ABSTRACT: Antimycins are a family of natural products possessing outstanding biological activities and unique structures, which have intrigued chemists for over a half-century. Of particular interest are the ring-expanded antimycins that show promising anticancer potential and whose biosynthesis remains uncharacterized. Specifically, neoantimycin and its analogs have been shown to be effective regulators of the oncogenic proteins GRP78/BiP and K-Ras. The neoantimycin structural skeleton is built on a 15-membered tetralactone ring containing one methyl, one hydroxy, one benzyl, and three alkyl moieties, as well as an amide linkage to a conserved 3-formamidosalicylic acid moiety. Although the biosynthetic gene cluster for neoantimycins was recently identified, the enzymatic logic that governs the synthesis of neoantimycins has not yet been revealed. In this work, the neoantimycin gene cluster is identified, and an updated sequence and annotation is provided delineating a nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS) hybrid scaffold. Using cosmid expression and CRISPR/Cas-based genome editing, several heterologous expression strains for neoantimycin production are constructed in two separate Streptomyces species. A combination of in vivo and in vitro analysis is further used to completely characterize the biosynthesis of neoantimycins including the megasynthases and trans-acting domains. This work establishes a set of highly tractable hosts for producing and engineering neoantimycins and their C11 oxidized analogs, paving the way for neoantimycin-based drug discovery and development.

Antimycin (ant)-type depsipeptides are a large family of natural products widely produced by filamentous Actinobacteria. Antimycins are the archetypal members of this family and have been known for almost 70 years. They possess potent cytotoxicity because they bind to and inhibit mitochondrial cytochrome c reductase, the terminal step in respiration; this bioactivity is linked to a conserved 3-mitochondrial cytochrome c reductase, the terminal step in potent cytotoxicity because they bind to and inhibit inhibitors of the Bcl-2/Bcl-X L-related antiapoptotic proteins ingredient in Fintrol. Recently, antimycins were identified as inhibitors of the Bcl-2/Bcl-X L-related antiapoptotic proteins that are overproduced by cancer cells and confer resistance to chemotherapeutic agents whose mode of action is activation of apoptosis; it was also established that this antagonism is independent of its respiratory inhibitory activity.

More than 40 naturally occurring derivatives of antimycin (nine-membered ring) have been observed, and so-called “ring-expanded” members of the family have also been described. These include JBIR-06 (12-membered ring), neoantimycin (15-membered ring), and respirantin (18-membered ring). The hybrid nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) pathway dictating the biosynthesis of antimycins was identified recently, which facilitated the subsequent identification of biosynthetic gene clusters (BGCs) for ring-expanded members of the family. The biosynthesis of all ant-type depsipeptides is directed by a hybrid NRPS/PKS pathway that utilizes a 3-formamidosalicylate starter unit, followed by l-threonine, various α-keto acids, and malonate derivatives to produce a linear depsipeptide, which is cyclized and released from the terminal module by a cis-acting thioesterase domain. The final cyclic depsipeptide is a macrocyclic ring varying in size depending on the number of NRPS modules, with each ring-expanded member of the family possessing one additional module. Neoantimycins (compounds 1–6; Figure 1) are of particular interest, because a C11 oxidized derivative of 3 named...
prunustatin A downregulates the expression of the major molecular chaperone GRP78/BiP, whose overproduction is required for cancer cell survival during hypoxic conditions caused by their rapid proliferation. Additionally, several neoantimycin derivatives displayed nanomolar potency in causing the mislocalization of oncogenic K-Ras, a GTPase that regulates cell growth and proliferation in numerous cancers. Thus, neoantimycin is an exciting anticancer lead compound for the treatment of diverse cancers. Natural product lead compounds are difficult to derivatize synthetically, and a powerful alternative approach to this is bioengineering. We therefore sought to robustly characterize the biosynthesis of neoantimycin as the first step toward establishing a platform for biologically expanding the chemical space of this compound class.

Figure 1. Biosynthesis of neoantimycins. (A) The neoantimycin biosynthetic gene cluster BGC in *Streptomyces orinoci* NRRL B-3379. The locations of Cosmid 69 and 813 are indicated by horizontal lines, and a double vertical hash indicates that the cosmid insert harbors additional DNA that falls outside the boundaries of this schematic. (B) The proposed biosynthetic pathway for neoantimycins. Structural variation arises from natural promiscuity of NRPS modules 3 and 6 as well as formylation of the starter unit. A, adenylation; T, thiolation; C, condensation; KR, ketoreductase; KS, ketosynthase; AT, acyltransferase; MT, methyltransferase; ACP, acyl-carrier protein; TE, thioesterase.

