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Insights into the Biosynthesis of 12-Membered Resorcylic Acid Lactones from Heterologous Production in *Saccharomyces cerevisiae*

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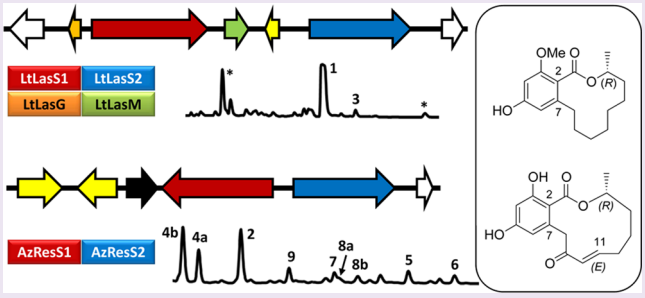
Supporting Information

ABSTRACT: The phytotoxic fungal polyketides lasiodiplodin and resorcylide inhibit human blood coagulation factor XIIIa, mineralocorticoid receptors, and prostaglandin biosynthesis. These secondary metabolites belong to the 12-membered resorcylic acid lactone (RAL_{12}) subclass of the benzenediol lactone (BDL) family. Identification of genomic loci for the biosynthesis of lasiodiplodin from *Lasiodiplodia theobromae* and resorcylide from *Acremonium zeae* revealed collaborating iterative polyketide synthase (iPKS) pairs whose efficient heterologous expression in *Saccharomyces cerevisiae* provided a convenient access to the RAL_{12} scaffolds desmethyl-lasiodiplodin and *trans*-resorcylide, respectively. Lasiodiplodin production was reconstituted in the heterologous host by co-expressing an *O*-methyltransferase also encoded in the lasiodiplodin cluster, while a glutathione-*S*-transferase was found not to be necessary for heterologous production. Clarification of the biogenesis of known resorcylide congeners in the heterologous host helped to disentangle the roles that biosynthetic irregularities and chemical interconversions play in generating chemical diversity. Observation of 14-membered RAL homologues during *in vivo* heterologous biosynthesis of RAL_{12} metabolites revealed "stuttering" by fungal iPKSs. The close global and domain-level sequence similarities of the orthologous BDL synthases across different structural subclasses implicate repeated horizontal gene transfers and/or cluster losses in different fungal lineages. The absence of straightforward correlations between enzyme sequences and product structural features (the size of the macrocycle, the conformation of the exocyclic methyl group, or the extent of reduction by the hrPKS) suggest that BDL structural variety is the result of a select few mutations in key active site cavity positions.



Benzenediol lactones (BDLs) are a growing class of fungal polyketide secondary metabolites defined by a 1,3-benzenediol moiety bridged by a macrocyclic lactone ring.[1] The intramolecular aldol condensation that forms the benzenediol ring of BDLs may occur between C-2 and C-7 to yield resorcylic acid lactones (RALs) or between C-8 and C-3 to produce dihydroxyphenylacetic acid lactones (DALs).[2,3] The best studied BDLs are RALs with a 14-membered macrocyclic ring (RAL_{14}) such as zearalenone, hypothemycin, and radicicol and DALs with a 12-membered ring (DAL_{12}) like 10,11-dehydrocurvularin (Figure 1). These BDLs are rich pharmacophores with an astonishing range of biological activities, including receptor agonist, mitogen-activated protein kinase inhibitory, anti-inflammatory, and heat shock response and immune system modulatory activities.[4,5]

The scaffolds of these fungal polyketides are biosynthesized by iterative polyketide synthases (iPKSs). These enzymes catalyze recursive, decarboxylative Claisen condensations of malonyl-CoA precursors to produce linear poly-β-ketoacyl intermediates using a single core set of ketoacyl synthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domains. Further maturation of the polyketide chain requires additional domains. BDL biosynthesis initiates with the assembly of a variably reduced linear polyketide intermediate on a highly reducing iPKS (hrPKS). In addition to the core set of domains, hrPKSs feature ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER) domains. These domains catalyze the

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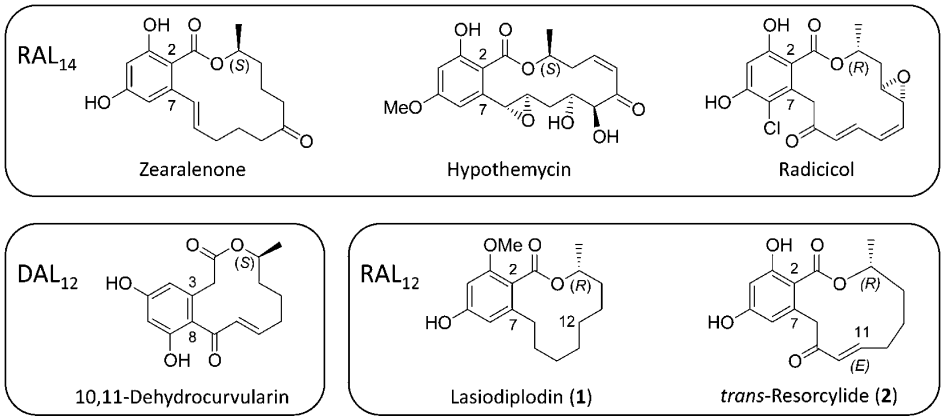

Figure 1. Resorcylic acid and dihydroxyphenylacetic acid lactones.

