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Intrinsic and Extrinsic Programming of Product Chain Length and Release Mode in Fungal Collaborating Iterative Polyketide Synthases

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Abstract

Combinatorial biosynthesis with fungal polyketide synthases (PKSs) promises to produce unprecedented bioactive “unnatural” natural products (uNPs) for drug discovery. Genome mining of the dothideomycete *Rhytidhysteron rufulum* uncovered a collaborating highly reducing PKS (hrPKS) – nonreducing PKS (nrPKS) pair. These enzymes produce trace amounts of rare S-type benzenediol macrolactone congeners with a phenylacetate core in a heterologous host. However, subunit shuffling and domain swaps with voucher enzymes demonstrated that all PKS domains are highly productive. This contradiction led us to reveal novel programming layers exerted by the starter unit acyltransferase (SAT) and the thioesterase (TE) domains on the PKS system. First, macrocyclic vs. linear product formation is dictated by the intrinsic biosynthetic program of the TE domain. Next, the chain length of the hrPKS product is strongly influenced *in trans* by the off-loading preferences of the nrPKS SAT domain. Last, TE domains are size-selective filters that facilitate or obstruct product formation from certain priming units. Thus, the intrinsic programs of the SAT and TE domains are both part of the extrinsic program of the hrPKS subunit, and modulate the observable metaprogram of the whole PKS system. Reconstruction of SAT and TE

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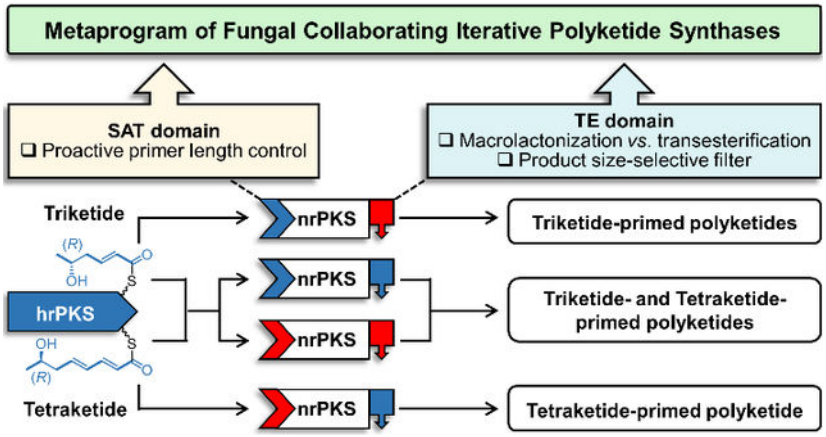
SUPPORTING INFORMATION

Supporting Information. Experimental and bioinformatic procedures, descriptions on the isolation and structure elucidation of compounds, spectroscopic data, supplementary figures including additional structures, product distributions during combinatorial biosynthesis, and phylogenetic trees.

phylogenies suggests that these domains travel different evolutionary trajectories, with the resulting divergence creating potential conflicts in the PKS metaprogram. Such conflicts often emerge in chimeric PKSs created by combinatorial biosynthesis, reducing biosynthetic efficiency or even incapacitating the system. Understanding the points of failure for such engineered biocatalysts is pivotal to advance the biosynthetic production of uNPs.

Graphical Abstract



INTRODUCTION

Polyketides constitute a large group of natural products with enormous structural diversity. In their natural contexts, they act as toxins, pigments, virulence factors, and effectors of communication and competition.[1] Many polyketides, together with their semisynthetic derivatives and analogs have been developed into selective chemical probes and prominent therapeutics.[2] In particular, fungal polyketide scaffolds have provided blockbuster drugs such as the anticholesteremic agent lovastatin, the immunosuppressant mycophenolic acid, and the antifungal griseofulvin.[3] Fungal polyketides are biosynthesized from short chain carboxylic acid monomers (typically, acetyl or malonyl-coenzyme A) through recursive, decarboxylative thio-Claisen condensations by multidomain monomodular megasynthases similar to a single module of the bacterial Type I modular PKSs.[4] However, unlike most modular PKSs, the fungal enzymes iteratively use a single set of ketoacyl synthase (KS), malonyl acyltransferase (AT) and acyl carrier protein (ACP) domains to catalyze all chain extension cycles (iterative PKSs).[3] Based on their domain content, fungal iterative PKSs are classified into three groups. Highly reducing PKSs (hrPKSs) harboring ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER) domains execute a cryptic biosynthetic program to selectively reduce the nascent β-keto moieties after each chain extension step into a β-alcohol, an alkene, or an alkane.[5–7] Partially reducing PKSs lack ER domains and afford relatively simple cyclic structures.[8] Finally, nonreducing PKSs (nrPKSs) lack all reductive domains, but incorporate a starter unit : ACP transacylase (SAT) domain for priming unit selection,[9,10] and a product template (PT) domain for regiospecific closure of the first aromatic ring.[10,11] In addition, nrPKSs typically feature a *C*-terminal product release domain, such as a C-C bond-forming thioesterase/Claisen cyclase that yields

terminal benzene moieties;[12] a Type I O-C bond-forming thioesterase (TE) that affords macrolactones, pyrones, complex esters or simple carboxylates;[13] or a reductive release domain that produces aromatic aldehydes or acylresorcinols.[12,14]

The biosynthesis of the majority of fungal polyketides requires a single iterative PKS each. However, some polyketides, including the congeners of the benzenediol lactones (BDLs), polylactones, sorbicillinoids, tricholignans and some azaphilones are assembled by a collaborating hrPKS - nrPKS pair acting in sequence.[13–15] In these quasi-modular systems the hrPKS produces a linear polyketide chain with an idiosyncratic reduction pattern and configuration (Fig. 1A). This priming unit is transferred by the SAT domain onto the downstream nrPKS for further chain elongation without reduction, and first ring aldol cyclization.[16] In the case of BDL congeners, chain release typically involves macrolactone formation.[17] Further tailoring reactions may then afford the mature bioactive molecules.[18–20] BDLs featuring a C2-C7 connectivity at the benzene ring (F-type cyclization) are the resorcylic acid lactones (RALs), while those having a C8-C3 connectivity (S-type cyclization) are known as dihydroxyphenylacetic acid lactones (DALs).[16,21] Natural RALs contain 12- or 14-membered macrocycles (RAL_{12} or RAL_{14}). These compounds are rich pharmacophores with wide-ranging biological activities, such as resorcylide, a RAL_{12} mineralocorticoid receptor antagonist; lasiodiplodin, a RAL_{12} prostaglandin biosynthesis inhibitor; radicicol, a RAL_{14} heat-shock protein 90 inhibitor; and hypothemycin, a RAL_{14} selective inhibitor of mitogen-associated protein kinases (SI Fig. S1). As opposed to RALs, Nature synthesizes a much more confined library of DALs, exemplified by sporostatin, a DAL_{10} kinase inhibitor, and 10,11-dehydrocurvularin, a DAL_{12} p97 inhibitor.[22,23] One hallmark of fungal iterative PKSs is their enigmatic programming rules that control starter unit selection; the extent of chain extension; the degree and configuration of β-keto reduction; and the chemo- and regio-specificity of ring closure. For BDL congeners, an additional level of programming complexity arises from the interactions of the BDL synthase subunits (i.e. the hrPKS and the nrPKS) as a result of the inter-protein handover of the priming unit.[24] Insights into the *intrinsic* biosynthetic programs of fungal iterative PKS domains (i.e. their catalytic competence towards a given intermediate), their *extrinsic* programming (i.e. domain interactions and the kinetic competition for a given intermediate) and the emergent *metaprogram* of the PKS (i.e. the overall observable program of the whole system) may accelerate the discovery of novel polyketide scaffolds, and guide the rational engineering of iterative PKSs for the production of “unnatural” natural products (uNPs).