Table 1. Proposed Functions of Proteins Encoded by the Neoantimycin Biosynthetic Gene Cluster

| gene | size, aa | deduced role | protein homologue* | accession number | identity/similarity %/% |
|------|----------|--------------|--------------------|-----------------|------------------------|
| NatA | 149      | extracytoplasmic function RNA polymerase sigma factor | AntA (*S. blastmyceticus*) | AGG37762.1 | 83/93 |
| NatB | 4542     | trimodular nonribosomal peptide synthetase | AntC (*S. blastmyceticus*) | AGG37764.1 | 68/76 |
| NatC | 1411     | unimodular polyketide synthase | hypothetical (*S. albus sp. albus*) | KUJ65684.1 | 75/82 |
| NatD | 2403     | unimodular nonribosomal peptide synthetase | putative peptide synthetase (*S. pyridomyceticus*) | AIF33080.1 | 44/55 |
| NatE | 66       | MbtH-like protein | MbtH family protein (*S. solWspMP-5a-2*) | SCD38000.1 | 69/78 |
| NatF | 342      | ketoreductase | NAD-dependent epimerase (*N. coensis*) | WP033408890.1 | 54/65 |
| NatG | 255      | thioesterase | hypothetical (*S. coaentaxia*) | WP049714988.1 | 69/78 |
| NatQ | 230      | phosphopantetheinyl transferase | EnID (*Streptomyces sp. TLI 146*) | PKV83804.1 | 59/66 |
| NatF | 495      | Acyl-CoA ligase | CoA ligase (*Streptomyces sp. ADM21*) | AIF33754.1 | 81/88 |
| NatG | 79       | peptidyl carrier protein | AntG (*S. blastmyceticus*) | AGG37769.1 | 96/96 |
| NatH | 339      | multicomponent oxygenase | PaaG (*S. albus sp. J1074*) | AG192189.1 | 84/91 |
| NatI | 97       | multicomponent oxygenase | antt (*S. blastmyceticus*) | AGG37771.1 | 82/91 |
| NatJ | 251      | multicomponent oxygenase | Paal (*S. lincolensis*) | WP067444180.1 | 72/80 |
| NatK | 165      | multicomponent oxygenase | PaaF (*Streptomyces sp. ERV7*) | OAR24946.1 | 81/89 |
| NatL | 366      | multicomponent oxygenase | AntL (*S. blastmyceticus*) | AGG37774.1 | 74/82 |
| NatN | 270      | tryptophan 2,3-dioxygenase | AntN (*S. blastmyceticus*) | AGG37776.1 | 79/87 |
| NatO | 274      | N-formylase | alpha/beta hydrolase (*Streptomyces sp. ERV7*) | WP067162889.1 | 81/86 |
| NatP | 416      | kynureninase | AntP (*S. blastmyceticus*) | AGG37778.1 | 80/87 |

*Results generated by BLASTP analysis.*
Here, we report the identification, cloning, and heterologous expression of the neoantimycin biosynthetic gene cluster from *Streptomyces orinoci* NRRL B-3379. We use our heterologous expression platform to establish the functionality of a trans-acting ketoreductase, NatF, and type II thioesterase, NatG, and combined with *in vitro* analyses, we also demonstrate that the geminal dimethyl moiety originates from an iteratively cis-acting methyltransferase domain within the NatC PKS. On the basis of our analyses, a biosynthetic pathway for neoantimycins is proposed and in the longer term our genetically tractable neoantimycin production platform can be used to facilitate the rapid generation of novel neoantimycin analogs.

**RESULTS AND DISCUSSION**

**Identification of the Neoantimycin ( *nat* ) Biosynthetic Gene Cluster.** The *nat* BGC was previously identified in the genome of *S. orinoci* NRRL B-3379; however, neither its DNA sequence nor the genome sequence was made publically available.11 The reported *nat* BGC and proposed biosynthetic pathway deviated considerably from what is known about the production of the related compound, antimycin. Two proteins essential for the biosynthesis of the starter unit 3-formamidosalicylate were missing: an orthologue of AntG, a discrete peptidyl carrier protein (PCP), and an orthologue of AntI, a constituent of the multicomponent oxygenase that converts tryptophan to 3-aminovaleric acid prior to N-formylation by AntQ.15,16 In addition, in the reported *nat* BGC, there was a redundancy of ketoreductase (KR) domains encoded by both a standalone gene (*natF*) and in the unimodular PKS gene (*natC*) and a redundancy of thioesterase domains encoded by both a standalone gene (*natG*) and in the NRPS gene (*natE*). The sixth module encoded by the NRPS genes (*natD* and *natE*) further showed abnormalities that this module lacked a typical condensation domain and the protein itself was disconnected such that the adenylation domain encoding gene would be divergently transcribed from the opposite strand of DNA (Figure S1).

