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

Wiebke Thiele,[a] Rita Froede,[b] Wolfgang Steglich,[b] and Michael Müller*[a]

Dedicated to Prof. Rolf Huisgen on his 100th birthday.

Dimeric polyketides are widespread fungal secondary metabolites. They occur in both ascomycetes and basidiomycetes and, therefore, across fungal phyla. Here we report the isolation of a new binaphthalene, named rufoschweinitzin, from the basidiomycete *Cortinarius rufoolivaceus*. Rufoschweinitzin consists of two symmetrically 4,4'-coupled torachrysone-8-O-methyl ether moieties. Furthermore, we have identified a binaphthalene biosynthetic gene cluster in an unrelated fungus, the ascomycete *Xylaria schweinitzii*. Heterologous expression of the encoded cytochrome P450 enzyme verified its coupling activity: dimerization of torachrysone-8-O-methyl ether led to the formation of rufoschweinitzin alongside a hitherto unknown regioisomer, now named alloschweinitzin. We have thus demonstrated enzymatic formation of the basidiomycete's metabolite rufoschweinitzin and made the regiochemistry of alloschweinitzin accessible with an ascomycete-derived enzyme.

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 biosynthe-

sis of the dimeric naphthopyrones nigerone (**5**) and auraspero-ne 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 torachrysone-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 pseudophleg-macin.[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 ^{13}C 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 ($\delta = 5.96$ ppm, $^4J = 2.5$ Hz and $\delta = 6.42$ ppm, $^4J = 2.5$ 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 torachrysone-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
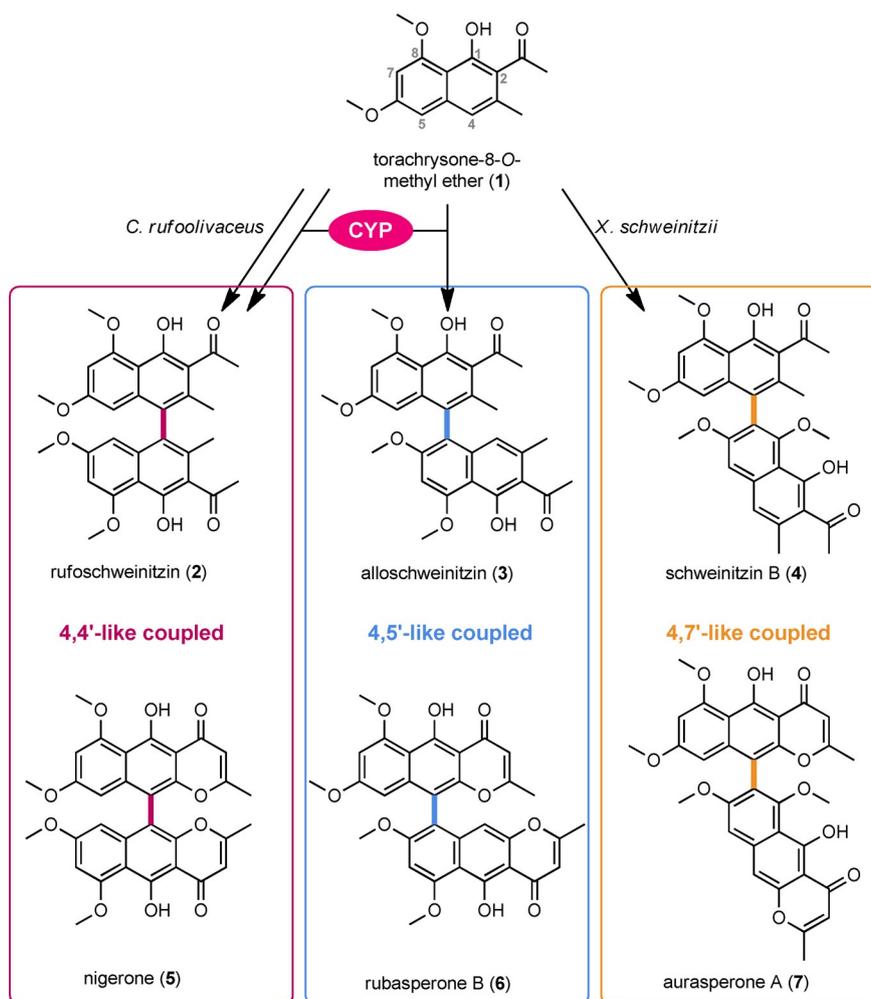
[a] W. Thiele, Prof. Dr. M. Müller
Institute of Pharmaceutical Sciences, Albert-Ludwigs-Universität Freiburg
Albertstrasse 25, 79104 Freiburg (Germany)
E-mail: Michael.mueller@pharmazie.uni-freiburg.de

[b] Dr. R. Froede, Prof. Dr. W. Steglich
Department of Chemistry, Ludwig-Maximilians-Universität München
Butenandtstrasse 5–13, Haus F, 81377 München (Germany)