formation of carbonyl, alcohol, alkene, or alkane functionalities by reducing the nascent β-ketones of the growing polyketide chain after each condensation, executing a cryptic biosynthetic program.[6] The polyketide product of the hrPKS is directly transferred to a second, nonreducing iPKS (nrPKS) by the starter AT (SAT) domain of the nrPKS.[6] After further elongations of the advanced starter unit without reduction, the product template (PT) domain of the nrPKS directs first ring closure by regiospecific aldol condensation. This condensation yields a resorcylic acid moiety (F-type folding, C2−C7 register) or a dihydroxyphenylacetic acid group (S-type folding, C8−C3 register).[7,8] Finally, the C-terminal thioesterase (TE) domain of the nrPKS catalyzes product release by using an alcohol functionality of the polyketide chain as the nucleophile for the formation of the BDL macrolactone. Alternative nucleophiles may include an intramolecular enol to yield α-pyrones or water or alcohols from the media to form acyl resorcylic acids, acyl dihydroxyphenylacetic acids, and their esters.[9,10] Thus, in contrast to most fungal polyketides that are assembled by a single iPKS, the biosynthesis of BDLs involves a collaborating hrPKS-nrPKS pair acting in sequence, forming a quasi-modular BDL synthase system.[11−14] iPKSs that produce the RAL_{14} and DAL_{12} subclass of BDLs have been characterized in the producer fungi by gene disruptions[11,12] and reconstituted both *in vivo* by heterologous expression in yeast and *in vitro* using isolated recombinant iPKS enzymes.[13,15,16] However, the biosynthesis of the 12-membered RAL (RAL_{12}) subclass of BDLs has not been characterized up till now, despite the important biological activities of these compounds and their potentially interesting biosynthetic mechanisms.

Among RAL_{12}, lasiodiplodin (Figure 1) and its congeners (12-, 13- or 14-hydroxy-, 13-oxo-, and 3-*O*-desmethyl-lasiodiplodin) have been isolated from the tropical/subtropical plant pathogen *Lasiodiplodia theobromae* (syns. *Botryodiplodia theobromae* and *Diplodia gossypina*, teleomorph *Botryosphaeria rodina*).[17−19] Lasiodiplodins have also been identified from *Syncephalastrum racemosum*[20] and various plant biomass sources potentially inhabited by fungi.[21] Lasiodiplodin congeners are plant growth regulators that induce potato microtuber formation.[17,18] They are also phytotoxic by blocking the electron transport chain in thylakoids at multiple targets that are different from those of current herbicides.[19] In addition to its phytotoxic activities, 3-*O*-desmethyl-lasiodiplodin inhibits prostaglandin biosynthesis[21] and displays nonsteroidal mineralocorticoid receptor inhibitory activity.[22] Lasiodiplodin has been reported to have antileukemic activities.[23]

Another RAL_{12}, *trans*-resorcylide (Figure 1), and its congeners (*cis*-resorcylide, 11-hydorxyresorcylide enantiomers, and their methyl esters) have been isolated from *Penicillium* spp.,[24] *Pyrenophora teres*,[25] and *Acremonium (Sarocladium) zeae*.[26] Resorcylides are phytotoxic as demonstrated in leaf-puncture wound assays and inhibit seedling root elongation.[24−26] *trans*-Resorcylide (but not the *cis* isomer) is a specific inhibitor of 15-hydroxyprostaglandin dehydrogenase, a key enzyme in prostaglandin catabolism.[27] *cis*-*R*-(−)-Resorcylide specifically inhibits blood coagulation factor XIIIa and may be advantageous to enhance fibrinolysis and resolve blood clots.[28]

The polyketide origin and the intermediacy of 9-hydroxydecanoic acid in the biosynthesis of lasiodiplodins have been demonstrated by feeding labeled precursors to *L. theobromae*.[29] Moreover, hydroxylation at the C-11 position in 11-hydroxylasiodiplodin was shown to occur after the completion of lasiodiplodin assembly on the iPKS. Fittingly, 7,9-dihydroxydecanoic acid was not utilized as a precursor by the producer fungus.[29]

In this work, we have cloned and sequenced the lasiodiplodin biosynthetic cluster from *L. theobromae* NBRC 31059 and the *trans*-resorcylide biosynthetic locus from *Acremonium zeae* NRRL 45893. We reconstituted the production of *trans*-resorcylide and its congeners and lasiodiplodin and some of its congeners in *Saccharomyces cerevisiae* by heterologous expression of the collaborating hrPKS-nrPKS gene pairs and tailoring enzymes if necessary. We have also isolated minor lasiodiplodin and resorcylide congeners revealing *in vivo* stuttering of iterative hrPKS and nrPKS enzymes in heterologous expression systems.

RESULTS AND DISCUSSION

Isolation of the Lasiodiplodin and Resorcylide Biosynthetic Loci. On the basis of established precedents for RAL_{14} and DAL_{12} production in fungi,[11−14] we hypothesized that the biosynthesis of the RAL12 phytotoxins lasiodiplodin and resorcylide may involve collaborating iPKSs. In the absence of genome sequences for any RAL_{12} producers, we utilized our previously described PCR-based strategy[14] to clone the lasiodiplodin and the resorcylide biosynthetic gene clusters. The resulting 30.1-kb (*L. theobromae*) and 28.4-kb (*A. zeae*) genomic loci encode putative proteins for one hrPKS-nrPKS pair each (LtLasS1-LtLasS2 and AzResS1-AzResS2, respectively, Figure 2A, Supplementary Table S2). The predicted lasiodiplodin cluster also features genes for a deduced *O*-methyltransferase (LtLasM), a putative glutathione-*S*-trans-

