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Enantioselective Phenol Coupling by Laccases in the Biosynthesis of Fungal Dimeric Naphthopyrones

Sebastian Obermaier,[+] Wiebke Thiele,[+] Leon Fürtges, and Michael Müller*

Dedicated to Ben Feringa and in memory of Hans Wynberg

Abstract: Biaryls are ubiquitous metabolites that are often formed by dimerization through oxidative phenol coupling. Hindered rotation around the biaryl bond can cause axial chirality. In nature, dimerizations are catalyzed by oxidative enzymes such as laccases. This class of enzymes is known for non-specific oxidase reactions while inherent enantioselectivity is hitherto unknown. Here, we describe four related fungal laccases that catalyze γ-naphthopyrone dimerization in a regio- and atropselective manner. In vitro assays revealed that three enzymes were highly *P*-selective (*ee* >95%), while one enzyme showed a remarkable flexibility. Its selectivity for *M*- or *P*-configured dimers varied depending on the reaction conditions. For example, a lower enzyme concentration yielded primarily *P*-ustilaginoidin A, whereas the *M*-atropisomer was favored at higher concentration. These results demonstrate an inherent enantioselectivity in an enzyme class that was previously thought to comprise only non-selective oxidases.

Laccases are copper-dependent one-electron oxidases that act on phenolic compounds, generating phenoxy radicals that undergo subsequent coupling reactions including C–C, C–O, and C–N bond formations. Typically, the outcome of these downstream reactions is not controlled by the laccase, thus leading to a mixture of regio- and stereoisomeric products.[1–4] Recent research on polyketide dimerization in fungi led to the discovery of regioselective laccases.[5,6] In these examples, the polyketide monomers possess several activated positions, of which only one is selected for coupling by a distinct laccase. In contrast to inherent regioselectivity, enantioselectivity has only been found when laccases were combined with other proteins.[7–9] Specifically, so-called dirigent proteins are able to mediate enantioselective coupling of radicals produced by a laccase.[10]

In this study, we focused on a group of laccases that catalyzes coupling reactions in the biosynthesis of ustilaginoidins, dimeric γ-naphthopyrones isolated from different fungal species.[11–15] The archetype is ustilaginoidin A (**1**), which is composed of two identical γ-naphthopyrone moieties (Figure 1). The other congeners differ from ustilaginoidin A (**1**) by Δ^2 double bond saturation, methylation at position 3, or a combination of both as in chaetochromin A (**2**).

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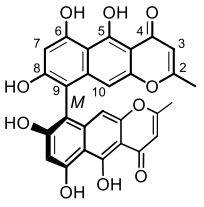
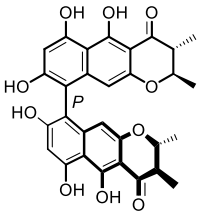
As they possess three possible positions for C–C bond formation (positions 7, 9, and 10), monomeric γ-naphthopyrones can theoretically be coupled to six regioisomeric dimers via oxidative phenol coupling. However, exclusively 9,9′-coupled dimers are found in ustilaginoidin producers, so the coupling was expected to proceed in a regioselective manner. Furthermore, the biaryl bond is axially chiral: the rotation of the naphthopyrone moieties is sterically hindered, resulting in two stable atropisomers. Whereas multiple chemically modified derivatives co-exist in most producer strains, the configuration of the biaryl axis is often the same for all congeners from one fungal strain (Figure 1). For example, more than 20 γ-naphthopyrone 9,9′-dimers have been found in *Ustilaginoidea virens*, all of which are *M*-configured, while for *Chaetomium arcuatum* only *P*-configured compounds are known (Figures S1–S3).[16–19] It may thus be concluded that the biosynthesis of these compounds proceeds via dimerization systems that favor one regio- and atropisomer. The complementarity of the *M*- and *P*-configured metabolites prompted us to investigate the enzymatic systems responsible for dimerization in ustilaginoidin biosynthesis and to characterize their selectivity.

Here, we present laccases that are regioselective and also dimerize γ-naphthopyrone monomers enantioselectively, favoring the formation of either *M*- or *P*-atropisomers. One of the enzymes revealed a marked flexibility, as the atropselectivity was reversed depending on the reaction conditions.



(*M*)-ustilaginoidin A
(*M*)-**1**



(*P*)-chaetochromin A
(*P*)-**2**

Figure 1. Ustilaginoidin A (**1**) from *Ustilaginoidea virens* with unmodified, achiral naphthopyrone moieties, naturally produced in *M*-configuration, and chaetochromin A (**2**) from *Chaetomium arcuatum*, produced in *P*-configuration.