The above peculiarities prompted us to resequence *S. orinoci* NRRL B-3379. The final genome assembly, which is available under GenBank accession PHNC01000000, revealed a 7,502,208 bp chromosome represented over 44 contigs. The *nat* BGC was identified by the genome mining package antiSMASH 3.0,17 whose identity was easily corroborated by manual identification of gene products orthologous to AntFGHIJKLNO from *S. albus*,9,18,19 which biosynthesize the 3-formamidosalicylate moiety15,16,20 present in both antimycins and compounds 1–3.

The ~38 kb *nat* BGC identified in this study harbors 18 genes with the deduced functionalities shown in Table 1 and is organized into four apparent transcriptional units (Figure 1). Interestingly, we identified three previously unreported genes: *natE*, *natQ*, and *natI*, which encode an MbtH-like protein, a phosphopantetheinyl transferase, and the missing constituent of the multicomponent oxygenase involved in 3-formamidosalicylate biosynthesis, respectively (Table 1, Figure 1). In addition, we were unable to identify a KR domain within the deduced amino acid sequence for NatC and, strikingly, identified the gene encoding the NatD NRPS as a contiguous unbroken coding sequence that harbored the anticipated condensation domain (Figures 1 and S1). While it is formally possible that the differences between our *nat* BGC and that reported previously could result from genetic rearrangements and/or deletions, it is more likely to be the consequence of incomplete annotation and/or genome assembly error(s) considering the previous study demonstrated the strain to produce neoantimycins.11

**Verification of Adenylation Domain Substrate Specificity.** To confirm that the various alkyl substitutions on the lactone ring are due to the promiscuity of the megasynthases, we tested the adenylation domain substrate specificity of the NatB and NatD NRPSs using an ATP/PPI exchange assay. NatB was truncated into three individual modules, NatB-B, -C, and -X, that contain biosynthetic modules 2, 3, and 4, respectively, and were overproduced and purified from the *E. coli* strain BAP1.21 Purified NatB-C showed activation of 3-methyl-2-oxobutanoic acid, 3-methyl-2-oxopentanoic acid, and pyruvate as expected by structural analysis of the naturally occurring neoantimycins, and NatB-X exhibited a strong activation of phenylpyruvate (Figure 2). Purified NatB-B curiously did not exhibit PPI exchange activity when incubated with its presumed substrate, L-threonine. However, the purified dimodule protein NatB-BC was able to activate L-threonine as well as 3-me-2-oxobutanoic acid, 3-me-2-oxopentanoic acid, and pyruvate (Figure 2). This suggests that individual
truncation and purification of module 2 did not yield an active adenylation domain, albeit the protein seemed to be solubly expressed from E. coli (Figure S2). The terminal module harbored by NatD overproduced and purified as above and exhibited PPI activity when incubated with the tested substrates 3-methyl-2-oxobutanoic acid and 3-methyl-2-oxopentanoic acid. The demonstrated substrate specificities of four adenylation domains are all consistent with the known molecular structures of neoantimycins.

**Cloning of the nat BGC and Heterologous Production of Neoantimycins by Streptomyces coeliker.** *S. orinoci* is genetically intractable, and therefore we pursued a heterologous expression strategy in order to study neoantimycin biosynthesis. We constructed a cosmids library and identified two overlapping cosmids, Cosmid 69 and Cosmid 813, which together span the entire nat BGC (Figure 1). In lieu of not capturing the entire gene cluster in one cosmid, we modified Cosmid 69 and Cosmid 813 to integrate into orthologous phage sites, which would abrogate the need to establish a contiguous gene cluster clone. Next, we replaced the native promoters of key loci in the gene cluster with strong constitutive ones to ensure expression of the gene cluster in a surrogate host (see Methods). In brief, Cosmid 69 was engineered such that natFG was expressed from the rpsL(XC) promoter (pRFSUL2), and Cosmid 813 was engineered such that natBCDE was expressed from the rpsL(XC) promoter and natA was expressed from the erm*E* promoter (pRFSUL3). On the basis of the promoter motifs recognized by its characterized ortholog (σ^nac^), we predict that σ^nac^ will activate expression of natQF’G’HIJKLNOP.^22^ Engineered cosmids pRFSUL2 and pRFSUL3 were mobilized to *S. coelicolor* M1146,^23^ and chemical extracts prepared from cointegrant and parental strains were evaluated by LC-HRMS for the presence of neoantimycins. As we anticipated, molecular formulas for neoantimycins such as 1–3 were only observed in chemical extracts prepared from M1146 harboring both pRFSUL2 and pRFSUL3, but not in extracts generated from the empty M1146 strain (Figure S3). Taken together, these data unambiguously establish the identity of the genes required for the biosynthesis of neoantimycins.