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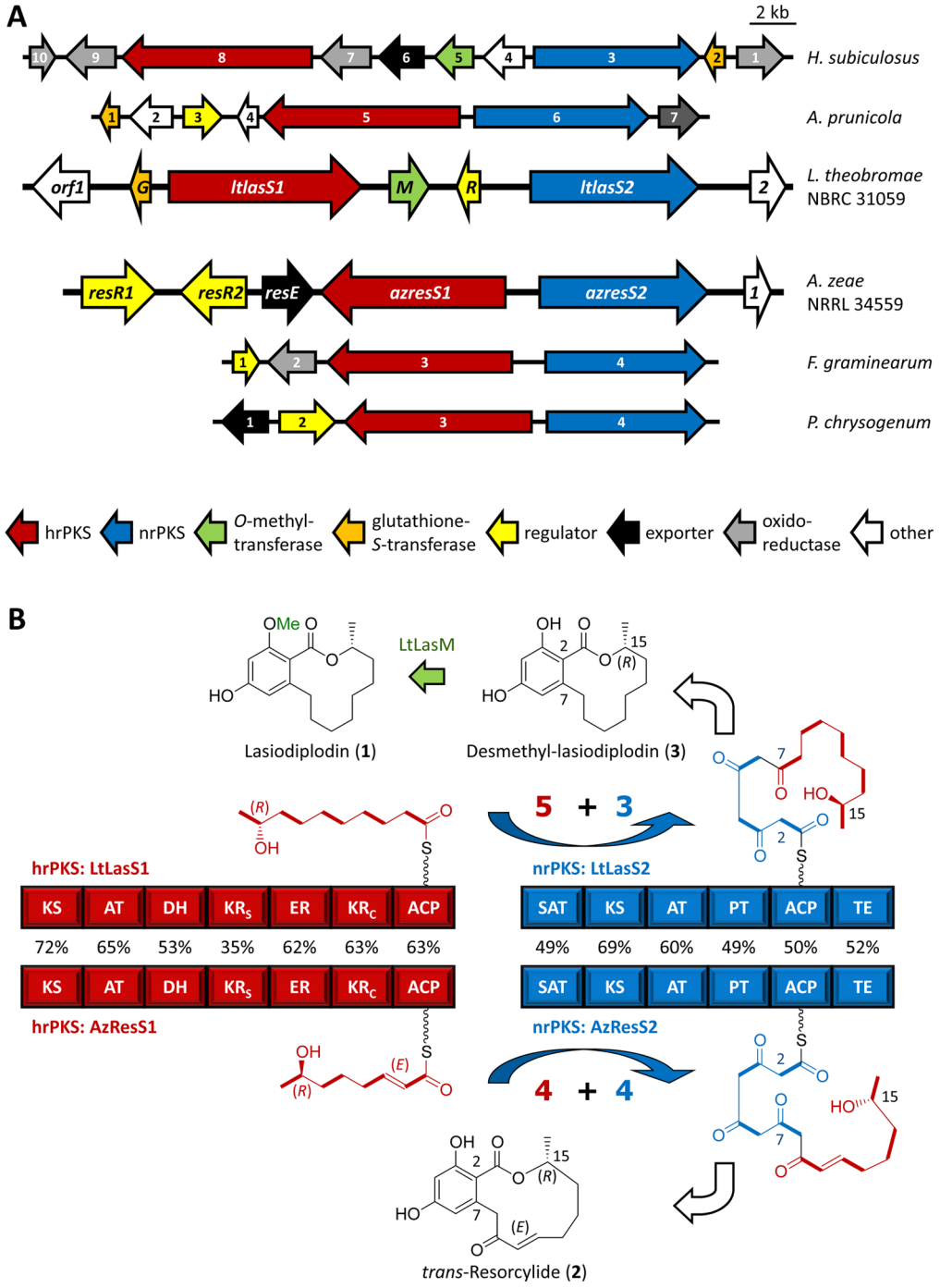

Figure 2. Biosynthesis of BDLs. (A) Biosynthetic loci for lasiodiplodin from *L. theobromae*, *trans*-resorcylide from *A. zeae*, hypothemycin from *H. subiculosus*, zearalenone from *F. graminearum*, and orphan BDL biosynthetic loci from *A. prunicola* and *P. chrysogenum*. (B) Models for lasiodiplodin and *trans*-resorcylide biosynthesis. 5 + 3 and 4 + 4 indicate the number of malonate-derived C_2 units (C−C bonds shown in bold) inserted by the hrPKS vs the nrPKS ("division of labor" or "split" by the collaborating iPKSs). Percentages show protein sequence identities for the indicated domains. KR_S, ketoreductase structural subdomain; KR_C, ketoreductase catalytic subdomain. For detailed annotations, see Supplementary Tables S2 and S3.

ferase (LtLasG), and a basic region leucine zipper domain family protein (LtLasR, Figure 2A, Supplementary Table S2, Supplementary Results). In addition to the iPKSs, the resorcylide cluster also encodes a predicted major facilitator superfamily transporter (AzResE) and a pair of GAL4-like transcriptional regulators (AzResR1 and AzResR2, Figure 2A, Supplementary Table S2, Supplementary Results). The predicted protein products of the surrounding genes of the two loci play no apparent roles in lasiodiplodin or resorcylide biosynthesis.

The sequenced lasiodiplodin and resorcylide loci show no synteny with the complete genomes of the closest relatives of the producer fungi in the JGI MycoCosm or with BDL clusters in GenBank whose iPKSs display the highest identities to LtLasS and AzResS (Figure 2A, Supplementary Tables S2 and S3). Nevertheless, orthologous iPKS pairs are present in these

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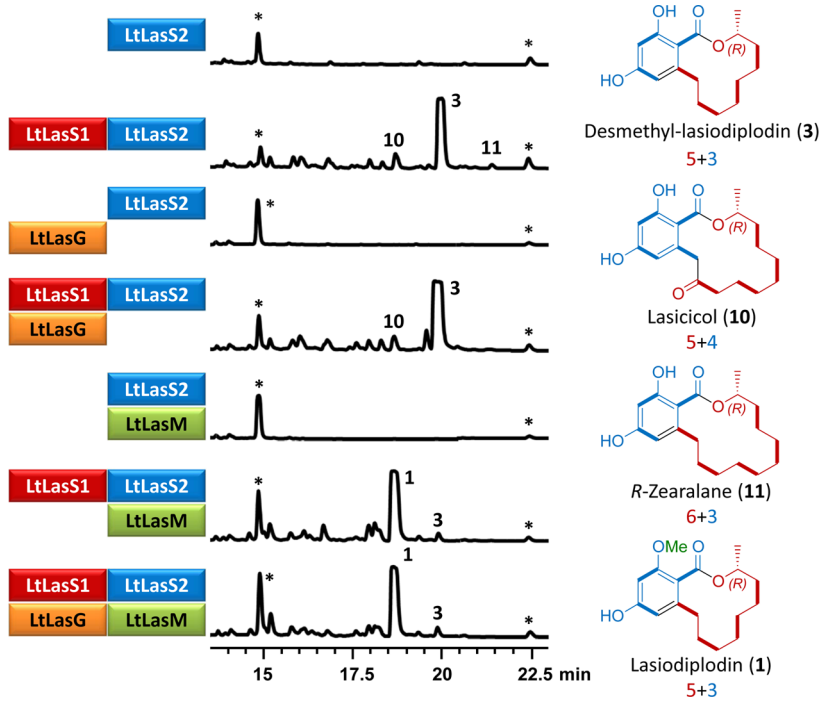

