Three Redundant Synthetases Secure Redox-Active Pigment Production in the Basidiomycete *Paxillus involutus*

**Highlights**
- Diarylcyclopentenone biosynthesis proceeds via atromentin as precursor
- Atromentin biosynthesis in *Paxillus involutus* is a parallelized process
- A thioesterase signature sequence indicative for quinone formation was identified
- Quinone synthetases are amenable to engineering by domain swaps

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**In Brief**
Diarylcyclopentenones, produced by the symbiotic fungus *Paxillus involutus*, are redox-active metabolites involved in carbon cycling as they serve Fenton-based decomposition of lignocellulose in forest ecosystems. Braesel et al. show that the fungus uses three enzymes in parallel to secure the key step in diarylcyclopentenone biosynthesis.
Three Redundant Synthetases Secure Redox-Active Pigment Production in the Basidiomycete *Paxillus involutus*

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### SUMMARY

The symbiotic fungus *Paxillus involutus* serves a critical role in maintaining forest ecosystems, which are carbon sinks of global importance. *P. involutus* produces involutin and other 2,5-diarylcyclopentenone pigments that presumably assist in the oxidative degradation of lignocellulose via Fenton chemistry. Their precise biosynthetic pathways, however, remain obscure. Using a combination of biochemical, genetic, and transcriptomic analyses, in addition to stable-isotope labeling with synthetic precursors, we show that atromentin is the key intermediate. Atromentin is made by tridomain synthetases of high similarity: InvA1, InvA2, and InvA5. An inactive atromentin synthetase, InvA3, gained activity after a domain swap that replaced its native thioesterase domain with that of InvA5. The found degree of multiplex biosynthetic capacity is unprecedented with fungi, and highlights the great importance of the metabolite for the producer.

### INTRODUCTION

The basidiomycete model fungus *Paxillus involutus* (poison pax mushroom, *Boletales*) displays a Janus-faced existence. It can cause a deadly hemolytic autoimmune reaction, infamously known as Paxillus syndrome, upon repeated ingestion of its carpophores. On the other hand, this species is of critical ecological importance, and thus represents one of the best-studied symbiotic fungi on a molecular, physiological, and environmental level. It forms symbiotic associations, so-called ectomycorrhizae, with various trees in managed and unmanaged forests, among them dominant and both silviculturally and economically important species such as spruce, pine, birch, poplar, and beech (Wallander and Söderström, 1999). A remarkable feature of *P. involutus* and numerous other species within the *Boletales* is their capacity to synthesize brightly colored aromatic pigments. These natural products fall into various subclasses, among them the pulvinic acids and the 2,5-diarylcyclopentenones, e.g., involutin and gyrocyanin (Edwards et al., 1967; Edwards and Gill, 1973; Besl et al., 1973; Steglich et al., 1977; Figure 1). These pigments display redox activity and are assumed to play a crucial role in the reduction of Fe$^{3+}$ during the Fenton-based decomposition of litter material by *P. involutus* (Eastwood et al., 2011; Shah, 2014). Decomposition of such material by ectomycorrhizal fungi are thought to play a key role in mobilizing nutrients embedded in recalcitrant organic matter complexes, thereby making them accessible to the host plant (Lindahl and Tunlid, 2015).

Details of the pigment biosynthesis have been profoundly studied on a chemical level (reviewed by Gill and Steglich, 1987 and Zhou and Liu, 2010). Stable-isotope labeling confirmed L-tyrosine as the metabolic origin and that the terphenylquinone atromentin (Figure 1) is the direct precursor of the pulvinic acids (Hermann, 1980; Gill and Steglich, 1987). However, the biogenesis of 2,5-diarylcyclopentenones has remained enigmatic, as two routes are conceivable that either include an atromentin-independent direct condensation of 4-hydroxyphenylpyruvic acid (Figure 1, route 1) or involve atromentin formation (route 2), which then undergoes oxidative ring contraction or conversion to atromentin acid to form the cyclopentenone skeleton.

Here, we report on the biosynthesis of diarylcyclopentenone pigments in *P. involutus* and show that atromentin is their metabolic precursor, which is biosynthesized in a multiplexed process. Evidence comes from (1) in vitro characterization of six wild-type and two artificially created chimeric quinone synthetases, (2) transcriptomic data, and (3) stable-isotope labeling to track the turnover of precursors by *P. involutus*. The chimeric quinone synthetases demonstrate that successful engineering of these enzymes by replacing an entire domain is feasible. We also characterized the *P. involutus* phosphopanteinyl transferase PptA, which converts the above quinone synthetases from the inactive apo into their functional holo forms.
RESULTS AND DISCUSSION