**Construction of a Chimeric Antimycin/Neoantimycin Biosynthetic Pathway in Streptomyces albus.** Although we demonstrated effective neoantimycin production using our engineered cosmids and M1146, the strain does not sporulate well, grows relatively slowly, and harbors several antibiotic resistance markers, which limits the introduction of DNA in future experiments. We therefore selected *S. albus* J1074 as a heterologous production platform on the basis that it grows rapidly and has genome editing tools such as the CRISPR/Cas9 system readily available. Since *S. albus* J1074 is a native antimycin producer possessing the 3-formamidosalicylate biosynthetic machinery, we subcloned natABCDE from Cosmid 813 into an *E. coli*-Streptomyces shuttle vector that additionally had natFG cloned under a constitutive erm*E* promoter. The resulting plasmid containing nat*A*-G was introduced into *S. albus* J1074 by conjugative transfer to generate the strain 5c-0. Analysis of the culture extracts of 5c-0 by LC-HRMS showed the heterologous production of all six of the neoantimycins that have previously been isolated from *S. orinoci*, and the titers of these compounds were comparable to those from the native producer (Figures 3 and S4). Successful combinatorial heterologous production of neoantimycins encouraged the creation of an unmarked heterologous host to facilitate future engineering efforts and remove background antimycin production. We used CRISPR/Cas9 genome editing to create an unmarked strain of *S. albus* J1074 (named 5c-1) in which antC was replaced by natB, and erm*E*p-driven natCDEFG was introduced into the intergenic space between antE and antF within the antimycin BGC (Figure 4). Chemical extracts prepared from 5c-1 demonstrated its ability to produce neoantimycins with a titer similar to that of the native producer *S. orinoci* (Figure 3). These data demonstrate that the NatB NRPS efficiently interacts with 3-formamidosalicylate-S-antG and is the first experimental evidence suggesting combinatorial bioengineering of ant-type depsipeptide biosynthesis may be possible.

Interestingly, the strain 5c-1 would promote the formation of a chimeric biosynthetic assembly line consisting of NatB and AntD (Figure 4), which would result in the production of 12-membered ring ant-type depsipeptides related to JBIR-06.24 We therefore closely inspected LC-HRMS data sets generated with the 5c-1 strain but were unable to detect molecular formulas consistent with variants related to JBIR-06, suggesting that both NatB and AntD do not interact to form a functional assembly line, or a dedicated thioesterase is needed to cyclize and release the 12-membered lactone ring. This conclusion is consistent with the lack of 12-membered ring compounds from the intermediate strains 5c-0, in which antC was replaced by natB (Figure 4). In addition, the production of antimycins (nine-membered ring) by 5c-0 and 5c-1 was not detected, demonstrating the lack of interactions between NatB and AntD for antimycin synthesis through module skipping.

**The Geminal-Dimethyl Moiety of Neoantimycins Is Generated by a cis-Acting Methyltransferase Domain That Functions Iteratively.** The placement of the geminal-dimethyl moiety observed at C12 of neoantimycins implicates involvement of the penultimate biosynthetic module encoded by the NatC PKS (Figure 1). Bioinformatics analysis of the NatC revealed the following domain composition, KS-AT-MT-ACP; however, the substrate specificity of AT could not be reliably predicted. Given the absence of other enzymes with predicted methyltransferase activity within the nat BGC, we hypothesized that the cis-acting MT domain generates the geminal-dimethyl moiety either by acting once upon a malonate unit or twice upon a malonate unit. In order
to distinguish between these possibilities, we overexpressed and purified NatC using E. coli BAP1 and performed a 14C gel autoradiography assay to determine if [2-14C]malonyl-CoA could be loaded onto the PKS. The results of this assay showed successful transfer of the radiolabel to NatC, indicating that the NatC-AT domain was functional in vitro and capable of recognizing malonyl-CoA (Figure 5A). As this did not exclude the possibility that methylmalonyl-CoA could also be utilized, we then fed [methyl-d3] L-methionine (the precursor of the predicted methyl group donor, S-adenosylmethionine (SAM)) to 4c-1. In this experiment, a single methylation would result in production of neoantimycins with molecular masses of M + 3, whereas a dimethylation would produce molecular masses of M + 6. LC-UV analysis of the resultant chemical extracts showed that the fermentation product profiles remained the same upon the feeding of unlabeled and labeled L-methionine (Figure S5), and LC-HRMS analysis showed the presence of M + 6 neoantimycins upon the feeding of [methyl-d3] L-methionine, indicating a dimethylation event. We also observed M + 3 neoantimycins upon the feeding of labeled L-methionine, which is most likely a consequence of incorporation of one labeled and one unlabeled methyl group (Figure 5B).

