Complete Reconstitution and Deorphanization of the 3 MDa Nocardiosis-Associated Polyketide Synthase

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Detailed methods, figures, and tables (PDF)
Abstract

Several *Nocardia* strains associated with nocardiosis, a potentially life-threatening disease, house a nonamodular assembly line polyketide synthase (PKS) that presumably synthesizes an unknown polyketide. 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 resorcyraldehyde 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 *in vitro* 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 identified. Analysis of orphan polyketide synthases (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 nocardiosis-associated polyketide (NOCAP) synthases. NOCAP synthases harbor *cis*- and *trans*-acyltransferases and are only found in strains of the actinomycete *Nocardia*, most of which are isolated from patients affected with nocardiosis, a serious pulmonary or systemic disease. The NOCAP synthase is clustered with sugar biosynthesis and transfer enzymes and is composed of four separate proteins containing nine PKS modules (eight of which are collinear) (Figure 1, Table S2). 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 infrequent features: (a) a naturally split (junction between KS/DH domains) and “stuttering” module capable of catalyzing three elongation and reductive cycles (module 5), (b) a terminal thioester reductase (TR), 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 surrogate primer unit octanoyl-CoA.

Building on our laboratory’s prior experience in functionally reconstituting the complete 6-deoxyerythronolide B synthase (DEBS) in *E. coli* and from purified protein components, we sought to deorphanize a prototypical NOCAP synthase outside of its genetically difficult and potentially hazardous 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 L (previously named “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 L fusion protein in *E. coli* and purified it to homogeneity (Figure S1). To identify 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 L without the ACP domain (KS<sub>L</sub>) and stand-
alone ACP_L (Figure S2). Apo-ACP_L was incubated with Sfp phosphopantetheinyl transferase\(^{11}\) and malonyl-CoA to obtain malonyl-S-ACP_L, which was then incubated with KS_L. LC-MS analysis revealed that malonyl-S-ACP_L was predominantly decarboxylated to acetyl-S-ACP_L in a KS_L-dependent manner (Figure 2a), suggesting that KS_L is able to decarboxylate malonyl-S-ACP_L to generate an acetyl unit for translocation to module 1. Interestingly, while KS_L appears functionally analogous to specialized KS_Q domains,\(^{12}\) its active site Cys residue is not replaced by a highly conserved Gln.

To assay the overall activity of modules L, 1, and 2, a bimodular protein (KS_L-AT_1-DH_1-KR_1-ACP_1-KS_2-DH_2-ER_2-KR_2) lacking ACP_2 was expressed and purified; separately, stand-alone holo-ACP_2 was also expressed and purified. These proteins were assayed via a phosphopantetheine (PPant) ejection assay\(^{13}\) in the presence of MBP–module L, truncated tAT (i.e., lacking its TE domain; see below paragraph), 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 Streptomyces coelicolor malonyl-CoA synthetase MatB\(^{14}\) 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 ER_0 from here onward). Together, these results confirm our hypothesis that module L–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 biosynthesis.\(^{15-18}\) This suggestion was consistent with our earlier observation that absence of the TE did not affect tAT activity, but use of truncated tAT in place of full-length tAT-TE resulted in a 2- to 10-fold decrease in product formation.\(^{5}\) To test this hypothesis, we incubated Sfp-derived acetyl-S-ACP_1, a stalled acyl-ACP surrogate, with either tAT-TE or tAT. LC-MS analysis uncovered that acetyl-S-ACP_1 was hydrolyzed to holo-ACP_1 in the presence of tAT-TE but not tAT (Figure 2c). Analogous radiolabeling experiments further verified the above findings (Figure S3). Together, these results provide strong evidence that this TE is a member of the “TEII” subfamily of thioesterases (designated TEII hereafter) that acts as a proofreading enzyme by hydrolyzing unproductive intermediates.

