Biosynthesis

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Regio- and Stereoselective Oxidative Phenol Coupling in *Aspergillus niger***

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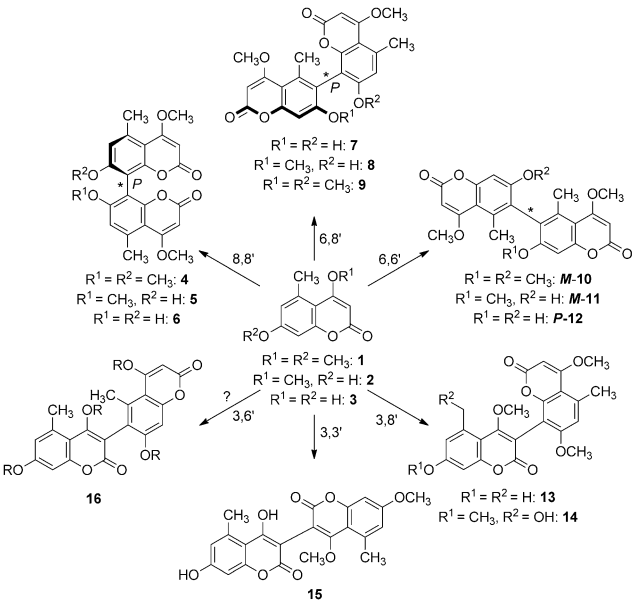
Dedicated to Professor Wolfgang Steglich

Since the first mention of the oxidative phenol coupling reaction by Pummerer and colleagues in 1925,[1] the mechanism which governs the regio- and stereoselectivity of the intermolecular phenol coupling in organisms has remained unclear. The first evidence for the involvement of cytochrome P450 enzymes was found for the biosynthesis of the benzylisoquinoline alkaloids salutaridine and berbamunine.[2] Davin et al. showed that the dimerization of (*E*)-coniferyl alcohol into (+)-pinoresinol in *Forsythia* sp. is controlled by a dirigent protein, although Freudenberg had postulated a fortuitous coupling of lignols to polymeric lignin.[3,4] The reaction is catalyzed by laccase or any other radical-forming oxidant, whereas the dirigent protein determines the selectivity of the coupling. In transformations with an enantiocomplementary dirigent protein from *Arabidopsis thaliana*, the laccase-catalyzed oxidative coupling gave (−)-pinoresinol.[5] However, homologues of the dirigent proteins are limited to spermatophytes. Thus, the question of which proteins mediate the regio- and stereoselective intermolecular phenol coupling in other organisms remains unsolved.[6]

The ascomycetes *Aspergillus* and *Emericella* produce a variety of bicoumarins putatively by dimerization of the monomeric coumarin siderin (**1**) or its demethyl derivatives **2** and **3**.[7] Twelve bicoumarins, **4**–**15**, representing five of the six possible regioisomeric dimers, have been isolated from diverse ascomycetes species so far (Scheme 1).



Scheme 1. Bicoumarins formed by the coupling of siderin (**1**) or its derivatives **2** and **3**. Kotanin (**4**), 7-demethylkotanin (**5**),[8] orlandin (**6**),[9] desertorin A–C (**7**-**9**),[10] isokotanin A–C (**10**–**12**),[11] 7-*O*-demethyl-3,8′-bisiderin (**13**),[12] aflavarin (**14**)[13] and bicoumanigrin (**15**).[14]

Herein, we report the identification and analysis of the biosynthetic cluster responsible for kotanin (**4**) production in *A. niger* FGSC A1180. Through homology modeling and substrate docking, a rationale for the regio- and stereoselective phenol coupling reaction was derived.