To corroborate the above findings and to unambiguously determine the malonyl-CoA utilized by NatC, we deactivated NatC-MT in 5c-1 by using CRISPR/Cas9 genome editing to replace a codon for a catalytically important histidine with asparagine (H1189N). 26 LC-HRMS analysis of chemical extracts generated from the resulting mutant strain (named 7a-3) showed the absence of neoantimycins and the presence of compounds with molecular masses consistent with a loss of the

Figure 4. Construction of strain 5c-1 using CRISPR/Cas9. The gene antC in the antimycin BGC of S. albus J1074 is replaced by natB to generate an intermediate strain 5c-0, followed by the insertion of natCDEFG to generate 5c-1.
geminal dimethyl moiety (Figures 5C and S6). Subsequent MS/MS analysis of these compounds in comparison to neoantimycins was indicative of des-geminal dimethyl neoantimycins (Figures S7 and S8). Taken together, these data indicate that NatC utilizes malonyl-CoA and NatC-MT acts iteratively to generate the geminal dimethyl moiety.

NatG Is a Proofreading Thioesterase and NatF Is a trans-Acting Ketoreductase. We next probed the "redundant" gene products NatG and NatF encoded in the nat BGC in comparison to the ant BGC. On the basis of our reannotation of nat BGC, we propose that, similar to AntM, NatF is likely a trans-acting ketoreductase that is responsible for the apparent regiospecific ketoreduction at C11. Further bioinformatics analysis of NatG shows that it belongs to InterPro Family IPR01223, which indicates that NatG is likely a proofreading or type II thioesterase. Many PKS and NRPS biosynthetic gene clusters harbor a gene specifying a type II thioesterase, where they have been shown to increase production levels by removal of aberrant thioester intermediates from the assembly line.27 We chose to establish the functionality of NatG and NatF in vivo and did so with our S. albus-based neoantimycin production platform. Two cosmids harboring either natABCDDEF or natABCDEF were constructed and mobilized to S. albus J1074 to generate strains 4c-2 and 4c-3, respectively. Chemical extracts from the resulting strains were analyzed by LC-UV and compared to the extracts generated from S. albus J1074 harboring natABCDDEF. Neoantimycins were still produced in the absence of natG albeit at a reduced titer, which is consistent with our hypothesis that NatG is a proofreading thioesterase (Figure 6). Conversely, neoantimycins were not observed in chemical extracts generated in the absence of natF; instead, a suite of compounds with UV absorption spectra identical to those of neoantimycins, but with shifted retention times, was observed (Figures 6 and S9). LC-HRMS and MS/MS analysis of these compounds were consistent with oxidized variants of neoantimycins, such as prunustatin A and neoantimycin H that were reported previously (Figures S10 and S11). The identity of o-3 was further confirmed by comparing to the authentic standard of prunustatin A. These results indicate that NatF is responsible for reduction of the keto group to a hydroxyl on C11. Since oxidized neoantimycins have shown interesting biological activities with prunustatin A established as a downregulator of the molecular chaperone BiP/GRP78,12 this work has also generated a useful strain that produces C11 oxidized neoantimycins exclusively.

