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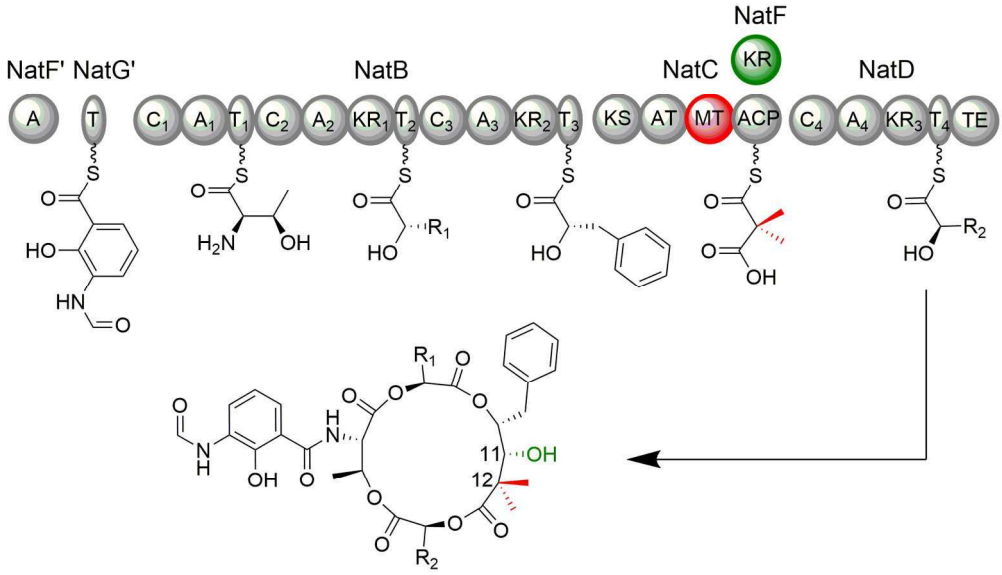

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Biosynthesis of the 15-membered ring depsipeptide neoantimycin

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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 anti-cancer 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 non-ribosomal 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.

Introduction

Antimycin (*ant*)-type depsipeptides are a large family of natural products widely produced by filamentous *Actinobacteria.*[1,2] Antimycins are the archetypal members of this family and have been known for almost 70 years.[3] They possess potent cytotoxicity because they bind to and inhibit mitochondrial cytochrome c reductase, the terminal step in respiration;[4] this bioactivity is linked to a conserved 3-formamidosalicylate moiety not observed elsewhere in nature. Antimycin is used commercially as a piscicide and is the active ingredient in Fintrol. Recently, antimycins were identified as inhibitors of the Bcl-2/Bcl-X_L-related anti-apoptotic 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.[5]

More than 40 naturally occurring derivatives of antimycin (9-memberd ring) have been observed and so-called 'ring-expanded' members of the family have also been described.[1] These include JBIR-06 (12-membered ring),[6] neoantimycin (15-membered ring),[7] and respirantin (18-membered ring).[8] The hybrid non-ribosomal peptide synthetase (NRPS) / polyketide synthase (PKS) pathway dictating the biosynthesis of antimycins was identified recently,[9] which facilitated the subsequent identification of biosynthetic gene clusters (BGCs) for ring-extended members of the family.[10,11] 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.[10]

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.[12] 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.[13,14] 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.

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 and a *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-aminosalicylate prior to *N*-formylation by AntO.[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 abnormities 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 re-sequence *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 moiety[15,16,20] present in both antimycins and compounds **1-3**.

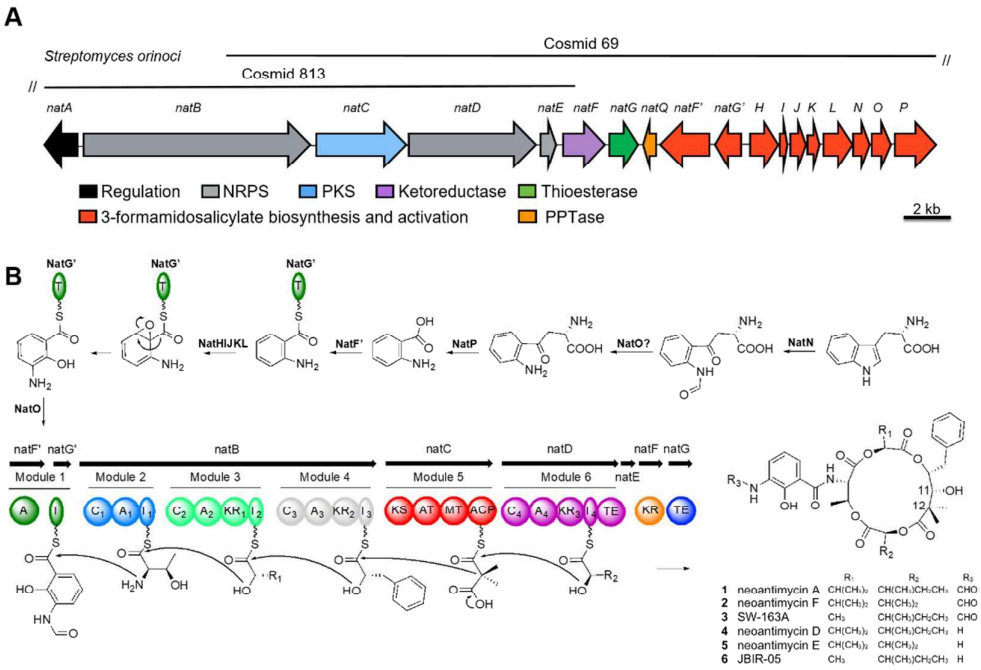

Figure 1. Biosynthesis of neoantimycins. (A) The neoantimycin biosynthetic gene cluster BGC in *Streptomyces orinoci* NRRL B-3379**.** The locations of Cosmid 69 and 813 are indicated by horizontal lines and double vertical hash indicates that cosmid insert harbors additional DNA that falls outside the 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.