We have been investigating the programming rules and exploring the promiscuity and plasticity of collaborating hrPKS – nrPKS pairs in fungal BDL synthases using in vivo combinatorial synthetic biology. We generated novel BDL scaffolds in the heterologous host *Saccharomyces cerevisiae* by BDL synthase subunit shuffling, combinatorial domain exchanges and rational engineering of the active site cavities of domains.[16,17,24,28] We also explored combinatorial BDL scaffold decoration with non-cognate tailoring enzymes.[29,30] Although product structures may be affected in artificial contexts such as some heterologous hosts or during in vitro reconstitution of biosynthetic enzymes[31], BDL synthases and similar fungal iterative PKSs show remarkably high programmatic fidelity upon heterologous expression in yeast or *Aspergillus* chassis.[3,14] Here we report the resuscitation of an unproductive orphan BDL synthase system, discovered by genome mining, by exploiting its

combinatorial biosynthetic potential. In addition to generating unexpected non-macrocyclic DAL congeners, our combinatorial synthetic biology approaches also reveal novel layers of programming exerted by the SAT and TE domains on the BDL synthase system, and demonstrate that evolutionary divergence in the intrinsic programs of iterative PKS programming subroutines may create conflicts in the metaprogram of the system and thus incapacitate these semi-modular synthases.

RESULTS

Functional Characterization of an Orphan DAL Biosynthetic Gene Cluster from *Rhytidhysteron rufulum*

During the course of our genome mining efforts, we encountered a BDL synthase biosynthetic gene cluster in the published genome sequence[25] of the plant pathogenic fungus *Rhytidhysteron rufulum* CBS 306.38 (Dothideomycetes, Hysteriales). This cluster harbors a pair of genes (*rrdalS1* and *rrdalS2*) that encode an orphan hrPKS – nrPKS pair (RrDalS1 – RrDalS2, Figure 1B and C).[25] These iterative PKS genes are bracketed by *rrE1* and *rrE2* encoding two major facilitator superfamily (MFS) exporters (Supporting Information, SI Table S1). However, no genes encoding potential polyketide decoration enzymes are present in this cluster, suggesting that the scaffold generated by RrDalS1 – RrDalS2 does not undergo post-PKS tailoring. Global sequence comparisons showed that RrDalS1 and RrDalS2 are orthologous to characterized BDL-producing hrPKSs and nrPKSs, respectively, with identities in the 55% to 66% range (SI Table S2). Detailed sequence comparisons indicated that the domains of RrDalS1 and RrDalS2 share the highest sequence identities with those of DAL-type BDL synthases, and predicted that $PT_{RrDalS2}$ catalyzes an S-type (C8-C3) aldol condensation to yield a DAL scaffold (SI Table S2).[16] Since DALs are relatively rare in Nature,[22] we set out to functionally characterize this BDL synthase using the heterologous host *Saccharomyces cerevisiae* BJ5464-NpgA.[26,27] Characterization of the native product of the system in *R. rufulum* was not attempted due to difficulties in obtaining the strain, the absence of information on the expression conditions of the cluster, and the lack of developed methods for the genetic manipulation of the fungus. Co-expressing the intron-free *rrdalS1* and *rrdalS2* genes synthesized with codon optimization for expression in yeast led to the production of several polyketide products in minute quantities insufficient for isolation (Entry #1 in Fig. 2 and SI Fig. S3). Thus, we opted for a combinatorial synthetic biology approach where RrDalS1 and separately RrDalS2 were paired with non-cognate BDL synthase partners from four model systems: AtCurS1 – AtCurS2 for 10,11-dehydrocurvularin biosynthesis,[20] AzResS1 – AzResS2 for *trans*-resorcylide production,[19] LtLasS1 – LtLasS2 for desmethyl-lasiodiplodin assembly,[19] and CcRadS1 – CcRadS2 for monocillin II synthesis.[18] These voucher systems offer pre-defined biosynthons that assemble to a variety of products with different scaffold types (DAL and RAL), macrolactone ring sizes (12- and 14-membered rings), macrolactone redox patterns (ketone, alkene, and alkane) and configurations (*R* or *S* configuration of the alcohol at the penultimate carbon).

First, the hrPKS RrDalS1 was co-expressed with the DAL-making nrPKS AtCurS2 in *S. cerevisiae.* The resulting heterocombination produced the same polyketides as those

obtained with the native *R. rufulum* PKS pair, but with an improved titer for compound **11** (Entry #3 in Fig. 2 and SI Fig. S3). Structure elucidation of **11** (Fig. 3) using HRESIMS, NMR, and other spectroscopic techniques showed that this compound is an acyl dihydroxyphenylacetic acid (ADA) ethyl ester featuring an (*R*,2*E*,4*E*)-7-hydroxyocta-2,4-dienoyl moiety, derived from the tetraketide priming unit **4Pc** (Fig. 2). The minor product **1b** and its hydrated analogues **2c**/**d** turned out to be ADA ethyl esters derived from the triketide **3Pa**, a priming unit that results from the incorporation of one fewer ketide units.[15,28,32] Thus, the increased productivity of **11** at the expense of **1b** and **2c/d** with AtCurS2 effectively reversed the proportion of triketide-derived vs. tetraketide-derived products (3:1 with the native pair, 1:3 with the heterocombination, Entries #1 vs. #3 in Fig. 2 and SI Fig. S3). To reveal the structural basis for this shift, we replaced the KS-AT-PT-ACP chassis of RrDalS2 with that of AtCurS2. The resulting chimera produced the same array of polyketides as the native pair when challenged with RrDalS1, but with an even lower productivity (Entry #2 in Fig. 2 and SI Fig. S3). This indicated that the RrDalS2 polyketide homologation machinery (KS, AT and ACP) and first ring cyclase (PT) are not the primary determinants for the observed low productivity of the *R. rufulum* BDL synthase. Moreover, the ratio of triketide vs. tetraketide-derived products was the same as that with the native RrDalS pair (3:1), indicating that the SAT and/or the TE domains are the decisive factor(s) that determine the length distribution of the RrDalS1-produced priming units.

To further probe the program of RrDalS1, we created heterocombinations with the RAL-producing nrPKSs AzResS2, LtLasS2, and CcRadS2 (Entries #4, #5 and #6 in Fig. 2 and SI Fig. S3). These RAL nrPKSs accepted tetraketide **4Pc** as the priming unit, with no **3Pa**-derived product evident, and carried out their characteristic biosynthetic program for chain elongation and formation of the benzene ring in the C2-C7 register. Off-program products were also obtained, however these all originated from anomalies in the chain elongation programs of the RAL-type nrPKSs and not that of RrDalS1. Thus, ARA ethyl ester **22** formed when AzResS2 conducted only three extension cycles instead of the on-program four, while isocoumarin **21** derived from the incorporation of four ketide units instead of the on-program three by LtLasS2 (Entries #4 and #5 in Fig. 2 and SI Fig. S3). The products were released by transesterification to ethanol produced by the yeast host (**1b**, **2c**/**d**, **11**, **20** and **22)**, or by formation of an α-pyrone (**21**) as a result of an intramolecular nucleophilic attack of the C-9 enol on the C-1 carbonyl. The absence of DAL or RAL products in these experiments may reflect the difficulty for the RrDalS1-derived short (**3Pa**) or very rigid (**4Pc**) acyl priming units to adopt a conformation conducive for macrolactone formation. Taken together, these heterocombination experiments show that RrDalS1 is a competent hrPKS that synthesizes tetraketide **4Pc** with an *R*-configured secondary alcohol and an α,β,γ,δ-unsaturated ketone functionality as its typical product, but may also afford the shorter homologue **3Pa**.

Next, we turned to clarify the biosynthetic program of RrDalS2 using heterocombinations with voucher hrPKSs. All four resulting noncognate pairs produced a panel of novel polyketides in low to fair amounts (Entries #11, #18, #25 and #31 in Fig. 2 and SI Fig. S4). RrDalS2 accepted the enantiomeric tetraketide priming units **4Pa** (from AtCurS1) and **4Pb** (from AzResS1), readily executed four rounds of chain elongation, faithfully carried out the

predicted S-type (C8-C3) aldol condensation, and exclusively released the final products as ADA alkyl esters with no macrolactones detectable (Entries #11 and #18 in Fig. 2 and SI Fig. S4). The expected α,β-enone functionality from **4Pa** or **4Pb** is transformed into β-hydroxyl or α-tetrahydropyranyl ketone moieties via spontaneous inter- or intramolecular oxy-Michael addition in the matured products. These compounds (Fig. 3, **7a**/**b**, **8a**/**b**, **9a** and **10a** from AtCurS1 – RrDalS2 vs. **7c**/**d**, **8c**/**d**, **9b**, and **10b** from AzResS1 – RrDalS2) are enantiomers in a pairwise manner due to the enantiomeric nature of **4Pa** and **4Pb**. Replacement of the KS-AT-PT-ACP chassis of RrDalS2 with that of AtCurS2 led to no change in the product profiles (Entries #12 and #19 in Fig. 2 and SI Fig. S4), although significantly improved overall productivity.