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Scheme 1. Structures of fungal secondary metabolites with a dimeric scaffold. Torachrysone-8-O-methyl ether (**1**) is dimerized to rufoschweinitzin (**2**) and alloschweinitzin (**3**) through the action of a CYP. Binaphthalene **2** was also isolated from *C. rufoolivaceus*, whereas schweinitzin B (**4**) was previously isolated from Vietnamese *X. schweinitzii*.[16]
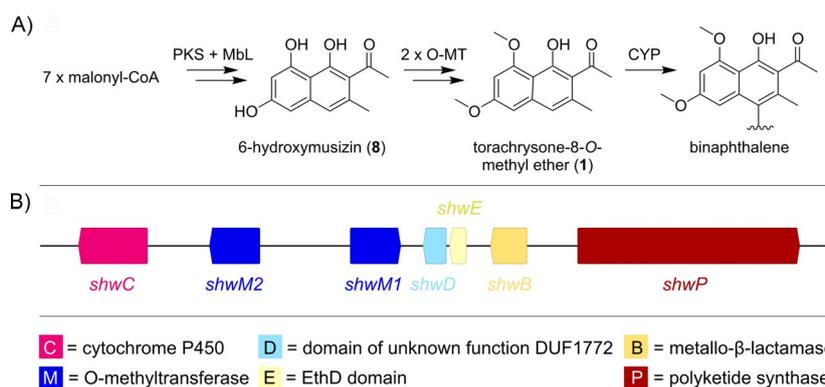
Figure 1. A) Proposed binaphthalene biosynthesis, involving a polyketide synthase (PKS), a metallo-β-lactamase (MbL), two O-methyltransferases (O-MTs), and a cytochrome P450 enzyme (CYP). B) Binaphthalene biosynthetic gene cluster identified in the genome of *X. schweinitzii* BCC 1337.

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-hydroxymusi-

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zin (**8**) after product release. This is methylated twice to give torachrysone-8-*O*-methyl ether (**1**), which is subsequently dimerized. On the basis of the similarity to the aforementioned naphthopyrones in terms of the methylation pattern and the regiochemistry, we anticipated two separate *O*-methyltransferases and a CYP taking part in the biosynthesis.

The identified BGC consists of seven genes, encoding an NR-PKS (ShwP) as core element. On the basis of a product template domain sequence alignment, ShwP can be grouped into PKS that exhibit a C6–C11 cyclization pattern.[17] 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.[11] 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 *Aspergillus niger*. For this, the genes were amplified from isolated gDNA, ligated into the fungal vector pSUC, and transformed into *A. niger* FGSC A1180. Spores of transformants were used to inoculate expression cultures that were utilized for RNA isolation. After cDNA synthesis, the spliced *O*-methyltransferase genes *shwM1* and *shwM2* were cloned into pET19b for expression in *E. coli*. Whole cells were fed with 6-hydroxymusizin (**8**), as this was anticipated to be the physiological substrate. LC-DAD-MS analysis of assay extracts confirmed their enzymatic activity.
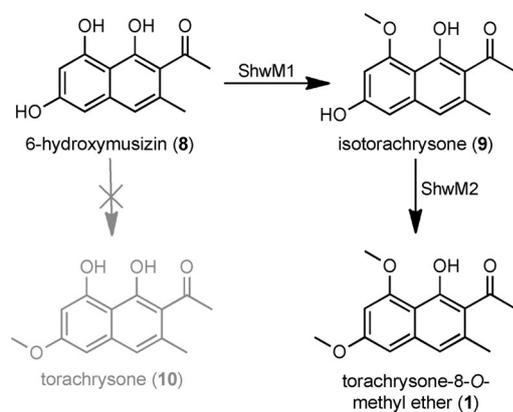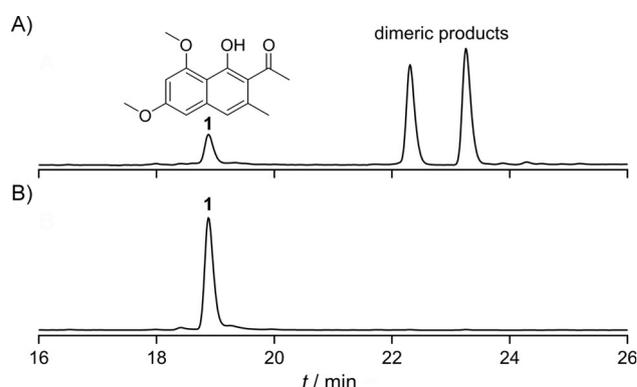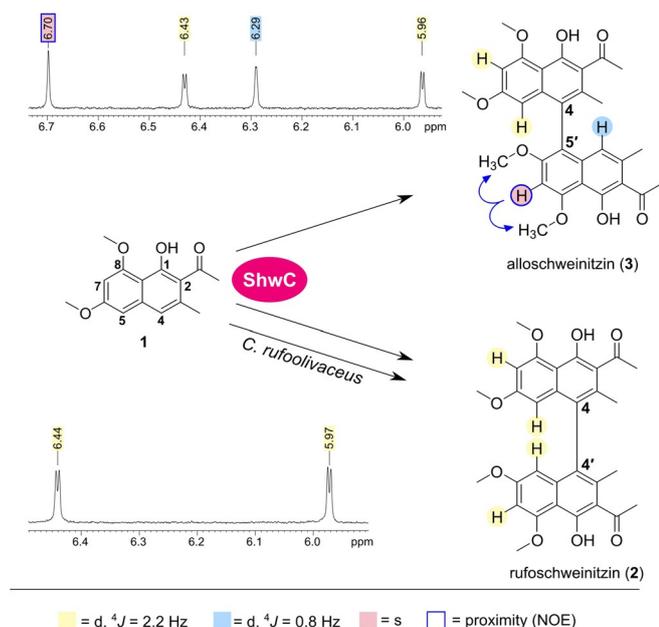
Treatment of **8** with ShwM1 alone led to a new peak in the LC-DAD-MS chromatogram with the corresponding *m/z* for a monomethylated 6-hydroxymusizin derivative—isotorachrysone (**9**, Scheme 2 and Figure S1)—whereas assays with only ShwM2 showed no activity. As the chromatographic data of the monomethylated 6-hydroxymusizin did not match those for the authentic torachrysone (**10**) reference, we concluded that the first methylation occurred at position 8-OH, leading to isotorachrysone (**9**). The combination of ShwM1- and ShwM2-producing cells resulted in the formation of torachrysone-8-*O*-methyl ether (**1**), which was identified by comparison with a chemically synthesized reference compound (Figure S1).