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 (**Figure 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]

Table 1. Proposed functions of proteins encoded by the neoantimycin biosynthetic gene cluster

Gene	Size, aa	Deduced Role	Protein Homolog*	Accession Number	Identity/ Similarity %/%
NatA	149	Extracytoplasmic function RNA polymerase sigma factor	AntA (*S. blastmyceticus*)	AGG37762.1	83/93
NatB	4542	Trimodular non-ribosomal 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 non-ribosomal peptide synthetase	Putative peptide synthetase (*S. pyridomyceticus*)	AEF33080.1	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. cosensis*)	WP033408890.1	54/65
NatG	255	Thioesterase	Hypothetical (*S. caatingaensis*)	WP049714988.1	69/78
NatQ	230	Phosphopantetheinyl transferase	EntD (*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	AntI (*S. blastmyceticus*)	AGG37771.1	82/91
NatJ	251	Multicomponent oxygenase	PaaI (*S. lincolnensis*)	WP067444180.1	72/80
NatK	165	Multicomponent oxygenase	PaaJ (*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.

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 contains biosynthetic modules 2, 3, and 4, respectively, and 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

harboured 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 specificity of four adenylation domains are all consistent with the known molecular structures of neoantimycins.

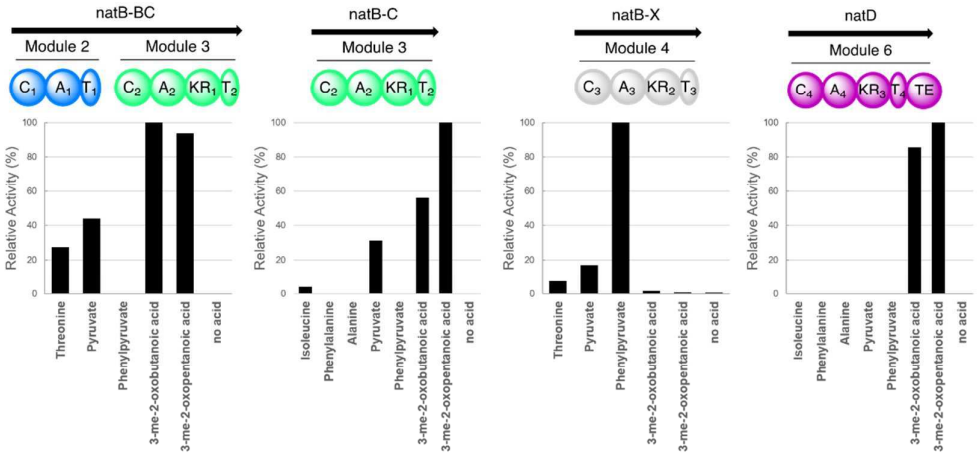

Figure 2. Analysis of NRPS adenylation domain activity. The four NRPS adenylation domains within the neoantimycin biosynthetic gene cluster were analyzed *in vitro* using ATP-[^{32}P]PPi exchange assays to verify utilization of the predicted substrate for each module. Values are displayed as relative activity normalized to 3-me-2-oxobutanoic acid (NatB-BC); 3-me-2-oxopentanoic acid (NatB-C); phenylpyruvate (NatB-X) and 3-me-2-oxopentanoic acid (NatD) dependent exchange corresponding to approximately 250k cpm.

Cloning of the *nat* BGC and heterologous production of neoantimycins by *Streptomyces coelicolor.* *S. orinoci* is genetically intractable and therefore we pursued a heterologous expression strategy in order to study neoantimycin biosynthesis. We constructed a cosmid 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 *ermE** promoter

(pRFSUL3). Based on the promoter motifs recognized by its characterised ortholog (σ^{AntA}) we predict that σ^{NatA} will activate expression of *natQF'G'HIJKLNOP*.[22] Engineered cosmids pRFSUL2 and pRFSRUL3 were mobilized to *S. coelicolor* M1146[23] and chemical extracts prepared from co-integrant and parental strains were evaluated by LC-HRMS for the presence of neoantimycins. As we anticipated, molecular formulae 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.

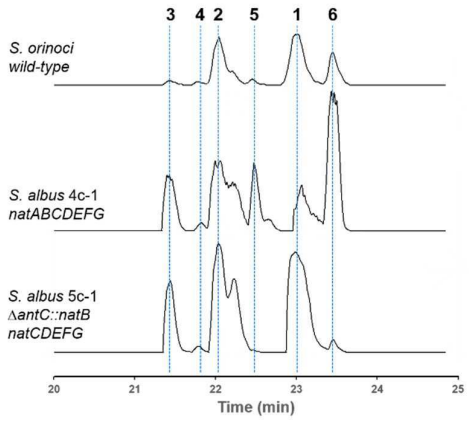

Figure 3. Heterologous production of neoantimycins. Extracted ion chromatograms show the production of neoantimycins by the wild-type *S. orinoci* and two engineered *S. albus* strains. The calculated mass with a 10 ppm error tolerance was used. Calculated masses: **1**, *m/z* 699.3124 $[M+H]^+$; **2**, *m/z* 685.2967 $[M+H]^+$; **3**, *m/z* 671.2811 $[M+H]^+$; **4**, *m/z* 671.3180 $[M+H]^+$; **5**, *m/z* 657.3018 $[M+H]^+$; **6**, *m/z* 643.2861 $[M+H]^+$.

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.[24] 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 *ermE** promoter. The resulting plasmid containing *natA-G* was introduced into *S. albus* J1074 by conjugative transfer to generate the strain 4c-1. Analysis of the culture extracts of 4c-1 by LC-HRMS showed the heterologous production of all six of the neoantimycins that have previously been isolated from *S. orinoci*, and the titres 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 *ermE**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 titre similar to that of the native producer *S. orinoci* (**Figure 3**). These data demonstrate that the NatB NRPS efficiently interacts with 3-formamidosalycilate-*S*-AntG and is the first experimental evidence suggesting combinatorial bioengineering of *ant*-type depsipeptide biosynthesis may be possible.

