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Diversity-Oriented Combinatorial Biosynthesis of Hybrid Polyketide Scaffolds from Azaphilone and Benzenediol Lactone Biosynthons

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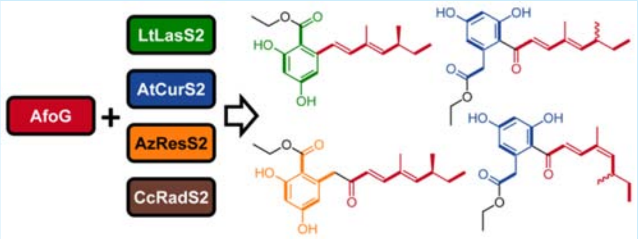
Supporting Information

ABSTRACT: Two disparate polyketide families, the benzenediol lactones and the azaphilones, are produced by fungi using iterative polyketide synthase (iPKS) enzymes consisting of collaborating partner subunits. Exploitation of this common biosynthetic logic using iPKS subunit shuffling allowed the diversity-oriented combinatorial biosynthesis of unprecedented polyketide scaffolds new to nature, bearing structural motifs from both of these orthogonal natural product families. Starter unit acyltransferase domain replacements proved necessary but not sufficient to guarantee communication between iPKS subunits.



Benzenediol lactones (BDLs) and azaphilones are disparate structural classes of fungal polyketide natural products with an impressive array of biological activities.[1,2] Thus, BDLs such as monocillin II (**1**, Figure 1), a resorcylic acid lactone (RAL) that is the precursor of radicicol (**2**), and the dihydroxyphenylacetic acid lactone (DAL) 10,11-dehydrocurvularin (**5**) all display anticancer and immune system modulatory activities.[1,3] Phytotoxic RALs like *trans*-resorcylide (**3**) and 3-*O*-desmethyllasiodiplodin (**4**) show mineralocorticoid receptor antagonist and prostaglandin biosynthesis inhibitory activities in mammals.[1] Pre-asperfuranone (**6**) is a biosynthetic intermediate of asperfuranone (**7**), an azaphilone that may find application as a synthetic precursor for lipoxygenase inhibitors to treat allergies, inflammation, and asthma.[2,4]

The biosynthesis of BDLs involves two sequentially acting iterative polyketide synthases (iPKSs).[5] First, a highly reducing iPKS (hrPKS) produces a reduced linear polyketide that is transferred to a nonreducing iPKS (nrPKS) by the starter unit:ACP transacylase (SAT) domain, where it primes further chain elongation without reductions (Figure 1). Next, the nrPKS product template (PT) domain forms a resorcylic acid (connectivity: C2−C7) or a dihydroxyphenylacetic acid ring (C3−C8). Last, the nrPKS thioesterase (TE) domain releases the BDL scaffold as a macrolactone. Azaphilones feature a pyranoquinone or furanoquinone bicyclic core derived from an nrPKS-produced acyl benzaldehyde (ABH).[2] For azaphilones such as **7**, ABH biosynthesis on the nrPKS is primed by an hrPKS-derived reduced polyketide chain in a sequential biosynthetic scheme analogous to that of BDLs (Figure 1).[6−8] Some other azaphilones use a convergent biosynthetic scheme, whereby the hrPKS product acylates a free pyranoquinone intermediate.[9−11] Chimeric BDL scaffolds were previously generated by us using iPKS subunit shuffling among noncognate BDL synthase (BDLS) hrPKS and nrPKS partners.[12] While domain replacements allowed the asperfuranone nrPKS to accept a single medium-chain fatty acid substrate offered either by the fatty acid synthase or another azaphilone hrPKS resident in *Aspergillus nidulans*, other attempts to force the enzyme to utilize alternative acyl substrates proved unproductive.[13,14] As opposed to generating chimeric products based solely on the BDLs or alternatively on the azaphilones, in this work, we investigated whether we can bridge these two nonorthologous natural product groups by leveraging their common sequential biosynthetic logic and produce chimeric scaffolds that fuse orthogonal biosynthons from the two groups in a diversity-oriented combinatorial biosynthetic scheme.

