Complete Reconstitution and Deorphanization of the 3 MDa NOCAP (NOCardiosis-Associated Polyketide) Synthase

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ABSTRACT: Several *Nocardia* strains associated with nocardiosis, a potentially life-threatening disease, house a non-modular assembly-line polyketide synthase (PKS) that presumably synthesizes an unknown natural product. Here, we report the discovery and structure elucidation of the NOCAP (NOCardiosis-Associated Polyketide) aglycone by first fully reconstituting the NOCAP synthase *in vitro* from purified protein components followed by heterologous expression in *E. coli* and spectroscopic analysis of the purified products. The NOCAP aglycone has an unprecedented structure comprised of a substituted resorcyaldehyde headgroup linked to a 15-carbon tail that harbors two conjugated all-*trans* trienes separated by a stereogenic hydroxyl group. This report is the first example of reconstituting a *trans*-acyltransferase assembly-line PKS either *in vitro* or in *E. coli*, and of using these approaches to “deorphanize” a complete assembly-line PKS identified via genomic sequencing. With the NOCAP aglycone in hand, the stage is set for understanding how this PKS and associated tailoring enzymes confer an advantage to their native hosts during human *Nocardia* infections.

Within the past decade, genomic sequencing has exposed many “orphan” biosynthetic gene clusters encoding assembly-line PKSs whose products have yet to be identified¹. Analysis of orphan PKSs has the potential to reveal new biosynthetic strategies as well as products with unprecedented structures and biological activities. Of particular interest to our laboratory is an intriguing family of orphan assembly-line PKSs termed NOCAP synthases. NOCAP synthases harbor *cis*- and *trans*-acyltransferases², and are invariably found in strains of the actinomycete *Nocardia* isolated from patients affected with nocardiosis, a serious pulmonary or systemic disease³⁻⁵ (Table S1). The NOCAP synthase is composed of four separate proteins (NOCAP_PKS1-4) containing nine PKS modules (eight of which are collinear) (Figure 1). Modules 1 and 3 possess their own acyltransferase domains, whereas the remaining modules require a *trans*-acyltransferase (*tAT*) to supply malonyl extender units. Notably, this PKS has three other unusual features: a) a “split and stuttering” module capable of catalyzing three elongation and reductive cycles (module 5)^{5,8,9}, b) a terminal thioester reductase (TR)¹⁰, and c) a thioesterase (TE) domain fused to the *tAT*. In a preliminary study, several unprecedented, albeit partially characterized, octaketide and heptaketide products were generated by incubating purified modules 4-8 with the surrogate primer unit octanoyl-CoA⁵. Building on our laboratory’s prior experience in functionally reconstituting the complete 6-deoxyerythronolide B synthase (DEBS) in *E. coli*⁶ and from purified protein components⁷, we sought to deorphanize a prototypical NOCAP synthase outside of its genetically difficult and potentially hazard-

ous natural host using both of these approaches. Here we report on the successful reconstitution of the entire assembly-line NOCAP synthase *in vitro* as well as in *E. coli*.

We hypothesized that the uncharacterized module “X” synthesizes a primer unit for the collinear assembly line comprised of modules 1-8. Accordingly, we expressed a soluble maltose-binding protein (MBP)-module X fusion protein in *E. coli*, and purified it to homogeneity (Figure S1). To identify the substrates and products bound to its acyl carrier protein (ACP) domain by intact protein LC-MS, we further expressed and purified two derivatives of this protein: MBP-module X without the ACP domain (KS_X), and the stand-alone ACP_X (Figure S2). Apo-ACP_X was incubated with the Sfp phosphopantetheinyl transferase¹¹ and malonyl-CoA to obtain malonyl-S-ACP_X, which was then incubated with KS_X. LC-MS analysis revealed that malonyl-S-ACP_X was predominantly decarboxylated to acetyl-S-ACP_X in a KS_X-dependent manner (Figure 2a), suggesting that KS_X is condensation-incompetent (hence the designation KS₀ from here onwards) but is able to decarboxylate malonyl-S-ACP_X to generate an acetyl unit for translocation to module 1. Interestingly, while KS_X appears functionally analogous to specialized KS_Q domains¹², its active site Cys residue is not replaced by a highly conserved Gln. KS₀ domains are prevalent in *trans*-AT PKSs, and lack the active site His residue located within the HGTGT motif²; however, KS_X also retains this conserved His residue.