Previously, we demonstrated that in *A. niger* the monomeric coumarin **2** is coupled regio- and stereoselectively exclusively to the 8,8′-bicoumarin *P*-(+)-**6**, and subsequent O-methylation forms *P*-(+)-kotanin [*P*-(+)-**4**] (see Scheme 2 for structures).[15] Our feeding experiments with α-D-[$^{13}C_6$]glucose proved the polyketidic origin of **4** and its precursors (see the Supporting Information). Fungal polyketides are usually produced by iteratively acting type I polyketide synthases (PKSs) which can be further subdivided by their domain organization and phylogeny into highly reducing, partially reducing, and nonreducing PKSs.[16] The two sequenced producers of **4**, *A. niger* ATCC 1015 and CBS 513.88, harbor about 35 PKS genes (see the Supporting

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Information).[17,18] Since no reduction steps are required for the formation of the monomeric coumarin from linear pentaketide, only nonreducing PKSs were considered as putative sources of the carbon backbone.[19] A phylogenetic analysis of the ketosynthase domains of *A. niger* ATCC 1015 PKS grouped six PKSs into the nonreducing clade with five having homologues in the CBS 513.88 strain.[18] An annotation of the genes adjacent to the six PKS genes revealed that only four of the clusters encoded enzyme candidates for the tailoring steps leading to **4**. These are cytochrome P450s and other monooxygenases (possibly catalyzing the phenol coupling reaction) and methyltransferases (see the Supporting Information).

The PKS cluster responsible for biosynthesis of **4** was identified by targeted gene deletion in the NHEJ-deficient *A. niger* strain FGSC A1180,[20] which also produces the dimeric coumarins **4**–**6** (Figure 1A). Disruption of the PKS gene ANI_1_2226184 (*ktnS*) led to complete loss in coumarin biosynthesis (Figure 1B).[21] A complementation of the deletion mutant by use of a fungal autonomous replicating plasmid[22] bearing the endogenous *ktnS* gene reestablished production of **4**–**6**.

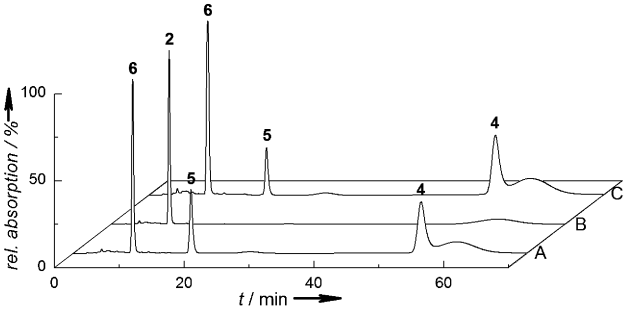


Figure 1. HPLC chromatograms of metabolite extracts of A) wild-type *A. niger* FGSC A1180, B) the *A. niger* FGSC A1180 Δ*ktnS* mutant, and C) the *A. niger* FGSC A1180 Δ*ktnS* mutant complemented with the endogenous gene *ktnS*.

A functional analysis of the genes in the putative biosynthesis cluster of **4** was also performed by targeted gene deletion. A disruption of the O-methyltransferase gene ANI_1_1410184 (*ktnB*) resulted in complete termination of the coumarin biosynthesis (see Figure S2 in the Supporting Information). Hüttel and Müller have shown that the methoxy group at C4 is a prerequisite for the oxidative phenol coupling reaction forming **6**, and suggests that the methyltransferase is involved in the initial O methylation of the PKS product **3**.[15] By complementing the deletion mutant with the endogenous gene, the biosynthesis of **4**–**6** was restored (see the Supporting Information). Furthermore, chemical complementation by feeding of the monomethylated precursor **2** to the Δ*ktnB* mutant proves the independence of the final O-methylation steps of KtnB (see Figure S3 in the Supporting Information).

A disruption of the cytochrome P450 monooxygenase gene ANI_1_2228184 (*ktnC*) also led to a breakdown of the biosynthesis of **4**–**6**. However, a new metabolite was found,