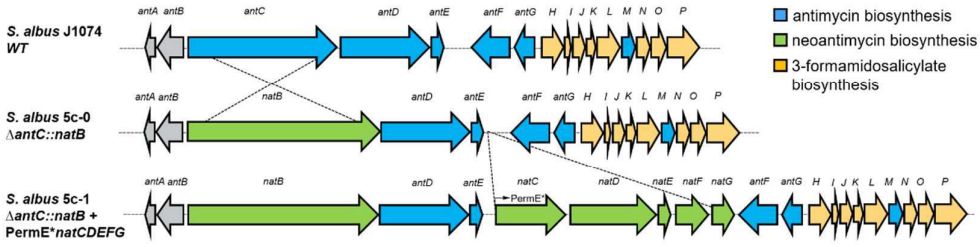

Figure 4. Construction of strain 5c-1 using CRISPR/Cas9. The gene *antC* in the antimycin BGC of *S. albus* J1074 is replaced by *natB* to generate an intermediate strain 5c-0, followed by the insertion of *natCDEFG* to generate 5c-1.

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.[6] We therefore closely inspected LC-HRMS datasets generated with the 5c-1 strain but were unable to detect molecular formulae

consistent with variants related to JBIR-06, suggesting that either 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 (9-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.[25] 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 methylmalonate 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 ^{14}C gel autoradiography assay to determine if [2-^{14}C]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 unlabelled and labelled 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 labelled L-methionine, which is most likely a consequence of incorporation of one labelled and one unlabelled methyl group (**Figure 5B**).

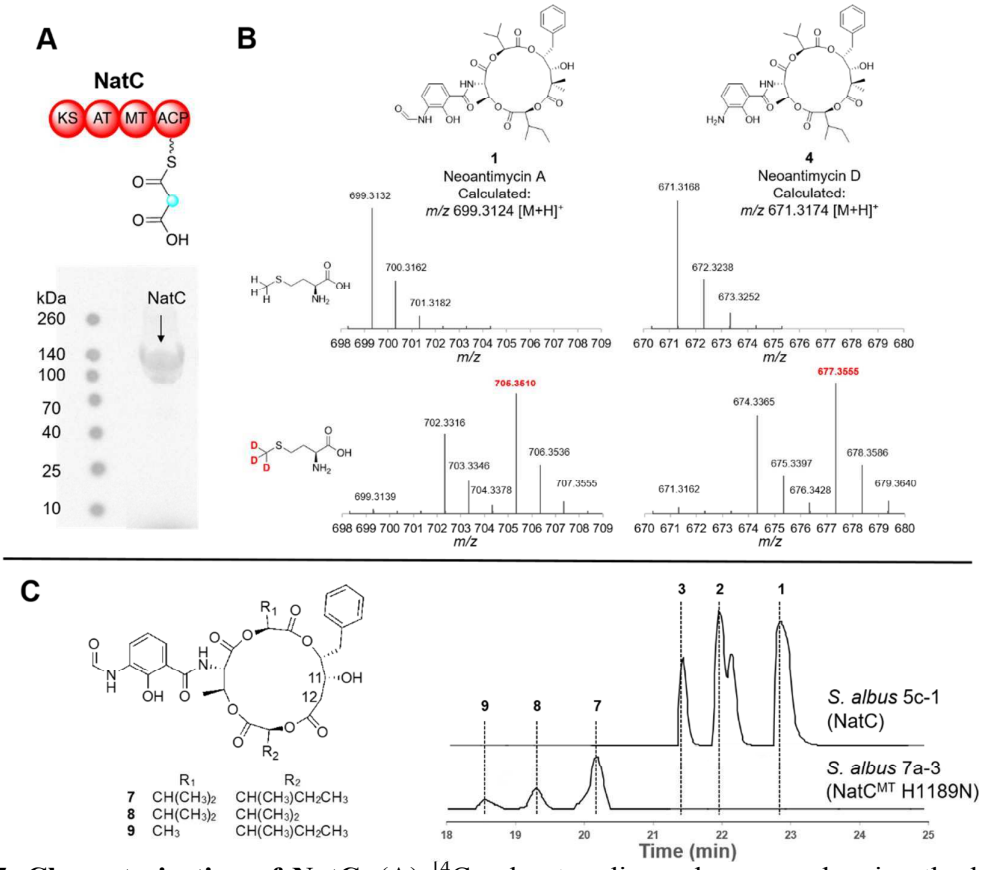

Figure 5. Characterization of NatC. (A) ^{14}C gel autoradiography assay showing the labeling of NatC by [2-^{14}C]malonyl-CoA. (B) Selected isotopic peak patterns of neoantimycins produced by cultures fed with unlabeled L-methionine (top) and [methyl-d_3] L-methionine (bottom). (C) Extracted ion chromatograms showing the production of neoantimycins and *des*-geminal dimethyl neoantimycins by engineered *S. albus* strains. The calculated mass with a 10 ppm error tolerance was used. Calculated masses: : **1**, *m/z* 699.3124 $[M+H]^+$; **2**, *m/z* 685.2967 $[M+H]^+$; **3**, *m/z* 671.2811 $[M+H]^+$; **7** *m/z* 671.2811 $[M+H]^+$; **8** *m/z* 657.2654 $[M+H]^+$; **9** *m/z* 643.2498 $[M+H]^+$. The proposed structures of **7-9** are shown to the left.

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 loss of the 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. Based on our re-annotation 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 harbouring either *natABCDEF* or *natABCDEG* were constructed and mobilized to *S. albus* J1074 to generate strains 4c-2 and 4c-3, respectively. Chemical extracts from the resulting strains were analysed by LC-UV and compared to the extracts generated from *S. albus* J1074 harbouring *natABCDEFG*. Neoantimycins were still produced in the absence of *natG* albeit at a reduced titre, 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**).[13] 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.

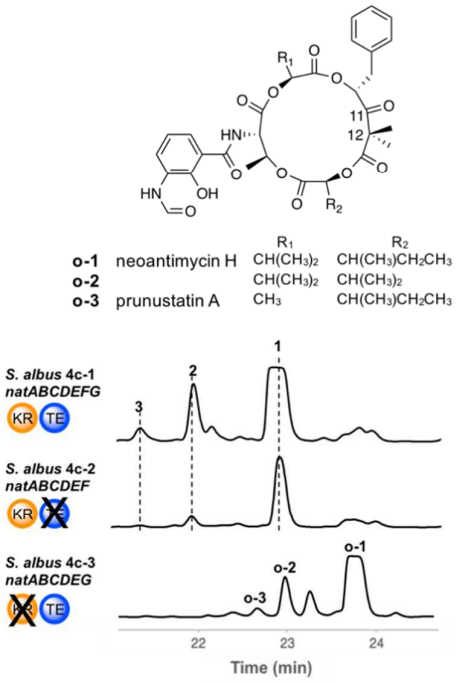

Figure 6. Characterization of NatF and NatG. HPLC-UV analysis of neoantimycins produced by engineered *S. albus* strains expressing different combinations of *natF* and *natG*. Chromatograms (320 nm) show neoantimycins (**1**–**3**) production by *S. albus* 4c-1 expressing *natABCDEFG* and reduced titers of neoantimycins by *S. albus* 4c-2 omitting *natG* that encodes a type II thioesterase. A new set of neoantimcyin derivatives was produced by *S. albus* 4c-3 omitting *natF* that encodes a ketoreductase.

