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Engineering of an "Unnatural" Natural Product by Swapping Polyketide Synthase Domains in *Aspergillus nidulans*

Ting Liu,[†] Yi-Ming Chiang,[†,‡] Amber D. Somoza,[∥] Berl R. Oakley,[§] and Clay C. C. Wang*[,†,∥]

[†]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, and [∥]Department of Chemistry, College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California 90089, United States

[‡]Graduate Institute of Pharmaceutical Science, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan

[§]Department of Molecular Biosciences, University of Kansas, 1200 Sunnyside Avenue, Lawrence, Kansas 66045, United States

Supporting Information

ABSTRACT: An StcA-AfoE hybrid polyketide synthase (PKS), generated by swapping the AfoE (asperfuranone biosynthesis) SAT domain with the StcA (sterigmatocystin biosynthesis) SAT domian, produced a major new metabolite with the same chain length as the native AfoE product. Structure elucidation allowed us to propose a likely pathway, and feeding studies supported the hypothesis that the chain length of PKS metabolites may be under precise control of KS and PT domains.

Fungal polyketides are produced by multidomain iterative type I polyketide synthases (PKSs). Fungal PKSs can be further grouped into non-reduced (NR), partially reduced (PR), and highly reduced (HR) PKS by examining the domains encoded in the gene.[1] NR-PKSs contain starter unit ACP transacylase (SAT), β-ketoacyl synthase (KS), acyl transferase (AT), product template (PT), and acyl carrier protein (ACP) domains at a minimum and may also contain methyltransferase (CMeT) and product-releasing domains such as thioesterase/Claisen-cyclase (TE/CLC), thioesterase (TE), and reductase (R). The SAT domain has been determined to possess remarkable selectivity toward starter units and to initiate polyketide synthesis by transferring the starter unit onto the ACP domain.[2] The PT domain was found to cooperate with the KS domain to exert precise control over product formation.[3] The KS domain extends the starter unit to a fixed length, and the PT domain transforms a reversible precursor to an irreversible form by specific aldol cyclization and aromatization, thereby enhancing the flux to products.[3] Recent crystallographic studies of the PksA PT monomer revealed that the PT catalytic pocket is comprised of three regions: (1) a phosphopantetheine (PPT)-binding region at the entry, (2) a hydrophobic hexyl-binding region at the bottom, perfectly adapting the hexanoyl head, and (3) a cyclization chamber between regions 1 and 2. Conformational changes of the PT domain lead to folding a fully extended C_{20}-precursor anchored by regions 1 and 2 to a "hairpin" conformation that fits and subsequently gets cyclized in the chamber.[4]

Through a genome mining effort in *Aspergillus nidulans*, our group was able to uncover the genes involved in asperfuranone biosynthesis, an azaphilone class of fungal natural product.[5] Azaphilones are structurally diverse polyketides that share a highly oxygenated bicyclic core and a chiral quaternary center. These polyketides are known for the reaction of their 4*H*-pyran motif with amines to produce the corresponding vinylogous γ-pyridones.[6] Mostly of fungal origin, these pigments display a broad range of biological activities, including inhibition of monamine oxidase, acyl-CoA:cholesterol acyltransferase, and cholesteryl ester transfer protein.[7] The asperfuranone biosynthesis pathway, similar to all azaphilones, involves two PKSs: the HR-PKS AfoG and the NR-PKS AfoE.[5] AfoG is responsible for the biosynthesis of the dimethyloctadienone moiety, which is loaded on the SAT domain of AfoE, extended with four malonyl-CoAs, and modified with one *S*-adenosyl methionine (SAM) (Figure 2, upper).[5] We reasoned that if we could engineer the NR-PKS AfoE to accept different starter units, we would have the opportunity to create new "unnatural" polyketide products *in vivo*. To test this hypothesis, we replaced the SAT domain in AfoE with the SAT domain from StcA, the NR-PKS in the sterigmatocystin (ST) biosynthesis pathway, which accepts the hexanoyl starter unit produced by the fatty acid synthase (FAS) encoded by *stcJ* and *stcK*.[8,9]

