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Biosynthesis of LL-Z1272β: Discovery of a New Member of NRPS-like Enzymes for Aryl-Aldehyde Formation

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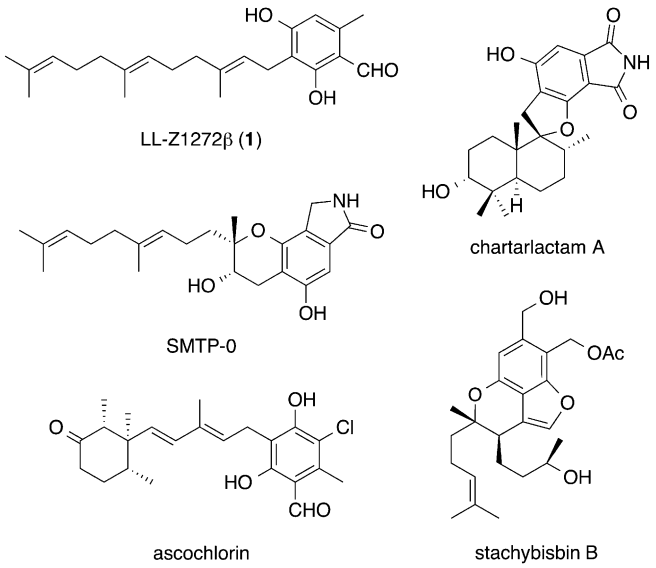
LL-Z1272β (**1**) is a prenylated aryl-aldehyde produced by several fungi; it also serves as a key pathway intermediate for many fungal meroterpenoids. Despite its importance in the biosynthesis of natural products, the molecular basis for the biosynthesis of **1** has yet to be elucidated. Here we identified the biosynthetic gene cluster for **1** from *Stachybotrys bisbyi* PYH05-7, and elucidated the biosynthetic route to **1**. The biosynthesis involves a polyketide synthase, a prenyltransferase, and a nonribosomal peptide synthetase (NRPS)-like enzyme, which is responsible for the generation of the aldehyde functionality. Interestingly, the NRPS-like enzyme only accepts the farnesylated substrate to catalyze the carboxylate reduction; this represents a new example of a substrate for adenylation domains.

Meroterpenoids are structurally diverse natural products with remarkable biological activities, and thus many scientists have attempted to elucidate the biosynthesis of these molecules.[1] LL-Z1272β (**1**, also known as ilicicolin B), a fungal meroterpenoid originally isolated from *Fusarium* sp.,[2] exhibits a variety of bioactivities, including antimicrobial and cytotoxic activities, as well as inhibitory activity against cytochrome bd quinol oxidase.[3] Additionally, **1** is considered to be a pathway intermediate in the biosyntheses of many meroterpenoids, including chartarlactams,[4] SMTPs,[5] ascochlorin,[6] bisabosquals,[7] and chartarutines[8] (Scheme 1). It is believed to be derived from orsellinic acid and farnesyl diphosphate (FPP), but no genetic or molecular basis for the biosynthesis of **1** has been reported. The elucidation of the biosynthesis of **1** at the molecular level should provide an opportunity to produce **1** by an engineered microorganism, as well as insights into the biosynthesis of related natural products.

LL-Z1272β (**1**) possesses an aryl-aldehyde moiety, which is common in fungal secondary metabolites. Most aryl-aldehydes are generated by a reducing (R) domain[9] that is covalently attached to the end of a non-reducing polyketide synthase (NR-PKS). However, Wang et al. recently reported a distinct aryl-aldehyde formation mechanism catalyzed by a nonribosomal peptide synthetase (NRPS)-like enzyme (ATEG_03630) that has only three domains: adenylation (A) domain, acyl carrier protein (ACP), and R domain.[10] 5-Methylorsellinic acid (5-MOA) is activated by the A domain and then loaded onto the ACP, where it undergoes reductive cleavage of the thioester by the R domain to afford the aldehyde group.



Scheme 1. LL-Z1272β (**1**) and selected fungal meroterpenoids putatively derived from **1**.

Previously, we isolated **1** from the wetland fungus *Stachybotrys bisbyi* PYH05-7 (10.3 mg per kg of rice medium), along with several compounds containing a seco-bisabosqual skeleton, such as stachybisbin B, which appeared to originate from **1** (Scheme 1).[3c] Here we describe the identification and characterization of the gene cluster for the biosynthesis of **1**. We elucidated the functions of the enzymes encoded by the gene cluster, and we show that the NRPS-like enzyme similar to ATEG_03630 accepts a farnesylated aryl acid to generate the aldehyde group of **1**.