Proposed biosynthetic pathway for neoantimycins. Based on 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-dioxyngenase (NatN) to produce *N*-formyl-L-kynurenine, which is converted to anthranilate by a housekeeping *N*-formylase or NatO and a kynureninase (NatP). Anthranilate is then activated by an acyl-ACP ligase, NatF' and loaded into its cognate carrier protein, NatG', followed by conversion to 3-aminosalicylate by a multicomponent oxygenase NatHIJKL and *N*-formylation by NatO. 3-formamidosalicyl-*S*-NatG' serves as a starting unit for the hybrid NRPS/PKS machinery and is first presented to the NatB NRPS. NatB possesses three modules organised as follows: C1-A1-T1-C2-A2-KR1-T2-C3-A3-KR2-T3. The A1 domain activates and loads L-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 L-Thr by C2. The A3 domain activates and loads phenyl pyruvate onto T3, which is stereospecifically reduced by KR2 and condensed with the aminoacyl thioester attached to T2. The NatC PKS harbours one module organized as follows: KS-AT-MT-ACP. AT transfers malonate to ACP followed by installation of the geminal dimethyl group by MT. Next, KS catalyzes decarboxylative condensation between geminal dimethyl malonate and the aminoacyl thioester on T3 of NatB. The NatD NRPS harbours 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 aminoacyl 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.

Methods

Growth media, strains and reagents. *Escherichia coli* strains were cultivated on Lennox agar (LA) or broth (LB) and *Streptomyces* strains were propagated on mannitol-soya flour agar or broth.[28] Culture media was supplemented with antibiotics as required at the following concentrations: apramycin (50 μg/ml), carbenicillin (100 μg/ml), hygromycin (75 μg/ml), kanamycin (50 μg/ml), nalidixic acid (25 μg/ml). Chemicals and media were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise stated. Phusion High-Fedelity 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 **Table 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 RSII 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, 14,938,895 250 bp paired-end Illumina reads were mapped to these contigs using the Geneious R8.1.19 implementation of Bowtie2. The final genome assembly is available under DDBJ/EMBL/GenBank accession 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 XL phage according to the manufacturer's instructions (Agilent Technologies). One thousand cosmid clones were screened for an insert spanning the neoantimycin BGC by PCR using primers RFS468 and RFS469 (which target the 3'end of *natB* and 5' end of *natC,* respectively) and

RFS519 and RFS520 (which target *natG*) (**Table S1**). The resulting cosmids, Cosmid 69 and Cosmid 813 were insert-end sequenced using primers RFS184 and RFS185 and mapped onto the *S. orinoci* genome using BLAST 2.2.29+.[29]

Construction of pUC19-PAprP, pUC19-PHygP and pRFSUL1. The recombineering PCR templates PAprP and PHygP were constructed as follows: (1) RFS406 and RFS407 were used to amplify the apramycin resistance gene and *oriT* from pIJ773[30] and the hygromycin resistance gene and *oriT* from pIJ10700[31], (2) RFS658 and RFS659 were used to PCR amplify the *rpsL*(XC) promoter from pCRISPomyces-,[24,32] (3) RFS667 and RFS668 were used to PCR amplify the *ermE** promoter from pSET152-*ermE*p,[33] and (4) RFS663 and RFS664 were used to linearize pUC19. The resulting PCR products were restricted with DpnI, gel purified and assembled using the NEB HiFi DNA Assembly kit. The resulting plasmids, pUC19-PAprP and pUC19-PHygP, contained FRT site-flanked apramycin and hygromycin resistance genes, respectively and divergently firing *rpsL*(XC) and *ermE** promoters. The plasmid pRFSUL1 was generated by RecET recombineering using *E. coli* strain GB05-red.[34] Briefly, oligonucleotides RFS448 and RFS449 were used to PCR amplify a ~2.2 kb fragment from pMS82, which contained the ΦBT1 integrase, *attP*, and a hygromycin resistance gene.[35] Recombineering with this PCR fragment replaced the *neo/kan* resistance gene present on the backbone of Supercos1 to generate pRFSUL1. The ΦBT1 integrase, *attP*, and a hygromycin resistance gene is released from pRFSUL1 by a SspI restriction digest and can be used to recombineer Supercos1 clones of interest for integration into *Streptomyces* chromosomes. The DNA sequences for pUC19-PAprP, pUC19-PHygP and pRFSUL1 are available at http://www.ryanseipkelab.com/tools.html.

Cosmid manipulations. Cosmid 69 and Cosmid 813 were engineered to integrate into *S. coelicolor* chromosomes using RecET recombineering with *E. coli* GB05-red and a ~5.2 kb and 5.4 kb SspI restriction fragment from pRFSUL1 and pIJ10702,[36] respectively. The resulting cosmids were named Cosmid 69-ΦBT1 and Cosmid 813-ΦC31. RecET recombineering with pUC19-PAprP and oligonucleotides DT133 and DT134 was used to modify Cosmid 69-ΦBT1 such that it only

harbored *natFGQF'G'HIJKLNOP* and that *natFG* expression was driven by the *rpsL*(XC) promoter. The resulting cosmid was named pRFSUL2. RecET recombineering with pUC19-PHygP and oligonucleotides DT132 and DT135 was used to engineer Cosmid 813-ΦC31 such that *natA and natB* were expressed from the *ermE** and *rpsL*(XC) promoters, respectively, to result in Cosmid813-ΦC31-mod. The *hygR* gene of the PHygP cassette was removed from Cosmid 813-ΦC31-mod by the Flp recombinase encoded by pCP20 as previously described[30] to result in pRFSUL3.