To identify the ustilaginoidin biosynthetic genes, we postulated a biosynthetic pathway as depicted in Scheme 1A (inferred from aurofusarin biosynthesis in *Fusarium graminearum*).[20] It included three enzymes: a non-reducing polyketide synthase (PKS), a dehydratase, and a laccase that acts as the coupling enzyme. By genome analysis of the ustilaginoidin producer *U. virens*, we identified a biosynthetic gene cluster (BGC) comprising genes for all these three expected enzymes.[21] We also found very similar putative BGCs in the

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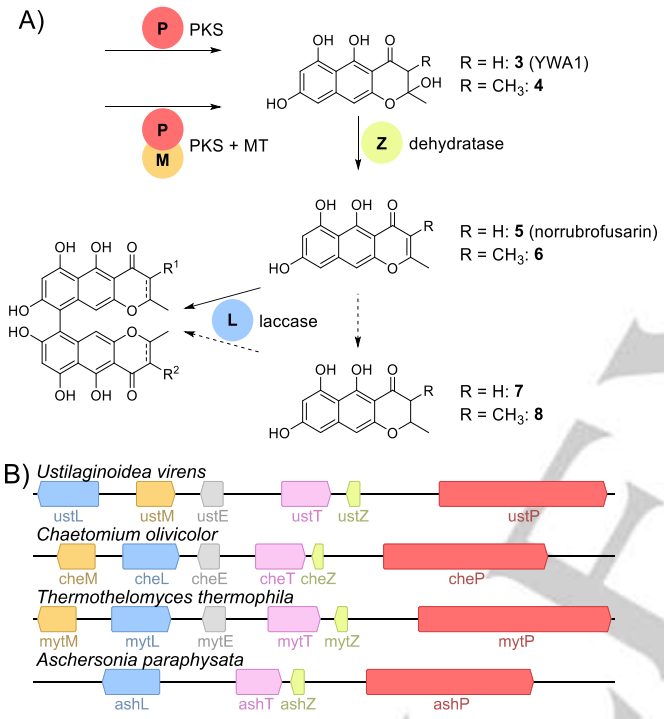
genomes of *Chaetomium olivicolor* and *Thermothelomyces* (*Myceliophthora*) *thermophila*.[22,23] *C. olivicolor* is closely related to the chaetochromin producer *C. arcuatum*, while *Thermothelomyces* species have not been observed to produce ustilaginoidins as yet. In all three investigated organisms, the BGCs are well-defined by a near-syntenic region of exactly six genes (Scheme 1B). In addition to the expected genes noted above, each BGC contains predicted genes coding for a methyltransferase with a phosphopantetheine attachment site, an efflux pump, and a protein with a phospholipid methyltransferase domain. Additionally, a shortened version of the above-mentioned BGCs was found in the genome of *Aschersonia paraphysata*. This gene cluster differs from the others in lacking homologs to the two genes with predicted methyltransferase domains.



Scheme 1. A) Proposed biosynthetic pathway of the ustilaginoidins. PKS: polyketide synthase; MT: methyltransferase. B) Biosynthetic gene clusters. The capital letter of the gene name and the color correspond to the predicted function or domains: M – methyltransferase with phosphopantetheine binding site (orange); L – phenol-coupling laccase (blue); E – phospholipid methyltransferase domain (gray); T – efflux transporter (pink); Z – dehydratase (yellow); P – polyketide synthase (red).

Based on the genes in the BGCs, we postulate a biosynthetic pathway as follows, using *U. virens* as the example.[24] The PKS UstP produces the γ-naphthopyrone precursor YWA1 (**3**), which is followed by a UstZ-catalyzed dehydration reaction to yield norrubrofusarin (**5**).[25] A key enzyme in the biosynthetic pathway is the laccase UstL, which dimerizes two norrubrofusarin molecules by oxidative phenol coupling. For the biosynthesis of 3-methylustilaginoidin derivatives such as chaetochromin A (**2**), we propose that a methylated derivative (**4**) of YWA1 (**3**) is required. The *C*-methylation is considered to be catalyzed by UstM, whose phosphopantetheine attachment site indicates that it acts on the growing polyketide chain before release of the product (see Supporting Information). For the biosynthesis of chaetochromin A (**2**), it is assumed that the saturation of the Δ^2 double bond takes place before dimerization.[24] It is probably catalyzed by an external reductase, as no candidate gene was identified within the BGC.