Co-expression of the *A. nidulans* asperfuranone hrPKS−nrPKS pair AfoG and AfoE[8] in *Saccharomyces cerevisiae* BJ5464-NpgA[15,16] led to the production of the expected ABH **6** (5.1 mg/L, Figure 2). ABH **6** was previously isolated from the azaphilone producer *Penicillium multicolor*[17] and from engineered *A. nidulans* strains.[4,7,8] *S. cerevisiae* strains expressing the AfoG−AfoE pair, or AfoE alone, also produced two ABHs primed by medium-chain fatty acids (**10**, 0.13 mg/L and **11**, trace

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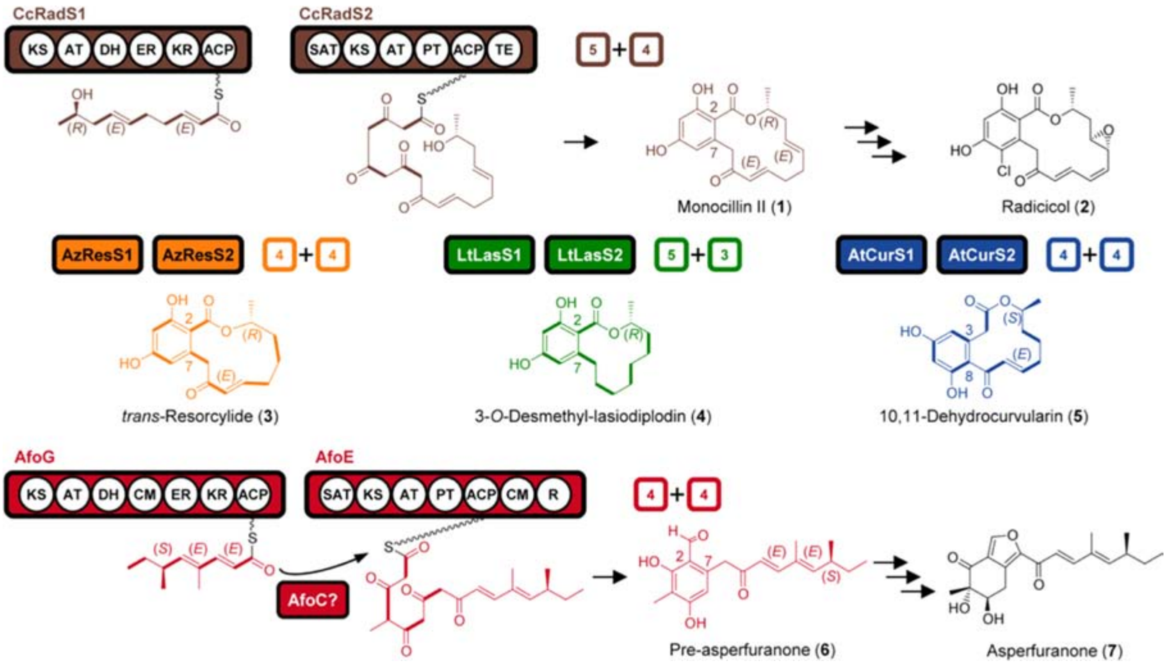

Figure 1. Biosynthesis of BDLs and the azaphilone asperfuranone. Colored numerals: the number of ketide (C2) units (C−C bond in bold) incorporated by the hrPKS vs the nrPKS ("split" of the iPKS pair). Key: ACP, acyl carrier protein; AT, malonyl-CoA:ACP acyltransferase; CM, C-methyltransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; KS, ketosynthase; PT, product template; R, reductive release; SAT: starter unit:ACP transacylase; TE, thioesterase.

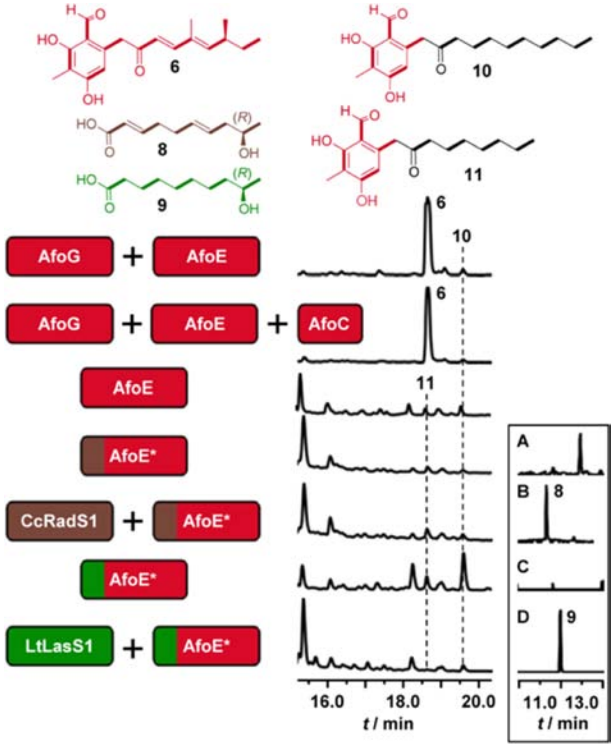

Figure 2. Combinatorial biosynthesis with BDL hrPKSs and AfoE. HPLC profiles recorded at 300 nm and LCMS-selected ion chromatograms (inset: (A and B) m/z 207 $[M + Na]^+$; (C and D) m/z 211 $[M + Na]^+$) of *S. cerevisiae* BJ5464-NpgA expressing hrPKS-nrPKS pairs. Unlabeled peaks: yeast metabolites unrelated to the iPKS products. Split-color boxes (*): replacement of SAT_{AfoE} by SAT_{BDLS} to enforce coupling.

