Enzymatic Formation of Rufoschweinitzin, a Binaphthalene from the Basidiomycete Cortinarius rufoolivaceus

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Naturally occurring binaphthalenes have attracted the interest of scientists due to their auspicious bioactivity and their similarity to BINOL-type catalysts.[1–5] Nevertheless, their biosynthesis has not been investigated as yet, especially with regard to the regio- and stereoselective oxidative phenol coupling of the monomeric precursors.

Recent research has demonstrated that different sets of enzymes are responsible for the dimerization of polyketides in fungi. Laccases were shown to mediate regioselective C–C bond formation, sometimes depending on auxiliary proteins to be active or to introduce stereoinformation at the biaryl axis.[6–9] Cytochrome P450 enzymes (CYPs) are also associated with phenol-coupling reactions. In the biosynthesis of bicoumarins, they control both regio- and stereoselectivity on their own, without the need for auxiliary proteins.[10,11] Further, CYPs are known to catalyze the coupling reaction in the biosynthesis of the dimeric naphthopyrones nigerone (5) and aurasperone A (7, Scheme 1).[12] These two metabolites are regioisomers, each exclusively formed by a distinct Aspergillus strain and not occurring as a regioisomeric mixture. This means that enzymes of one class (CYPs) can catalyze selective dimerizations leading to different regioisomers. The mechanisms behind this are unknown.

Here, the isolation and characterization of a new binaphthalene, named rufoschweinitzin (2), from the basidiomycete Cortinarius rufoolivaceus are presented. Furthermore, we also demonstrate enzymatic formation of 2 through the action of a CYP that is encoded in a biosynthetic gene cluster (BGC) identified in the genome of the ascomycete Xylaria schweinitzii (BCC 1337). Besides the coupling activity of the CYP, we have verified the activity of two other encoded enzymes—namely, two O-methyltransferases—through heterologous expression. As hypothesized, the two O-methyltransferases methylate the monomeric precursor 6-hydroxymusizin (8) to provide torachryson-8-O-methyl ether (1). The CYP-catalyzed coupling of the latter leads to the formation of two binaphthalenes: one identified as rufoschweinitzin (2) and the other as a hitherto unknown binaphthalene, named alloschweinitzin (3), that matches the regiochemistry of rubasperone B (6) and pseudophlegmacin.[13,14]

The basidiomycete C. rufoolivaceus is known to produce hetero- and homodimeric polyketides. Fractionation of the fruiting body extract led us to the discovery of an uncharacterized metabolite.[15] This metabolite, named rufoschweinitzin (2), shows seven signals in the 1H NMR spectrum and 15 signals in the 13C NMR spectrum (see the Supporting Information). As mass spectrometry clearly indicates the existence of a dimer, 2 was assumed to be coupled symmetrically. The presence of two aromatic protons, each giving a doublet in the 1H NMR spectrum (δ = 5.96 ppm, J = 2.5 Hz and δ = 6.42 ppm, J = 2.5 Hz), corroborated the symmetrical 4,4′-coupling arrangement. Accordingly, 2 represents a regioisomer of schweinitzin B (4), which was isolated from fruiting bodies of the Vietnamese ascomycete X. schweinitzii.[16]

As it would be responsible for a key step in the biosynthesis of such binaphthalenes, the identification of a phenol-coupling enzyme capable of catalyzing the dimerization of torachryson-8-O-methyl ether (1) became a priority. Recently, the two ascomycete CYPs BfoB and AunB were shown to dimerize the naphthopyrone rubrofusarin B, either to nigerone (5) or to aurasperone A (7).[12] These two metabolites reflect the regiochemistry of rufoschweinitzin (2) and schweinitzin B (4), respectively, so a CYP-catalyzed dimerization was supposed, at