Buoyed by the reconstitution of modules L, 1, and 2, we endeavored to reconstitute \textit{in vitro} the complete NOCAP synthase. To overcome its exceptionally large size (the synthase’s homodimeric mass approaches 3 MDa), multi-modular proteins were dissected into smaller unimodular or bimodular proteins that could easily be expressed in \textit{E. coli}.\(^{7}\) 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 noncovalent interactions between successive modules on a PKS assembly line.\(^{19-21}\) NocapA 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-KS_5) as the third protein. Separately, NocapB was dissociated into two proteins: DH-ACP-KR tridomain of module 5 fused to complete module 6 (DH_5-ACP_5-KR_5-module 6) and a bimodular protein composed of modules 7 and 8 along with the terminal TR domain (modules 7-8-TR) (Figures 3a, S1).
These five NOCAP synthase-derived proteins were mixed with MBP-module L, tAT-TEII, malonyl-CoA, NADPH, and S-adenosyl methionine. To confirm that products originated from the assembly line PKS, [2-\textsuperscript{13}C]-, [1,3-\textsuperscript{13}C\textsubscript{2}]-, or \textsuperscript{13}C\textsubscript{3}-malonyl-CoA was used in place of malonyl-CoA in parallel reactions.

By high-resolution MS, we identified polyketide \textbf{1} with a molecular formula of C\textsubscript{23}H\textsubscript{26}O\textsubscript{4} (observed [M – H]\textsuperscript{−} m/z 365.1762, theoretical [M – H]\textsuperscript{−} m/z 365.1753, 2.5 ppm) (Figure 3c). The observation of +11, +11, and +22 mass shifts for 1 in mixtures containing [2-\textsuperscript{13}C], [1,3-\textsuperscript{13}C\textsubscript{2}], and [1\textsuperscript{3}C\textsubscript{3}] malonyl-CoA, respectively, indicated that \textbf{1} traversed the entire polyketide synthase and underwent three rounds of chain elongation, ketoreduction, and dehydration at module 5 (Figures 1, S4, and S5). Because biosynthesis of 1 requires the entire assembly line PKS, we propose that \textbf{1} is the aglycone product of the NOCAP synthase. A closely related polyketide \textbf{2} was detected that had presumably undergone one fewer round of chain elongation, ketoreduction, and dehydration than \textbf{1} (molecular formula C\textsubscript{21}H\textsubscript{24}O\textsubscript{4}, observed [M – H]\textsuperscript{−} m/z 339.1604, theoretical [M – H]\textsuperscript{−} m/z 339.1596, 2.4 ppm) (Figures S6-S8). Its MS/MS fragmentation pattern matched well with that of 1, leading us to hypothesize that an upstream module was “skipped” during biosynthesis of \textbf{2}. Two more minor polyketides, \textbf{3} and \textbf{4}, were identified with MS/MS fragmentation patterns noticeably different than 1 and 2 (Figures S9-S14). We hypothesize that 3 and 4 are 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 \textit{E. coli} as a heterologous host for scalable polyketide biosynthesis.\textsuperscript{6} Informed by the \textit{in vitro} reconstitution experiments summarized above, we engineered three plasmids with compatible antibiotic resistance markers and origins of replication that collectively encode the pathway. To exclude the possibility that matched docking domains facilitate chain translocation between modules 2 and 4, dissociated NocapA proteins were fused with orthogonal docking domains and NocapB was left intact. Plasmid pCK-KPY222 encoded modules 1 and 2 as one bimodular protein and module 3. Plasmid pCK-KPY259 encoded module 4-KS\textsubscript{5} and intact NocapB, and pCK-KPY178 encoded tAT-TEII and MBP-module L (Figures 3b, 3). To enhance the malonyl-CoA pool in \textit{E. coli}, pCK-KPY178 also encodes MatB and \textit{Rhizobium leguminosarum} malonate carrier protein MatC.\textsuperscript{22,23} Gratifyingly, \textit{E. coli} BAP1[pCK-KPY222/pCK-KPY259/pCK-KPY178] produced \textbf{1} and \textbf{2} (Figures 3c, S15). \textit{E. coli}-derived \textbf{1} and \textbf{2} had the same MS/MS fragments as \textbf{1} and \textbf{2} produced \textit{in vitro}. Derivatization of \textbf{1} and \textbf{2} with Girard’s reagent T\textsuperscript{24} confirmed the presence of an aldehyde (Figures S22-S25). 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 \textit{E. coli}, 3 and 4 were not further characterized.