Figure 3. Reconstitution of lasiodiplodin biosynthesis *in vivo*. Product profiles (HPLC traces recorded at 300 nm) of *S. cerevisiae* BJ5464-NpgA[31] co-transformed with the genes for the indicated *L. theobromae* proteins. ∗, yeast metabolites unrelated to the iPKS products.

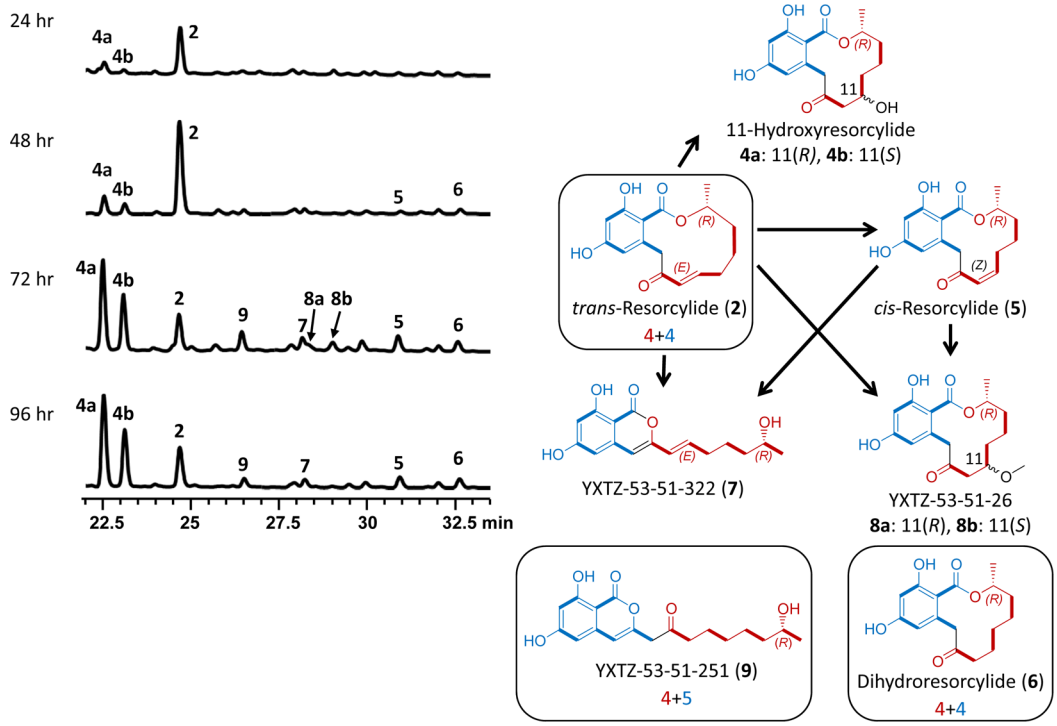

Figure 4. Time-course analysis and interconversion of resorcylide congeners. Product profiles (HPLC traces recorded at 300 nm) of *S. cerevisiae* BJ5464-NpgA[31] co-transformed with *azresS1* and *azresS2* and induced for BDL production for the indicated amount of time.

fungi in different genomic contexts. *Fusarium graminearum* and *Hypomyces subiculosus* are the producers of the known RAL_{14} zearalenone and hypothemycin, respectively.[11,13] Although *Aplosporella prunicola* and *Penicillium chrysogenum* are not known to produce BDLs, their orphan BDL loci are predicted here to encode the biosynthesis of RALs.[2]

Heterologous Production of Desmethyl-lasiodiplodin and *trans*-Resorcylide. To establish cluster identity in the absence of available transformation systems for *L. theobromae* and *A. zeae*, heterologous production of lasiodiplodin and resorcylide was attempted.[13,15,16,30] Thus, intron-less versions of the *ltlasS1* and the *ltlasS2* genes on one hand and those of the *azresS1* and *azresS2* on the other were introduced into *S.*

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cerevisiae BJ5464-NpgA using YEpADH2p-derived expression vectors.[14,16,31] The expected RAL_{12} products, desmethyl-lasiodiplodin (**3**) and *trans*-resorcylide (**2**) were produced in good yields by the recombinant strains (isolated yields: 10 mg/L for **3** and 8 mg/L for **2**) (Figures 3 and 4). Minor BDL and isocoumarin products were also observed in both fermentations (see below).

In the absence of their hrPKS partners, heterologous hosts expressing BDL nrPKSs have been observed to produce acyl resorcylic or acyl dihydroxyphenylacetic acids by the chance utilization of various short chain fatty acyl thioesters.[10,14,32] However, we have detected no such products in fermentations with *S. cerevisiae* strains expressing LtLasS2 or AzResS2 alone (Figure 3 and Supplementary Figure S1). Supplementation of these cultures with various short chain (C_6–C_{12}) fatty acids did not lead to the production of polyketides either. The lack of utilization of host-derived or supplemented fatty acids may be due to the more stringent substrate selectivity of these nrPKSs or, in the case of the fed substrates, excessive catabolism or a failure in uptake/compartmentalization by the yeast host.