These data show that ShwM1 and ShwM2 selectively catalyze the first and the second methylation step at positions 8-OH and 6-OH, respectively, of 6-hydroxymusizin (**8**), yielding torachrysone-8-*O*-methyl ether (**1**, Scheme 2), which was anticipated to be the substrate for ShwC. Hence, we produced ShwC heterologously; the gene was amplified from gDNA and cloned into the sucrose-inducible vector pSUC. *Aspergillus aculeatus* served as host organism, as it is known to be eligible for CYP production and testing.[12] In a whole-cell approach, the substrate was incubated with ShwC-producing cells, and the extract was analyzed by LC-DAD-MS. As expected, we observed the dimerization of torachrysone-8-*O*-methyl ether (**1**). Remarkably, two products were formed, each with an *m/z* corresponding to the mass of the expected dimer (Figure 2).

The appearance of two dimer peaks suggested the formation of two bi(torachrysone-8-*O*-methyl ether) 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 ($\delta = 9.67$ 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 ($\delta = 5.96$ ppm, $^4J = 2.2$ Hz

Scheme 2. Methylation of 6-hydroxymusizin (**8**) in the presence of ShwM1 leads to a monomethylated intermediate (isotorachrysone, **9**) that is subsequently methylated through the action of ShwM2 to yield torachrysone-8-*O*-methyl ether (**1**). No formation of torachrysone (**10**) was observed.

Figure 2. HPLC-DAD chromatograms ($\lambda = 350$–430 nm). A) Enzymatic conversion of **1** in the presence of ShwC yielded two dimeric products. B) The negative control (empty vector expression culture) showed only the substrate **1**.

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Figure 3. Dimerization of **1** to alloschweinitzin (**3**) and rufoschweinitzin (**2**) in the presence of ShwC. Binaphthalene **2** is also produced by *C. rufoolivaceus*. Structures were elucidated by NMR analysis; significant signals of aromatic protons in the ^{1}H NMR spectra are shown on the left.

Figure 4. CD spectra (CH$_3$OH) of isolated (from *C. rufoolivaceus*, ——) and enzymatically formed (——) rufoschweinitzin (**2**).
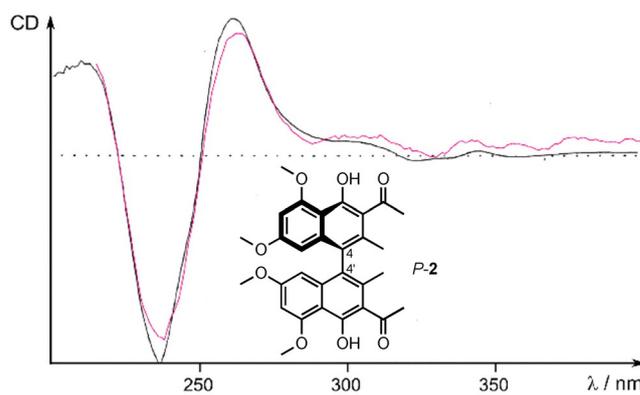
and $\delta = 6.43$ ppm, $^4J = 2.2$ Hz). Consequently, the signal for H-4 with an allylic coupling to the methyl group in **1** is missing (NMR data for **1** are given in the Supporting Information). Because the dimer is unsymmetrical, the coupling position of the other moiety could be either 5′ or 7′. Both coupling positions would lead to the observed doublet at 6.29 ppm ($^4J = 0.8$ Hz), which could be assigned to H-4′, and the aromatic singlet at 6.70 ppm as there is no coupling across C4′a.

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 S6).

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).[15]

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 atropisomer. All spectra exhibit a positive first and a negative second cotton effect, so the 1-1′-binaphthalene system is twisted clockwise (Figures 4, S9 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.[13,25]

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.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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