Our analysis of the metabolism profile of wild-type *A. nidulans* shows that the ST biosynthesis pathway is highly expressed under normal laboratory culture conditions. This means that it should not be necessary to artificially turn on the expression of the two FAS genes *stcJ* and *stcK* for the production of the hexanoyl starter unit. However, it would be necessary to delete *stcA* so the hexanoyl starter unit is not consumed by the ST pathway. Metabolic profiles of an *stcA*Δ mutant showed that the production of ST was abolished in either inducing or non-inducing conditions (Figure S1, Supporting Information), and we used the *stcA*Δ strain for our domain swap experiments. We also observed that production of ST and terrequinone (TQ) in controls was decreased under the inducing conditions (Figure S1B, Supporting Information), probably due to growth inhibition of cyclopentanone that was used to induce the *alcA* promoter.

Next, we replaced the SAT domain of AfoE with the SAT domain from StcA. Since we did not know the optimal junction position of AfoE, we used the Udwary−Merski algorithm (UMA)[10] to predict the linker region between the SAT and KS domains of AfoE and selected three sites, E386, Q410, and R424 (numbered as sites 1−3), out of the linker as the SAT junction sites. Since the SAT domain of PksA, the homologue of StcA in *A. parasiticus*, has been previously cloned from three sites as individually expressed,[2] we selected three corresponding sites,

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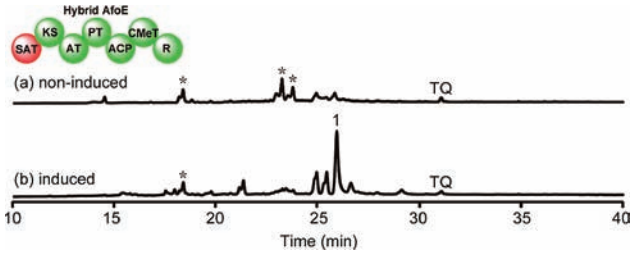


Figure 1. HPLC profiles of extracts of *A. nidulans stcAΔ, hybrid B2* double mutant under non-inducing (a) and inducing (b) conditions as detected by UV absorption at 254 nm. The *y*-axis of each profile was at the same order of magnitude. *: metabolites that are non-specific to this study.

K357, T363, and D379, on StcA (labeled as sites A−C) as the SAT junction sites, based on protein sequence alignment between StcA and PksA (Figure S2, Supporting Information). Additionally, an inducible *alcA* promoter was used to conditionally turn on the hybrid AfoE since the asperfuranone gene cluster is normally silent. Together, nine hybrid constructs (A1, A2, A3, B1, B2, B3, C1, C2, C3) were generated for metabolic analysis.

LC-DAD-MS analysis of the metabolites of the *stcAΔ, hybrid* (*A1*−*C3*) double mutants revealed that all nine constructs were capable of producing **1** under induction, which was a major metabolite for most of the constructs (Figure 1 and Figure S3, Supporting Information). To elucidate the structure of **1**, the compound was isolated and purified from a large-scale culture of the *stcAΔ, hybrid B2* strain. HRESIMS (*m/z* 303.1245, $[M - H]^-$) and ^{13}C NMR spectroscopic data provided the molecular formula $C_{17}H_{20}O_5$ for **1**, representing eight indices of hydrogen deficiency (IHD). ^1H, ^{13}C, and HMQC NMR spectra showed signals for an aliphatic side chain [δ_H 0.89 (3H, t, J = 6.4 Hz, H_3-16), 1.27−1.32 (6H, H_2-13, H_2-14, and H_2-15), and 1.66 and 1.74 (each 1H, m, H_2-12)], one downfield methyl group (δ_H 1.99; δ_C 7.3), one oxygen-bearing methine (δ_H 5.02; δ_C 68.3), and two aromatic protons [δ_H 7.36 and 8.06 (each 1H, s)] (Table S3, Supporting Information). Besides the aliphatic side chain, the ^{13}C NMR spectrum also revealed that **1** contains four olefins (δ_C 111.4, 119.6, 125.0, 125.4, 130.7, 138.5, 155.2, and 158.7) and two carbonyl groups (δ_C 181.0 and 185.6). This, together with the fact that **1** has eight IHD, suggested that **1** contains a naphthaquinone chromophore. Tetrahydrosclerotoquinone, an alkali-degraded product of tetrahydrosclerotiorin,[11] has a UV−vis absorption maximum similar to that of **1** (Figure S4, Supporting Information), indicating that **1** has a naphthaquinone chromophore similar to that of tetrahydrosclerotoquinone. HMBC correlations of **1** allowed us to fully construct its structure as a 1,4-naphthaquinone derivative (Figure S5, Supporting Information).