Comparative Bioinformatic and Functional Analysis of the RAL_{12} iPKSs. The predicted LtLasS1 and AzResS1 enzymes are orthologous (Figure 2B) and share identical domain composition (KS-AT-DH-KR_S-ER-KR_C-ACP) with each other and the characterized BDL hrPKSs.[11−14] These hrPKSs synthesize the starter acyl chains whose lengths determine the sizes of the bridging macrocycles of BDLs, while their variably reduced β-carbons dictate the shape and reactivity of these macrolactones. AzResS1 is expected to yield the tetraketide 7(*R*)-hydroxyoct-2(*E*)-enoic acid (Figure 2B), the C-7 enantiomer of the tetraketide biosynthesized by the dehydrocurvularin hrPKS AtCurS1.[14] LtLasS1 is predicted to produce the pentaketide 9(*R*)-hydroxydecanoic acid, a reduced analogue of the radicicol/monocillin II pentaketide advanced starter unit.[12,16] These hydroxycarboxylic acid products remain parked on the hrPKS as acyl-ACP thioesters pending transfer to the BDL nrPKS,[6] with any acids released by infrequent spontaneous hydrolysis expected to be catabolized by the host. Accordingly, expression of LtLasS1 or AzResS1 in *S. cerevisiae* without their cognate nrPKS partners did not yield any detectable shunt metabolites (results not shown).

While the LtLasS1 and AzResS1 DH domains are programmed not to reduce the β-hydroxy moiety at the diketide stage, the KR domains are expected to be active after each extension step. This program yields a secondary alcohol at the penultimate carbon of the polyketide chain that will be used by the TE domain of the nrPKS for macrolactone closure. The stereochemical outcome of the KR-catalyzed reduction of the nascent β-ketobutyryl-ACP after the first chain extension determines the configuration of the exocyclic methyl group of BDLs. This stereocenter is *R* in radicicol, but *S* in hypothemycin, zearalenone, and dehydrocurvularin (Figure 1), highlighting a strict and chain length specific stereocontrol exercised by these enzymes.[16,30] The absolute configurations of the C-15 positions of the resorcylide congeners had not been determined previously from *A. zeae* NRRL 45893 but were assumed to be 15(*S*) based on resorcylides from *Penicillium* sp.[24,26] However, total synthesis of *S*-dihydroresorcylide unambiguously showed that the natural dihydroresorcylide from *A. zeae* is in fact the C-15 epimer (i.e., 15*R*).[33] Similarly, in contrast to earlier reports on the absolute configuration of C-15 being *S*,[34] most lasiodiplodin congeners from *L. theobromae* have later been described to feature a 15*R* geometry.[17] Considering these ambiguities, we have used optical rotation and circular dichroism data to unequivocally establish 15*R* as the configuration of the exocyclic methyl groups for both *trans*-resorcylide (**2**) and desmethyl-lasiodiplodin (**3**) isolated from recombinant yeast. In agreement, **2** from *A. zeae* and **1** and **3** from *L. theobromae* displayed optical rotations and/or specific Cotton effects identical to those of the recombinant products. Similarly, all congeners of *trans*-resorcylide and desmethyl-lasiodiplodin isolated from recombinant yeast strains (see below) yielded optical rotation values and specific Cotton effects with the same sign as those of **2** and **3**. Global comparison of the amino acid sequences of the KR_C domains of BDL hrPKSs and comparisons with KR signature motifs in bacterial modular PKSs were uninformative, preventing primary sequence-based predictions of the stereochemical programming of the KR reactions.[14,35]

The ER domain of LtLasS1 is unique among the characterized BDL synthase hrPKSs as this domain is programmed to generate an alkane functionality at the last extension. All other known BDL synthases, including AzResS1, are programmed not to act at this stage, and as a result, lasiodiplodins and the recently described opioid receptor binder neocosmosins[36] are the only 12- or 14-membered BDLs that lack an (*E*) double bond at the Southern face of their unmodified polyketide scaffolds. Global alignments of the ER domains of the known BDL synthases again failed to account for this programming difference.

LtLasS2 and AzResS2 are orthologous to characterized BDL nrPKSs[11−14] and feature identical domain compositions (SAT-KS-AT-PT-ACP-TE, Figure 2B). Their SAT domains[37] transfer the pentaketide (LtLasS2) or the tetraketide (AzResS2) from the ACP of their cognate hrPKS partners to initiate three (LtLasS2) or four (AzResS1) additional chain extension cycles (Figure 2B). As in the majority of nrPKS SAT domains in GenBank, the LtLasS1 SAT domain features a Cys, His active site dyad (Cys121, His246), while a Ser, His dyad is present in the active site of all other characterized BDL nrPKS (AzResS1: Ser133, His258).[37] Global comparisons of the BDL hrPKSs and nrPKSs and separate alignments of their KS domains fail to cluster these enzymes according to the number of the extension cycles they are expected to conduct, nor do the sequences of the nrPKS SAT domains provide immediate clues for their substrate length preferences. Thus, the length of the linear polyketide product intermediate (octaketide for lasiodiplodin, resorcylide, and dehydrocurvularin and nonaketide for hypothemycin, radicicol, and zearalenone) and consequently the size of the BDL macrocyclic ring remain unpredictable from primary sequences alone. Evolution of substrate and product specificities in these orthologous enzymes may involve keyhole surgery of binding pocket residues by Nature.[2,38]

Following the assembly of the linear polyketide chain, the nrPKS PT domains catalyze a regioselective aldol condensation that yields the BDL aromatic ring.[7,8,39] PT domains in RAL synthases direct an "F-type" folding[3] of the polyketide and catalyze aldol condensation in the C2−C7 register. In contrast, DALs derive from an atypical C8−C3 cyclization, resulting from "S-type" folding[3] of the nascent polyketide. Irrespective of the register of the aldol cyclization, all BDL PT domains fall into the same clade in multiple sequence alignments.[7,14,39] However, the regiochemical outcome of the PT-catalyzed cyclizations can be predicted (and reprogrammed) using a set of three diagnostic residues.[2] The PT domains of LtLasS2 and AzResS2 contain the expected Tyr, Phe, Leu signature for C2−

C7 condensations (LtLasS2: Y1443, F1563, L1571; AzResS2: Y1540, F1664, L1672), leading to the formation of their resorcylic acid moieties.

