated.

antiSMASH server;[24] (ii) the *A. niger* genes *ktnS* and *ktnC* were used as the probes for a BLAST search.[27] Six genes coding for five PKS and one cytochrome P450 enzyme were found. The best hits, a PKS (DesS) and a cytochrome P450 (DesC) enzyme (59% and 44% identity, respectively), were located adjacently. Moreover, we identified a putative *O*-methyltransferase (DesB) that flanked DesS (Table 1), thus completing the essential parts of a putative desertorin biosynthetic gene cluster.

Functional Expression of *E. desertorum* DesC. The putative bicoumarin synthase DesC was expressed in the same way as *A. niger* KtnC. The *S. cerevisiae* cells harboring the construct pESC-HIS::*desC* and the plasmid pESC-HIS (negative control) were cultivated in induction medium, and 7-demethylsiderin was fed. After 4 days of cultivation, a single product peak was detected by HPLC-DAD only in the cells harboring *desC* (Figure 3). The product displayed the typical UV spectrum of these coumarins and appeared at the retention time of desertorin A. MS analysis of the product indicated an $[M + H]^+$ ion (m/z 411) corresponding to a bicoumarin. The feeding of [^{13}C-2]-labeled 7-demethylsiderin resulted in the expected peak for the $[M + H]^+$ ion of the adduct at m/z 413.

The dimeric product was purified by semipreparative HPLC and analyzed by ^{13}C NMR spectroscopy. As expected for an unsymmetric coupling, two distinct signals for the carboxylic

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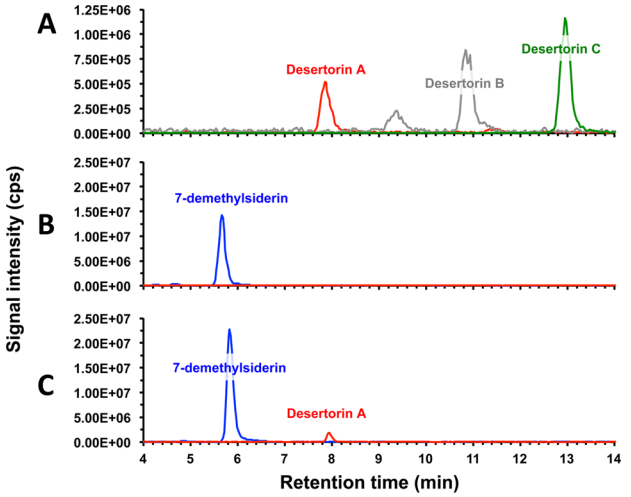

Figure 3. HPLC-MS analysis of the DesC activity: extracts from (A) *E. desertorum*, (B) *S. cerevisiae*::pESC-HIS negative control, and (C) *S. cerevisiae*::pESC-HIS::*desC*. The coumarins were detected by single-ion monitoring at *m*/*z* 207 for the substrate 7-demethylsiderin (blue), 411 for desertorin A (red), 425 for desertorin B (gray), and 439 for desertorin C (green). The tentative assignment of the peak at 10.9 min to desertorin B is based on UV spectra and MS analysis.

carbons (^{13}C-2,2′: 162.23 and 162.27 ppm) were detected (Figure S2). The data agreed well with the chemical shifts reported for the chemically synthesized 6,8′-dimer desertorin A.[39]

The substrate specificity of DesC was assayed using the same monomeric coumarins as listed for KtnC. Again, this exposed a stringent substrate specificity for DesC, as none of the monomers was converted into a dimer.

In order to elucidate the stereoselectivity of the dimerization catalyzed by DesC, the resulting product was analyzed by CD spectroscopy. The dimer was optically active, and its absolute configuration was assigned as *M* by comparison with desertorin A isolated from *E. desertorum* (Figure 2B).[31] The enzyme DesC was therefore proven to be regio- and stereoselective.

In summary, KtnC and DesC dimerize the same monomeric precursor, 7-demethylsiderin. A partial conversion of the substrate (3−7% at 0.5 mM substrate concentration, see Figures 1 and 3; 35−55% at 0.05 mM substrate concentration) by whole-cell biocatalysis was estimated. Some of the reasons of the limited turnover could be the use of a heterologous CPR, or the insufficient permeability of the cells to the substrates. Because this yield did not preclude the structural characterization of the products, further optimization was not performed. In both cases, the coupling is asymmetric, yielding the atropisomers *P*-orlandin and *M*-desertorin A, respectively. Although both enzymes catalyze a homocoupling, the regioselectivity is different: KtnC yields a symmetric dimer and DesC catalyzes an unsymmetric coupling (Scheme 1).