To substantiate the proposed ustilaginoidin biosynthetic pathway, the product of the *U. virens* polyketide synthase UstP was characterized. For this, *ustP* was heterologously expressed in *Aspergillus aculeatus*; the recombinant fungus produced a yellow-green pigment, which was isolated. HPLC and NMR analysis revealed that it was YWA1 (**3**), confirming our hypothesis regarding the first step in the biosynthetic pathway (Figure 2A).

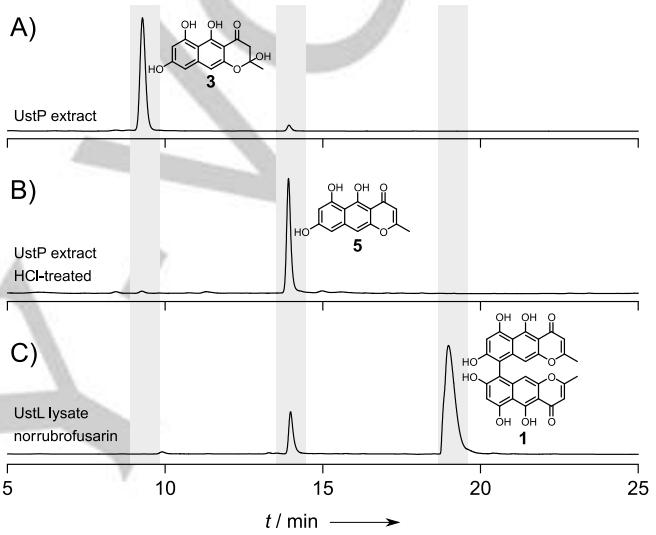


Figure 2. HPLC chromatograms. A) Extract of *Aspergillus aculeatus ustP*, producing YWA1 (**3**) and traces of norrubrofusarin (**5**). B) The same extract after treatment with hydrochloric acid to obtain **5**. C) In vitro coupling of **5** with UstL to yield ustilaginoidin A (**1**).

The essential step in the biosynthetic pathway is the phenol-coupling reaction leading to the dimeric ustilaginoidin scaffold. The complementary *M*- and *P*-selective coupling systems in *U. virens* and *C. arcuatum* allowed us to investigate the stereochemistry of this key reaction. A stereochemical control is conceivable by two different modes: an inherent selectivity of the coupling enzyme or an unknown additional factor such as a dirigent protein. To clarify this, the four laccase genes were expressed in *Aspergillus niger* and the enzymes were tested in vitro. Norrubrofusarin (**5**) was used as substrate because it was anticipated to be the direct precursor of ustilaginoidin A (**1**). Substrate **5** could be prepared in a biomimetic approach by dehydration of YWA1 (**3**) obtained from the recombinant PKS UstP (Figure 2B). Furthermore, norrubrofusarin (**5**) has the advantage that it is achiral and cannot induce stereoselectivity in the coupling reaction.

In an initial experiment, both culture supernatant and lysate (cell-free extract) of *A. niger ustL* expression cultures were used to assay enzymatic activity. Despite the predicted extracellular localization of the protein, no activity was found in the culture supernatant. For the assay of the lysate, however, the HPLC-MS

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chromatogram revealed a peak corresponding to the *m*/*z* of the ionized dimer ustilaginoidin A (**1**), which was not present in the negative controls (Figure S4). Consequently, lysate was utilized for the subsequent in vitro assays, which were carried out in sodium citrate buffer at 25 °C. Norrubrofusarin (**5**) dissolved in dimethyl sulfoxide (DMSO) was added. All four laccases produced the same new compound.

To determine the structure of the new product, enzymatic conversions were performed on a milligram scale. Norrubrofusarin (**5**) was quantitatively converted by successive addition of laccase-containing lysate. The ethyl acetate extract of the reaction mixture was used without further purification to acquire ^{1}H, ^{13}C, and 2D NMR spectra, which matched published spectra of ustilaginoidin A (**1**).[26] Both HPLC and NMR analysis indicated that the enzymatic conversion yielded only one product and was thus 9,9'-regioselective (Figures 2C and S8–S11).