Genetic and Transcriptional Characterization of invA Genes

Diarylcyclopentenone natural products originate from the shikimic acid pathway that is likely extended by an AtrD-like transaminase activity (Schneider et al., 2008) to produce 4-hydroxyphenylpyruvic acid from L-tyrosine. However, the mechanistic basis for the condensation of two 4-hydroxyphenylpyruvic acid units into the diarylcyclopentenone scaffold has remained obscure. Two routes appear plausible (Figure 1, routes 1 and 2), which either require or bypass atromentin as a precursor. In the atromentin-dependent scenario that requires atromentin synthetase activity (route 2), atromentin may undergo oxidative ring contraction to yield the cyclopentenone scaffold in a one-step process, or is converted into atromentic acid, i.e., a butenolide, which then serves as intermediate for cyclopentenone formation (Gill and Steglich, 1987). An alternative biosynthetic scenario (route 1) includes a direct decarboxylative condensation (black and red arrows between 4-hydroxyphenylpyruvic acid units) into gyrocyanin, i.e., the common diarylcyclopentenone intermediate. Experimentally verified route 2 (blue) involves symmetric condensation (black and blue arrows between 4-hydroxyphenylpyruvic acid units) into atromentin, followed by oxidative ring contraction to yield gyrocyanin. Dotted arrows indicate a possible shunt within route 2 via atromentic acid.

Figure 1. L-Tyrosine-Derived Paxillus Pigments
Schematic of pigment biosynthesis. L-Tyrosine is deaminated to 4-hydroxyphenylpyruvic acid by a PLP-dependent transaminase, followed by covalent tethering as a thioester and oxoester onto the thiolation and thioesterase domains, respectively, of the InvAx synthetases (x = 1, 2, 5). Two alternative theoretical routes may complete cyclopentenone biosynthesis. Route 1 (red) includes direct decarboxylative condensation (black and red arrows between 4-hydroxyphenylpyruvic acid units) into gyrocyanin, i.e., the common diarylcyclopentenone intermediate. Experimentally verified route 2 (blue) involves symmetric condensation (black and blue arrows between 4-hydroxyphenylpyruvic acid units) into atromentin, followed by oxidative ring contraction to yield gyrocyanin. Dotted arrows indicate a possible shunt within route 2 via atromentic acid.
superfamily (orf11–orf15), an oxidoreductase (orf1), a glycosyltransferase (orf5), an aldo-keto reductase (orf10), and an alcohol dehydrogenase (orf12).

Transcriptomic analysis identified invA1 to invA6 as expressed genes, when the fungus is grown in axenic culture, regardless of the media tested. Analysis of relative transcript levels showed a high abundance of invA1, invA2, and invA5 transcripts, and moderate levels of invA6 mRNA. In contrast, invA3 and invA4 were only poorly expressed. When using media containing organic nitrogen, we found a more than 13-fold and 15-fold higher number of transcripts encoding invA1 and invA5, respectively, compared with invA3 or invA4. The transcripts of invA1 and invA5 were about 5-fold more abundant than those of invA6, and 1.4-fold and 2-fold higher compared with invA2. The experimentally verified cDNA sequences for invA1 and invA3 deviate from the predicted sequences deposited on the JGI server. For these genes, additional 673- and 595-bp introns, respectively, were erroneously predicted in the center portion of the reading frame. By amplification of partial sequences of genomic and cDNA, we showed that the protein sequence of InvA3 is one amino acid shorter than predicted.

The genes invA1 possesses three and invA2–invA4 four introns, which are between 50 and 61 bp in length (Figure 2B). The intron positions are conserved in the InvA orthologs atrA and greA. However, these genes are interrupted by a fifth intron in their 3’-terminal portions. For invA3 and invA6, erroneously spliced transcripts were found that either included retained introns or erroneously removed exonic sequences. The resulting shifted reading frames lead to premature stop codons and, consequently, non-functional proteins. Transcript variants of a gene may be interpreted as a posttranscriptional regulatory mechanism (Conti and Izaurralde, 2005) and are also described for the basidiomycetes Phanerochaete chrysosporium and Armilaria mellea (Larrondo et al., 2004; Misiek and Hoffmeister, 2008).

**P. involutus 4’-Phosphopantetheinyl Transferase**

For enzymatic activity, multidomain biosynthesis enzymes, such as peptide and quinone synthetases, strictly require posttranslational modification by 4’-phosphopantetheinyl (4’-PPT) transfer to a conserved serine residue within the T domain. This 4’-PPT transfer is catalyzed by dedicated transferases (so-called Sfp-type 4’-PPTase). As no such enzyme of basidiomycete origin has yet been described or characterized, we carried out a tblastn search across the *P. involutus* genome, using the sequence of *Aspergillus nidulans* 4’-PPTase NpgA (Keszenman-Pereyra et al., 2003) as query. A gene encoding a 263-amino-acid putative Sfp-type 4’-PPTase (OMIM: 174503) was identified on scaffold 3 of the *P. involutus* genome. The 1,004-bp gene includes four introns and is referred to as pptA hereafter. PptA shares 54% identical amino acids with a predicted 4’-phosphopantetheinyl transferase of *Gloeophyllum trabeum* ATCC 11539 (accession number NCBI: XP_007861898).