We performed whole-genome sequencing of *S. bisbyi* PYH05-7 to obtain the biosynthetic gene cluster for **1** and its downstream metabolites. Given the structure of **1**, we initially reasoned that orsellinaldehyde is the specific precursor of **1**, and therefore searched for a gene encoding a protein homologous to the known NR-PKS orsellinaldehyde synthase (PkfA),[9b] which has an R domain at its C terminus. We found one gene encoding a product with high similarity to PkfA among the predicted proteins of *S. bisbyi* PYH05-7. However, the gene product (designated as StbA) lacks an R domain but possesses a thioesterase (TE) domain, which hydrolyzes the thioester bond and releases the polyketide chain from the enzyme to yield a carboxyl group. This clearly indicates that StbA is not

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responsible for the formation of orsellinaldehyde. However, investigation of the region flanking *stbA* revealed a gene encoding a homologue of ATEG_03630 (*stbB*), as well as a putative UbiA-like prenyltransferase (PT) gene (*stbC*), which is often found in fungal meroterpenoid biosynthesis and is responsible

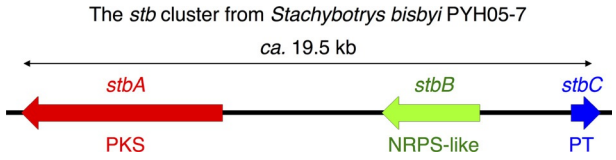


Figure 1. The *stb* cluster.

for farnesylation or geranylgeranylation (Figure 1; GenBank accession LC125467). The domain organization of StbB (A-ACP-R) was the same as that of ATEG_03630,[10] and therefore we supposed that StbA synthesizes orsellinic acid, which is then accepted by StbB for transformation into orsellinaldehyde. Finally, StbC would perform farnesylation to provide **1**. We were not able to find putative tailoring genes for the biosynthesis of the downstream metabolites in the flanking regions of the cluster, thus indicating that they are in another region of the genome.

In order to investigate the biosynthetic pathway and to analyze the functions of the enzymes encoded by the *stb* cluster, we introduced and heterologously expressed the *stb* genes in *Aspergillus oryzae* NSAR1,[11] which has been successfully applied in biosynthetic studies of fungal natural products.[12] When the PKS gene *stbA* was expressed alone, the transformant produced the single product **2** (Figure 2, trace ii; not found in the control strain transformed with an empty vector). In order to isolate the new metabolite, the transformant was cultivated in induction medium for three days. The culture broth was extracted with ethyl acetate and subjected to silica gel column chromatography. After further purification by preparative HPLC, NMR and MS analyses (Table S3, Figures S4 and S5 in the Supporting Information) confirmed that **2** was orsellinic

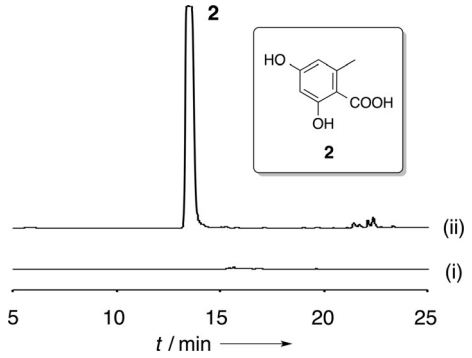


Figure 2. HPLC profiles (254 nm) of culture supernatant extract of *A. oryzae* transformants harboring i) empty vector and ii) *stbA*.

acid,[13] and therefore StbA is indeed the orsellinic acid synthase.

We then sought to clarify the function of the NRPS-like protein StbB, by coexpressing *stbA* and *stbB*. To our surprise, the transformant did not produce any metabolites other than **2**

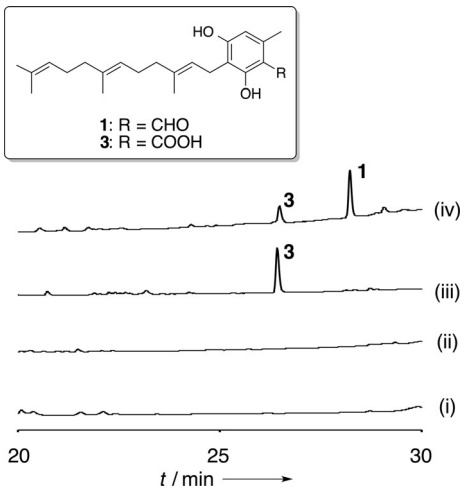
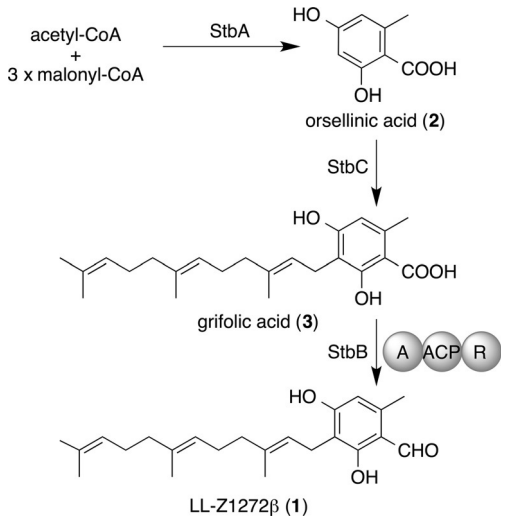


Figure 3. HPLC profiles (254 nm) of mycelial extract of *A. oryzae* transformants harboring i) *stbA*, ii) *stbA* and *stbB*, iii) *stbA* and *stbC*, and vi) *stbA*, *stbB*, and *stbC*.