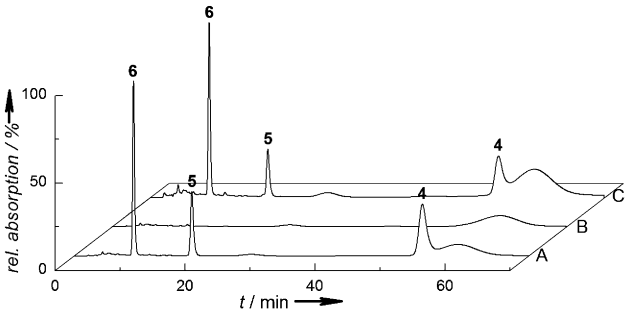
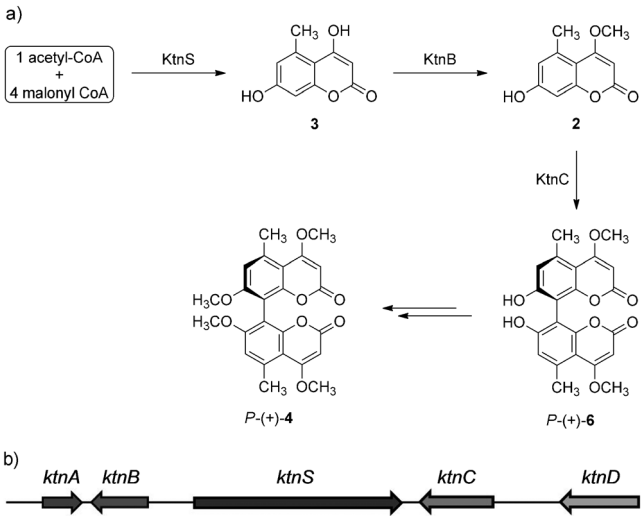


Figure 2. HPLC chromatograms of metabolite extracts of A) wild-type *A. niger* FGSC A1180, B) the *A. niger* FGSC A1180 Δ*ktnC* mutant, and C) the *A. niger* FGSC A1180 Δ*ktnC* mutant complemented with the endogenous gene *ktnC*.

and was identified by NMR spectroscopy to be the monomeric precursor **2** (Figure 2B and the Supporting Information). The production of the dimeric coumarins **4**–**6** could be restored by complementation with the endogenous cytochrome P450 gene *ktnC* (Figure 2C). Disruption of other neighboring genes had no effect on the biosynthesis of *P*-(+)-**4**.



Scheme 2. a) Proposed pathway for the biosynthesis of *P*-(+)-**4** in *A. niger* FGSC A1180. b) the gene cluster of *A. niger* CBS 513.88.

Hence, a pathway for the biosynthesis of **4** is formulated as follows: the pentaketidic dihydroxycoumarin **3** is synthesized by the PKS KtnS and O-methylated to give **2** by the O-methyltransferase KtnB. Then, an oxidative phenol coupling forms *P*-(+)-**6** and is catalyzed by the cytochrome P450 monooxygenase KtnC (Scheme 2). The sole occurrence of **2** in the cytochrome P450 deletion experiment suggests steady state kinetics in the course of the biosynthesis of **4**, whereas the generation of the carbon backbone by the PKS KtnS probably represents the rate-limiting step.

To gain first insights into the mechanism by which the cytochrome P450 KtnC controls the regio- and stereoselectivity of the phenol coupling reaction, we performed docking

experiments with the monomeric coumarin **2**, as well as with the dimeric product **6**. Molecular docking was performed with a homology model of the enzyme calculated by the software "I-TASSER" and "Glide".[23,24]

Docking of the dimeric *P*-(+)-**6** resulted in considerably better Glide-Scores than docking of *M*-(−)-**6** (see the Supporting Information). This data suggests that specific coupling of monomeric **2** forming the *P*-(+)-**6** enantiomer is due to stereoselective catalysis by the enzyme. The high-scoring conformation in direct proximity to the heme prosthetic group (Figure 3) was selected for a comparison with the docked reactants.

Docking of two molecules of coumarin **2** resulted in a geometry similar to that of the dimer, as shown in the superposition of both structures (Figure 4). As a result of the rigid conformation of the monomers, C8 and C8′ converge to a distance of 3.6 Å while having a torsion angle of 66°. The distance of C8 proximal to the heme prosthetic group is 5.6 Å.

Oxidizing enzymes which catalyze intermolecular biaryl couplings are well known. In particular, multicopper enzymes like laccases, and iron-dependent enzymes such as peroxidases and cytochrome P450 oxygenases, have been reported.[25] For the latter, an intermolecular coupling reaction with a substrate similar to **2**, but without regio- and stereochemical control, has been described by Zhao et al.[26] The structure of CYP158A2 from *Streptomyces coelicolor* A3(2) was resolved without substrate and when complexed with two flaviolin molecules. In contrast to the docking conformations described herein, the two substrate molecules formed a quasi-planar stack parallel to the heme prosthetic group, thus resulting in random coupling to 3,3′- and 3,8′-biflaviolin as well as a noncharacterized trimer (see Figure S4 in the Supporting Information). Nevertheless, the distances between the substrate **17** and the prosthetic group are similar to our model: the distance between the monomeric precursors is 3.7 Å, and the interspace between the lower **17** and the heme is 4.6 Å.