**Biochemical Characterization of Putative Synthetases**

The apo-proteins InvA1–A6 were heterologously produced in *Escherichia coli* KRX and SoluBL as N-terminally hexahistidine-tagged fusion proteins (Figure S1). Substrate specificity, temperature, and pH optima of the A domains of apo-InvA1–A6 were...
characterized using the ATP-[³²P]pyrophosphate radiolabel exchange assay.

As *Paxillus* pigments originate from the shikimic acid pathway, aromatic 2-oxo acids and aromatic L-amino acids were tested as potential substrates, along with pyruvic acid and L-alanine as controls. Strongest pyrophosphate exchange by InvA₁–A₃ and InvA₅ was observed with 4-hydroxyphenylpyruvic acid (InvA₁: 487,600 cpm; InvA₂: 255,600 cpm; InvA₃: 325,200 cpm; InvA₅: 241,100 cpm; [Figure 3]). 4-Hydroxyphenylpyruvic acid is the expected initial building block for diarylcyclopentenones, irrespective of the biosynthetic route. With the pyrophosphate exchange normalized with respect to 4-hydroxyphenylpyruvic acid, phenylpyruvic acid was turned over between 5.8% and 37.4%, and L-tyrosine between 2.1% and 26%, respectively. However, these compounds and a subsequently tested extended set of potential substrates, i.e., pools comprising 37 compounds (L-amino acids, α-keto acids, and aromatic carboxylic acids, Table S2) were rejected by InvA₄ and InvA₆ (<2,000 cpm; [Figures 3 and S1]). Therefore, their substrate preferences remain unknown but are incongruent with those of the other tested InvA enzymes. Alternatively, InvA₄ and InvA₆ may have lost enzymatic activity. Crystallography and biochemical evidence have established ten key amino acid residues within the primary amino acid sequence of A domains that are most likely involved in substrate recognition (Stachelhaus et al., 1999). This sequence signature, also referred to as non-ribosomal code, of the InvA enzymes is V-A-E-F-S-G-G-A-C-K, except for InvA₄, whose sequence bears a glycine instead of a glutamic acid at the third position (V-A-G-F-S-G-G-A-C-K). This signature is conserved in the biochemically characterized atromentin synthetases AtrA and GreA (Schneider et al., 2008; Wackler et al., 2012). Therefore, our biochemical results confirm this signature as specific non-ribosomal code for aromatic α-keto acid-activating synthetases. Optimal pyrophosphate exchange with InvA₁–A₃ and InvA₅ took place at 20°C (InvA₂, InvA₃) and 25°C (InvA₁, InvA₅), and at pH 7.2, 7.6, 6.8, and 7.4 for InvA₁, InvA₂, InvA₃, and InvA₅, respectively.

InvA₁–A₃ and InvA₅ were treated in vitro with the heterologously produced 4’-PPTases Svp (Sanchez et al., 2001) and, in separate reactions, with *P. involutus* PptA ([Figure S1]), to produce the *holo*-enzyme. *Holo*-InvA enzymes were separately incubated with 1.8 mM 4-hydroxyphenylpyruvic acid under optimum conditions in Mg²⁺-containing Tris buffer and 2.5 mM ATP. After 16 hr of incubation, the reactions containing InvA₁, InvA₂, and InvA₅, respectively, turned violet, irrespective of the 4’-PPTase used for priming.

In negative controls without ATP or a 4’-PPTase, this coloration was not observed. The UV-visible spectrum, high-resolution mass (found *m/z* 323.0555 [M – H]⁻, calculated for C₁₈H₁₂O₆: *m/z* 323.0561 [M – H]⁻), and retention time of the colored compound were identical to an authentic atromentin sample ([Figure 4]). We therefore conclude that InvA₁, InvA₂, and InvA₅ are atromentin synthetases, which is consistent with earlier reports that *P. involutus* does have the capacity to produce atromentin (Bresinsky and Rennschmidt, 1971; Bresinsky, 1974).

Usually, fungi rely on one set of small-molecule biosynthetic pathway genes per haploid genome. A three-fold redundantly secured natural product biosynthesis pathway is unprecedented with fungi, but points to an indispensable ecological role of the diarylcyclopentenone products, likely to degrade organic matter in forest ecosystems. This feature is somewhat reminiscent of a duplicated cluster of genes in *Aspergillus flavus*, which encode the production of the same set of piperazines that are essential for sclerotia formation (Forseth et al., 2013).