amounts, Figure 2). Indeed, **10** was previously isolated from *A. nidulans* upon exchange of SAT_{AfoE} (Supporting Information).[13,14] Thus, similar to BDL nrPKSs,[18,19] the azaphilone nrPKS AfoE also routinely engages in a metabolic cross-talk with the fatty acid biosynthesis of its host cell.

AfoC, a putative esterase/lipase, was speculated to transfer dimethyloctanoate from AfoG to AfoE (Figure 1).[7] Similarly, the AfoC orthologue AzaC was suggested to off-load the AzaB hrPKS product during azanigerone biosynthesis in *A. niger*.[9] In contrast, Cox et al. proposed that AfoC plays a role in the formation of the furan ring of **7**.[20,21] Considering the controversial role of AfoC, we coexpressed *afoC* with *afoG* and *afoE* in *S. cerevisiae* BJ5464-NpgA. However, the yield of **6** did not increase, nor were any additional metabolites produced (Figure 2). Thus, AfoC is neither necessary nor does it contribute to the formation of **6** in the heterologous system. This argues against the proposed role of AfoC in chain transfer,[7] but it is in agreement with the direct transfer of acyl chains between collaborative iPKSs.[6,22] Meanwhile, the absence of new metabolites was not surprising since furan ring formation requires a more advanced precursor than **6**.

Pairing CcRadS1, AzResS1, LtLasS1, or AtCurS1 with AfoE yielded none of the expected hybrid ABHs even after SAT_{AfoE} was exchanged for the cognate SAT_{BDLS} (Figure 2). Failure to obtain the expected products with the AfoE−SAT_{BDLS} hybrids was not due to generally disabled nrPKSs, since these enzymes still produced the fatty acyl ABHs **10** (0.08−0.5 mg/L) and **11** (0.03−0.1 mg/L) when expressed in the host without hrPKS partners. Importantly, the fatty acyl chains that were readily utilized to generate **10** and **11** are of the same lengths as those generated by our BDL hrPKSs (CcRadS1 and LtLasS1: pentaketides as in **10**; AzResS1 and AtCurS1: tetraketides as in **11**). Remarkably, the only difference between the accepted decanoate for **10** and the rejected (9*R*)-hydroxydecanoate (**9**) from LtLasS1 is the presence of the Ω-1 hydroxyl group in the

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latter. Thus, AfoE refuses BDL acyl chains not simply because of their lengths but because they contain more oxidized carbon centers. We had previously shown that noncognate BDLS subunits readily generate hybrid polyketides in vivo if their collaboration is enforced by SAT exchanges.[12] In contrast, these and other experiments[13] show that the replacement of the SAT domain alone is not always sufficient to guarantee communication between noncognate iPKSs. For AfoE, and by extension other nrPKSs, the rest of the chassis retains a decisive role in substrate utilization.

Unexpectedly, pairing of CcRadS1 with AfoE−$\mathrm{SAT_{CcRadS2}}$ and that of LtLasS1 with AfoE-$\mathrm{SAT_{LtLasS2}}$ led to the production of the hrPKSs-derived pentaketides (**8**, 0.3 mg/L and **9**, 3 mg/L, Figure 2). In contrast, the tetraketides of AzResS1 and AtCurS1 could not be detected upon pairing those enzymes with their cognate AfoE−$\mathrm{SAT_{BDLS}}$. Compounds **8** and **9** were not detectable when CcRadS1 or LtLasS1 was expressed alone in the host. Pairing of these enzymes with the native AfoE or with their cognate BDL nrPKS partners similarly failed to afford these compounds.[23] To the best of our knowledge, release and prominent accumulation of the reduced carboxylic acids from hrPKS subunits of collaborating iPKS systems has not been reported previously.