Figure 1. (a) Prototypical NOCAP synthase biosynthetic gene cluster from *N. puris*. (b) Biosynthesis of **1** by the NOCAP synthase. Key: KS, ketosynthase; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; ER, enoylreductase; cMT, C-methyltransferase; ACP, acyl carrier protein; TR, thioester reductase; and TEII, thioesterase II.

To assay the overall activity of modules X, 1 and 2, a bi-modular protein (KS₁-AT₁-DH₁-KR₁-ACP₁-KS₂-DH₂-ER₂-KR₂) lacking ACP₂ was expressed and purified; separately, stand-alone *holo*-ACP₂ was also expressed and purified (Figure S3). The two proteins were assayed via a phosphopantetheine (PPant) ejection assay¹³ in the presence of MBP-module X, truncated *tAT* (i.e., lacking its TE domain) and appropriate substrates. In this and all subsequent assays utilizing malonyl-CoA, this labile substrate was generated *in situ* by adding malonic acid, CoASH, ATP and the *Streptomyces coelicolor* malonyl-CoA synthetase MatB¹⁴ to the reaction mixture. Instead of detecting the anticipated hex-4-enoyl-PPant species, sorbyl-PPant was the major observed product (Figure 2b), implying that the enoylreductase (ER) domain of module 2 is inactive (designated ER₀ from here onwards). Together, these results confirm our hypothesis that module X-module 1-module 2 comprise the first three modules for initiation of NOCAP biosynthesis.

We hypothesized that the TE domain of the *tAT*-TE protein hydrolyzes acyl-ACPs under conditions of “stalled” polyketide biosynthesis⁵. This suggestion was consistent with our earlier observation that use of the truncated *tAT* in place of the full-length *tAT*-TE resulted in a two-to-ten-fold decrease in product formation⁵. To test this hypothesis, we incubated Sfp-derived acetyl-S-ACP₁ – a stalled acyl-ACP surrogate – with either *tAT*-TE or *tAT*. LC-MS analysis uncovered that acetyl-S-ACP₁ was hydrolyzed to *holo*-ACP₁ in the presence of *tAT*-TE but not *tAT* (Figures 2c, S4). Analogous radiolabeling experiments further verified the above findings (Figure S5). Together,

these results provide strong evidence that this TE is a member of the “TEII” sub-family of thioesterases (designated TEII hereafter) that acts as a proofreading enzyme by hydrolyzing unproductive intermediates.

Buoyed by the reconstitution of modules X, 1 and 2, we endeavored to reconstitute *in vitro* the complete NOCAP synthase. To overcome its exceptionally large size (the synthase’s homodimeric mass approaches 3 MDa), NOCAP_PKS₁ was expressed and purified as three stand-alone proteins: modules 1 and 2 as one bimodular protein, module 3 as a unimodular protein, and module 4 along with the KS domain of module 5 (module 4-KS₅) as the third protein. Separately, NOCAP_PKS₂ was dissociated into two proteins: the DH-ACP-KR tridomain of module 5 fused to the complete module 6 (DH₅-ACP₅-KR₅-module 6), and a bimodular protein comprised of modules 7 and 8 along with the terminal TR domain (modules 7-8-TR) (Figures 3a, S1). To facilitate intermodular chain translocation between separated modules, each protein was fused to complementary N-terminal and/or C-terminal docking domains from DEBS that have previously been shown to facilitate non-covalent interactions between successive modules on a PKS assembly line^{16,17}. These five NOCAP synthase-derived proteins were mixed with MBP-module X, *tAT*-TEII, malonyl-CoA, NADPH and *S*-adenosyl methionine. To confirm that products originated from the assembly-line PKS, [2-¹³C]-, [1,3-¹³C₂]- or [¹³C₃]-malonyl-CoA was used in place of malonyl-CoA in parallel reactions. By high-resolution MS, we identified a polyketide **1** with a molecular formula of C₂₃H₂₆O₄ (observed [M-

H^+ m/z 365.1762, theoretical $[\text{M}-\text{H}]^-$ m/z 365.1753, 2.5 ppm) (Figure 3c).