The LtLasS1 – RrDalS2 noncognate pair showed low productivity (Entry #25 in Fig. 2 and SI Fig. S4), but afforded the expected DAL lasilarin (**14**) and its ADA ester analogue (**16**), both derived from the on-program pentaketide priming unit of LtLasS1 (**5Pa**). Nevertheless, the main product was *epi*-curvularin (**6**, Fig. 3), a DAL derived from the off-program, shorter tetraketide priming unit **4Pd**. Replacing the KS-AT-PT-ACP chassis of RrDalS2 with that of AtCurS2 again increased productivity (Entry #26 in Fig. 2 and SI Fig. S4), revealing additional minor pentaketide or tetraketide-derived ADA ethyl esters such as **19** (from priming unit **5Pc**), **12** and **13** (from **4Pd**) and **7c/d** and **8d** (from **4Pb**). While these minor products increased the share of ADA esters at the expense of macrocyclic products, priming unit chain length preferences remained largely unchanged with the chimera. Curiously, this heterocombination also produced (*R*)-9-hydroxydecanoic acid (**23**, Fig. 3), the free on-program pentaketide priming unit **5Pa** of LtLasS1. Release of hrPKS products by hydrolysis was observed earlier[28] and may reflect a proofreading process (presumably by the TE) that removes unproductive intermediates from the nrPKS.

The CcRadS1 – RrDalS2 noncognate pair was unproductive, but could be revived by enforcing subunit coupling via $SAT_{RrDalS2}$ replacement with $SAT_{CcRadS2}$ (Entry #31 in Fig. 2 and SI Fig. S4). While overall productivity remained very low, this domain replacement nevertheless allowed product formation from **5Pb**, the on-program pentaketide primer from CcRadS1. However, ADA ethyl ester **17** and its hydrated analogues (**18a**/**b**) were the dominant products, with the expected DAL_{14} radilarin (**15**) barely detectable. Like compounds **7a/b** and **7c/d**, the isomeric mixture **18a**/**b** was generated by spontaneous hydration of the α,β-unsaturated bond of the enone. The diastereomers **18a** and **18b** were separated on a chiral column and their absolute configurations were assigned by ECD/TDDFT calculations as 11*S*,17*R* and 11*R*,17*R*, respectively (Fig. 3, SI Fig. S8.4). Replacing the KS-AT-PT-ACP chassis of RrDalS2 with that of AtCurS2 led to the production of the same pentaketide priming unit-derived products in trace amounts (Entry #32 in Fig. 2 and SI Fig. S4).

Taken together, these heterocombination experiments show that RrDalS2 is a competent nrPKS that is programmed to append an S-type (C8-C3) tetraketide biosynthon to a tetraketide priming unit, and release the product predominantly as an ADA ester. Pentaketide priming units are disfavored, but may allow the synthase to afford minute amounts of macrocyclic (DAL_{14}) products. DAL_{12} products are also possible when using a completely saturated, highly flexible tetraketide priming unit such as **4Pd**. The KS-AT-PT-ACP chassis

seems largely irrelevant in determining product distribution between macrocycles and linear products, or amongst compounds derived from priming units of different lengths, emphasizing that the chassis does not influence priming unit selection or chain termination chemistry.

SAT Domains as Proactive Logic Gates

Our subunit shuffling approach revealed that the individual RrDalS subunits are catalytically competent enzymes; uncovered their likely biosynthetic programs; and yielded a panel of unprecedented polyketides. Importantly, these experiments showed that AtCurS2 and RrDalS2 are orthologous DAL synthases that extend various priming units by incorporating four more malonate equivalents; however, these enzymes also show idiosyncratic differences in their chain initiation and termination chemistries. Considering the well-established role of SAT domains in priming unit loading,[9,10] we created hybrid RrDalS2 / AtCurS2 enzymes by swapping their SAT domains, and interrogated these chimeric enzymes by offering them the priming units produced by RrDalS1 or the voucher hrPKSs.

Grafting the $SAT_{RrDalS2}$ domain onto AtCurS2 led to surprisingly large shifts in the product distributions of all noncognate pairs, clearly favoring shorter, and if possible, triketide priming units (Entries #7, #14, #21, #28 and #34 in Fig. 2 and SI Fig. S5). Thus, with RrDalS1 as the partner, the formerly not isolable triketide-primed compounds **1b** and **2c**/**d** became the exclusive products, delivered at fair titers (Entry #7 vs. Entry #3 in Fig. 2 and SI Fig. S5). Compounds **2c** and **2d** are C-11 epimeric ADA ethyl esters that feature a 1,3-diol moiety in the acyl sidechain (Fig. 3). The configuration of C-11 was determined as 11*S* for **2c** and 11*R* for **2d** by ECD/TDDFT calculations (SI Fig. S8.1), considering that the secondary alcohol at C-13 should adopt an *R* configuration as was seen in compound **11**. Purified **1b** was rapidly converted to a mixture of **1b** and **2c**/**d** in aqueous solution, suggesting that it has an α,β-enone functionality that is prone to spontaneous oxy-Michael addition.

When pairing with AtCurS1 or AzResS1, the chimeric AtCurS2($SAT_{RrDalS2}$) accepted the enantiomeric tetraketide starter units **4Pa** or **4Pb**, and predominantly released the products as DALs (**4a**/**b** and **5a**–**d**) (Entries #14 and #21 in Fig. 2 and SI Fig. S5). However, triketide-primed polyketides **1a**, **2a**/**b** and **3** (for AtCurS1), and **1b** and **2c**/**d** (for AzResS1) were also detected. Compounds **2b** and **2a** were elucidated to have 11*R*,13*S* and 11*S*,13*S* configurations, respectively. Thus, these are the enantiomers of **2c** and **2d** in a pairwise manner (Fig. 3, SI Fig S8.1 and Table S5.2). Similar to **2c** and **2d**, the OH-11 of **2b** and **2a** also result from spontaneous hydration of the *E*-double bond in **1a**. Compound **3** is an ADA ethyl ester with a 5-hydroxyhexanoyl side chain (Fig. 3). We reckon that the corresponding highly saturated triketide priming unit **3Pc** is another off-program product of AtCurS1 rather than originating from a fortuitous modification by the host, considering that we have never observed analogous reductions by endogenous yeast enzymes.

The product profile of LtLasS1 – AtCurS2($SAT_{RrDalS2}$) also changed appreciably compared to LtLasS1 – AtCurS2 (Entries #28 vs. #27 in Fig. 2 and SI Fig. S5). Instead of the on-program pentaketide-primed lasilarin (**14**), the tetraketide **4Pd**-derived DAL_{12} *epi*-curvularin

(**6**) became the major product. Triketide **3Pa**-derived **1b** and **2c**/**d** appeared as minor products, in amounts far exceeding that of the on-program pentaketide-derived compound. Surprisingly, CcRadS1 – AtCurS2($SAT_{RrDalS2}$) successfully generated the on-program pentaketide-derived compounds **15** and **18a**/**b** (Entry #34 in Fig. 2 and SI Fig. S5). However, tetraketide **4Pc** and triketide **3Pa** were also apparently downloaded by the nrPKS, as evidenced by the appearance of **11** and **2c**/**d** in substantial amounts. Taken together, these SAT swap experiments show that $SAT_{RrDalS2}$ is selecting shorter priming units when those are readily offered by the hrPKS partner, and further, it forces the handover of derailed, off-program priming units in a proactive manner.

The complementary experiment (replacing $SAT_{RrDalS2}$ with $SAT_{AtCurS2}$) was unproductive despite our success with the $SAT_{CcRadS2}$ donor using an analogous switchover site. Varying the switchover site could not rescue productivity either (four additional, plausible sites were investigated, located in front of, within, or after the SAT/KS linker region, as described in the SI Supplementary Methods), regardless of the hrPKS partner used for the resulting chimeras. Considering that our previous experiments clearly show that both constituent parts of these chimeras (i.e. the $SAT_{AtCurS2}$ domain and the KS-AT-PT-ACP-TE segment of RrDalS2) are catalytically competent with the hrPKS-derived priming units, we hypothesize that unfavorable inter-domain structural interactions prevented product turnover with RrDalS2($SAT_{AtCurS2}$).