Construction 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 pIB139[37] 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 natE-Duet-F/Duet-Mbth-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.[24] Two pCRISPomyces-2 plasmids (pJL129 and pJL134) were generated using Golden Gate and Gibson Assembly as previously described.[24] 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-genera conjugation as previously described.[28] Temperature sensitive pCRISPomyces-2 plasmid was removed from apramycin-resistant transconjugants by culturing at 37°C. Replacement of *antC* by *natB* in the correct locus within the chromosome was verified by PCR and resulted in the generation a strain we named 5c-0: *S. albus* J1074 Δ*antC::natB*. Next, pJL129 plasmid was mobilized to *S. albus* J1074 + PermE* *natCDEFG* and processed as above in order to generate a strain we named 5c-1: *S. albus* J1074 Δ*antC::natB* + PermE* *natCDEFG*. In order to introduce the H1189N

mutation a third pCRISPomyces -2 plasmid, pDS,90 was created by adapting pJL129 to insert *natCDEFG* with the mutant *natC*. To construct pDS90 pJL129 was digested with EcoR321 and BseJI 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_BseJ1_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 DNA sequencing and subsequently used as above to generate *S. albus* J1074 Δ*antC::natB* + PermE* *natCDEFG* + $natC^{MT}$H1189N, the methylation deficient neoantimycin producer, named 7a-3.

Analysis of *S. coelicolor* strains. Engineered cosmids pRFSUL2 and pRFSUL3 were mobilized to *S. coelicolor* M1146 by cross-genera conjugation from *E. coli* ET12567/pUZ8002 as previously described.[28] M1146 strains were cultured in 10 ml LB while shaking (200 rpm) at 30 ºC for 3 days at which point the entire culture was added to mannitol-soya flour broth (50 ml in a 250-ml flask) and incubated at 30 ºC while shaking (200 rpm). After eight 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.[33]

Analysis of *S. albus* strains. *S. albus* strains were first grown in a 2 mL tryptic soy broth seed culture and inoculated at 1% inoculum into a 25 mL mannitol-soya flour broth. Cultures were grown for five days at 30°C, 150 rpm. Mycelia was removed by centrifugation and the supernatant was extracted with two volumes of ethyl acetate and dried with $MgSO_4$ 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 x 100 mm) and in each case a linear gradient of 25-95% CH_3CN with 0.1% formic acid (vol/vol) over 20 min in H_2O with 0.1% formic acid (vol/vol) 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-B, 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, 250 rpm until an OD_{600} of 0.5. Cultures were then put on ice for 10 minutes before induction with 120 mM IPTG. Induction of gene expression lasted for 16 hours at 16°C, 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 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 hour, 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 hour at 4°C. The protein resin 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 final concentration of 8%. Proteins were stored at -80°C or used immediately for *in vitro* assays.

Isotope-labeled precursor feeding experiments. *S. albus* J1074 4c-1 was cultured for the production of neoantimycins as described above, and 24 h after inoculation of the seed culture into 25 mL of MS with apramycin (100 μg/mL) and kanamycin (100 μg/mL), either unlabeled L-methionine or [methyl-d3] L-methionine was added to cultures to a final concentration of 1 g/L. Compound extraction and LC-HRMS analysis was performed as described above.

^{14}C Gel Autoradiography. Assays were performed in 10 μL of 50 mM HEPES (pH 8.0) containing 1 mM TCEP, 4 mM ATP, 4 mM $MgCl_2$, 1 mM CoA, 0.13 mM [2-^{14}C]malonic acid (0.1 mCi/mL; American Radiolabeled Chemicals), 25 μM MatB (malonyl-CoA synthetase), and 90 μM NatC. Reactions were incubated for 2 h at room temperature 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 phosphorimager (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 mM $MgCl_2$) containing 1 mM TCEP, 5 mM ATP, 1 mM tetrasodium pyrophosphate (Na_4PPi), 5 mM substrate and 5 μM enzyme. Before the addition of enzyme $Na_4[^{32}P]$-PPi was added to a final intensity of ~2.5 x10^6 cpm/mL. Reactions were allowed to proceed for two hours at 25°C and then quenched by the addition of 500 μL of charcoal (3.6% w/v activated charcoal, 150 mM Na_4PPi, 5% $HClO_4$). Samples were centrifuged and supernatant was discarded. To remove residual free [^{32}P]PPi the pellet was washed twice with wash solution (0.1 M Na_4PPi, 5% $HClO_4$). The pellet was resuspended in 500 μL water and added to scintillation fluid at a final volume of 5 mL. Radioactivity was measured using a Beckman LS 6500 scintillation counter.

Supporting Information

Oligonucleotides, DNA constructs, and bacterial strains used, annotation of the revised neoantimycin gene cluster, SDS-PAGE analysis of purified proteins, compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Biosynthesis of the 15-membered ring depsipeptide neoantimycin

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Supporting information

Supplementary Tables

Supplementary Figures

Table S1. Oligonucleotides used in this study.