Having established that laccases are the coupling enzymes, we focused on the stereochemistry of the biaryl axis. Based on the configuration of ustilaginoidins isolated from the respective source organisms, we expected that UstL would yield an *M*-configured dimer, while CheL should give the *P*-product. Circular dichroism (CD) spectroscopy was used to determine the stereochemistry of the ustilaginoidin A samples from enzymatic conversions.[16] For a subset of samples, the enantiomeric excess (*ee*) was quantified by chiral-phase HPLC after derivatization (Figure S5). This data was then used to calibrate CD signals of non-derivatized samples to approximate their *ee* values.

The chiroptical data confirmed the expectation for the *M*- and *P*-selective laccases UstL and CheL, respectively. The two laccases MytL and AshL, for which we had no information about a natural product, were both *P*-selective (Figure 3A). As both *M*- and *P*-atropselectivities were observed using different enzymes under otherwise identical conditions, an unidentified factor from the heterologous host inducing the regio- and stereoselectivity can be excluded.

Unexpectedly, the *ee* values of ustilaginoidin A (**1**) from UstL-catalyzed transformations varied between individual experiments, which led us to test the influence of different reaction conditions on the stereochemistry for all enzymes. We probed the influence of an elevated reaction temperature, to 40 °C, as well as an increased concentration of the co-solvent DMSO. The three enzymes CheL, MytL, and AshL were *P*-selective (71–99% *ee*) under all tested conditions. The fourth enzyme, UstL, for which strict *M*-selectivity was expected initially, exhibited a pronounced dependence on the reaction conditions. Both at 40 °C and at the increased DMSO concentration, ustilaginoidin A (**1**) was produced with an excess of the *P*-atropisomer (8% and 39% *ee*, respectively). The *M*-atropisomer was only favored under the original assay conditions at 25 °C (21% *ee*, Figure S6).

Furthermore, the initial experiments indicated that the protein concentration might influence the atropselectivity of UstL. To test this, the amount of lysate in the total reaction volume was varied. Five dilutions were tested for all four enzymes, corresponding to a 250-fold difference between the highest and the lowest concentration. The latter was chosen so that the activity was still detectable. Again, the three enzymes CheL, MytL, and AshL showed almost complete *P*-selectivity (84–99% *ee*). A distinct decrease in selectivity was only observed for the lowest lysate concentration (Figure 3B).

UstL, however, produced both atropisomers in varying amounts. Each concentration step resulted in an incremental decrease of the *P*-atropisomer leading to a reversal from *P*- to *M*-selectivity (Figure 3B). With the lowest concentration of UstL, an *ee* of 32% for the *P*-atropisomer was determined, while higher concentrations of UstL gave an *ee* of around 55% for the *M*-atropisomer. These experiments were repeated with enriched enzyme obtained by fractionated acetone precipitation. The results were congruent with those of the lysate assays. Again, a dependence of the atropselectivity on the enzyme concentration was observed, substantiating the findings on the flexibility of UstL (Figure S7).