TE Domains Are Logic Gates for Product Conformation

In addition to their differences in priming unit selection, AtCurS2 and RrDalS2 also diverge in their product release modes. Thus, AtCurS2 overwhelmingly produces DALs (Entries #13, #27 and #33 in Fig. 2 and SI Fig. S5), unless the priming unit is too rigid for macrolactone formation (Entry #3 in Fig. 2 and SI Fig. S5). In contrast, RrDalS2 typically delivers the final products as ADA alkyl esters (Entries #11, #18 and #31 in Fig. 2 and SI Fig. S4), unless encountering an especially flexible priming unit whose ω−1 alcohol moiety is able to redirect product release towards intramolecular cyclization (Entry #25 in Fig. 2 and SI Fig. S4). Considering the established gatekeeping roles of TE domains in product release,[17,33] we reasoned that these discrepancies may reflect differences in the intrinsic programs of the TE domains in the two nrPKSs. Thus, we created RrDalS2 / AtCurS2 chimeras by swapping their TE domains.

As expected, chain extension and S-type first ring formation remained unaffected in AtCurS2($TE_{RrDalS2}$). When challenged with voucher hrPKSs, priming unit selection also remained conservative, matching the expected, on-program tetraketides or pentaketides routinely offered by the partner enzymes (Entries #15, #22, #29, and #35 in Fig. 2 and SI Fig. S6). Importantly, heterocombination of the chimera with RrDalS1 failed to afford any triketide-derived products (Entry #8 in Fig. 2 and SI Fig. S6), in spite of the reasonable productivity of this noncognate BDL synthase, and the compatibility of $SAT_{AtCurS2}$ with such short priming units (Entry #3 in Fig. 2 and SI Fig. S5). The most important consequence of the TE replacement was the dramatic alteration of the mode of product release with all hrPKS partners, with predominant production of ADA alkyl esters at the expense of DAL macrocycles (Entries #8, #15, #22, #29, and #35 in Fig. 2 and SI Fig. S6).

Replacement of the TE domain of RrDalS2 with $TE_{AtCurS2}$ in the complementary experiment markedly shifted the product distribution towards macrocyclic DALs at the expense of ADA analogues (Entries #16, #23, #30, #36 and #37 in Fig. 2 and SI Fig. S6). As expected, macrocycle formation remained out of reach with triketide priming units (Entry #9 in Fig. 2 and SI Fig. S6). However, the combination with the cognate hrPKS partner RrDalS1 was remarkably productive. This pair not only produced 70 times the amount of polyketides obtained from the admittedly dismal RrDalS1 – RrDalS2 native pair, but was also by far the most productive BDL synthase, outperforming even the native AtCurS1 – AtCurS2 pair by well over 30%. In contrast, polyketide productivity was strongly diminished when the chimera was coupled to AtCurS1 (Entry #16 in Fig. 2 and SI Fig. S6). Surprisingly, a low level of heterocoupling with CcRadS1 was also observed even without SAT domain replacement (Entry #36 in Fig. 2 and SI Fig. S6). Nevertheless, the CcRadS1 – RrDalS2($TE_{AtCurS2}$) pair remained a marginal producer, affording only trace amounts of the expected DAL_{14} product, radilarin (**15**). Instead, the chain length fidelity of CcRadS1 broke down, and the product profile became dominated by polyketides derived from off-program, shortened priming units, such as the ADA ethyl ester (**11**) from tetraketide primer **4Pc**, and ADA ethyl esters (**2c**/**d**) from triketide **3Pa**. In fact, hrPKS fidelity is compromised in most pairings with RrDalS2($TE_{AtCurS2}$). Thus, RrDalS1 appears to be an efficient triketide (and not a tetraketide) synthase that affords **3Pa** exclusively; AtCurS1 and AzResS1 offer 4:1 mixtures of tetraketides and triketides instead of tetraketides only; and LtLasS1 generates mostly a tetraketide and even a triketide instead of its native pentaketide (Entries #9, #16, #23 and #30 in Fig. 2 and SI Fig. S6).

The striking productivity improvement upon introducing $TE_{AtCurS2}$ into RrDalS2 led us to consider that $TE_{RrDalS2}$ may be mismatched with the nrPKS chassis.[34] Thus, this TE may have “expected” an F-type (C2-C7) resorcylate intermediate instead of the S-type (C8-C3) phenylacetate offered by the rest of RrDalS2. Thus, we grafted $TE_{RrDalS2}$ onto the AzResS2 chassis, and paired this chimera with RrDalS1, AtCurS1 and AzResS1 (Entries #10, #17 and #24 in Fig. 2 and SI Fig. S6). The resulting noncognate BDL synthases were all barely functional, producing minute amounts of the isocoumarins **21**, **24a**, **24b** or **25** (Fig. 3) derived from the on-program priming units offered by the hrPKSs, and feature an F-type resorcylic acid motif afforded by $PT_{AzResS2}$. These isocoumarins may result from the spontaneous release of the enthalpically and entropically favored pyrone from the ACP-bound, stalled resorcylate intermediates that are not accepted by the grafted $TE_{RrDalS2}$.[17,34] The complementary experiment (introducing $TE_{AzResS2}$ into RrDalS2) provided only unproductive BDL synthase heterocombinations. This may indicate that $TE_{AzResS2}$ is unable to release a phenylacetate product, while spontaneous pyrone formation is not possible for S-type intermediates.[17,34]

Taken together, these TE swaps indicate that the intrinsic program of $TE_{RrDalS2}$ favors ADA ester formation regardless of product chain length, but it is not amenable to release F-type (resorcylate) intermediates. In contrast, $TE_{AtCurS2}$ is programmed to generate macrolactones, and is permissive towards products from shortened priming units transferred by the short chain-preferring $SAT_{RrDalS2}$.

DISCUSSION

Understanding the points of failure for engineered biocatalysts such as iterative PKSs originating from domain replacements or subunit shuffling is pivotal to advance the biosynthetic production of unnatural natural products (uNPs). To improve our design of custom biocatalytic machines for combinatorial synthetic uNP production, structural enzymology studies would need to predict folding deficiencies and protein-level domain interaction failures in engineered systems.[35–37] At the same time, it is necessary to understand the intrinsic programming (the catalytic competencies of domains for an intermediate) and the extrinsic programming (domain-level interactions and the kinetic competition of various catalytically competent domains for the same intermediate) of PKSs, and the "metaprogram" emerging from all these subroutines. A novel avenue for developing such an understanding became evident when we encountered a seemingly derelict, largely unproductive natural BDL synthase system from *Rhytidhysteron rufulum*. To our surprise, subunit shuffling and domain exchanges in a combinatorial context revealed that all constituent parts of the RrDalS system were functional and highly efficient in certain contexts. Nevertheless, the natural combination of these parts was almost completely unproductive in the heterologous host. In effect, Nature provided us with a natural example of an iterative PKS system with a faulty overall (meta)program.

To decipher the programming of the RrDalS system, we first paired the two subunits with voucher BDL synthase partners. Next, we built nrPKS chimeras by exchanging domains between RrDalS2 and AtCurS2, a highly productive DAL-producing nrPKS orthologue of RrDalS2 with subtle but consistent programming differences in priming unit selection and product release mechanisms. Based on previous studies,[14] we presumed that these differences would largely be determined by the SAT and the TE domains, with the nrPKS KS-AT-ACP polyketone homologation chassis and the PT domain exercising only marginal influence over the programmed outcomes. The resulting RrDalS2/AtCurS2 hybrid enzymes were challenged with voucher hrPKSs providing priming units with different lengths, reduction levels, and ω−1 alcohol configurations. Priming units of different length and functionalization have been seen to provoke (compensatory) changes in product length by the acceptor PKSs in bacterial iterative modules,[38] Type II PKSs[39] and fungal iterative PKSs.[32,40] However, in our extended set of experiments the nrPKS chain length program remained remarkably constant, regardless of the priming unit utilized (from triketides to pentaketides with different functional groups). Thus, the observed product structures could be easily ascribed to the engineered programming alterations upon SAT and TE exchanges. These experiments revealed several novel programming constraints that determine the overall metaprogram of BDL synthases, and by extension, those of similar PKS-containing natural or engineered biosynthetic systems.