Primer	Sequence (5' -> 3')	Description
supercos1-seq- F2	GCCACCTGACGTCTAAGAAA	initial sequencing primers for primer walking
supercos1-seq-R	GAA TGAACAA TGGAAGTCAA	
antC-up-R	cgtcggtccttcctctgctg ccgacgatcgtcctcgttgc	cloning of pCRISPomyces2-dantCgRNA-2kb
antC-down-F	gcaacgaggacgatcgtcgg cagcagaggaaggaccgacg	
antC-up-F	tcggttgccgccgggcgtttttttatctaga caggtcgttcatcgcctgct	cloning of pCRISPomyces2-dantCgRNA-2kb-(*natB)*
antC-down-R	gcggccttttttacggttcctggcctctaga agcgggaggacggtgtcgac	
antC-natB-up-R	agtgcgccccggatttccat gacaccaaccctcggttgcg	cloning of pCRISPomyces2-dantCgRNA-2kb-*natB*
antC-natB-F	cgcaaccgagggttggtgtc atggaaatccggggcgcact	
antC-natB-R	cgtcggtccttcctctgctg TCAGCCATGGTGAGAGGTGT	
antC-natB- down-F	ACACCTCTCACCATGGCTGA cagcagaggaaggaccgacg	
natB-R2	cctcgacctcctccaccacg	cloning of pCRISPomyces2-dantCgRNA-2kb-*natB* and expression of NatB-Mod 1&2
natB-F3	cagccatgagatagaggccg	
natB-R4	gtacggggcagggtggagac	
natB-F5	caggccgtactggtctccac	
dantC-gRNA construct	gagacatctttgaagacaaacgcCCCTCCTGTGCCCCGGAAAGgtttt agagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcac cgagtcggtgctttttttagcataaccccttggggcctctaaacgggtcttgaggggttttttg gctgctccttcggtcggacgtgcgtctacgggcaccttaccgcagccgtcggctgtgcg acacggacggatcgggcgaactggccgatgctgggagaagcgcgctgctgtacggcg cgcaccgggtgcggagcccctcggcgagcggtgtgaaacttctgtgaatggcctgttcg gttgctttttttatacggctgccagataaggcttgcagcatctgggcggctaccgctatgat cggggcgttcctgcaattcttagtgcgagtatctgaaaggggatacgcTCGGTCCTT TC CTCTGCTGCGgtttaa**gtcttc**tttcacgtggc	gRNA sequence for pJL134 Δ*antC::natB* insertion. Restriction endonuclease sites are bolded and spacer sequences are underlined.
antEF-gRNA-F2	ACGCaccggagaagagacgagggc	Cloning of gRNA for pJL129 insertion of *natCDEFG*
antEF-gRNA-R2	AAACgccctcgtctcttctccggt	
dantC-F2	gttcgtggcgcgagtccacc	confirmation of the *S. albus* J1074 Δ*antC* and Δ*antC::natB* mutants
dantC-R2	ctgaccggccgtacggactc	
dantC-B-R2	cagaactcccggtcgcgctc	
pIB-natF-F	GTTGGTAGGATCCA CATATG AGAAGGGAG CGGACATAC atgaaactcctgatcatcgg	Cloning of *natF/G/FG* into pIB139
natF-int-F	cctgaccagacaggccgaactg	
natF-int-R	cttccaggacgctcagttcg	
pIB-natG-R	gattacgaattcgatatcgc tcagggcagccgggcgccgg	
pIB-natF-R	gattacgaattcgatatcgc tcactgcggctgctttccct	

pIB-natG-F	GTTGGTAGGATCCA CATATG AGAAGGGAG CGGACATAC gtgagcaccaccgacctgat	
pSET152L-apr- cos-F	CTCCCCAGCAGGCAGAAGTATGCAAAGCA TGCAT agatccttttggttcatgtg	Cloning of pIB139-*natF/G/FG* into cos813
pIB-cos-R	GGCGGGACTATGGTTGCTGACTAATTGAGA TGCAT gattacgaattcgatatcgc	
natE-Duet-F	tatAGATCTcgtgctggaccggcacccg	Confirmation of uptake of heterologous expression vectors
natB-F1b	atcaccatcatcaccacagccaggatcc gaattc t atgtctgttcacgaggccgc	Expression of NatB-BC
natB-R3	cgtccagcagcacacaggtg	
natB-F4	acatggtgcccagcacctgt	
natB-R5b	tttctgttcgacttaagcattat gcggccgc aagctta ccggacggcgggcaccagcg	
natBX-pET24-F	gtttaactttaagaaggagatataCATATG CCGGCGCCGGGCACCCAGGA	Expression of Natb-X
natBX-pET24-F3	gtttaactttaagaaggagatataCATATG CTGTCGCTGGCCCAGTCCCG	
natB_modB_F_BglII	AAAagatctA atggaaatccggggcgcact	Expression of NatB-B
natB_modB_R_T_HindIII	TTTaagctt tcacagccgggcggcgatcccgg	
natB_modC_F_start_BglII	AAAagatctA cccgccgacgggcacaccgc	Expression of NatB-C
natB_modC_R_end_HindIII	TTTaagctt tcaccggacggcgggcaccagcg	
natC-pET30-F	GGT ATT GAG GGT CGC atggctgagcccaccgccca	Expression of NatC
natC-pET30-R	AGA GGA GAG TTA GAG CC tcagccgcgccgggcgctgc	
natDE-pET24-F	gtttaactttaagaaggagatataCATATG CCAACCCCCGTAGGCCG	Expression of NatD
natDE-pET24-R	atctcagtggtggtggtggtggtgCTCGAG cgcgggggttccttccaggg	
Duet-Mbth-F	AAA CATAtgacatccaccagtcccttc	Coexpression of NatE with NatD
Duet-Mbth-R	TAT AGATCTcatgccacggcctccggg	
NatC_KS_seq_F	AGCGTCTGCGTCCAGACCAC	Confirmation of pCRISPomyces genome editing
NatC_MT_seq_F	TCATGCGCTGATCGTCGGCC	
NatC_EcoRV_seq_R	GCCGATGTCGCTGGCCGCCA	
pJL129_EcoRV_F	GACGGGAAGACCGATATCACCCGGGCCGCC	Cloning and verification of pCRISPomyces-NatC-MTH1189N
pJL129_BseJI_R	CGCGGGAGAGGATGCCCATCGACCAGCCGT	
H1189N_F2	GTCGCCTACAACGTGCTGAACGCCACCCCGGACCTGC GCC	