Proposed Biosynthetic Pathway for Neoantimycins. On the basis of the above in vivo and in vitro analysis, we propose a complete neoantimycin biosynthetic pathway as follows (Figure 1): it begins with the opening of the indole ring of tryptophan by a tryptophan 2,3-dioxgenase (NatN) to produce N-formyl-L-tryptophan, which is converted to anthranilic acid by a housekeeping enzyme (NatM). Anthranilic acid is then activated by an acetyl-CoA ligase, NatF', and loaded into its cognate carrier protein, NatG', followed by conversion to 3-aminosalicylate by a multicomponent oxygenase NatHIJKL and 3-formylation by NatO. 3-Formamidosalicylic acid serves as a starting unit for the hybrid NRPS/PKS machinery and is first presented to the NatB NRPS. NatB possesses three modules organized as follows: C1-A1-T1-C2-A2-KR1-T2-C3-A3-KR2-T3. The A1 domain activates and loads i-Thr onto T1 followed by condensation with 3-formamidosalicylate by C1 to form an amide bond. The A2 domain activates and loads pyruvate, 3-methyl-2-oxobutanoic acid, or 3-methyl-2-oxopentanoic acid onto T2, which is subsequently stereospecifically reduced by KR1 and condensed with i-Thr by C2. The A3 domain activates and loads phenyl pyruvate onto T3, which is stereospecifically reduced by KR2 and condensed with the aminoclay thioester attached to T2. The NatC PKS harbors one module organized as follows: KS-A1-MT-ACP. AT transfers malonate to ACP followed by installation of the geminal dimethyl group by MT. Next, KS catalyzes decarboxylation condensation between geminal dimethyl malonate and the aminoclay thioester on T3 of NatB. The NatD NRPS harbors one module organized as follows: C4-A4-KR3-T4-TE. The A4 domain activates and loads 3-methyl-2-oxobutanoic acid or 3-methyl-2-oxopentanoic acid onto T4, which is stereospecifically reduced by KR3 and condensed with the aminoclay thioester attached to NatC-ACP prior to macrolactone cyclization and release of the 15-membered ring by NatD-TE. The trans-acting ketoreductase NatF reduces the C11 ketone to a hydroxyl, most likely acting on an intermediate tethered to the assembly line. In conclusion, our characterization and strain development within this work pave the way for rational reprogramming of the neoantimycin assembly line toward the biosynthesis of neoantimycin analogs.

![Figure 6](image-url) Characterization of NatF and NatG. HPLC-UV analysis of neoantimycins produced by engineered S. albus strains expressing different combinations of natF and natG. Chromatograms (320 nm) show neoantimycin (1–3) production by S. albus 4c-1 expressing natABCDDEF and reduced titers of neoantimycins by S. albus 4c-2 omitting natG that encodes a type II thioesterase. A new set of neoantimycin derivatives was produced by S. albus 4c-3 omitting natF that encodes a ketoreductase.
following concentrations: apramycin (50 μg/mL), carbenicillin (100 μg/mL), hygromycin (75 μg/mL), kanamycin (50 μg/mL), and nalidixic acid (25 μg/mL). Chemicals and media were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise stated. Phusion High-Fidelity PCR Master Mix (NEB) was used for PCR reactions. Restriction and ligation enzymes were purchased from New England Biolabs unless otherwise stated. Oligonucleotides were purchased from Integrated DNA Technologies and are described in Table S1. The DNA constructs and bacterial strains used in this study are listed in Tables S2 and S3, respectively.

**Genome Sequencing.** *S. orinoci* NRRL B-3329 was obtained from the United States Department of Agriculture ARS stock center. *S. orinoci* chromosomal DNA was sequenced by the Earlham Institute (Norwich, UK) using the Pacific Biosciences and Illumina MiSeq platforms. The hierarchical genome assembly process (HGAP) was applied to two RSH SMRT cells worth of sequencing data, which generated 44 contigs comprised of ~7.5 Mb of DNA sequence. In order to correct errors originating from PacBio sequencing, 14,938,895 raw PacBio and Illumina reads are available under Short Read PHNC01000000 and consists of 44 contigs comprised of 7,502,208 bp. The raw PacBio and Illumina reads are available under Short Read Archive accessions SRR6318812 and SRR6318811, respectively.

**Cosmid Library Construction and Screening.** A Supercos1 cosmid library was constructed from *S. orinoci* NRRL B-3329 genomic DNA partially digested with Sau3AI and packaged into Gigapack III. The resulting cosmids, Cosmid 69 and Cosmid 813, were insert-end engineered to integrate into chromosomes. The DNA sequences for pUC19-PAprP, pUC19-PHygP, and pFRSUL1 were used to amplify a 2.2 kb fragment from pMS82, which contained the *rpsL* (XC) promoter from pSET152 and the *ermE* (XC) promoter from *S. albus* Δ*ermE*; *natB* and *kan* genes were placed on the backbone of Supercos1 to generate pRFSUL1. The resulting cosmids were named pRSUL2.