(Figure 3 and Figure S1, traces i and ii), thus suggesting that StbB does not accept **2** for conversion into the corresponding aldehyde. Intriguingly, the strain coexpressing *stbA* and *stbC* (putative prenyltransferase gene) contained a new hydrophobic metabolite in its mycelia (**3**, Figure 3, lane iii). After large-scale cultivation and isolation, **3** was identified as the farnesylated form of **2** (grifolic acid),[14] by NMR and MS analysis (Table S4, Figures S6 and S7). This led us to hypothesize that StbB uses **3**, not **2**, as a substrate for the reduction of the carboxylate. We thus constructed a transformant containing all three of the genes, and analyzed the resulting metabolites. As expected, this generated the new compound (**1**, 13.0 mg per L culture; Figure 3, trace vi), which was identified as LL-Z1272β[3c] (Table S4, Figures S8 and S9). The production of **1** by these three enzymes established its complete biosynthetic pathway, which involves the unexpected reduction of the farnesylated aryl acid to form the aldehyde functionality (Scheme 2).

Because **1** is considered to be the biosynthetic precursor of many meroterpenoids in various fungi, we speculated that gene clusters homologous to the *stb* cluster should be widely distributed within the fungal kingdom, so we searched for *stb* homologues in publicly available genome sequences. *Stachybotrys chartarum* IBT 7711[15] and *Stachybotrys chlorohalonata* IBT 40285[15] have clusters similar to the *stb* cluster (Figure S2), and both have a PKS, an NRPS-like protein, and a UbiA-like prenyltransferase genes. Some strains of *S. chartarum* reportedly produce meroterpenoids that seem to be derived from **1**, such as chartarutines[8] and chartarlactams,[4] and therefore this cluster is also expected to be responsible for the production of **1** in *S. chartarum*.



Scheme 2. Proposed biosynthetic pathway of LL-Z1272β (**1**).

One of the most interesting discoveries in this study was the NRPS-like enzyme StbB responsible for aldehyde formation in LL-Z1272β biosynthesis. Although NRPS-like enzymes similar to StbB are frequently found in fungal secondary metabolism, most of the characterized ones belong to subtype I NRPS-like proteins (domain organization: A-ACP/PCP-TE).[10] The characterization of StbB thus provides new insight into the biosynthesis involving the less-characterized subtype II NRPS-like proteins with an R domain. The substrate specificity of StbB is noteworthy. Numerous A domains with distinct substrate preferences have been reported, and aryl acids are common substrates.[16] StbB, however, represents a very rare case, as the A domain requires prenylation before accepting its substrate. For example, tryptophan is dimethylallylated before entering the NRPS assembly line in cyclomarin/cyclomarazine biosynthesis,[17] but to the best of our knowledge, this is the first report of an A domain that activates a farnesylated molecule. Because A domains are central for diversity in NRPS-derived natural products, the discovery of this new A domain could expand the opportunities to provide novel molecules by engineered biological systems.

A recent phylogenetic analysis of fungal adenylate-forming reductases revealed that these enzymes can be grouped into four classes.[18] Although StbB and ATEG_03630 belong to class III (aryl acid reductases), they display clearly distinct substrate specificities, as ATEG_03630 is known to accept orsellinic acid (**2**) as well.[19] In order to provide a rational explanation for the specificity, we compared the specificity-conferring sequences[20] of the A domains of the proteins. L334 and H358 in ATEG_03630 are substituted with glycine and phenylalanine, respectively, in StbB, as well as in the other two highly homologous proteins mentioned above (Figure S3). Significantly, the H358A mutation in ATEG_03630 altered its substrate specificity;[19] the fact that StbB has phenylalanine at this position could explain the distinct substrate preference. However, this should be confirmed by mutational experiments and/or X-ray crystallographic studies.

In summary, we have identified the biosynthetic gene cluster for the production of LL-Z1272β (**1**) and characterized the enzymes in the pathway by heterologous expression in *A. oryzae*. We demonstrated that the NPRS-like enzyme StbB is responsible for aldehyde formation from the farnesylated aryl acid substrate, thus indicating a wide occurrence of adenylate-forming reductases with a variety of substrate specificities in natural product biosynthetic pathways. Future studies will focus on characterization of StbB and a search for the tailoring enzymes that transform **1** into more-complex meroterpenoid molecules.

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