Pairing the asperfuranone hrPKS AfoG with CcRadS2, AzResS2, LtLasS2, or AtCurS2 led to no product formation. However, AfoG heterocombinations with chimeric BDL nrPKSs featuring $\mathrm{SAT_{AfoE}}$ proved to be more successful (Figure 3). First, all of these heterocombinations afforded the expected tetraketide product of AfoG as a free carboxylic acid in substantial amounts (**12**, 0.2−0.7 mg/L). The highest yielding strain, AfoG−AtCurS2−$\mathrm{SAT_{AfoE}}$, also produced the phenethyl ester of **12** in good amounts (**13**, 0.3 mg/L). Compounds **12** and **13** were not detected in strains expressing either the native Afo pair or the AfoG−BDLS pairs without $\mathrm{SAT_{AfoE}}$. Efficient production of **8**, **9**, and **12** suggests that these polyketides are off-loaded from the hrPKS by the cognate SAT domain, resulting in a SAT oxoester ($\mathrm{SAT_{CcRadS2}}$) or thioester ($\mathrm{SAT_{LtLasS2}}$, $\mathrm{SAT_{AfoE}}$).[22] In the absence of a productive interaction with the nrPKS KS domain, these esters afford the free carboxylic acids **8**, **9**, and **12** by hydrolysis. Release of **12** from $\mathrm{SAT_{AfoE}}$ (or from $\mathrm{ACP_{AfoG}}$) may also be facilitated by 2-phenylethanol, a phenylalanine catabolic product of the yeast host, leading to the formation of **13**. In either case, the reductive release domain of AfoE is not expected to be involved, since $\mathrm{R_{AfoE}}$ would produce aldehydes and not carboxylic acids.

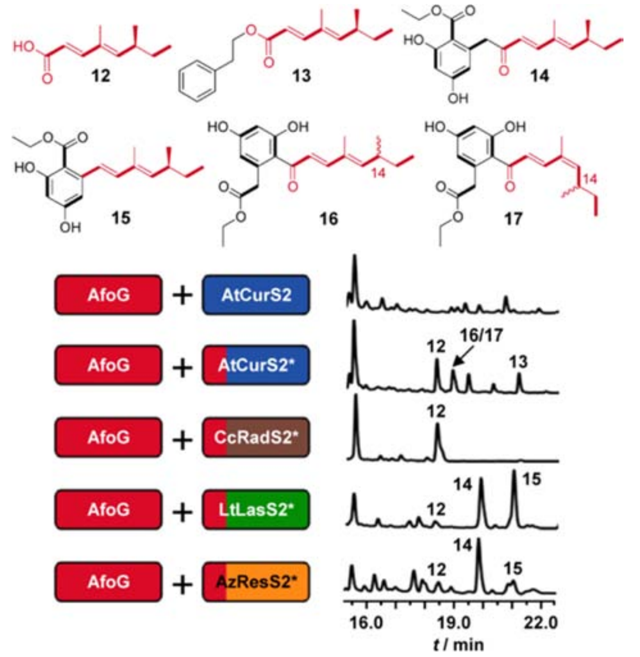

Figure 3. Combinatorial biosynthesis with AfoG and BDL nrPKSs. HPLC traces were recorded at 300 nm for *S. cerevisiae* BJ5464-NpgA cotransformed with hrPKS−nrPKS pairs. Unlabeled peaks: yeast metabolites unrelated to iPKS products. Split-color boxes (*): replacement of $\mathrm{SAT_{BDLS}}$ by $\mathrm{SAT_{AfoE}}$.

While **12** was the sole product of the AfoG−CcRadS2−$\mathrm{SAT_{AfoE}}$ pair, the strains with the three other AfoG−BDLS−$\mathrm{SAT_{AfoE}}$ heterocombinations produced the ethyl ester derivatives of the expected hybrid acyl benzenediol acids (ABAs, Figure 3). These ABA ethyl esters all feature the dimethyl tetraketide biosynthon of asperfuranone. This substrate was accepted by the BDLS nrPKSs, successfully extended with the appropriate number of ketide (C_2) units, and cyclized in the PT-programmed register to produce the expected resorcylic or dihydroxyphenylacetic acid rings. Considering the substantial steric bulk introduced by the two methyl substituents in the branched acyl chain, this represents a remarkable flexibility on the part of the BDL nrPKSs. Nucleophilic attack by ethanol from yeast metabolism was the favored mechanism for the release of these ABAs, since hydrolysis of the TE oxoesters to produce free ABAs or pyrone formation with the C9 self-enol was not evident.[19] Thus, the strain expressing the AfoG−LtLasS2−$\mathrm{SAT_{AfoE}}$ heterocombination produced 0.2 mg/L of the heptaketide **15**. This ABA ethyl ester displays the expected 4 + 3 "split" (i.e., the AfoG-derived dimethyl tetraketide was extended by three additional ketide units by the nrPKS) and features the predicted resorcylic acyl ring (C2−C7 first ring connectivity established by $\mathrm{PT_{LtLasS2}}$[23,24]). In addition to **15**, the strain also produced the octaketide **14** (0.12 mg/L) with an apparent 4 + 4 split. Stuttering (incorporation of an extra ketide unit) by LtLasS2 was seen previously.[12,23] Surprisingly, the strain expressing the AfoG−AzResS2−$\mathrm{SAT_{AfoE}}$ pair yielded the same two products: **14** (0.2 mg/L) with the expected 4 + 4 split and **15** (0.05 mg/L), with an apparent 4 + 3 split. While AzResS2 is programmed to incorporate four ketide units and may also stutter,[23] production of the heptaketide **15** requires AzResS2 to "sputter" (incorporate one fewer ketide units). Sputtering has been observed with the zearalenone nrPKS[18] as well as with a chimera of the asperfuranone nrPKS AfoE.[13]