Product formation was not observed with InvA₃, whereas the ATP-[³²P]pyrophosphate exchange assay verified that the A domain is active and adenylates 4-hydroxyphenylpyruvic acid. Therefore, we hypothesized that the TE domain that would...
catalyze symmetric condensation of two 4-hydroxyphenylpyruvic acid units into a substituted benzoquinone may be inactive. Furthermore, we assumed that replacing the InvA3 TE domain by an active *P. involutus* atromentin synthetase might reconstitute its functionality. To test this hypothesis, we constructed a chimeric gene (invA335) that encoded the InvA3 A-T didomain fused to the InvA5 TE domain. For negative control, we also created the inverse chimera, termed invA553, encoding a synthetase composed of the InvA5 A-T didomain and the InvA3 TE domain. For product formation, either chimera was heterologously produced as hexahistidine fusion in *E. coli* (for SDS gel picture, see Figure S1), converted in vitro into the holo form by 4'-PPTases Svp or PptA, and incubated under optimum conditions, as described above.

High-performance liquid chromatography (HPLC) analysis clearly showed atromentin production in vitro by the chimera InvA335, whereas the chimeric synthetase InvA553 did not produce atromentin, or any other compound, in detectable amounts (Figure 4). This result demonstrates that the wild-type InvA3 enzyme is inactive because of its non-functional TE domain, and gains full functionality when the TE domain is replaced. Engineering of quinone synthetases has not been reported as yet. Our results demonstrate that they are amenable to domain swaps. This finding may open up new avenues of research to engineer multimodular enzymes, as quinone synthetases represent a simple model for the study of interdomain interactions that involve TE domains.

**Signature Sequence of Quinone-Forming Thioesterase Domains**

Numerous non-ribosomal peptide synthetases (NRPSs) rely on a TE domain for product release (Schwarzer et al., 2003). Crystallographic analysis of the surfactin A synthetase SrfA-C and bioinformatics analyses revealed that TE domains belong to the α/β-hydrolase superfamily (Bruner et al., 2002; Samuel et al., 2006; Cantu et al., 2010). The TE domain catalytic triad is composed of the residues Ser80, Asp107, and His207 (numbering according to Bruner et al., 2002). The InvA synthetases show strictly conserved serine and histidine residues, with the second residue of the triad being variable (Table 1). InvA3, InvA4, and InvA6 harbor the conserved aspartate residue. In those enzymes that showed quinone synthetase activity, i.e., InvA1, InvA2, and InvA5, this residue was replaced by an asparagine (Table 1). We compared the sequences of all characterized quinone and furanone synthetases available from the literature, which include NPS3, TdiA, Atra, GreA, Bthi0204, EchA, RalA, and MicA (Eastwood et al., 2011; Schneider et al., 2007, 2008; Wackler et al., 2011, 2012; Biggins et al., 2011; Zhu et al., 2014; Yeh et al., 2012), along with TE domains of multimodular NRPSs, and identified a dichotomous motif pattern. Quinone synthetases, including InvA1, InvA2, and InvA5, consistently...
showed a Ser/Asn/His triad, with the asparagine being followed by a branched-chain aliphatic amino acid (leucine or isoleucine) and two proline residues. Synthetases catalyzing furanone assembly (RalA and MicA) and thioesterases of macrolacton/macrocycle-forming peptide synthetases SrfTE, FenTE, TycTE, and GrsTE (Bruner et al., 2002; Samel et al., 2006; Trauger et al., 2000; Hoyer et al., 2007) showed the regular Ser/Asp/His triad (Table 1). The aspartate residue within the catalytic triad showed product formation (data not shown). We therefore conclude that the adenylation and thioesterase domains of InvA6 are inactive. Taken together, these findings support our in vitro data of InvA3, InvA4, and InvA6 as not being relevant for atromentin production.

**Stable-Isotope Labeling**

InvA1, InvA2, and InvA5 were shown to possess atromentin synthetase activity, while InvA3 turned functional only after artificially replacing its TE domain, probably because of the aforementioned deviating TE motifs. InvA4 and InvA6 did not accept 4-hydroxyphenylpyruvic acid but did show product formation (data not shown). We therefore conclude that the adenylation and thioesterase domains of InvA6 are inactive. Taken together, these findings support our in vitro data of InvA3, InvA4, and InvA6 as not being relevant for atromentin production.