Product release from LtLasS2 and AzResS2 is catalyzed by O−C bond-forming TE domains generating RAL_{12} macrocycles using 15-OH as the nucleophile.[10,32] The biosynthesis of isocoumarin congeners **7** and **9** on the resorcylide nrPKS (see below) involves the attack of the C-9 enol on the TE oxoester intermediate, leading to α-pyrone formation.

Minor Congeners Reveal Stuttering in RAL_{12} Synthases. To our surprise, careful examination of fermentation extracts of *S. cerevisiae* [YEpLtLasS1, YEpLtLasS2] revealed two minor RAL_{14} products (**10** and **11**, Figure 3, isolated yields 0.3 and 0.1 mg/L, respectively). A minor product with the same planar structure as **10** has been described from *Monocillium nordinii* as "nordinone".[40] However, the steroid 11α-hydroxy-17,17-dimethyl-18-norandrosta-4,13-dien-3-one has a precedent for this name[41] so we have renamed the 17(*R*) isomer of the RAL_{14} compound as lasicicol (**10**). Product **11** is the 17(*R*) isomer of the known semisynthetic estrogen antagonist *S*-zearalane.[41] Both lasicicol (**10**) and *epi*-zearalane (**11**) are nonaketides, unlike lasiodiplodin, the major octaketide product of the LtLasS system. The presence of the C-9-oxo group in lasicicol and its absence in *epi*-zearalane indicates a different biosynthetic origin for the extra acetate equivalent incorporated into these compounds (Figure 3). Thus, biosynthetic precedents[11−16] dictate that lasicicol is derived from the same pentaketide starter as lasiodiplodin, but extended with four malonate units by the LtLasS2 nrPKS (a 5 + 4 division between hrPKS and nrPKS for lasicicol, instead of a 5 + 3 split as in lasiodiplodin). In contrast, *epi*-zearalane is expected to be produced with a 6 + 3 split: a hrPKS-derived hexaketide extended with three malonates by the nrPKS. We have not detected the production of **10** and **11** in *L. theobromae*.

Investigation of *S. cerevisiae* [YEpAzRESS1, YEpAzRESS2] failed to reveal the production of resorcylide congener RAL_{14} products. However, a minor nonaketide isocoumarin product, YXTZ-53-51-251 (**9**) was apparent in extended fermentations (>72 h., isolated yield 0.7 mg/L, Figure 4). Product **9** is apparently derived from the "normal" tetraketide starter of resorcylides by the addition of five malonyl units. Thus this molecule originates from a 4 + 5 division between the hrPKS and nrPKS, as opposed to the 4 + 4 split for *trans*-resorcylide (**2**). We were unable to detect the production of **9** in *A. zeae*.

Therefore, both the hrPKS (for *epi*-zearalane in the lasiodiplodin system) and the nrPKS (for lasicicol in the lasiodiplodin system and **9** in the resorcylide system) of the BDLSs may be able to produce a homologous polyketide with one extra acetate equivalent, a process referred to as "stuttering" for modular PKSs.[42] Stuttering may yield minor natural congeners or could be a programmed event that yields the main metabolite.[42,43] Stuttering may be provoked by unnatural intermediates during combinatorial biosynthesis[44] or result from imbalances in precursor supply during *in vitro* synthesis with purified enzymes.[32] The biosynthesis of minor RAL_{14} products in our heterologous host but not in the native producer fungi may reflect the differential abundance of accessible malonyl-CoA precursors in these cells, and the influence of precursor load on iPKS chain length control.

Formation of Known RAL_{12} Congeners. The lasiodiplodin cluster includes two genes encoding potential tailoring enzymes. LtLasM is a 398-amino-acid protein similar to *S*-adenosylmethione-dependent *O*-methyltransferases (OMT) involved in secondary metabolism (pfam00891), including the *H. subiculosus* hypothemycin OMT. Co-expression of LtLasM with LtLasS1 and LtLasS2 in yeast led to the almost complete conversion of **3** to lasiodiplodin (**1**) (Figure 3, isolated yield of 10 mg/L). Thus, co-expression of LtLasS1, LtLasS2, and LtLasM is sufficient to reconstitute the biosynthesis of lasiodiplodin in the yeast heterologous host and confirms that LtLasM is the dedicated OMT responsible for the methylation of the C-3 phenolic alcohol. Interestingly, Hpm5, the OMT from the hypothemycin cluster methylates the C-5 phenolic hydroxyl of the corresponding RAL, providing an alternative site-specific processing enzyme for future combinatorial biosynthesis. Pairing of the LtLasM OMT with the resorcylide iPKSs did not yield methylated resorcylides, indicating that the presence of the resorcylate carboxylic acid moiety is not sufficient for substrate recognition with this enzyme (Supplementary Figure S1).

LtLasG is a putative glutathione-*S*-transferase. The orthologous Hpm2 from the hypothemycin cluster facilitates the isomerization of the 7′,8′-*trans* double bond of aigialomycin through conjugation with glutathione, and this process appears coupled to the excretion of the resulting *cis*-isomer, hypothemycin.[13] Co-expression of LtLasG in *S. cerevisiae* [YEpLtLasS1, YEpLtLasS2] or in *S. cerevisiae* [YEpLtLasS1, YEpLtLasS2, YEpLtLasM] did not yield any new RAL, nor did it facilitate the accumulation of desmethyl-lasiodiplodin or lasiodiplodin in the culture supernatants (Figure 3). Pairing of LtLasG with the resorcylide iPKSs did not alter the product fingerprint in that system either (Supplementary Figure S1). Thus, the endogenous yeast transporters are versatile enough to export resorcylide and lasiodiplodin congeners (this work), as well as other natural and hybrid BDLs.[2,13−16,32]

The production of the 12-, 13-, or 14-hydroxy and the 13-keto congeners of lasiodiplodin that were isolated from *L. theobromae* fermentations but not from our recombinant yeast strains may be ascribed to oxidative tailoring of the primary products 3-*O*-desmethyl-lasiodiplodin and lasiodiplodin by additional enzymes encoded at different loci in the fungal genome and/or to spontaneous oxidations in fungal fermentations.[29]