**Constructions of Neoantimycin-Producing *S. albus* Strains.** *S. orinoci* genomic DNA was used as a template for PCR amplification of *natF/G/FG*; the resulting PCR product was cloned into pBl139 by Gibson assembly. The resulting plasmid was then amplified by PCR and further cloned into the NsiI/CIP-digested cosmid 813 containing *natA−E* using Gibson assembly. Subsequently, the constructs were electroporated into *E. coli* WM6026 and used for conjugation with *S. albus* J1074. Transconjugants were selected by apramycin and kanamycin resistance and confirmed by PCR using the nat-E-Duet/F-Duet-Mdh-R primers. The resulting strains were 4c-1, 4c-2, and 4c-3. To construct the CRISPR/Cas9 generated strain 5c-1, the neoantimycin biosynthetic genes were introduced into the *S. albus* J1074 chromosome using the recently described pCRISPomyces-2 system. Two pCRISPomyces-2 plasmids (pJL129 and pJL134) were generated using Golden Gate and Gibson Assembly as previously described. pJL129 was used to introduce *natCDEFG* under the control of the *ermE* promoter in between *antE* and *antF*. pJL134 was used to replace *antC* from the antimycin BGC with *natB*. First, pJL134 was mobilized to *S. albus* J1074 by cross-genotype conjugation as previously described. Temperature sensitive pCRISPomyces-2 plasmids was removed from apramycin-resistant transconjugants by culturing at 37 °C. Replacement of *antC* by *natB* in the correct locus within the chromosomal plasmid was verified by PCR and resulted in the generation of a strain we named 5c-0: *S. albus* J1074 Δ*antC::natB*. Next, pJL129 plasmid was mobilized to *S. albus* J1074 + *ermE* *natCDEFG* and processed as above in order to generate a strain we named 5c-1: *S. albus* J1074 Δ*antC::natB* + *ermE* *natCDEFG*. In order to introduce the H1189N mutation, a third pCRISPomyces −2 plasmid, pDS80, was created by adapting pJL129 to insert *natCDEFG* with the mutant *natC*. To construct pDS90, pJL129 was digested with EcoR321 and BseFI restriction enzymes, and the resulting 26.7 kb linear plasmid was gel purified. A single base pair change in the MT domain was introduced by overlap PCR from *S. orinoci* genomic DNA using pJL129_EcoRV_F/H1189N_R and H1189N_F/pJL129_BseFI_R. A three-piece Gibson assembly was used to introduce the overlapping PCR products to the digested pJL129 plasmid. The integrity of the resulting plasmid was verified by PCR sequencing and subsequently used as above to generate *S. albus* J1074 Δ*antC::natB* + *ermE* *natCDEFG* + *H1189N*, the methyl-ation deficient neoantimycin producer, named 7a-3.

**Analysis of *S. coelicolor* Strains.** Engineered cosmids pRSUL2 and pRSUL3 were mobilized to *S. coelicolor* M1146 by cross-genera conjugation from *E. coli* ET12567/pUZ8002 as previously described. *S. albus* strains were cultured in 10 mL of LB while shaking (200 rpm) at 30 °C for 3 days, at which point the entire culture was added to manitol-soya flour broth (50 mL in a 250-mL flask) and incubated at 30 °C while shaking (200 rpm). After 8 days of growth, bacterial cells were removed by centrifugation, and all of the culture supernatant was extracted once with two volumes of ethyl acetate and concentrated in vacuo. The residue was resuspended in 0.3 mL of methanol (100%). Two microliters of methanolic extract were injected into a Bruker MaXis Impact TOF mass spectrometer equipped with a Dionex Ultimate 3000 HPLC exactly as previously described.

**Analysis of *S. albus* Strains.** *S. albus* strains were first grown in a 2 mL of tryptic soy broth seed culture and inoculated at 1% inoculum into a 25 mL manitol-soya flour broth. Cultures were grown for 5 days at 30 °C and 150 rpm. Mycelia were removed by centrifugation, and the supernatant was extracted with two volumes of ethyl acetate and dried with MgSO4 before rotary evaporation. Dried extracts were resuspended in methanol and analyzed via LC-HRMS or LC-UV-MS. LC-HRMS analysis was performed on an Agilent 6520 Accurate-Mass Q-TOF LC-MS, and LC-UV-MS analysis was performed on an Agilent 6120 Single Quadrupole LC/MS with a 1260 series DAD. Each
instrument was equipped with an Agilent Eclipse Plus C18 column (4.6 × 100 mm), and in each case a linear gradient of 25–95% CH3CN with 0.1% formic acid (v/v) over 20 min in H2O with 0.1% formic acid (v/v) at a flow rate of 0.5 mL/min was used. A culture equivalent of 100 μL was injected. A collision energy of 20 V was used for all HRMS/MS experiments.