H1189N_R2	GGCGCAGGTCCGGGGTGGCGTTCAGCACGTTGTAGGCGAC	
MT_pJL129_gib_F	CTGGCGGGCACCGAGGTCCT	
MT_pJL129_gib_R	GCACCGGGCGGCGTAGGCGA	
MT_H1189N_Crispr_check	agcaggaacagcgcaccgcc	
DT132	CGGCTATGGGAAAGGCGTGCGGGTCCTTCGACGTACTCACATGGGGCCTCCTGTTCTAGA	PCR: *natAB* promoter engineering
DT133	CCTCCAGCGCCTCGCCCCCGGCCGGGTGCCGGTCCAGCACTACGTCTCCGTCGTCTACTC	PCR: *natFG* promoter engineering
DT134	CGGACACATAGTCCCGGGTGCCGGGCAGTTGCAGGATCACATGGGGCCTCCTGTTCTAGA	PCR: *natFG* promoter engineering
DT135	CGCCCGACAGCGGGCGGCCGGCGGCCTCGTGAACAGACATTACGTCTCCGTCGTCTACTC	PCR: *natAB* promoter engineering
RFS406	TGTAGGCTGGAGCTGCTTC	PCR: *aprR* and *hygR* cassette from pIJ773 and pIJ10700
RFS407	ATTCCGGGGATCCGTCGAC	PCR: *aprR* and *hygR* cassette from pIJ773 and pIJ10700
RFS546	CTGGGTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGCCTCGCCGTCGCAGAAC	PCR: ΦBT1 integrase, *attP* and *hygR* from pMS82
RFS547	ACCACAGAAGTAAGGTTCCTTCACAAAGATCCGGACCAAACTACAGCGCCGCAAGCTCCC	PCR: ΦBT1 integrase, *attP* and *hygR* from pMS82
RFS468	TGCACATGGCTGAGGTAG	PCR: *natBC* cosmid screening
RFS469	TGGAGACCGATGGCCTGG	PCR: *natBC* cosmid screening
RFS519	GCACCACCGACCTGATC	PCR: *natG* cosmid screening
RFS520	ATCGCCAGTACCGCCTC	PCR: *natG* cosmid screening
RFS663	ACTGGCCGTCGTTTTACAAC	PCR: linearization of pUC19
RFS664	GAATTCGAGCTCGGTACCCG	PCR: linearization of pUC19
RFS665	GTTGTAAAACGACGGCCAGTCATTACGTCTCCGTCGTCTA	PCR: *rpsL*(XC) promoter from pCRISPomyces-2
RFS666	GAAGCAGCTCCAGCCTACAGCCCTGCAGGCGGAAGTCAG	PCR: *rpsL*(XC) promoter from pCRISPomyces-2
RFS667	GGTCGACGGATCCCCGGAATAGCCCGACCCGAGCACGCGC	PCR: *ermE** promoter from pSET152*ermE*p
RFS668	CGGGTACCGAGCTCGAATTCCATATGGGGCCTCCTGTTCT	PCR: *ermE** promoter from pSET152*ermE*p

Table S2. Cosmids and plasmids used in this study.

Cosmid	Description [a]	Reference
Supercos1	Cosmid backbone for *S. orinoci* cosmid library; $Carb^R$, Kan^R	Stratagene
Cosmid 69	Supercos1 derivative harboring a partial *natB* gene and *natCDEFGHIJKLMNOPQR* genes; $Carb^R$, Kan^R	This study
Cosmid 69-ΦBT1	Cosmid 69 derivative engineered to integrate into the ΦBT1 *attB* site; $Carb^R$, Hyg^R	This study
Cosmid 813	Supercos1 derivative containing harboring *natABCDEF*; $Carb^R$, Kan^R	This study
Cosmid 813-ΦC31	Cosmid 813 derivative engineered to integrate into the ΦC31 *attB* site; $Carb^R$, Apr^R	This study
Cosmid 813-ΦC31-mod	Cosmid 813-ΦC31derivative with *natA* and *natB* expression controlled by *rpsL*(XC) and *ermE** promoters, respectively; $Carb^R$, Apr^R, Hyg^R	This study
pRFSUL2	Cosmid 69-ΦBT1 derivative with *natEF* expression controlled by *rpsL*(XC); $Carb^R$, Apr^R, Hyg^R	This study
pRFSUL3	Cosmid 813-ΦC31-mod derivative with the *hygR* gene removed by the Flp recombinase; $Carb^R$, Apr^R	This study
cos813-pIB139-natFG	SuperCos1/pIB139 for Heterologous expression of neoantimycins in *S. albus* J1074	This study
cos813-pIB139-natF	SuperCos1/pIB139 for Heterologous expression of neoantimycins in *S. albus* J1074	This study
cos813-pIB139-natG	SuperCos1/pIB139 for Heterologous expression of neoantimycins in *S. albus* J1074	This study
Plasmid	**Description [a]**	**Reference**
pCRISPomyces-2	pGM1190 derivative harboring the CRISPR/Cas9 machinery; Apr^R	6
pJL134: pCRISPomyces2-dantCgRNA-2kb-natB	pCRISPomyces-2 derivative for Construction of *S. albus* J1074 ΔantC::natB mutant	This study
pJL129: pCRISPomyces2-PermE*natCDEFG	pCRISPomyces-2 derivative for Insertion of *natCDEFG* into *S. albus* J1074	This study
pDS90: pCRISPomyces2-natCMTH1189N	pCRISPomyces-2 derivative for Construction of MT deficient NatC in *S. albus* J1074 5c-1	This study
pCDFDuet-1	Expression plasmid	Novagen
pCDF-natE	pCDFDuet-1 derivative for Purification of NatD from *E. coli*	This study
pET24b	pET21abcd(+) derivative with Kan^R	Novagen

pET24b-natD	pET24 derivative for Purification of NatD from *E. coli*	This study
pET30a	Expression plasmid	Novagen
pET30-natB-mod1&2	pET30 derivative for Purification of NatB (Mod 1&2) from *E. coli*	This study
pET30-natB-mod3	pET30 derivative for Purification of NatB (Mod 3) from *E. coli*	This study
pET30-natC	pET30 derivative for Purification of NatC from *E. coli*	This study
pSET152*ermE*p	pSET152 derivative containing *ermE**p cloned into the EcoRV-EcoRI sites; Apr^R	7
pIJ773	ReDirect PCR template plasmid harbouring an apramycin resistance cassette; $Carb^R$, Apr^R	8
pIJ10700	ReDirect PCR template plasmid harbouring a hygromycin resistance cassette; $Carb^R$, Hyg^R	9
pIJ10702	Supercos1 derivative harboring the ΦC31 integrase, *attP*, *oriT* and apramycin resistance gene from pSET152; $Carb^R$, Apr^R	10
pMS82	pGEM7 derivative that integrates into the ΦBT1 *attB* site in *Streptomyces*; Hyg^R	11
pRFSUL1	Supercos1 derivative harboring the ΦBT1 integrase, *attP*, *oriT* and hygromycin resistance gene from pMS82; $Carb^R$, Hyg^R	This study
pUC19	General cloning plasmid; $Carb^R$	New England Biolabs
pUC19-PAprP	pUC19 derivative harbouring an apramycin resistance gene flanked by FRT sites and divergently firing *rpsL*(XC) and *ermE** promoters; $Carb^R$, Apr^R	This study
pUC19-PHygP	pUC19 derivative harbouring a hygromycin resistance gene flanked by FRT sites and divergently firing *rpsL*(XC) and *ermE** promoters; $Carb^R$, Hyg^R	This study
pUZ8002	Encodes conjugation machinery for mobilization of plasmids from *E. coli* to *Streptomyces;* Kan^R	4

[a] Carb, carbenicillin; Apr, apramycin; Hyg, hygromycin, Kan, kanamycin; Cam, chloramphenicol; *oriT*, origin of conjugal transfer

Table S3. Bacterial strains used in this study.