Structure elucidation allowed us to propose a putative biosynthetic pathway of **1** (Figure 2, lower). The hexanoyl starter unit loaded from StcJ/StcK is elongated to a hexaketide intermediate. After C2−C7 cyclization, benzaldehyde intermediate **2** is reductively released from the R domain of the hybrid AfoE. Intramolecular Knoevenagel condensation followed by tautomerization produces naphthol **3**, which is then oxidized to become **4**. Birchall et al. have observed a rapid oxidation of a similar naphthol with **3** to 1,4-naphthaquinone.[12] After reduction of **4**, possibly by an endogenous reductase, **1** is generated. Interestingly, **1** and the native AfoE product, asperfuranone, both have

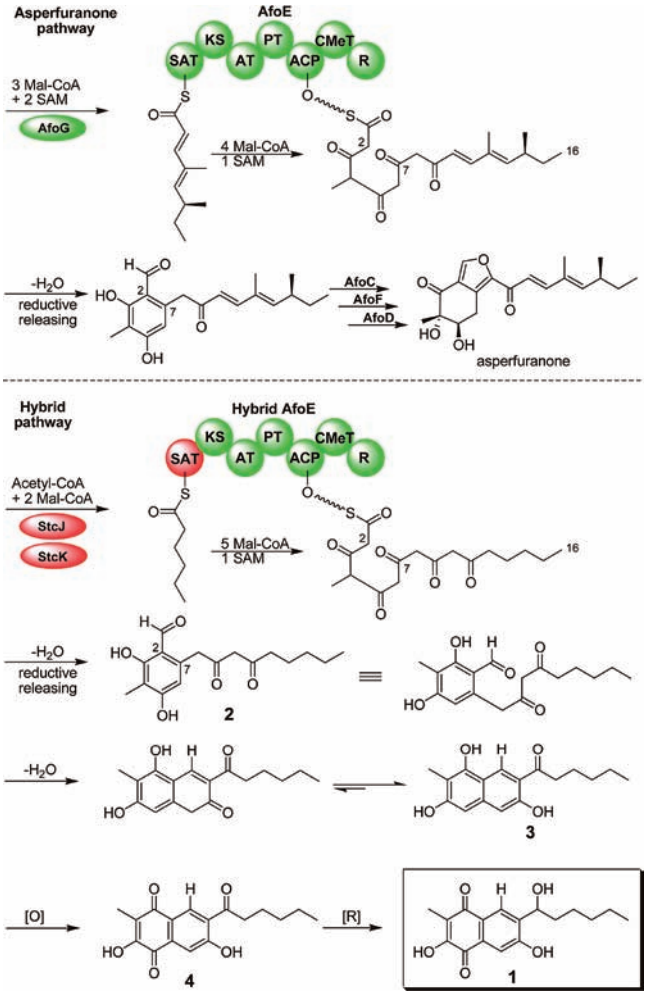
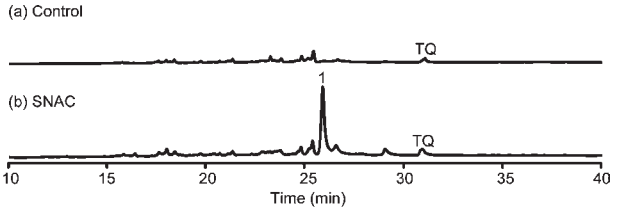


Figure 2. Proposed biosynthetic pathway of asperfuranone and **1** from native AfoE (upper) and hybrid AfoE (lower).

C_{16} chain length, despite having been initiated with starter units of different lengths. Thus, it appears that the final chain length, not the number of extension rounds, was fixed in the precursor elongation, seemingly supporting the idea of Crawford et al.[3] that the KS domain elongates the precursor to a fixed length, followed by cyclization by the PT domain, which stabilizes the precursor and drives the production flux.