**Table 1. Catalytic Triads of InvA1–A6, Other NRPS-like Enzymes, and NRPSs, Based on Alignment of Their TE Domains with GENEIOUS Software, Version 7.1.4, Using SrfTE as Reference**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Catalytic Triad</th>
<th>Product</th>
<th>Protein Accession #/NCBI Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>InvA3</td>
<td><em>Paxillus involutus</em></td>
<td>GYSYG…IID…-I-H-H-T</td>
<td>unknown</td>
<td>127833</td>
</tr>
<tr>
<td>InvA4</td>
<td><em>Paxillus involutus</em></td>
<td>GYSYG…IID…-MI-H-H-T</td>
<td>127875</td>
<td></td>
</tr>
<tr>
<td>InvA6</td>
<td><em>Paxillus involutus</em></td>
<td>GYSYG…LDIDIPP…H-H-D-T</td>
<td>69026</td>
<td></td>
</tr>
<tr>
<td>InvA1</td>
<td><em>Paxillus involutus</em></td>
<td>GYSYG…LNIIPP…H-Q-H-T</td>
<td>166672</td>
<td></td>
</tr>
<tr>
<td>InvA2</td>
<td><em>Paxillus involutus</em></td>
<td>GYSYG…LNIIPP…H-Q-H-Y</td>
<td>69019</td>
<td></td>
</tr>
<tr>
<td>InvA5</td>
<td><em>Paxillus involutus</em></td>
<td>GYSYG…LNIIPP…H-Q-Y-T</td>
<td>77684</td>
<td></td>
</tr>
<tr>
<td>AtrA</td>
<td><em>Tapinella panuoides</em></td>
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<td></td>
</tr>
<tr>
<td>GreA</td>
<td><em>Streptomyces griseus</em></td>
<td>GYSYG…LNIIPP…H-Q-H-Y</td>
<td>AFB76152</td>
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<tr>
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<td>EG023141.1</td>
<td></td>
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<tr>
<td>TdiA</td>
<td><em>Aspergillus nidulans</em></td>
<td>GYSFG…SWNLIPP…H-Q-A-H-Y</td>
<td>CBF80711.1</td>
<td></td>
</tr>
<tr>
<td>Bthl0204</td>
<td><em>Burkholderia pseudomallei</em></td>
<td>GYSYG…SFNNLP…H-Q-E-H-Y</td>
<td>CAH57575</td>
<td></td>
</tr>
<tr>
<td>EchA</td>
<td><em>Streptomyces sp. L235</em></td>
<td>GYSYG…SFNNLP…H-Q-P-H-Y</td>
<td>YP_008789943</td>
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</tr>
<tr>
<td>RalA</td>
<td><em>Ralstonia solanacearum</em></td>
<td>GYSYG…LSIADDAPPV…H-Q-E-H-T</td>
<td>AEC03966.1</td>
<td></td>
</tr>
<tr>
<td>GrsB</td>
<td><em>Bacillus brevis</em></td>
<td>GYSYG…LSFDFV…-YW…H-Q-S-N</td>
<td>CAA43838</td>
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<tr>
<td>TyC</td>
<td><em>Bacillus brevis</em></td>
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<td>O30409</td>
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<tr>
<td>FenB</td>
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<td></td>
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<tr>
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<td><em>Bacillus subtillis</em></td>
<td>GYSAG…MVDSS…-YK…H-Q-G-T-H-A-E</td>
<td>WP_029878554</td>
<td></td>
</tr>
</tbody>
</table>

After Bruner et al. (2002).

The residues of the catalytic triad Ser80, Asp107, and His207 (numbering according to SrfTE) are marked in bold. Enzymatic function deduced from indirect evidence, as these enzymes have not yet been characterized biochemically.
chromatographic and mass spectrometric characteristics with a synthetic reference (see Supplemental Experimental Procedures). Involutin was found only in non-deuterated form (m/z 313.0723 [M – H]-), likely due to the mycelial homogenate failing to reduce gyrocyanin into chamonixin (Figure 1), with the latter only being detectable in negligible traces and in non-deuterated form by mass spectrometry.

3′,3″,5′,5″-D4-atromentin was also added to intact P. involutus cultures at 1.5 mM final concentration. As fungi usually secrete atromentin and/or its follow-up products out of the cells and given the competing cellular biosynthesis of non-labeled atromentin, we expected very minor quantities of deuterated atromentin being taken up and catalytically converted by the cells, and being secreted back into the medium. We therefore resorted to single-ion monitoring high-resolution electrospray ionization mass spectrometry (HRESI-MS) for analysis. The mass spectrometric data (Figure S2) supported the above findings, as they demonstrated the presence of tetradeuterated gyrocyanin and gyroporin as well. We also detected the mass (m/z 301.1017 [M – H]-) of tetradeuterated chamonixin (Besl et al., 1980), the biosynthetic follow-up product downstream of gyrocyanin. Unlabeled involutin, which is three biosynthetic steps away from atromentin, we expected very minor quantities of deuterated atromentin being taken up and catalytically converted by the cells, and being secreted back into the medium. We therefore resorted to single-ion monitoring high-resolution electrospray ionization mass spectrometry (HRESI-MS) for analysis. The mass spectrometric data (Figure S2) supported the above findings, as they demonstrated the presence of tetradeuterated gyrocyanin and gyroporin as well. We also detected the mass (m/z 301.1017 [M – H]-) of tetradeuterated chamonixin (Besl et al., 1980), the biosynthetic follow-up product downstream of gyrocyanin. Unlabeled involutin, which is three biosynthetic steps away from atromentin, was expected to be present in negligible traces and in non-deuterated form by mass spectrometry.