PKS Gene Clusters in Bicoumarin Producers. In order to obtain a holistic picture of bicoumarin biosynthesis in fungi, we searched for further homologous gene clusters in the published genomes. *A. niger* and *E. desertorum* each produce only one regioisomer. Of the various fungi described as producing exclusively 8,8′-bisiderins, the genome sequence of *A. clavatus* NRRL1 (=CBS 513.65) is publicly available.[34−36] One PKS gene cluster was identified whose biosynthetic gene organization is very similar to that of *A. niger* (Table 1). The sequences of the PKS and cytochrome P450 enzymes shared high identity with KtnS (73%) and KtnC (71%), respectively.

Aspergillus flavus is a well-characterized fungus as it represents a major threat to agriculture and human health. 8,8′-Bisiderins have been isolated from the sclerotia of *A. flavus* growing on corn kernels.[37] In addition, the dimeric coumarin aflavarin, structurally related to kotanin but linked in a 3,8′-fashion and further modified, was identified. Even though the only strain of *A. flavus* (NRRL 3357) whose genome is available was until now not described as a bicoumarin producer, one PKS gene cluster containing the essential parts for the biosynthesis of bicoumarins was identified in its genome sequence (Table 1).

As a close relative of *A. flavus*, *A. alliaceus* (both *Aspergillus* spp. section Flavi)[40] produces at least two dimeric regioisomers of siderin. The 8,8′-bisiderins kotanin, demethylkotanin as well as the 6,6′-bisiderins isokotanins A, B, and C have been isolated from *A. alliaceus* NRRL 315 (=DSM 813).[38] The genome of this strain was sequenced and assembled to 1311 scaffolds (≥0.3 kb; N_{50}, 74 kb) with a total size of ≈40 Mb. The *A. flavus* sequences from the PKS gene cluster were used as the probes to identify homologous PKS, cytochrome P450 enzymes, methyltransferases, and *O*-methyltransferases. One PKS gene cluster bearing the essential parts for bicoumarin biosynthesis was identified. The organization is identical to the PKS gene cluster from *A. flavus* (Table 1). Despite different regioisomeric bicoumarins produced by *A. alliaceus*, only one cytochrome P450 enzyme was identified, which shared 55% identity with KtnC from *A. niger* (Table S1).

In addition to the known bicoumarin-producing fungi, a putative biosynthetic gene cluster was found in *A. oryzae*. This fungus has not been reported to produce bicoumarins and the cluster might be silent (see SI discussion). A phylogenetic tree was assembled from the cytochrome P450 protein sequences showing the relationships between KtnC from *A. niger*, DesC from *E. desertorum*, and homologous putative cytochrome P450 enzymes from *A. clavatus*, *A. flavus*, *A. oryzae*, and *A. alliaceus* (Table 1).

Based on amino acid sequence information and by comparison with cytochrome P450 enzymes with known three-dimensional structure, we identified the conserved heme-binding and EXXR motifs in the six bicoumarin synthases (Table S3).[41−43] The heme-binding loop usually contains the consensus sequence F-x-x-G-x-R-x-C-x-G.[41] The six dimerizing enzymes exhibit this sequence with slight modifications (F-S-H-G-x-x-x-C-P-A), whereas the CPA pattern in the CXG position is found in only 1.25% of the fungal P450 enzymes.[44] The EXXR motif shows the patterns ETFR and ETCR, which are also unusual in fungal cytochrome P450 enzymes (0.74% and 0.07%, respectively).[44] The third conserved motif of cytochrome P450 enzymes (A/G^{n-1}-G^{n}-x-x-T^{n+3}) is usually located in the central part of the I-helix, where the threonine T^{n+3} mediates the delivery of catalytically necessary protons to promote the scission of the O−O bond.[45] Notably, all six bicoumarin synthases lack this motif at the expected position of the alignment. Instead, a conserved sequence, Y/F-H-F-x-L, was identified in the same region (Table S3). We assume that the deviation from the typical I-helix might be a characteristic of phenol coupling enzymes.

In summary, six PKS biosynthetic gene clusters for the biosynthesis of bicoumarins were identified in fungi by in vivo and in silico studies. The protein sequences of the PKS enzymes are highly similar to that of KtnS (≥59% identity in all

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cases, Table S2) and the cytochrome P450 enzymes share ≥42% identity (Table S1). For *A. niger* and *E. desertorum*, the different selectivity was proven to be due solely to the respective cytochrome P450 enzyme.