To verify this hypothesis, we investigated whether other metabolites generated from the hybrid AfoE also featured C_{16} chain length by feeding deuterium-labeled starter units. We deleted *stcK* and *stcJ*, which are required for hexanoyl starter production, to generate a *stcAΔ, hybrid B2, stcKΔstcJΔ* triple mutant and conducted feeding studies with hexanoyl-*N*-acetylcysteamine (hexanoyl-SNAC), which is an analogue to hexnoyl-CoA, and D_{11}-hexanoyl-SNAC. The deletion of *stcK/stcJ* resulted in the loss of **1**, which was restored upon feeding of hexanoyl-SNAC. These results convincingly confirm that **1** originated from the hybrid PKS (Figure 3A). Besides **1**, eight other metabolites were also detected by comparing the extract profiles of SNAC-fed and D_{11}-SNAC-fed cultures, and they were shown to carry 16−19 carbons, based on high-resolution mass spectrometry results (Figure 3B and Figure S6, Supporting Information). The presence of C_{19} metabolites could be explained by a sixth extension round with malonyl-CoA. The C_{16} and C_{18} metabolites were

A



B

[M-H]-	t_R /min	m/z	m/z (D_{11} form)	%*
$C_{17}H_{19}O_5$ (**1**)	26.2	303.1245	314.1922	77.2
$C_{16}H_{19}O_6$	23.4	307.1187	318.1871	3.0
$C_{17}H_{17}O_5$	31.3	301.1078	312.1758	6.9
$C_{19}H_{17}O_5$	29.4	325.1083	336.1768	5.1
$C_{19}H_{21}O_6$	25.5	345.1344	356.2024	3.1
$C_{17}H_{21}O_4$	24.7	289.1357	300.2133	0.7
$C_{17}H_{23}O_5$	21.0	307.1553	318.2233	1.2
$C_{17}H_{21}O_6$	25.9	321.1350	332.2028	1.4
$C_{18}H_{23}O_7$	20.8	351.1441	362.2120	1.4

Figure 3. (A) HPLC profiles of extracts of *A. nidulans stcA*Δ, *hybrid B2*, *stcJ*Δ*stcK*Δ triple mutant fed with DMSO (a) and hexanoyl-SNAC (b), as detected by UV absorption at 254 nm. The *y* axis of each profile was at the same order of magnitude. (B) Summary of metabolites produced by hybrid AfoE based on feeding experiments. Each row lists the predicted ion formula, retention time (t_R), *m*/*z* of unlabeled form, *m*/*z* of D_{11}-incorporated form, and overall percentage of each metabolite in negative ionization mode. *: assuming all molecules are equally ionizable in negative mode.

possibly derived from C_{17} and C_{19} due to poor functioning of the CMeT domain or post-decarboxylation. However, the fact that up to 90% of metabolites were C_{17}, i.e., a C_{16} chain length, provided strong evidence that the length of metabolites is most likely under strict control of the AfoE KS and PT domains. Tang's group observed a similar length control based on data obtained when they fed octanoyl-CoA to PKS4 *in vitro*.[13]

For years, major obstacles in domain engineering have rested in the uncertainty of domain boundaries and the time-consuming traditional cloning workflow. Recent technological advances, such as the UMA algorithm[6] and the development of genetic engineering protocols,[11] allowed a precise and fast strategy for domain swap. We presented here our work on NR-PKS SAT domain swap experiments in *A. nidulans*. The successful swap between StcA and AfoE led to the production of compound **1**, which had the same length as native AfoE product. Feeding studies with deuterium-labeled hexanoyl-CoA analogues in a hexanoyl starter-deficient mutant restored the production of **1** and verified that chain length of the metabolites may be strictly determined by the KS and PT domains.

■ ASSOCIATED CONTENT

Supporting Information. Supplemental methods, *A. nidulans* strains and primers used in this study; HPLC profiles of extracts of the *stcA*Δ strain; protein sequence alignment of PksA and StcA; HPLC profiles of extracts of the *stcA*Δ; *hybrid* (*A1*−*C3*) strains; results of feeding studies in the *stcA*Δ, *hybrid B2*, *stcK*Δ*stcJ*Δ strain; ^{1}H and ^{13}C NMR data, UV−vis and ESIMS spectra, HMBC correlations, ^{1}H NMR spectrum, ^{13}C NMR spectrum of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
clayw@usc.edu

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