Figure 5. Chromatographic Analysis of the Isotope-Labeling Experiment

(A) Tetradeuterated atromentin, added to P. involutus mycelial homogenate. (B and C) Authentic atromentin (B) and synthetic gyrocyanin (C). Chromatographic signals: tR = 25.7 min for involutin (compound 1); tR = 38.9 min for gyroporin (2); tR = 52.3 min for gyrocyanin (3); tR = 60.4 min for atromentin (4). Detection wavelength was λ = 254 nm. Mass spectra of mycelial homogenate supplemented with unlabeled atromentin are shown in Figure S2.

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atromentin, and atromentic acid, which is a direct follow-up product of atromentin, were present in traces (Figure S2). Our data cannot fully rule out a route involving atromentic acid, i.e., a butenolide intermediate. However, our data strongly favor the latter model, since merely traces of atromentic acid were detectable by HRESI-MS, and diacylcyclopentenone biosynthesis in P. involutus proceeds through the terphenylinouquinine atromentin. This compound represents the common intermediate of both the atromentic acid/pulvinic acid and the thelephoric acid family of fungal pigments. Our results expand its significance by adding the cyclopentenones as another atromentin-derived class of ecologically relevant basidiomycete natural products.

**SIGNIFICANCE**

The basidiomycete *P. involutus* represents a model organism for ectomycorrhizae. These tree-fungus symbioses are critical for functional forest ecosystems, which are carbon ism for ectomycorrhizae. These tree-fungus symbioses are maintained carbon cycling. This chemistry requires redox-active 2,5-diarylcyclopentenone pigments, e.g., involutin. Our study demonstrates that the biosynthetic key intermediate is the terphenylinouquinine atromentin, which is supplied in a parallelized physiological process. The highly and simultaneously expressed genes *invA1*, *invA2*, and *invA5* encode functional three-domain quinone synthetases to secure atromentin biosynthesis. A three-fold multiplex natural product pathway is unprecedented with fungi, yet underlines the ecological significance of environmentally relevant redox-active cyclopentenones.

**EXPERIMENTAL PROCEDURES**

**General Procedures and Culture Conditions**

*P. involutus* ATCC 200175 was routinely grown at 23°C as still culture on liquid modified Melin-Norkrans (MMN) medium. Molecular genetic procedures were carried out according to the manufacturers’ instructions (Fermentas, NEB, Promega) or as described below. Chemicals were purchased from Alfa Aesar, Sigma-Aldrich, Roth, and VWR, except [32P]pyrophosphate, which was obtained from PerkinElmer, and deuterated -tyrosine (ring-3.5-D2, 98%), which was purchased from Cambridge Isotope Laboratories. The genomic sequence of *P. involutus* ATCC 200175 is available through the Mycobios genome portal at the Joint Genome Institute (Kohler et al., 2015).

**Cloning of invA1-invA6 and pptA cDNAs**

The SV total RNA isolation system (Promega) was used to purify total RNA from *P. involutus* mycelium. First-strand synthesis was primed with a 16-mer oligo(dT)-primer (40 pmol) and carried out in the supplied buffer, with MgCl2 3 mM MgCl2, 0.2 mM each dNTP, 40 pmol (each) primer (Table S1), and 1 U Phusion DNA polymerase in the supplied buffer and with the following thermocycling parameters: 30 s at 98°C; 30 cycles of 98°C for 10 s, 48°C, 57°C, and 64.5°C, respectively, for 15 s, and 72°C for 45 s (invA3 TE domain), 90 s (invA5 A-T didomain), and 3 min (pET28β); and a terminal hold for 5 min at 72°C. The primers introduced restriction sites into the PCR products, which were cloned into expression vectors pET28b and pRSETb, respectively, to create plasmids pJB051 (to express invA5), pJB064 (invA4), pJB078 (invA1), pJB080 (invA6), and pJB082 (invA3). Codon-optimized cDNAs of *invA2*, *invA3*, *invA6*, and *pptA* were synthesized by a commercial vendor (GenScript). The cDNAs of invA2 and pptA were ligated to the *NdeI* and BamHI sites of pET28b to create pJB053 (to express invA2) and pJB062 (pptA), respectively. The invA6 cDNA was ligated to the *NdeI* and BamHI sites of pET28b, to create plasmid pJB066, and later excised and ligated into equally cut pCodi-1 to generate plasmid pT206. The invA3 cDNA was cloned into the *Nhel* and BamHI sites of expression vector pRSETb to create plasmid pJB063.