Product Release Modes Are Determined by the Intrinsic Program of the TE Domain

In fungal nrPKSs, O-C bond-forming TE domains dictate product release modes that generally correlate with the phylogenetic grouping of the nrPKS.[14] Thus, these TEs may release products by hydrolysis (such as with orsellinic acid synthases) or 6-membered pyrone ring formation (such as with the cercosporin synthase).[41] An intriguing example is a

group of TEs that catalyze cross-coupling (transesterification) to a different, complex biosynthon.[42] However, TE-catalyzed product release for BDLs is generally thought to favor macrocyclization, with occasional hydrolysis or pyrone formation taken as an indication of the bypass of a TE that is unable to process a particular substrate.

In our experiments, $TE_{AtCurS2}$ efficiently facilitated the intramolecular marocyclization of octa- or nonaketide scaffolds derived from priming units **4Pa**, **4Pb**, **4Pd**, **5Pa**, and **5Pb**, showing permissiveness for the length, redox pattern and ω−1 alcohol configuration of the acyl chain. In contrast, the preferred mode of release for $TE_{RrDalS2}$ is intermolecular transesterification to a short-chain alcohol from primary metabolism to afford ADA esters, even when $TE_{AtCurS2}$ releases the very same carbon skeleton overwhelmingly as a macrocycle (cf. Entries #15 and #13, #11 and #16 and so on, Fig. 2). $TE_{RrDalS2}$ is still able to catalyze macrolactonization with a few scaffolds derived from pentaketide primers, or those originating from the very flexible, saturated tetraketide **4Pd**. However, macrocycles remain minor products even in these cases (Entries #29, #31, #32, and #35), and/or the overall product yield drops considerably (cf. Entries #25 and #26). Thus, preference for macrocycle vs. linear product formation in BDL synthases is a part of the intrinsic program of competent TE domains, and may be determined by the shape, charge distribution and volume of the TE cyclization chamber. Products derived from triketide primers, and those from the rigid tetraketide **4Pc** could not be released as macrolactones by either $TE_{AtCurS2}$ or $TE_{RrDalS2}$. Inside the active site cavity, the acyl tails of these polyketides may be unable to fold back to use their distal alcohol for a nucleophilic attack on the Ser oxoester. Modulation of the TE catalytic chamber may then allow different BDL synthase systems to occupy varying positions on the macrocycle – linear product spectrum. Meanwhile, the active site of $TE_{RrDalS2}$ must still be accessible to simple alcohols (but not to water) for product release as ethyl-, isobutyl-, or phenethyl esters.

SAT Domains Are Proactive Selectors Contributing to the hrPKS Metaprogram

In vitro reconstitution of the dissected aflatoxin biosynthetic nrPKS has indicated that the SAT domain and the polyketide homologation machinery were remarkably tolerant towards a range of artificial priming units.[40] Similar results from the chaetoviridin or the asperfuranone collaborating iterative PKSs[32,43] and the hypothemycin BDL synthase pair,[13] together with the in vivo permissiveness of SAT domains during subunit shuffling[24] were interpreted to mean that the intrinsic program of the SAT domains in collaborating, quasi-modular systems (such as FAS – nrPKS or hrPKS – nrPKS pairs) involves little discrimination towards the priming unit presented by the hrPKS.[40] Thus, SAT domains have been described to resemble “passive docking domains that enable vectorial substrate transfer in modular PKSs”.[36]

Our current results show that the SAT of AtCurS2 (and that of AzResS2) was indeed behaving as a promiscuous, permissive catalyst. Thus, $SAT_{AtCurS2}$ faithfully transferred the on-program tetraketide or pentaketide priming units offered by the hrPKS noncognate partners, with the exception of **5Pb**, the priming unit from CcRadS1. On the contrary, $SAT_{RrDalS2}$ displayed a clear intrinsic selectivity, consistently choosing shorter, and if possible triketide priming units at the expense of longer, especially pentaketide ones (Entries

#7, #9, #14, #16, #21, #23, #28, #30, #34 and #36, Fig. 2). Although $SAT_{RrDalS2}$ could also accept tetraketides or even pentaketides to a lesser extent (including small amounts of **5Pb** from CcRadS1), it behaved in a proactive way and forced the hrPKS partner to offer derailment products. Thus, $SAT_{RrDalS2}$ downloaded large amounts of premature priming units from all hrPKS partners that were one or even two ketide units shorter, and less reduced, than the on-program products of the hrPKSs. Such versatility of the voucher hrPKSs to assemble polyketides of irregular lengths and redox patterns has not been observed before, and indicates that their biosynthetic output is strongly influenced by the off-loading preferences of the SAT domain. Thus, SAT domains are not "passive docking domains",[36] but follow an intrinsic program to discriminate priming units according to their lengths. What's more, this intrinsic program then becomes a part of the extrinsic metaprogram of the hrPKSs in collaborating systems.

$SAT_{RrDalS2}$ typically intercepted the programmed elongation and reduction sequence of the hrPKS before the ER-catalyzed reduction step one or two extension cycles before product maturation (priming units **3Pa**, **3Pb**, **4Pb**, **4Pc**). Interception before the ER recreated the α,β-enone functionality of the full-length, RrDalS1-generated tetraketide priming unit **4Pc**, indicating a strong preference for this motif by $SAT_{RrDalS2}$. Moreover, the same priming unit (**3Pa**) was downloaded by $SAT_{RrDalS2}$ from three different hrPKSs with distinct product types (tetraketide synthase AzResS1 and two pentaketide synthases, LtLasS1 and CcRadS1). Forced handover of intermediates before chain extension but after full reduction (**4Pd** in Entries #25, #26, #28 and #30; **3Pc** in Entries #14 and #16) or before β-keto processing (**5Pc**, Entry #26) were also observed, although less frequently. Parallel work with dissected modules from Type I modular PKSs showed that heterologous TE domains may similarly intercept polyketide intermediates before the KR-catalyzed β-keto reduction.[44]

The role of downstream, off-loading domains in the chain length control of hrPKSs has been extensively investigated in hrPKS-NRPS (nonribosomal peptide synthetase) hybrid enzymes for acyltetramic acid or lovastatin biosynthesis. While the NRPS condensation (C) domain shows considerable selectivity during acyltetramic acid biosynthesis,[45] it only acts when the hrPKS module exhausted all its synthetic possibilities.[46] A closer parallel to the proactive off-loading activity of $SAT_{RrDalS2}$ is found in soppiline B biosynthesis where a downstream chalcone synthase actively intercepts and offloads a shorter, premature polyketide from the partner hrPKS.[47]

Taken together, our results support and extend a model in which the hrPKS metaprogram (the overall program of the whole enzyme) emerges from the kinetic competition of domains that are catalytically competent for the ACP-bound intermediates.[36,46] In BDL synthases and similar collaborating systems, kinetic competition between the hrPKS *cis*-acting domains KS, KR, DH and ER is supplemented *in trans* by that of the SAT domain from the nrPKS subunit. This multipartite competition is responsible for setting the biosynthetic trajectories of the nascent β-keto intermediates on the hrPKS, channeling them towards β-keto processing, further chain extension, or transacylation to the nrPKS partner.