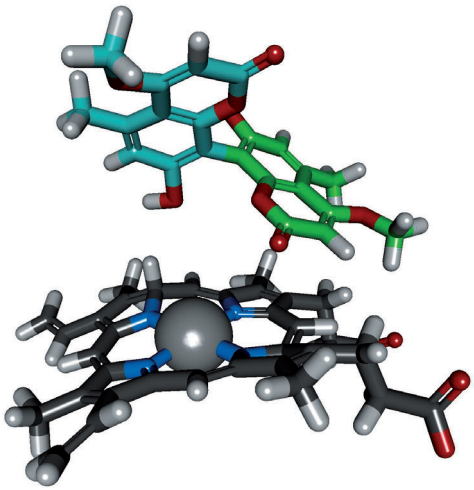


Figure 3. Calculated geometry of *P*-(+)-**6** within the active site of the modelled three-dimensional structure of the cytochrome P450 monooxygenase KtnC.

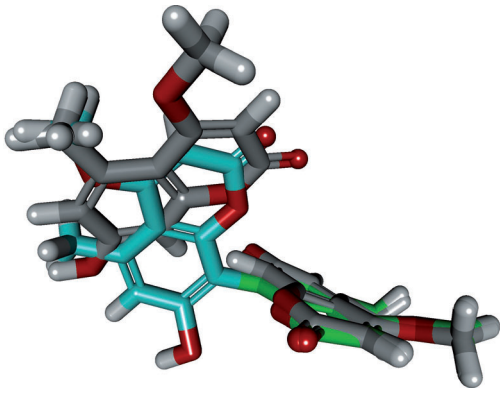


Figure 4. Superposition of the calculated conformation of *P*-(+)-**6** and two molecules of monomer **2** in the active site of the cytochrome P450 monooxygenase KtnC.

According to our model, the regioselectivity of the coupling as well as the stereoselectivity of the biaryl axis depends on the substrate orientation in the active site of cytochrome P450 monooxygenase KtnC. A fixation of the substrate molecules **2** results in approximation of C8 and C8′, thus allowing the regioselective coupling reaction. Thereby, the torsion angle of 66° supports the formation of the *P*-enantiomer.

In summary, we have identified and analyzed the biosynthetic cluster responsible for kotanin production. Through homology modeling and substrate docking, a rationale for the regio- and stereoselective phenol coupling reaction was derived.

Experimental Section

For metabolite identification, *A. niger* FGSC A1180 extracts were analyzed by HPLC on an Agilent 1200 Station (Agilent Technologies, Santa Clara, USA), equipped with a LiChrospher 100 RP18, 250 mm × 3 mm column, eluent: 0.1 % acetic acid/35 % acetonitrile, 400 μL min^{-1}, compounds were detected with a UV/Vis DAD at a wavelength of $\lambda = 310$ nm (BW = 15 nm).

Synthesis protocols for the reference compounds can be found in the literature.[15,27]

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[1] R. Pummerer, H. Puttfarcken, P. Schopflocher, *Ber. Dtsch. Chem. Ges.* **1925**, *58*, 1808–1820.
[2] M. H. Zenk, R. Gerardy, R. Stadler, *J. Chem. Soc. Chem. Commun.* **1989**, 1725–1727.
[3] L. B. Davin, H. B. Wang, A. L. Crowell, D. L. Bedgar, D. M. Martin, S. Sarkanen, N. G. Lewis, *Science* **1997**, *275*, 362–366.
[4] K. Freudenberg, *Nature* **1959**, *183*, 1152–1155.
[5] B. Pickel, M. Constantin, J. Pfannenstiel, J. Conrad, U. Beifuss, A. Schaller, *Angew. Chem.* **2010**, *122*, 207–209; *Angew. Chem. Int. Ed.* **2010**, *49*, 202–204.
[6] S. Ralph, J.-Y. Park, J. Bohlmann, S. D. Mansfield, *Plant Mol. Biol.* **2006**, *60*, 21–40.