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least in the case of dimerization in ascomycetes. The genome of X. schweinitzii BCC 1337 from Thailand was hence sequenced on an Illumina platform and bioinformatically investigated. Indeed, we identified a putative BGC (annotated as shw, Figure 1B) that encodes the required enzymes for binaphthalene biosynthesis as depicted in Figure 1A. An iterative nonreducing polyketide synthase (NR-PKS) is necessary to form the heptaketidic backbone that is cyclized to yield 6-hydroxymusim.
As previously described and verified in vitro, this type of iterative type I NR-PKS typically lacks a thioesterase domain, thus making it dependent on an external metallo-β-lactamase (ShwB)\(^{[17,18]}\). Furthermore, two O-methyltransferases (ShwM1 and ShwM2) and a CYP (ShwC) are encoded in this BGC. The sequence of ShwC contains a conserved heme-binding motif, only differing at position 2 from the consensus pattern that is provided by PROSITE (L instead [SGNH]), whereas the typical A/G-G-X-X-T motif of the I-helix is lacking\(^{[19,20]}\). This discrepancy is seen in other phenol-coupling CYPs as well, and is therefore proposed to be a characteristic\(^{[21]}\). Further genes in the BGC encode an EthD-like domain (ShwE) and a domain of unknown function (DUF1772, ShwD). ShwE possibly acts as a decarboxylase, whereas ShwD could possess an oxidative activity\(^{[21–24]}\). To verify the BGC function, the two O-methyltransferases ShwM1 and ShwM2 were heterologously produced in *Escherichia coli* BL21 Gold(DE3). Because *X. schweinitzii* BCC 1337 does not actively transcribe the genes under laboratory conditions, cDNA was obtained by first expressing the genes in *A. niger* and cDNA was obtained by first expressing the genes in *Aspergillus aculeatus*. The appearance of two dimer peaks suggested the formation of two bi-torachrysone-8-O-methyl ether (1) regioisomers. NMR experiments provided the necessary information for complete structure elucidation (Figures S3–S8). The earlier eluting dimer shows 14 signals in the \(^1\)H NMR spectrum (Figure S3), indicating an unsymmetrically coupled product. Two singlets (δ = 9.67 and 10.05 ppm) were assigned to the phenolic hydroxy group of each half of the molecule, whereas four singlets were assigned to the methoxy groups. Moreover, four singlets originate from the methyl groups. The aromatic protons correspond to four signals, one singlet and three doublets (Figure 3), thus indicating that one monomeric moiety is coupled at position 4, with the aromatic protons at positions 5 and 7 giving rise to two doublets (δ = 5.96 ppm, \(^3J =2.2\) Hz).
and $\delta = 6.43$ ppm, $J = 2.2$ Hz). Consequently, the signal for H-4 with an allylic coupling to the methyl group in 1 is missing (NMR data for 1 are given in the Supporting Information). Because the dimer is unsymmetrical, the coupling position of the other moiety could be either 5' or 7'. Both coupling positions would lead to the observed doublet at 6.29 ppm ($J = 0.8$ Hz), which could be assigned to H-4', and the aromatic singlet at 6.70 ppm as there is no coupling across C4'.

To differentiate between the possible 4,5'- and 4,7'-coupling arrangements, an NOE experiment was performed on the aromatic singlet with $\delta = 6.70$ ppm. The NOE signals show proximity of this proton to two methoxy groups, hence confirming its position at C-7' and thus the formation of a 4,5'-coupled binaphthalene, which we have named alloschweinitzin (3, Figures 3 and 56).

The later eluting dimer shows seven signals in the $^1$H NMR spectrum that exactly match the NMR data of the isolated rufoschweinitzin (2, Figure S7).¹³

Hence, the identified CYP gives two bi(torachrysone-8-O-methyl ether) regioisomers: rufoschweinitzin (2), symmetrically coupled at positions 4 and 4', and the hitherto unknown alloschweinitzin (3), unsymmetrically coupled at positions 4 and 5' (Figure 3).

The CD spectra of both the isolated and the enzymatically produced binaphthalenes 2 and 3 each show the excess of one atroposmer. All spectra exhibit a positive first and a negative second cotton effect, so the 1-1'-binaphthalene system is twisted clockwise (Figures 4, 59 and S10). Because the monomer 1 contains no stereochemical information, and just two out of (at least) three possible products—2 to 4—are formed atroposelectively, ShwC must control the regio- and stereochemistry of the oxidative phenol coupling.

To investigate the impact of the substrate’s methylation pattern on the dimerization reaction, we tested 6-hydroxymusizin (8) and torachrysone (10) for conversion in the presence of ShwC. No coupling activity was observed at all (Figure S11), so we propose that the methylation pattern is crucial for the dimerization of polyketides in ascomycetes.

In summary, the new binaphthalene rufoschweinitzin (2) was isolated from the basidiomycete C. rufoolivaceus. Furthermore, we also identified a BGC that allows the production of such binaphthalenes in the genome of X. schweinitzii BCC 1337. With three encoded enzymes in hand, we demonstrated the biosynthesis of rufoschweinitzin (2) and its regioisomer alloschweinitzin (3) in ascomycetes. With our findings, we can link the regiochemistry of 4,4'- and 4,5'-like coupled dimers (2 and 3, respectively) to CYP-catalyzed dimerizations, and thus most probably also the 4,7'-like regiochemistry of schweinitzin B (4), thus, we predict CYP involvement in the biosynthesis of other 4,5'-like coupled biaryls such as the rubasperones or the asperinines from ascomycetes.¹¹,²²

Nevertheless, it is questionable whether the type of coupling enzyme remains the same across fungal phyla; no homologous enzymes have so far been found in basidiomycetes or attributed to dimerization reactions. This might indicate that convergent evolution has resulted in two completely different enzymatic setups to produce one and the same secondary metabolite in two different organisms, as in the case of rufoschweinitzin from a basidiomycete and from an ascomycete.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: binaphthalenes · biosynthesis · dimerization · phenol coupling · regioselectivity

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