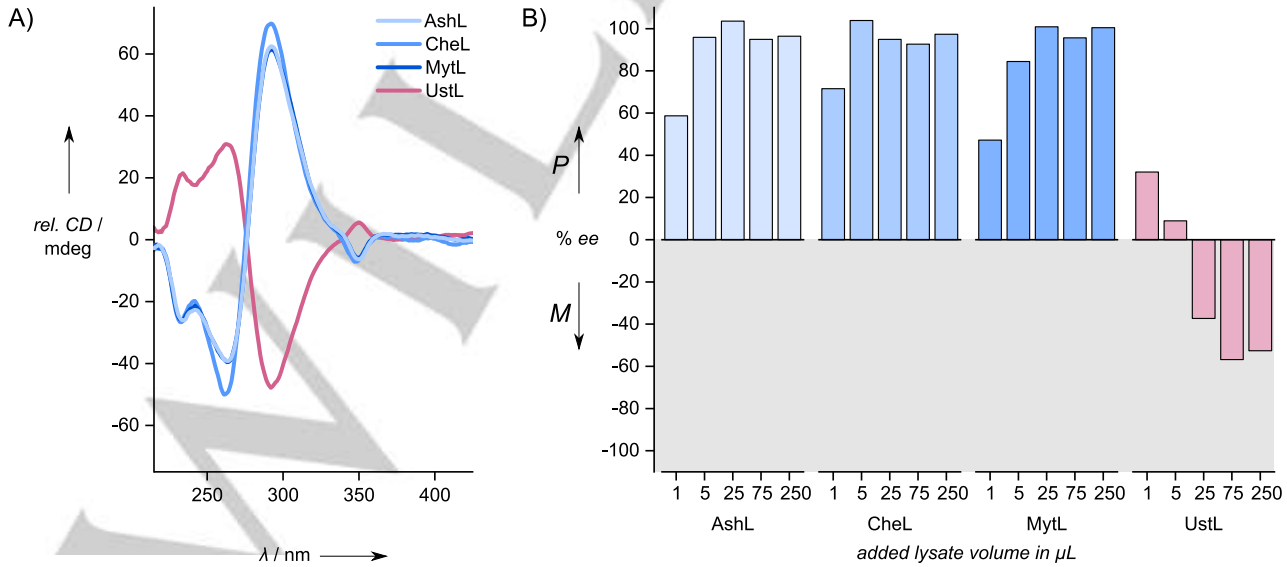

Figure 3. A) Example CD spectra of ustilaginoidin A (**1**) samples from laccase-catalyzed coupling. Spectra are normalized to a UV absorbance of 1.0 at 288 nm. B) Direct CD measurement of *ee* values of ustilaginoidin A formed by in vitro phenol coupling with the laccases AshL, CheL, MytL, and UstL. The amount of lysate was varied in a total reaction volume of 251 µL.

Until now, enantioselective phenol-coupling reactions have been mainly associated with cytochrome P450 enzymes. Even in biosynthetic pathways of other dimeric γ-naphthopyrones similar to ustilaginoidins, cytochrome P450 enzymes catalyze the dimerization reactions.[27] However, the group of laccases described here possesses an unprecedented selectivity toward the formation of the biaryl bond, making this a remarkable example of convergent evolution.

In summary, we have demonstrated inherent atropselectivity for four phenol-coupling laccases; their function does not depend on any dirigent proteins from the source organisms. Three of the laccases were consistently *P*-atropselective under all tested conditions, while one enzyme (UstL) was modifiable, enabling access to both enantiomers of the product. This illustrates the tendency of nature towards diversity-oriented biosynthesis.[28] Yet, the biological relevance of flexibility for *U. virens* remains to be examined, as its natural products occur in one fixed configuration. Although UstL was the only laccase that substantially changed selectivity, it is possible that this flexible atropselectivity is also present in the other three laccases, only not observed under the tested conditions. The factors affecting the selectivity, such as possible concentration-dependent oligomerization states of the laccases, remain to be investigated. We expect that stereoselectivity in laccases is not unique to polyketide dimerization and that further specialized laccases will be identified in various metabolic pathways.

Acknowledgements

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Keywords: atropisomerism • dimeric polyketides • enantioselectivity • oxidoreductases • phenol coupling

[1] F. Xu, *Biochemistry* **1996**, *35*, 7608–7614.

[2] M. A. Constantin, J. Conrad, U. Beifuss, *Green Chem.* **2012**, *14*, 2375–2379.

[3] C. Engelmann, S. Illner, U. Kragl, *Process Biochem.* **2015**, *50*, 1591–1599.

[4] T. Kudanga, B. Nemadziva, M. Le Roes-Hill, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 13–33.

[5] M. Kawaguchi, T. Ohshiro, M. Toyoda, S. Ohte, J. Inokoshi, I. Fujii, H. Tomoda, *Angew. Chem. Int. Ed.* **2018**, *57*, 5115–5119.

[6] L. Fürtges, S. Obermaier, W. Thiele, S. Foegen, M. Müller, *ChemBioChem* **2019**, DOI 10.1002/cbic.201900041.

[7] I. Effenberger, B. Zhang, L. Li, Q. Wang, Y. Liu, I. Klaiber, J. Pfannstiel, Q. Wang, A. Schaller, *Angew. Chem. Int. Ed.* **2015**, *54*, 14660–14663.

[8] J. Hu, F. Sarrami, H. Li, G. Zhang, K. A. Stubbs, E. Lacey, S. G. Stewart, A. Karton, A. M. Piggott, Y.-H. Chooi, *Chem. Sci.* **2019**, *10*, 1457–1465.

[9] A. S. Urquhart, J. Hu, Y.-H. Chooi, A. Idnurm, *bioRxiv Microbiol.* **2019**, DOI 10.1101/601716.

[10] L. B. Davin, H.-B. Wang, A. L. Crowell, D. L. Bedgar, D. M. Martin, S. Sarkanen, N. G. Lewis, *Science* **1997**, *275*, 362–367.