**Construction of Chimeric Genes**

The codon-optimized version of gene *invA3* was inserted into the *Nhel* and BamHI sites of expression vector pET28b to create plasmid pJB059. Subsequently, the portion of pJB059 encoding the TE domain of invA3 was replaced by the corresponding portion of invA5 (taken from plasmid pJB051) using the *KpnI* and BamHI restriction sites. The created chimeric gene invA335 was cloned into expression vector pRSETb to create pJB074. It encodes a chimeric synthetase composed of the InvA3 adenylation-thiolation (A-T) didomain and the InvA5 thiostreloase (TE) domain. A gene for the inverse chimera (InvA553, InvA5 A-T didomain fused to the InvA3 TE domain) was constructed using the Gibson Assembly Master Mix (New England Biolabs) using plasmids pJB051 and pJB059 as templates, to yield plasmid pJB075. The reactions (50 μl) consisted of 1.5–2.5 mM MgCl2, 0.2 mM each dNTP, 20 pmol (each) primer (Table S1), and 1 U Phusion DNA polymerase in the supplied buffer and with the following thermocycling parameters, to create plasmid pJB075: 30 s at 98°C; 30 cycles of 98°C for 10 s, 48°C, 57°C, and 64.5°C, respectively, for 15 s, and 72°C for 45 s (invA3 TE domain), 90 s (invA5 A-T didomain), and 3 min (pET28β); and a terminal hold for 5 min at 72°C. To create the chimeric gene invA556 (encoding the InvA3 A-T didomain fused to the InvA6 TE domain), plasmid pJB048 (invA5, cloned into pRSETb) was used. The gene portion encoding the TE domain of invA6 was excised by *ClaI* and BamHI restriction from pJB066 and ligated to pJB048, cut equally, to yield pJB071. The complete chimeric gene invA556 was then cloned between the *Ndel* and BamHI sites of pET28b, which yielded the final expression plasmid pJB072.

**Heterologous Gene Expression and In Vitro Enzyme Characterization**

N-Terminally hexahistidine-tagged InvA2, InvA4, InvA5, InvA553, InvA556, and PptA were produced in *E. coli* KRX, transformed with plasmids pJB053, pJB064, pJB051, pJB075, pJB072, and pJB062, respectively. Expression of genes *invA1*, *invA3*, *invA6*, and *invA335* was accomplished in *E. coli* SoluBL transformed with plasmids pJB078, pJB063, pT026, and pJB074, respectively. For details on gene expression and protein purification, see Supplemental Experimental Procedures. To characterize the InvA1-A6 adenylation domains, the ATP-[32P]pyrophosphate exchange assay was used as described by Schneider et al. (2006), 4-Hydroxypyruvylpyruvate, or other substrates (Table S2) to determine substrate specifities, were added at 1 mM final concentration. To determine optima, the temperature was varied from 10°C to 35°C, and the pH from 6.0 to 8.2 with phosphate or Tris buffers, covering overlapping pH ranges.

**In Vitro Biotransformation**

Conversion of apo-enzymes into their holo form was catalyzed by the phosphopantetheinyl transferases Svp (Sanchez et al., 2001) or PptA. 0.5 μM of the respective apo-enzyme and 0.5 μM Svp or PptA were incubated for 30 min at 20°C–25°C in 75 mM Tris-HCl buffer (pH 6.8–7.6), and 120 μM coenzyme A as donor substrate. Product formation was accomplished in 500-μl reactions, containing 75 mM Tris-HCl buffer (pH 6.8–7.6, according to the optimum conditions), 5 mM MgCl2, 125 mM EDTA, 2.5 mM ATP, 0.5 μM InvA, and 1.8 mM 4-hydroxypyruvic acid, at 20°C–25°C for 16 hr. The reaction mixtures were extracted twice with an equal volume of ethyl acetate, and the organic extract was concentrated under reduced pressure. For analytical HPLC (see below), the extracts were dissolved in methanol.