DISCUSSION

A broad variety of biaryls is produced by fungi; however, in most cases, the biarylic coupling step in their biosynthesis is still enigmatic. The cytochrome P450 enzyme KtnC from *A. niger* was functionally expressed in *S. cerevisiae*. Through in vivo experiments, we have demonstrated that KtnC selectively catalyzes the coupling of 7-demethylsiderin to orlandin. Chiang and co-workers have reported that the majority of UV-active metabolites produced by *A. niger* ATCC 11414 in solid GMM medium are dimeric naphtho-γ-pyrones, with only minor production of kotanin.[46] The polyketide synthase (AlbA) for the synthesis of the monomeric precursor of naphtho-γ-pyrones was identified. Its deletion did not deactivate the biosynthesis of kotanin, which agrees with our previous finding that another PKS enzyme (KtnS) is responsible for kotanin biosynthesis.[20] The search for enzymes responsible for the dimerization of the naphtho-γ-pyrones was not successful and these enzymes are likely to be located separate from the gene cluster.[46] Although it cannot be fully excluded that KtnC also catalyzes the coupling of the naphtho-γ-pyrones, the stringent substrate specificity of the enzyme strongly suggests that this is not the case.

One of the most interesting aspects of the biaryl coupling is that many products are axially chiral. The isolation of chiral bicoumarins from fungi was reported several decades ago.[34,32] The cytochrome P450 enzyme involved in the coupling step in *A. niger* was recently identified by our group.[20] Now, we have identified the origin of the regio- and stereoselectivity. The KtnC- and DesC-catalyzed phenol coupling was performed in *S. cerevisiae* in the absence of other proteins from the producer fungi, and only the *P*-8,8′- or the *M*-6,8′-coupling product was detected. Thus, it has been demonstrated that the distinctive linkages are due to the exquisite selectivity of the cytochrome P450 enzymes. Recent reports show that the interaction of the CPR with the cytochrome P450 enzyme can determine the outcome of the reaction.[47,48] Indeed, the berbamunine synthase from *Berberis stolonifera* showed different selectivity depending on the source of CPR.[18,19] Our experiments have demonstrated that KtnC and DesC act selectively even when the CPR is contributed from the host, *S. cerevisiae*.

The different regioselectivity of KtnC and DesC is also noteworthy. A noncatalytic preference for 8,8′-dimerization can be excluded by the activity of DesC, which generates the 6,8′-dimer, and also by the asymmetric induction. While *A. niger*, *A. clavatus*, and *E. desertorum* comply with the principle "one strain, one regioisomer", *A. alliaceus* is known to produce two dimeric regioisomers of siderin.[38] *A. alliaceus* DSM 813 has one biosynthetic gene cluster homologous to the kotanin gene cluster of *A. niger*. It is plausible that this gene cluster codes for different regioisomeric bisiderins. Consequently, in terms of selectivity, a more promiscuous cytochrome P450 enzyme than KtnC can be expected from this gene cluster. This assumption is supported by the fact that no homologous genes were found elsewhere in the genome.

Most members of the cytochrome P450 enzyme superfamily function as monooxygenases.[49] The enzymes KtnC and DesC are phenol-coupling cytochrome P450s with the characteristic heme-binding domain and the EXXR motif in the sequence. The alignment of the bicoumarin synthases described herein revealed a lack of the A/G^{n-1}-G^{n}-x-x-T^{n+3} motif. In some proteins (EryF, CYP158A1, and CYP158A2), the requirement for threonine in the n+3 position is bypassed by substrate-assisted proton delivery.[45] The biflaviolin synthases CYP158A1 and CYP158A2 catalyze the intermolecular dimerization of flaviolin in *Streptomyces coelicolor* A3(2) and are, until now, the only intermolecular dimerizing enzymes with known three-dimensional structure.[15,50] Here, T^{n+3} is replaced by an alanine residue whose amide nitrogen is rotated toward the heme pocket, providing hydrogen bonds to water molecules. It is possible that the eukaryotic bicoumarin synthases also employ a substrate-assisted proton delivery. Future work on the elucidation of the three-dimensional structures of the proteins and site-directed mutagenesis will bring insights into the mechanism of the cytochrome P450 phenol-coupling enzymes and also into the structural features that determine coupling regio- and stereoselectivity. The deviation from the conserved I-helix motif together with the rare patterns in the heme-binding domain and EXXR motif described in this work could be used to identify fungal phenol coupling cytochrome P450 enzymes in a biosynthetic gene cluster.

In conclusion, we have shown that KtnC from *A. niger* and DesC from *E. desertorum* catalyze the regio- and stereoselective intermolecular coupling of 7-demethylsiderin to *P*-orlandin and *M*-desertorin A, respectively. Via in silico analysis of the genomes of bicoumarin-producing fungi, we have identified two homologous gene clusters likely involved in the synthesis of bicoumarins by *A. flavus* and *A. alliaceus*. We have demonstrated that eukaryotic cytochrome P450 enzymes catalyze biaryl coupling reactions with high selectivity, irrespective of any additional proteins. Our approach for the identification of this pool of enzymes will also be informative for the understanding of the mechanism of oxidative phenol coupling by cytochrome P450 enzymes from the fungal and plant kingdoms, as many dimeric biaryl compounds isolated from organisms of either kingdom show identical or similar structures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06776.

Experimental details and supplementary data (PDF)

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Notes

The authors declare no competing financial interest.

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