SAT and TE Domain Interactions Influence the BDL Synthase Metaprogram

The TE domain has emerged as one of the most important decision gates in polyketide biosynthesis.[17,27,44,48,49] In a combinatorial context, an appropriate BDL synthase TE domain may confer high productivity, but an incompatible TE may render the same hrPKS – nrPKS unproductive.[17] Apart from proofreading during chain extension, TE domains monitor the formation[50] and the register[17,34] of the first aromatic ring as stringent logic gates. However, macrocycle-forming BDL synthase TEs are permissive towards the configuration of the nucleophilic alcohol, and the oxidation state of the acyl chain.[17,34] TE domains are also permissive for the length of the product (typically within one ketide unit), with the KS as the primary determinant for nrPKSs product size.[14] Correspondingly, the chain length of the nrPKS biosynthon did not vary with AtCurS2 or RrDalS2, irrespective of the SAT or TE domains or the hrPKS subunit partners used. In contrast, the TE domains substantially modulated the distribution of products derived from different priming units, and thus moderated or intensified the effects of SAT domain exchanges. Thus, $TE_{AtCurS2}$ is a permissive gatekeeper that successfully releases heptaketide, octaketide and nonaketide products derived from triketide, tetraketide or pentaketide priming units, respectively. In particular, $TE_{AtCurS2}$ facilitates the efficient production of triketide-primed polyketides when this domain and $SAT_{RrDalS2}$ are appended to an AtCurS2 or RrDalS2 chassis (Entries #7, #9, #14, #16, #21, #23, #28, #30, #34 and #36, Fig. 2). In contrast, $TE_{RrDalS2}$ is much less efficient in releasing triketide-primed products when present in the same enzyme assemblies as $SAT_{RrDalS2}$, triggering large shifts towards longer polyketide homologues, and depressing the overall product yield (e.g. Entries #2 vs. #7, #1 vs. #9, #12 vs. #14, Fig. 2). On the other hand, appropriate combinations of SAT and TE domains significantly enhance overall product yields. Thus, when $SAT_{RrDalS2}$ is matched with $TE_{AtCurS2}$ in the RrDalS2 chassis, the production of **1b** and **2c**/**d** with RrDalS1 as the partner increases seventyfold as compared to the native RrDalS1 – RrDalS2 pair (Entries #9 vs. #1, Fig. 2). These observations indicate that TE domains work as size-selective filters in BDL synthases. Thus, the intrinsic TE program modulates the observable metaprogram of the hrPKS subunits by facilitating or obstructing product formation from certain priming units. For example, RrDalS1 would be perceived as a pure triketide synthase when combined with an nrPKS chassis featuring $SAT_{RrDalS2}$ and $TE_{AtCurS2}$. However, the same hrPKS would be categorized as a mixed triketide/tetraketide synthase when paired with an analogous nrPKS equipped with $TE_{RrDalS2}$ (cf. Entries #7 and #9 with #1 and #2, Fig. 2). This apparent control of the hrPKS chain length manifests at a remote level, since the TE (as opposed to the SAT) is not in a kinetic competition for hrPKS-bound intermediates, but monitors advanced intermediates on the nrPKS ACP after chain extension and first ring cyclization. Since the metaprogram of the hrPKS, and in turn that of the whole BDL synthase is an emergent property that derives from the intrinsic selectivities of the constituent domains, their extrinsic interactions and kinetic competition for the same ACP-bound intermediates, the result of an engineered change affecting only a few factors is rarely clear-cut. Instead, multiple products are biosynthesized, and complex patterns emerge depending on the overall biosynthetic context (such as in chimeric PKSs, or when an engineered nrPKS is paired with different hrPKS partners).

Evolutionary Perspectives on BDL Synthase Metaprogram Emergence

To obtain a perspective on the evolution of BDL synthase programming, we inferred the evolutionary history of O-C bond-forming Type I nrPKS TE domains using the C-C bond-forming thioesterase/Claisen cyclase domains as the outgroup, and cross-referenced the resulting phylogenetic tree with the products of the synthases, if known. BDL-type TEs form a large superfamily (Fig. 4), accompanied by basal clades with TE domains for the hydrolytic release of orsellinic acid (OA); OA cross-coupling to other scaffolds; TEs affording polylactones; and two other clades with no known metabolic products. The BDL TE superfamily neatly splits into two sister clades according to the register of the first aromatic ring. This dichotomy is in agreement with a previously observed functional correlation between PTs and TEs from F-type (C2-C7) vs. S-type (C8-C3) systems, whereby neither TE group can macrocyclize intermediates with the opposite type of aromatic ring.[17,34] Accordingly, a PT phylogenetic tree constructed from the same nrPKSs shows a very similar clading pattern, with the F-type vs. S-type register as its main organizing principle (SI Fig. S7). Further, F-type PTs and TEs both branch to RAL macrocycles where the nrPKS extends the priming unit through three additional extension cycles (X+3 split); RAL macrocycles with an X+4 split; pyrones with a C2-C7 first aromatic ring; and a group with unknown products where F-type cyclization is clearly indicated by diagnostic PT residues.[16] For S-type PTs and TEs, clades for DAL macrocycles and unknown C8-C3 products are observable. The PT and TE domains of RrDalS2 are ensconced in a clade with no other functionally characterized synthases. This clade is sister to those of macrocyclic DALs, and may include other cross-coupling, ADA ester-synthesizing enzymes. Thus, PT and TE domains in BDL synthase nrPKSs follow similar evolutionary trajectories, reflecting the close collaboration of these domains in product formation.

The inferred evolutionary history of nrPKS SAT domains shows surprising differences compared to the TE and PT reconstructions (Fig. 4). Although the BDL superfamily is still distinct and accompanied by similar basal clades, there is no clean dichotomy for F- vs. S-type product skeletons. The BDL SAT domain superfamily is not organized according to the chain length or the redox/stereochemical characteristics of the priming units either. Instead, the RrDalS2 ADA ester clade, and another S-type (C8-C3) clade with unknown products “invades” the RAL X+4 group (Fig. 4), a pattern also apparent in a phylogenetic tree of entire hrPKS subunits (SI Fig. S7). This indicates that in BDL synthase systems, SAT domains and hrPKSs follow similar evolutionary trends, consistent with the role of the SAT domains in creating the hrPKS metaprogram. However, considering the divergence of the TE (PT) and the SAT (hrPKS) trees, these programming subroutines may travel different evolutionary trajectories. This may lead to structural innovations in BDL biosynthesis. However, this disjointed evolution also poses the danger of developing a programmatic conflict among BDL synthase subroutines and even a breakdown of the overall metaprogram, as is often seen with chimeric synthases inadvertently constructed from ill-matched parts.[46] We believe the native RrDalS system is a victim of such a fate: the RrDalS2 SAT (and its hrPKS partner) developing a strong preference for triketide priming units, while the TE domain drifting towards a preference for tetraketide-initiated products. The emerging metaprogram of the BDL synthase thus becomes self-contradictory, leading to

an unproductive tug-of-war between the SAT and the TE, nearly incapacitating the RrDalS system in the heterologous host.

CONCLUSIONS

A combination of rational domain swaps with subunit shuffling in a heterologous host allowed the revitalization of a seemingly derelict BDL synthase system identified by genome mining in *R. rufulum*, and generated a library of structurally diversified DALs and ADA esters in isolable amounts. Although the structure of the native product of the BDL synthase system in *R. rufulum* remains uncharacterized, our synthetic biology experiments in *S. cerevisiae* further expanded the chemical diversity of the relatively rare S-type fungal BDL congeners by 23 compounds which are new to Nature. Even more importantly, our results revealed constraints and programming complexities that genetic engineers would need to take into account when designing iterative PKS genetic machines for combinatorial synthetic microbiology.

Combinatorial biosynthetic production of uNPs in native or heterologous hosts relies on the promiscuity and plasticity of natural product biosynthetic machineries such as iterative PKSs. Structure-guided active site cavity engineering, and swaps of sub-domains, domains, or even subunits presupposes that protein-protein interactions among engineered and native units will remain operational, and the altered product intermediates will be acceptable to all functional units. Previous experiments with fungal dual PKS systems suggested that the constituent hrPKS and nrPKS enzymes are autonomous, and assemble their own biosynthons in a largely independent manner. Successful subunit collaboration depends on an appropriate SAT domain functionally coupling these synthases into a BDL synthase system. Our current findings demonstrate a complementary, but more dynamic scenario where the overall BDL synthase metaprogram emerges from the intrinsic substrate specificities of the individual domains, and from the extrinsic interactions and kinetic competition among catalytically competent domains for the growing polyketide intermediates. Thus, the growing hrPKS biosynthon can be intercepted *in trans* by a downstream SAT domain with a strict intrinsic substrate preference, resulting in the facile transfer of a shortened priming unit, often with an altered reduction pattern, to the nrPKS. Successful product formation is gated by the terminal TE domain that not only monitors the formation of the first aromatic ring, but also works as a size selectivity filter. Thus, the size of the priming unit (the hrPKS chain length metaprogram) and that of the overall product (the BDL synthase chain length metaprogram) involves the complex interplay of *cis*-acting and *trans*-acting subroutines from both the hrPKS and the nrPKS. In addition to its contribution to chain length determination, the intrinsic program of the TE domain also sets the overall product shape by adopting a download mode from a repertoire that includes macrocycle formation, hydrolysis, transesterification, and pyrone formation. It is instructive that PT and TE domains on one hand, and SAT domains and hrPKS subunits on the other travel related evolutionary paths in native BDL synthase systems. However, it is also instructive that divergence of these evolutionary paths may lead to unproductive combinations even in nature, as exemplified by the BDL synthase system of *R. rufulum*. To avoid such a fate, combinatorial synthetic microbiology must carefully characterize and