[7] a) P. Venturella, A. Bellino, F. Piozzi, *Tetrahedron Lett.* **1974**, *15*, 979 – 981; b) H. Hussain, J. Hussain, A. Al-Harrasi, K. Krohn, *Tetrahedron* **2012**, *68*, 2553 – 2578.
[8] a) G. Büchi, D. H. Klaubert, R. C. Shank, S. M. Weinreb, G. N. Wogan, *J. Org. Chem.* **1971**, *36*, 1143 – 1147; b) G. Büchi, K. C. Luk, B. Kobbe, J. M. Townsend, *J. Org. Chem.* **1977**, *42*, 244 – 246.
[9] H. G. Cutler, F. G. Crumley, R. H. Cox, O. Hernandez, R. J. Cole, J. W. Dorner, *J. Agric. Food Chem.* **1979**, *27*, 592 – 595.
[10] K. Nozawa, H. Seyea, S. Nakajima, S. Udagawa, K. Kawai, *J. Chem. Soc. Perkin Trans. 1* **1987**, 1735 – 1738.
[11] J. A. Laakso, E. D. Narske, J. B. Gloer, D. T. Wicklow, P. F. Dowd, *J. Nat. Prod.* **1994**, *57*, 128 – 133.
[12] K. Nozawa, S. Nakajima, K. Kawai, S. Udagawa, M. Miyaji, *Phytochemistry* **1994**, *35*, 1049 – 1051.
[13] M. R. TePaske, J. B. Gloer, D. T. Wicklow, P. F. Dowd, *J. Nat. Prod.* **1992**, *55*, 1080 – 1086.
[14] J. Hiort, K. Maksimenka, M. Reichert, S. Perovic-Ottstadt, W. H. Lin, V. Wray, K. Steube, K. Schaumann, H. Weber, P. Proksch, R. Ebel, W. E. G. Muiller, G. Bringmann, *J. Nat. Prod.* **2004**, *67*, 1532 – 1543.
[15] W. Hüttel, M. Müller, *ChemBioChem* **2007**, *8*, 521 – 529.
[16] R. J. Cox, *Org. Biomol. Chem.* **2007**, *5*, 2010 – 2026.
[17] H. J. Pel et al., *Nat. Biotechnol.* **2007**, *25*, 221 – 231.
[18] K. M. Fisch, A. F. Gillaspy, M. Gipson, J. C. Henrikson, A. R. Hoover, L. Jackson, F. Z. Najar, H. Wägele, R. H. Cichewicz, *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 1199 – 1213.
[19] R. D. Lapper, *Tetrahedron Lett.* **1974**, *15*, 4293 – 4296.
[20] V. Meyer, M. Arentshorst, A. El-Ghezal, A. C. Drews, R. Kooistra, C. A. van den Hondel, A. F. J. Ram, *J. Biotechnol.* **2007**, *128*, 770 – 775.
[21] As a result if sequence identities of > 99 % between the analyzed FGSC A1180 and CBS 513.88 genes, the CBS 513.88 nomenclature was adopted for the FGSC A1180 genes.
[22] H. Kuwayama, S. Obara, T. Morio, M. Katoh, H. Urushihara, Y. Tanaka, *Nucleic Acids Res.* **2002**, *30*(E2), 1 – 5.
[23] Y. Zhang, *Proteins Struct. Funct. Genet.* **2007**, *69*, 108 – 117.
[24] Glide, version 5.6, Schrödinger, LLC, New York, NY, **2010**.
[25] a) I. Fujii, H. Iijima, S. Tsukita, Y. Ebizuka, U. Sankawa, *J. Biochem.* **1987**, *101*, 11 – 18; b) A. M. Mayera, R. C. Staples, *Phytochemistry* **2002**, *60*, 551 – 565; c) K. Yamanaka, K. S. Ryan, T. A. M. Gulder, C. C. Hughes, B. S. Moore, *J. Am. Chem. Soc.* **2012**, *134*, 12434 – 12437.
[26] B. Zhao et al., *J. Biol. Chem.* **2005**, *280*, 11599 – 11607.
[27] W. Hüttel, M. Nieger, M. Müller, *Synthesis* **2003**, 1803 – 1808.