The resorcylide cluster contains no genes for the tailoring of *trans*-resorcylide (**2**), the apparent main product in the yeast host. Nevertheless, resorcylide congeners (*cis*-resorcylide **5**, 11-hydorxyresorcylide enantiomers **4a** and **4b**, dihydroresorcylide **6**, and 11-methoxyresorcylide enantiomers **8a** and **8b**) have been isolated from the producer fungi[26] and were also recovered from our heterologous yeast producer strain (Figure 4). We also detected the production of small amounts of two isocoumarins, YXTZ-53-51-322 (**7**) and YXTZ-53-51-251 (**9**). A time-course analysis of the fermentation with *S. cerevisiae* co-expressing AzResS1 and AzResS2 (Figure 4) shows that *trans*-resorcylide (**2**) is the main product up till 48 h after the induction of polyketide production. However, 11-hydroxy-resorcylides (**4a** and **4b**) become dominant by 72 h, with congeners **5** to **9** accumulating at low yields. Extending the fermentation to 96 h and beyond did not alter the product ratios any further.

Incubation of purified **2** or **5** with the untransformed yeast host or with the uninoculated fermentation medium led to the gradual appearance of **7**, **8a**, and **8b** (Figure 5). *trans*-Resorcylide **2** was readily converted to *cis*-resorcylide **5** regardless of the presence of the yeast host, but the reverse reaction (conversion of **5** to **2**) was not observed.

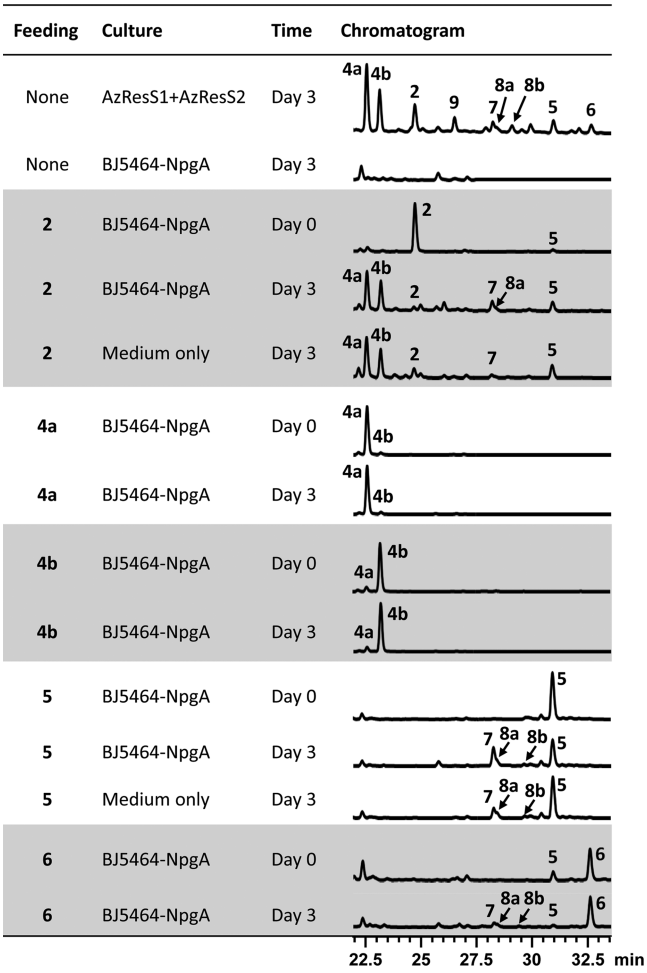

Figure 5. (Bio)conversion of resorcylide congeners. Product profiles (HPLC traces recorded at 300 nm) of *S. cerevisiae* BJ5464-NpgA[31] cultures or media only, supplemented with the purified resorcylide congener at 0.025 mg mL^{-1} at Day 0 and incubated for the indicated amount of time.

Hydroxyresorcylides **4a** and **4b** formed from *trans*-resorcylide (**2**) but not from *cis*-resorcylide (**5**) regardless of the presence of the yeast host. 11-Hydroxyresorcylides (**4a** and **4b**) and dihydroresorcylide (**6**) remained stable in the presence of yeast cells or culture media. The appearance of minor amounts of **7**, **8a**, and **8b** in incubations of **6** with or without the cells is probably due to the conversion of **5**, present as a contaminant in our preparations of **6**. Formation of isocoumarin **9** from purified **2**, **4a**, **4b**, **5**, or **6** was not detected in these bioconversion experiments (Figure 5). Taking these observations together, the biogenesis of the resorcylide congeners may be summarized as follows (Figure 4). The primary product of the AzResS1-AzResS2 PKS system is *trans*-resorcylide **2**. *cis*-Resorcylide **5** is formed by the spontaneous isomerization of the enone in the presence of light.[24] The appearance of the 11-hydroxyresorcylides (**4a** and **4b**, respectively) in approximately 3:2 enantiomeric ratio in longer fermentations and in incubations of **2** with or without the yeast host indicates that water addition is spontaneous in the production medium. 11-Methoxyresorcylides **8a** and **8b** may be formed by methanol addition during workup of the extracts. Such conversions are precedented by the formation of 11-hydroxycurvularin and 11-methoxycurvularin enantiomers from 10,11-dehydrocurvularin in fermentations of *Alternaria cinerariae* and recombinant yeast.[14] The isocoumarin **7** is produced from **2** by base-catalyzed enolization of the α-aryl ketone followed by attack of the resulting enol on the lactone, leading to the opening of the macrolactone ring and formation of the more thermodynamically stable α-pyrone ring. Dihydroresorcylide (**6**) is a genuine alternative biosynthetic product of AzResS1-AzResS2, originating by an off-program reduction at the tetraketide stage by the hrPKS ER domain. Finally, the isocoumarin **9** is a shunt product of the resorcylide iPKS system, resulting from a "stuttering" of the nrPKS (see earlier), followed by product release via α-pyrone formation.[10]