standardize biosynthetic parts, and devise iterative PKS metaprograms from mutually compatible subroutines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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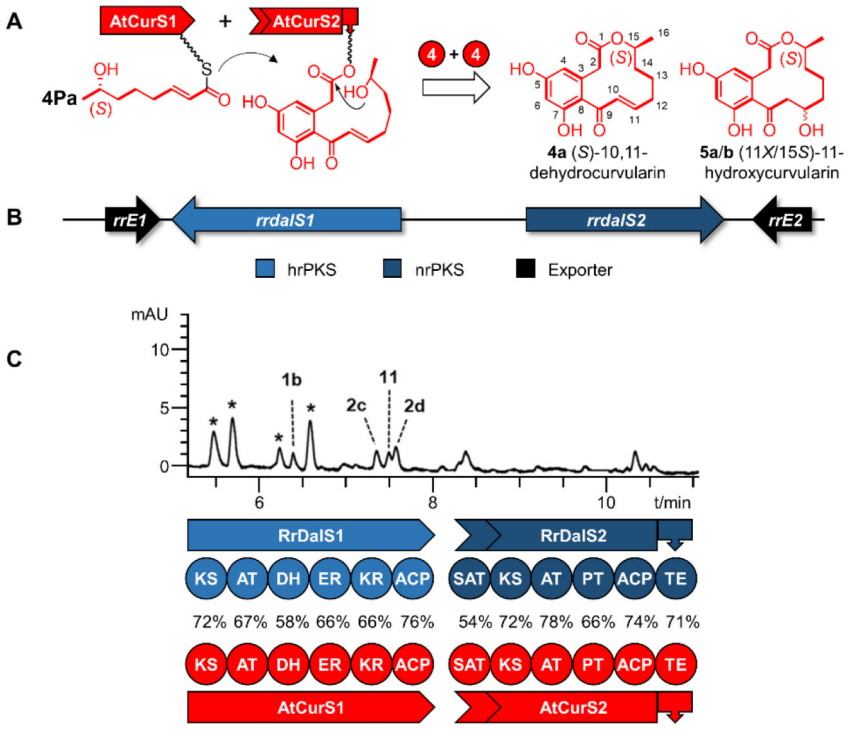

Figure 1. Biosynthesis of DALs.

A. Scheme for the biosynthesis of the DAL_{12} 10,11-dehydrocurvularin (**4a**) and its spontaneous hydration products 11-hydroxycurvularins (**5a/b**) by the collaborating hrPKS – nrPKS pair AtCurS1 – AtCurS2.[20] The tetraketide **4Pa** is transferred from AtCurS1 to AtCurS2 by the SAT domain, and used as a priming unit to initiate four more cycles of chain extension ("4+4"), followed by PT-catalyzed S-type (C8-C3) aldol cyclization and aromatization, and TE-catalyzed macrolactone formation. **B**. Schematic representation of the orphan *rrdal* biosynthetic gene cluster of *Rhytidhysteron rufulum* CBS 306.38.[25] **C.** Comparison of the domain architectures of the RrDalS1 – RrDalS2 and the AtCurS1 – AtCurS2 hrPKS – nrPKS pairs (percent amino acid sequence identities of the domains are shown), and the product profile (reversed-phase LC-MS trace recorded at 300 nm with a photodiode array detector) of a representative culture of *S. cerevisiae* BJ5464-NpgA[26,27] expressing RrDalS1 and RrDalS2. Peaks labeled with a *star* are unidentified host metabolites. See Fig. 3 for product structures.

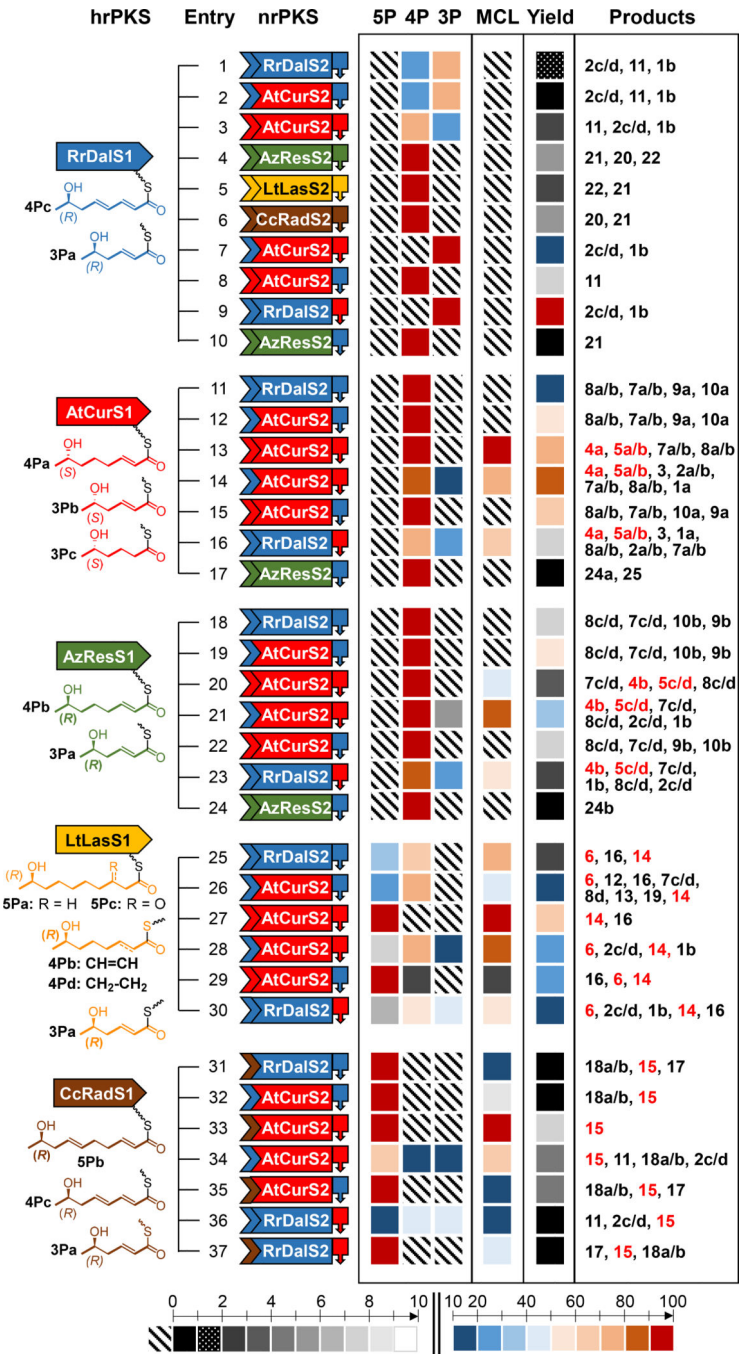

Figure 2. Combinatorial biosynthesis with BDL synthase heterocombinations.

The deduced structures of the priming unit thioesters are presented below the cartoons of the appropriate hrPKSs. nrPKS cartoons show the SAT domains (*chevron*), the KS-AT-PT-ACP chassis (*rectangle*), and the Type I TE domains (*block arrow*), color-coordinated with the hrPKSs. *Blue,* RrDalS; *red*, AtCurS; *green*, AzResS; *yellow*, LtLasS; *maroon*, CcRadS. Products are listed in the rightmost column in the order of decreasing abundance; compound numbers in *red* indicate macrocycles. See Fig. 3 for product structures. *First heatmap block*, distribution (in percentages) of pentaketide (5P), tetraketide (4P), and triketide (3P)-primed

polyketides among all detected products of the strain expressing the hrPKS – nrPKS combination. *Second heatmap block,* relative quantification (in percentage) of all macrocyclic (MCL) products among all detected products of the strain. *Third heatmap block,* relative overall polyketide productivity (in percentage) of the strain, compared to the overall polyketide productivity of the strain expressing RrDalS1 – RrDalS2($TE_{AtCurS2}$) (the most productive BDL synthase combination in these experiments). The *heatmap color code* is given at the bottom of the figure, *crosshatch* indicates no production of the given product type. Fermentations were conducted in triplicates and repeated a minimum of three times (n=9, means are shown), as described in SI Materials and Methods.