Figure 2. *In vitro* characterization of modules X, 1-2 and tAT-TEII. (a) Extracted ion chromatograms (EICs) of the 11⁺ charge states for malonyl-S-ACP_x and acetyl-S-ACP_x in the presence or absence of KS₀. (b) EICs of malonyl-PPant and sorbyl-PPant ejected from malonyl-S-ACP₂ and sorbyl-S-ACP₂, respectively, in the presence or absence of module X. (c) EICs of the 11⁺ charge states for acetyl-S-ACP₁ and holo-ACP₁ in the presence or absence of either tAT or tAT-TEII.

The observation of +11, +11 and +22 mass shifts for **1** in mixtures containing [2-¹³C], [1,3-¹³C₂] and [¹³C₃] malonyl-CoA, respectively, indicated that **1** traversed the entire polyketide synthase and underwent three rounds of chain elongation, ketoreduction and dehydration at module 5 (Figures 1, S6-7). Because biosynthesis of **1** requires the entire assembly-line PKS, we propose that **1** is the aglycone product of the NOCAP synthase. A closely related polyketide **2** was detected that had presumably undergone one fewer round of chain elongation, ketoreduction and dehydration than **1** (molecular formula C₂₁H₂₄O₄, observed $[\text{M}-\text{H}]^-$ m/z 339.1604, theoretical $[\text{M}-\text{H}]^-$ m/z 339.1596, 2.4 ppm) (Figures S8-10). Its MS/MS fragmentation pattern matched well with that of **1**, leading us to hypothesize that an upstream module was “skipped” during the biosynthesis of **2**. Two more minor polyketides **3** and **4** were identified with MS/MS fragmentation patterns noticeably different than **1** and **2** (Figures S11-16). **3** and **4** are most likely premature polyketides with a pyrone moiety that originated from spontaneous release after module 7 extension and C-1-C-5 oxygen lactonization.

For definitive structural analysis, we sought to produce the NOCAP synthase products by using *E. coli* as a heterologous host for scalable polyketide biosynthesis⁶. Informed by the *in vitro* reconstitution experiments summarized above, we engineered three plasmids with compatible antibiotic resistance markers and origins of replication that collectively encode the pathway. Plasmid pCK-KPY222 encoded modules 1-2 as one bimodular protein and module 3. Plasmid pCK-KPY259 encoded module 4-KS₅ and the intact NOCAP_PKS₂, and pCK-KPY178 encoded the tAT-TEII and MBP-module X proteins (Figures 3b, S17). To enhance the malonyl-CoA pool in *E. coli*, pCK-KPY178 also encodes MatB and the *Rhizobium leguminosarum* malonate carrier protein MatC^{18,19}. Gratifyingly, *E. coli* BAP1[pCK-KPY222/pCK-KPY259/pCK-KPY178] produced **1** and **2** (Figures 3c, S18-23). *E. coli*-derived **1** and **2** had the same MS/MS fragments as **1** and **2** produced *in vitro*. Derivatization of **1** and **2** with Girard’s reagent T²⁰ confirmed the presence of an aldehyde (Figures S24-27). **3** and **4** were much lower in abundance from extracts of this strain, suggesting that these metabolites do not arise under physiological conditions, but are only synthesized under conditions with excess substrates. Because of their scarcity in *E. coli*, **3** and **4** were not further characterized.

We isolated **1** and **2** as faint yellow solids from 4 L of *E. coli* BAP1[pCK-KPY222/pCK-KPY259/pCK-KPY178] using lipid extraction with methyl *tert*-butyl ether/methanol²¹, C₁₈ solid-phase extraction and UV-absorbance-guided semi-preparative HPLC (Figures S28-30). A number of 1D- and 2D-NMR experiments (¹H, COSY, TOCSY, HSQC, HMBC, NOESY and ROESY) allowed us to fully elucidate their chemical structures (Figures 4, S31-62; Table S2). For **1**, COSY, TOCSY and HMBC experiments established carbon-carbon connectivity from C-1 (195.1 ppm) to C-22 (18.3 ppm) as well as the phenolic moiety resulting from C-2-C-7 aldol condensation. These spectra also revealed a pair of conjugated trienes, one synthesized by modules 1-3

of the biological role of its fully decorated natural product. Such efforts are compellingly motivated by the statistically significant but nonetheless correlative occurrence of this PKS only in strains associated with clinical cases of nocardiosis.

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Notes

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