**Overproduction and Purification of Recombinant Protein.** The NRPS components, NatB and NatD, and the PKS NatC were cloned and purified as follows. NatD and NatC were PCR amplified from *S. orinoci* genomic DNA as intact proteins. NatB was separated into three individual modules and PCR amplified as NatB-A, NatB-C, and NatB-X as well as a fourth construct containing the first two modules, NatB-BC from *S. orinoci* genomic DNA. Purified PCR constructs were cloned into either pET-30 or pET-24b using Gibson assembly or restriction enzyme digestion and quick ligation. Plasmids were verified by sequencing and transformed into BAP1 cells for protein production. Expression strains were grown in 0.7 L of LB supplemented with 50 μg/mL of kanamycin at 37 °C and 250 rpm until an OD600 of 0.5. Cultures were then put on ice for 10 min before induction with 120 mM IPTG. Induction of gene expression lasted for 16 h at 16 °C and 200 rpm. The cells were then harvested by centrifugation (6000 rpm, 15 min, 4 °C), and supernatant was removed. The pellet was resuspended in 30 mL of lysis buffer (25 mM HEPES, pH 8, 0.5 M NaCl, 5 mM imidazole) and homogenized using an Avestin homogenizer. The insoluble fraction was removed by centrifugation (15,000 rpm, 1 h, 4 °C), and the supernatant was filtered with a 0.45 μm filter before batch binding. Ni-NTA resin (Qiagen) was added to the filtrate at 2 mL/L of cell culture, and samples were allowed to nutate for 1 h at 4 °C. The protein mixture was added to a gravity filter column, and the flow through was discarded. The column was then washed with approximately 24 mL of wash buffer (25 mM HEPES, 300 mM NaCl, pH 8) until untagged proteins were removed, determined by Bradford assay. Tagged protein was then eluted in approximately 18 mL of elution buffer (25 mM HEPES, 100 mM NaCl, 250 mM imidazole, pH 8). Complete elution was determined by Bradford assay. Purified proteins were then concentrated and exchanged into appropriate buffer (25 mM HEPES, 100 mM NaCl, pH 8) using Amicon ultra filter units. After two rounds of exchange and concentration, pure protein was removed, and glycerol was added to a final concentration of 8%. Proteins were stored at −80 °C or used immediately for in vitro assays.

**Isotope-Labeled Precurso*r Feeding Experiments.** *S. albus* J1074 4c-1 was cultured for the production of neoantimycins as genomic DNA. Puriﬁed proteins were removed, determined by Bradford assay. Tagged protein mixture was added to a gravity filter column, and the flow through was discarded. The column was then washed with approximately 24 mL of wash buffer (25 mM HEPES, 300 mM NaCl, pH 8) until untagged proteins were removed, determined by Bradford assay. Tagged protein was then eluted in approximately 18 mL of elution buffer (25 mM HEPES, 100 mM NaCl, 250 mM imidazole, pH 8). Complete elution was determined by Bradford assay. Purified proteins were then concentrated and exchanged into appropriate buffer (25 mM HEPES, 100 mM NaCl, pH 8) using Amicon ultra filter units. After two rounds of exchange and concentration, pure protein was removed, and glycerol was added to a final concentration of 8%. Proteins were stored at −80 °C or used immediately for in vitro assays.

**14C Gel Autoradiography.** Assays were performed in 10 μL of 50 mM HEPES (pH 8.0) containing 1 mM TCEP, 4 mM ATP, 4 mM MgCl2, 1 mM CoA, 0.13 mM [2-14C]malonic acid (0.1 μCi/mL; American Radiolabeled Chemicals), 25 μM MatB (malonyl-CoA synthetase), and 90 μM NatC. Reactions were incubated for 2 h at RT and quenched with an equal volume of 1X SDS sample buffer before SDS-PAGE analysis with a 4–15% TGX gel (Criterion). The gel was subsequently dried for 2.5 h at 50 °C and then exposed on a storage phosphor screen (20 × 25 cm; Molecular Dynamics) for 2–3 days. Phosphor images were captured using a Typhoon 9400 phosphor-imager (Storage Phosphor mode, best resolution, 50 μm resolution; Amersham Biosciences).

**ATP-PPi Exchange Assays.** Substrate speciﬁcity assays were performed in 100 μL of reaction buffer (50 mM Tris-HCl pH 8.2, 2 mM MgCl2) containing 1 mM TCEP, 5 mM ATP, 1 mM tetrasodium pyrophosphate (Na2PPi), 5 mM substrate, and 5 μM enzyme. Before the addition of enzyme, Na2[32P]PPi was added to a ﬁnal intensity of ~2.5 × 1010 cpm/mL. Reactions were allowed to proceed for 2 h at 25 °C and then quenched by the addition of 500 μL of charcoal (3.6% w/v activated charcoal, 150 mM Na2PPi, 5% HClO4). Samples were centrifuged, and supernatant was discarded. To remove residual free [32P]PPi, the pellet was washed twice with wash solution (0.1 M Na2PPi, 5% HClO4). The pellet was resuspended in 500 μL of water and added to scintillation fluid at a ﬁnal volume of 5 mL. Radioactivity was measured using a Beckman LS 6500 scintillation counter.

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