**Synthesis of Deuterated Atromentin**

The synthesis of deuterated 4-hydroxypyruvic acid was carried out according to a described method (Munde et al., 2013). The synthesis of
deuterated atromentin was carried out in analogy to the in vitro atromentin formation (see above). A reaction mixture (8 ml) consisted of 75 mM Tris-HCl buffer (pH 7.2), 10 mM MgCl₂, 125 mM EDTA, 5 mM ATP, 4 µM InvA1, 4 µM Syp, 114 µM coenzyme A, and 3 mM deuterated 4-hydroxyphenylpyruvic acid. It was incubated for 16 hr at 25 °C and subsequently extracted three times with an equal volume of ethyl acetate. The extract was dissolved in methanol, and purification was accomplished by preparative HPLC (see below).

**Stable-Isotope Labeling**

For supplementation with stable-isotope-labeled atromentin, *P. involutus* was grown in Petri dishes on a layer of glass beads immersed in liquid medium (van Schöll et al., 2006). A monolayer of autoclaved 4-mm glass beads was poured into the bottom of a 9-cm Petri dish and 10 ml of MMN medium was added. A mycelial plug was cut from the mycelial margin of a culture actively growing on MMN agar and transferred to the center of the glass-bead plate. After 9 days of incubation at 18 °C–20 °C in the dark, the MMN medium was removed. The glass beads and the mycelium were washed with sterile MilliQ water, and 10 ml of MMN medium without a nitrogen source was added. After 24 hr, the mycelium was washed with sterile MilliQ water. Subsequently, 10 ml of maize hot extract supplemented with glucose (final concentration 2.5 g/l, pH 4) was added (Rineau et al., 2012; Shah et al., 2013). The culture was incubated for another 7 days at 18 °C–20 °C in the dark. For the first feeding experiment with homogenized mycelium, ten plates of *P. involutus* were cultivated as described above. The mycelium was then homogenized in 100 ml of 75 mM Tris-HCl buffer (pH 7.2) supplemented with 1 mM PMSF, using an Ultra-Turrax T25 basic (IKA). Unlabeled or 3°,3°,5°,5°–D₄-atromentin was added to 30 ml of homogenized mycelium at a final concentration of 1 mM. As control, another 30 ml was left without any labeled compounds. The reactions were incubated for 24 hr at room temperature and then extracted three times with 30 ml of ethyl acetate. The organic extracts were concentrated under reduced pressure. The extracts were redissolved in methanol for analytical HPLC (see below). For the second labeling experiment, six plates of *P. involutus* were cultivated as described above. Two plates were supplemented with 3°,3°,5°,5°–D₄-atromentin (final concentration: 1.5 mM, added together with the maize hot extract). Another two plates without labeled compounds served as control. The cultures were incubated for another 7 days at 18 °C–20 °C in the dark, before the media were extracted three times with ethyl acetate and further processed as described above.

**Chromatography and Mass Spectrometry**

HPLC analysis of in vitro product formation was performed on an Agilent 1200 system equipped with a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm particle size). Solvent A was 0.1% (v/v) trifluoroacetic acid in water, and solvent B was methanol. The solvent gradient was: initial hold at 5% B, for 0.5 min, linear gradient from 5% to 90% B within 14.5 min, held at 90% B for 2 min, increased to 100% B within 0.5 min, and held for 4.5 min, at a flow rate of 1.0 ml/min. The detection wavelength range was 200–600 nm. The detection wavelength range was 200–600 nm. The detection wavelength range was 200–600 nm.

**Transcriptomic Analysis**

Fungal mycelia were grown as described above, but using BSA (16% [w/w] N) as sole nitrogen source, or organic matter extracts (forest hot extract, maize hot extract, and maize compost). For each treatment there were three biological replicates with three Petri dishes per replicate. The biomass was collected and immediately dropped into a clean mortar filled with liquid nitrogen, and homogenized using a pestle. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) with the RLC buffer and the on-column DNase treatment according to the manufacturer. Total RNA was eluted in diethylpyrocarbonate-treated H₂O and stored at −20 °C until use. For quality assessments, all samples were inspected using an RNA 6000 Nano kit on an Agilent 2100 Bioanalyzer. The microarray analysis was performed using already published data including six biological replicates on the reference MMN medium that are available at NCBI GEO (accession numbers NCBI-GEO: GSM848412–GSM848414 and GSM848421–GSM848423), as well as three replicates each for forest hot extract (GSM848415–GSM848417), maize hot extract (GSM848418–GSM848420), maize compost (GSM848424–GSM84842), and BSA (GSE47836) (Rineau et al., 2012; Shah et al., 2013).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.08.016.

**AUTHOR CONTRIBUTIONS**

J.B. performed molecular biological and biochemical experiments and chromatographic analyses; S.G. synthesized gyrocyanin; F.S. and A.T. generated and interpreted the transcriptomic data; D. Heine provided critical input on the synthesis of deuterated atromentin; J.T. cloned and expressed the invA6 gene; D. Hoffmeister designed the study and co-wrote the paper together with C.H., P.S., and A.T.

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