Conclusions. Identification of the biosynthetic gene clusters for lasiodiplodin and resorcylide provides access, for the first time to the best of our knowledge, to the iPKS systems responsible for the production of the RAL_{12} subclass of BDLs. Exposing the apparent origins of resorcylide and lasiodiplodin congeners isolated from many fungal (and bulk plant material) sources helps to disentangle the roles that biosynthetic and chemical interconversions play in generating chemical diversity in these fungi. The close global sequence similarities of BDL synthases in the RAL_{14}, DAL_{12}, and RAL_{12} structural classes suggest that all BDL synthases are orthologous and have been the subject of repeated horizontal gene transfers and/or cluster losses during the evolution of different fungal lineages. The absence of straightforward correlations between primary amino acid sequences of key functional iPKS domains on one hand and BDL structural features (macrocyclic ring size, exocyclic methyl group conformation, or the extent of reduction at the macrolide β-carbons) on the other implicate that BDL structural variety emerges by a select few mutations in key active site cavity positions, as recently validated by us for PT domains[2] and implied by combinatorial biosynthesis with alternative TE domains.[10,45] Observation of RAL_{14} homologues during *in vivo* heterologous production of RAL_{12} metabolites extends previous observations of "stuttering" by modular PKSs[43,44] to the realm of iPKSs in fungi. Together with earlier *in vitro* observations,[32] stuttering in collaborating iPKSs indicates that precursor accessibility may override chain length control in both hrPKSs and nrPKSs. The successful heterologous production of BDLs foretells the possibility of combinatorial biosynthesis of novel BDL analogues and congeners by overexpression of heterologous iPKS pairs in various combinations, a possibility that we will address in a separate communication in due course. Such experiments will help to further exploit these interesting scaffolds against different human disease targets.

METHODS

Strains and Culture Conditions. *Acremonium zeae* NRRL 45893 (Ascomycota, Sordariomycetes, Hypocreomycetidae, Hypocreales) and *Lasiodiplodia theobromae* NBRC 31059 (Ascomycota, Dothideomycetes, Botryosphaeriales, Botryosphaeriaceae) were maintained on potato dextrose agar (PDA, Difco) at 28 °C. *E. coli* DH10B and plasmid pJET1.2 (Fermentas) were used for routine cloning and sequencing, while *E. coli* Epi300 and fosmid pCCFOS1 (Epicentre) were utilized for genomic library construction. The compatible yeast–*E. coli* shuttle vectors pRS425,[46] YEpADH2p-FLAG-URA and YEpADH2p-FLAG-TRP[14] were used to express BDL biosynthetic genes in *Saccharomyces cerevisiae* BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2-Δ1 trp1 pep4::HIS3 prb1 Δ1.6R can1 GAL*),[31] as described.[2,10,14]

Cloning, Sequencing, and Sequence Analysis. Degenerate primer pairs[14] were used to amplify KS- and AT-encoding regions of BDL iPKSs, using chromosomal DNA of *A. zeae* NRRL 45893 and *L.*

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theobromae NBRC 31059 as the templates. Specific primers (Supplementary Table S1) against amplicons encoding iPKS regions showing >50% identity to BDL iPKSs were used to screen libraries raised in pCC1FOS (Epicentre) by PCR. Fosmids AzP3B3, AzP22E9, AzP39H7, and AzP26A7 for *A. zeae* and separately fosmids LtP20C5, LtP40A8, and LtP69D4 for *L. theobromae* were mixed in equimolar ratios, and the two mixtures were sequenced using IonTorrent technology with the 314 chip (Life Technologies, Inc.). Initial assembly of the ~200 bp reads was done with Newbler (Roche Diagnostics), followed by further iterations of assemblies with SeqMan NGen 3.1.2 (DNASTAR). Finishing was done using Sequencher 5.0 (GeneCodes Corp.), with further Sanger sequencing of the fosmids. HMM-based gene models were built with FGENESH (Softberry). The UMA algorithm was used to predict domain boundaries in PKSs.[47]

Production of Polyketides in Engineered Yeast Strains. Details of the construction of BDL expression plasmids are described in the Supplementary Methods. Three to five independent *S. cerevisiae* BJ5464-NpgA transformants were used to survey polyketide production by each recombinant yeast strain. Fermentations with representative isolates were repeated at least three times to confirm results and scaled up to isolate products.[2,14] Cultivation of yeast strains, bioconversion experiments, extraction of polyketides, and routine analysis of extracts by reversed phase HPLC were done as described.[2,14] HPLC conditions for the analysis of resorcylide analogs were 5% CH_3CN in H_2O for 5 min, a linear 5−95% gradient of CH_3CN in H_2O over 10 min, and 95% CH_3CN in H_2O for 10 min; flow rate of 0.8 mL min^{-1}; Kromasil C18 column (5 μm, 250 mm × 4.6 mm); detection at 300 nm.

Chemical Characterization of Polyketide Products. Accurate mass measurements were performed with matrix assisted laser desorption/ionization (MALDI) on a Bruker Ultraflex III MALDI TOF-TOF instrument. Low-resolution mass measurements were done on an Agilent 6130 Single Quad LC-MS. ^1H, ^{13}C, and 2D NMR (COSY, HSQC, HMBC) spectra were obtained in CD_3OD or C_5D_5N on a JEOL ECX-300 spectrometer. Optical rotations were recorded on a Rudolph Autopol IV polarimeter using a 10-cm microcell. Circular dichroism (CD) spectra were acquired with a JASCO J-810 instrument. See the Supplementary Methods for details.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Molecular cloning procedures, isolation and structure characterization for compounds **1**—**11**, and additional bioinformatic analyses. This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

The nucleotide sequence of the resorcylide and the lasiodiplodin loci appear in GenBank under accession numbers KJ434939 and KJ434938, respectively.

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Notes

The authors declare no competing financial interest.

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