[11] S. Shibata, Y. Ogihara, A. Ohta, *Chem. Pharm. Bull.* **1963**, *11*, 1179–1182.

[12] G. Tertzakian, R. H. Haskins, G. P. Slater, L. R. Nesbitt, *Proc. Chem. Soc.* **1964**, 195.

[13] M. Matsumoto, H. Minato, E. Kondo, T. Mitsugi, K. Katagiri, *J. Antibiot.* **1975**, *28*, 602–604.

[14] S. Sekita, K. Yoshihira, S. Natori, *Chem. Pharm. Bull.* **1980**, *28*, 2428–2435.

[15] X. Kong, X. Ma, Y. Xie, S. Cai, T. Zhu, Q. Gu, D. Li, *Arch. Pharmacal Res.* **2013**, *36*, 739–744.

[16] K. Koyama, S. Natori, Y. Iitaka, *Chem. Pharm. Bull.* **1987**, *35*, 4049–4055.

[17] S. Natori, K. Koyama, *Chem. Pharm. Bull.* **1987**, *35*, 578–584.

[18] J. H. Cardellina, V. I. Roxas-Duncan, V. Montgomery, V. Eccard, Y. Campbell, X. Hu, I. Khavrutskii, G. J. Tawa, A. Wallqvist, J. B. Gloer, et al., *ACS Med. Chem. Lett.* **2012**, *3*, 387–391.

[19] W. Sun, A. Wang, D. Xu, W. Wang, J. Meng, J. Dai, Y. Liu, D. Lai, L. Zhou, *J. Agric. Food Chem.* **2017**, *65*, 5151–5160.

[20] J.-E. Kim, K.-H. Han, J. Jin, H. Kim, J.-C. Kim, S.-H. Yun, Y.-W. Lee, *Appl. Environ. Microbiol.* **2005**, *71*, 1701–1708.

[21] T. Kumagai, T. Ishii, G. Terai, M. Umemura, M. Machida, K. Asai, *Genome Announc.* **2016**, *4*, 851–874.

[22] Concordia University, "Genozymes Project Public Genomes", can be found under http://www.fungalgenomics.ca/, **2014**.

[23] R. M. Berka, I. V. Grigoriev, R. Otillar, A. Salamov, J. Grimwood, I. Reid, N. Ishmael, T. John, C. Darmond, M. C. Moisan, et al., *Nat. Biotechnol.* **2011**, *29*, 922–929.

[24] During preparation of this manuscript, Li et al. published knockout studies on ustilaginoidin biosynthesis genes in *U. virens*. The genes are identical to the *ust* BGC covered in this work. Y. Li, M. Wang, Z. Liu, K. Zhang, F. Cui, W. Sun, *Environ. Microbiol.* **2019**, DOI 10.1111/1462-2920.14572.

[25] R. J. N. Frandsen, C. Schütt, B. W. Lund, D. Staerk, J. Nielsen, S. Olsson, H. Giese, *J. Biol. Chem.* **2011**, *286*, 10419–10428.

[26] S. Lu, W. Sun, J. Meng, A. Wang, X. Wang, J. Tian, X. Fu, J. Dai, Y. Liu, D. Lai, et al., *J. Agric. Food Chem.* **2015**, *63*, 3501–3508.

[27] S. Obermaier, M. Müller, *Manuscript submitted for publication* **2019**.

[28] R. D. Firn, C. G. Jones, *Nat. Prod. Rep.* **2003**, *20*, 382.

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Layout 1:

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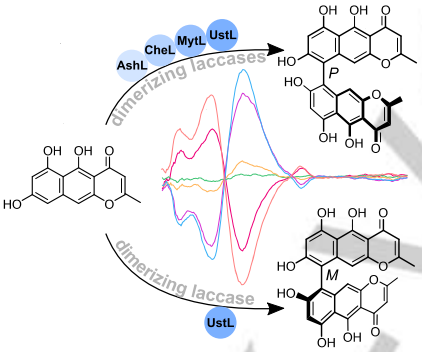
Lonesome laccases: Several fungi selectively produce atropisomers of biarylic polyketides. Having identified laccases as the dimerizing enzymes, we demonstrated their inherent enantioselectivity, favoring either the *M*- or *P*-atropisomer. One enzyme even reversed its atropselectivity upon changed reaction conditions. Contrary to previous assumptions, our findings show that some laccases can be atropselective without the help of dirigent proteins.



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