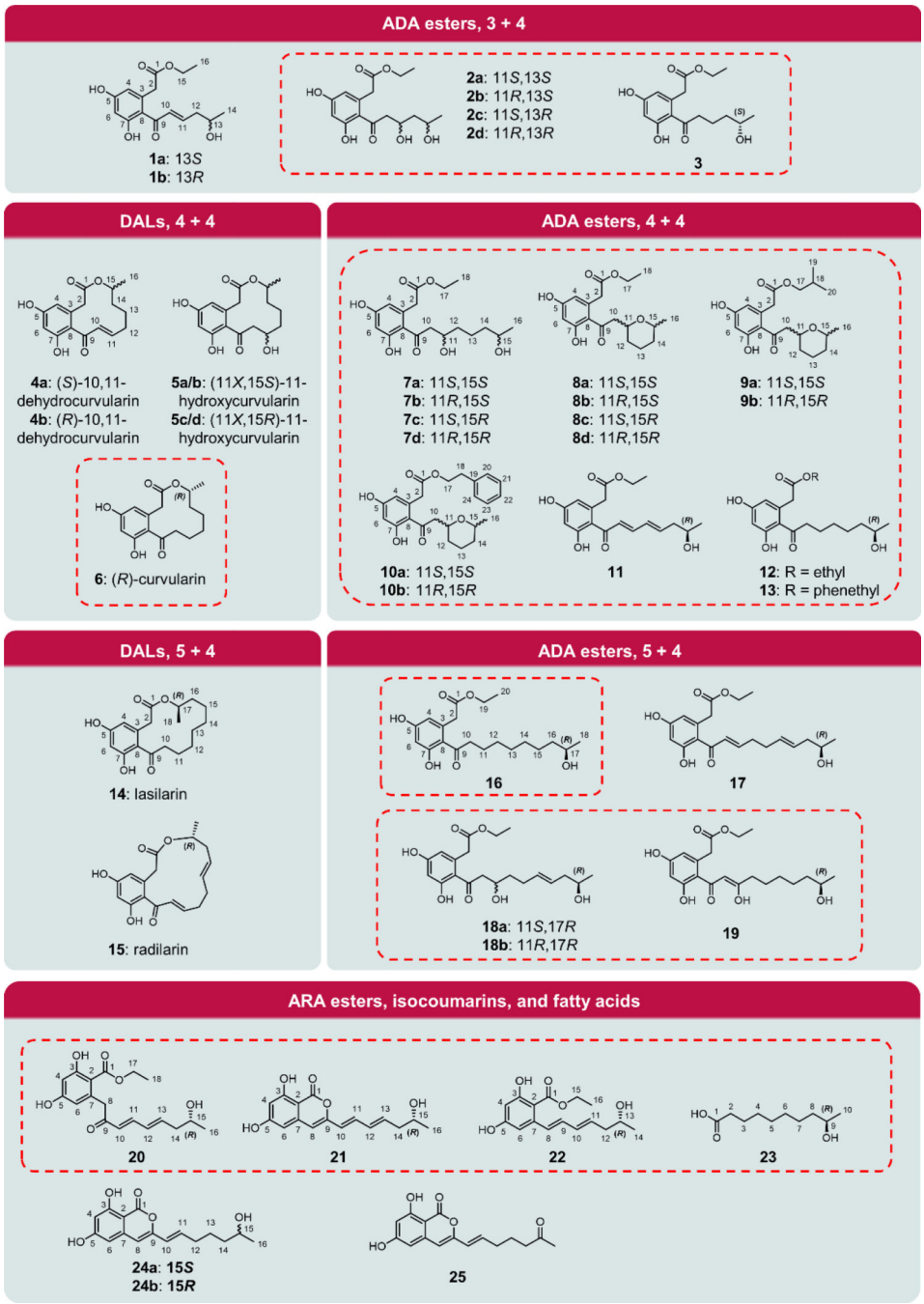

Figure 3. Chemical structures of detected products.

Compounds in dashed boxes were isolated and identified on the basis of their spectroscopic data. Other products were identified by their LC-MS spectra and comparisons with isolated standards.

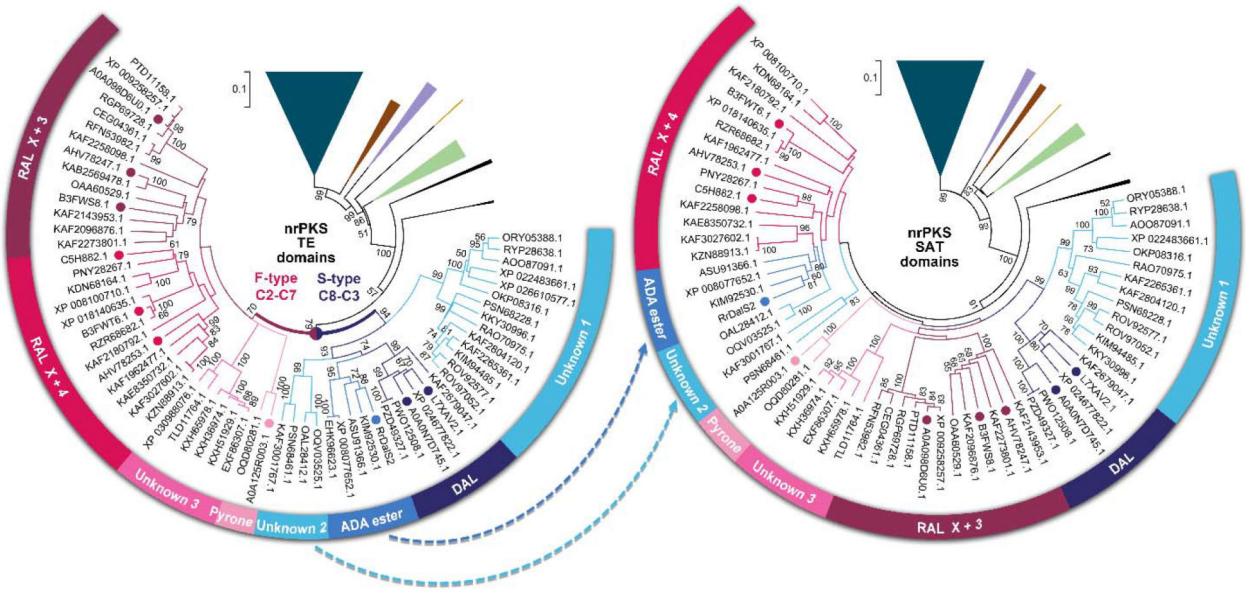

Figure 4. Phylogenetic trees for nrPKS TE and SAT domains.

nrPKSs with O-C bond-forming Type I TEs were collected from NCBI GenBank in a comprehensive search (>40% amino acid identity, >80% coverage to “bait” sequences), aligned using MEGA 7, and their evolutionary histories were inferred with the neighbor joining method. nrPKSs with thioesterase/Claisen cyclase domains were used as the outgroup (*dark teal triangle*). The percentage (if >50%) of replicate trees where the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branches basal to BDL synthases were collapsed and shown as colored wedges (*brown*, collaborating hrPKS – nrPKS systems with a discrete TE protein; *purple,* TEs from orsellinic acid hybrid metabolite synthases; *yellow*, polylactone TEs; *green*, TEs from orsellinic acid synthases; *black*, TEs from synthases with unknown products). Colored dots represent TEs from voucher BDL synthase systems (*maroon*, zearalenone,[51] lasiodiplodin[19] and hypothemycin[52] synthases; *red*, resorcylide[19] and monocillin II[18,52] synthases; *pink*, cladosporin[53] synthase; *blue*, RrDalS2, *navy*, dehydrocurvularin[20,54] synthases). *Blue arrows* indicate the relocation of the ADA ester and the unknown S-type clade into the RAL X+4 clade.