We isolated \textbf{1} and \textbf{2} as faint yellow solids from 4 L of \textit{E. coli} BAP1[pCK-KPY222/pCK-KPY259/pCK-KPY178] using lipid extraction with methyl tert-butyl ether/methanol,\textsuperscript{25} C\textsubscript{18} solid-phase extraction, and UV-absorbance-guided semipreparative HPLC, with yields on the order of 1–10 mg/L (Figures S26-S28). A number of 1D and 2D NMR experiments (\textsuperscript{1}H,
COSY, TOCSY, HSQC, HMBC, NOESY, and ROESY) allowed us to fully elucidate their chemical structures (Figures 4, S29-S60; Table S3).

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 and the other by module 5. The aldehyde substituent at C-1 shows that 1 and 2 were released from the assembly line by the terminal TR domain. We observed expected hydroxyl substituents at C-3 (163.6 ppm, module 8), C-5 (160.8 ppm, module 7), and C-15 (71.9 ppm, module 4), a singlet methyl substituent at C-6 (114.7 ppm, module 6), and a terminal doublet methyl (C-22 for 1, C-20 for 2, module L). ROESY analysis of 1 and NOESY analysis of 2 verified that all of their double bonds have trans stereoconfigurations as predicted by bioinformatic analysis. To determine the absolute configuration of the stereocenter set by module 4’s KR domain, we converted the C-15 hydroxyl substituent of 2 to a Mosher ester. Mosher ester analysis with COSY confirmed that the absolute configuration at C-15 is R, also as predicted by bioinformatic analysis (Figures S61-S64). Unlike 1, compound 2 featured a conjugated diene—not triene—in its “tail”. We therefore hypothesized that a combination of the dissociated-by-design nature of module 3 and broad substrate tolerance of the KS domain of module 4 permitted facile chain translocation of a growing polyketide chain from module 2 to module 4 despite mismatched docking domains. Indeed, E. coli that does not express module 3 only produced 2, substantiating our hypothesis that biosynthesis of 2 involves bypassing module 3 (Figure S65). Collectively, these spectroscopic efforts validated 1 as the aglycone product of the NOCAP synthase.

This report represents two milestones. First, we describe for the first time the full in vitro reconstitution of an assembly line PKS that is predominantly comprised of trans-AT modules. trans-AT PKSs represent over 23% of all sequenced assembly line PKSs according to a recent survey and display remarkable architectural diversity; however, the understanding of trans-AT PKSs has significantly lagged that of cis-AT PKSs. Based on this report, the NOCAP synthase is a promising model for analyzing the structure–function relationships of trans-AT PKSs. Second, this work concludes the first example of polyketide discovery by reconstituting orphan assembly line PKSs in vitro. In principle, the methodology described here could be applied to other orphan polyketides, especially those synthesized in low abundance or from unculturable organisms. The discovery and structure elucidation of 1 will also allow us to turn our attention to substantiating its presence in Nocardia and the ultimate characterization 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 in strains associated with clinical cases of nocardiosis.

Supplementary Material

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**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.
Figure 2.
In vitro characterization of modules L, 1, and 2 and tAT-TEII. (a) Extracted ion chromatograms (EICs) of 11+ charge state for malonyl-S-ACP$_L$ and acetyl-S-ACP$_L$ in the presence or absence of: KS$_L$. (b) EICs of malonyl-PPant and sorbyl-PPant ejected from malonyl-S-ACP$_2$ and sorbyl-S-ACP$_2$, respectively, in the presence or absence of module L. (c) EICs of 11+ charge states for acetyl-S-ACP$_1$ and holo-ACP$_1$ in the presence or absence of either tAT or tAT-TEII
Figure 3.
Reconstitution of the NOCAP synthase (a) in vitro and (b) in E. coli. (c) EICs of 1 and 2 from either in vitro reactions or E. coli pellet extracts. As a negative control, modules 1 and 2 (as one bimodular protein) were omitted.
Figure 4.
Structures of 1 and 2 assembled from 2D NMR data.