Pairing AfoG with AtCurS2-$\mathrm{SAT_{AfoE}}$ provided the C12(13) *cis/trans* (1:0.7) isomeric and C14 racemic mixture of the ADA ethyl esters **16** and **17** (combined yield: 0.4 mg/L). We propose that the *E*-C12(13) double bond in (14*S*)-**16** (originating as the *E*-C4(5) double bond of the AfoG-derived biosynthon[4,8]) may undergo spontaneous *E*/*Z* isomerization during fermentations and/or workup of the extracts to yield **16** and **17**. Similar isomerizations were also detected with the chaetochiversins and paraphaeosporins in fermentations with *Chaetomium chiversii* and *Paraphaeosphaeria quadriseptata*.[25] The absence of *Z* isomers of **6**, **13**, and **14** suggests a possible role that the phenolic OH at *ortho* and *para* positions to the acyl chain in **16** may play to facilitate the enolization of the conjugated C9 ketone, as a prelude toward *E*/*Z* isomerization at C12(13) with concomitant racemization at C14.

Diversity-oriented combinatorial biosynthesis with fungal iPKSs promises to expand the polyketide structural space toward unprecedented carbon skeletons that may be exploited for drug discovery. Previously, we showed that subunit shuffling among the orthologous BDLS family of collaborating iPKSs is a facile approach to generate such biosynthetic diversity.[12] The work presented here attempted to extend this methodology toward nonorthologous collaborating iPKSs and thus merge two disparate polyketide families, the BDLs and the asperfuranones. The asperfuranone nrPKS AfoE turned out to be a fastidious catalyst accepting only straight-chain fatty acids by cross-talk with the host primary metabolism but rejecting polar acyl chains from four different BDL hrPKSs in spite of SAT exchanges. Accumulation of the hrPKS products as free acids in these hybrid systems indicated that SAT domain-catalyzed transfer of the hrPKS products is a necessary but not sufficient precondition of further chain extension by the nrPKS. This further confirms a proposed gating function practiced by the KS[26] and/or other domains of the nrPKS chassis.[19] Meanwhile, release of the hrPKS products also provides a scalable method to synthesize substituted medium chain fatty acids (such as **8**, **9**, and **12**) in biological systems for use as synthetic building blocks. Gratifyingly, productive interactions emerged between the hrPKS AfoE and three of the four BDL nrPKSs, enforced with SAT exchanges. This led to the incorporation of unprecedented, orthogonal, branched acyl chains into BDL-related ARA and ADA ethyl esters new to nature. Both AfoE and the BDL nrPKSs retained their intrinsic biosynthetic programs and built their idiosyncratic biosynthons so that the chimeric system combined into novel unnatural products such as **14**−**17**. The foreign substrate nevertheless led to compensatory adjustments in the overall size of the polyketide products[14] by provoking stuttering or sputtering of the nrPKS. In conclusion, this work suggests that continued expansion of the polyketide space by iPKS engineering will require, beyond transplanted SAT domains, a better understanding of the multistep, sequential decision gating functions of nrPKSs, and an improved aptitude to manipulate the factors that determine the acceptability of orthogonal acyl chains transferred to hybrid nrPKSs for further chain extension.[27]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00110.

> Complete description of methods and additional results; tables and figures, including structure elucidation for compounds **6** and **8**−**17**; proposed mechanism for isomerization and racemization of **16** and **17** (PDF)

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Notes

The authors declare no competing financial interest.

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