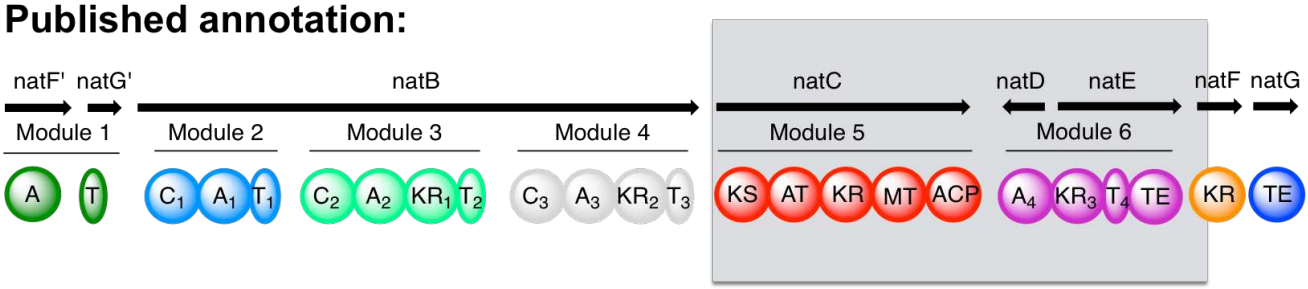
Strain	Description [a]	Reference
Streptomyces		
M1146	*S. coelicolor* M145 harboring mutations in the biosynthetic pathways for: actinorhodin, undecylprodigiosin, calcium-dependent antibiotic and coelimycin	1
M1146/Cosmid813-ΦC31-Cosmid69-ΦBT1	M1146 *attB* ΦC31::Cosmid813-ΦC31 *attB* ΦBT1::Cosmid69-ΦBT1; $Carb^R$, Apr^R, Hyg^R	This study
M1146/pRFSUL2-pRFSUL3	M1146 *attB* ΦC31::Cosmid813-ΦC31-mod *attB* ΦBT1::Cosmid69-ΦBT1-mod; $Carb^R$, Apr^R, Hyg^R	This study
S. albus J1074	Derivative of *S. albus* G lacking the *salI* restriction system	2

S. orinoci NRRL B-3379	Native producer of neoantimycins	3
S. albus J1074 4c-1	*S. albus* strain harboring *natABCDEFG* as an integrated construct; Apr^R, Kan^R	This study
S. albus J1074 4c-2	*S. albus* starin harboring *natABCDEF* as an integrated construct; Apr^R, Kan^R	This study
S. albus J1074 4c-3	*S. albus* starin harboring *natABCDEG* as an integrated construct; Apr^R, Kan^R	This study
S. albus J1074 5c-1	*S. albus* strain generated through CRISPR/Cas9 engineering to replace *antC* with *natB* and insert *natCDEFG* under control of P*ermE**; *S. albus* J1074 Δ*antC::natB* + PermE**natCDEFG*	This study
S. albus J1074 5c-0	*S. albus* strain generated through CRISPR/Cas9 engineering to replace *antC* with *natB S. albus* J1074 Δ*antC::natB*	This study
S. albus J1074 7a-3	CRISPR/Cas9 generated *S. albus* J1074 further engineered to introduce H1189N mutation; *S. albus* J1074 Δ*antC::natB* + P*ermE*natC*$^{MT\ H1189N}$*DEFG*	This study
Escherichia coli		
ET12567	Non-methylating host for transfer of DNA into *Streptomyces* spp. (*dam, dcm, hsdM*); Cam^R	4
XL1-Blue MR	Cosmid library host strain	Agilent Technologies
GB0R-red	Host for RecET recombination	1
XL10-Gold	General cloning host	Agilent Technologies
XL1-Blue	General cloning host	Agilent Technologies
BL21-Gold(DE3)	Overproduction of recombinant proteins	Agilent Technologies
BAP1	Derivative of BL21(DE3) harboring the *sfp* PPTase; used for overproduction of megasynthases NatB-BC, NatB-X, NatC	5

[a] Carb, carbenicillin; Apr, apramycin; Hyg, hygromycin, Kan, kanamycin; Cam, chloramphenicol; *oriT*, origin of conjugal transfer

S. orinoci Neoantimycin Biosynthetic Gene Cluster

Published annotation:



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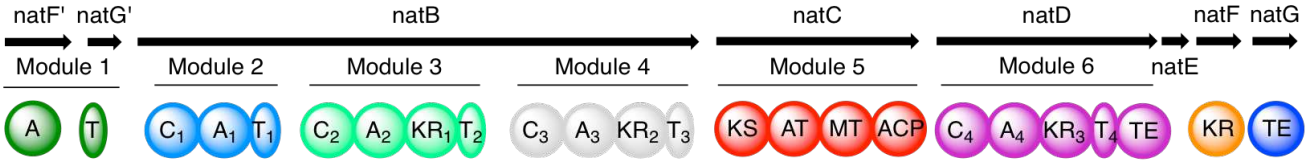


Figure S1. Revised sequence annotation for the neoantimycin biosynthetic gene cluster. The gene encoding the *natC* PKS does not harbor a KR domain and the terminal NRPS *natD* is contained within one contiguous coding sequence with domain organization C4-A4-KR3-T4-TE. Additionally, located between the terminal NRPS and *natF* is a gene encoding an mbtH-like protein which is denoted *natE*.

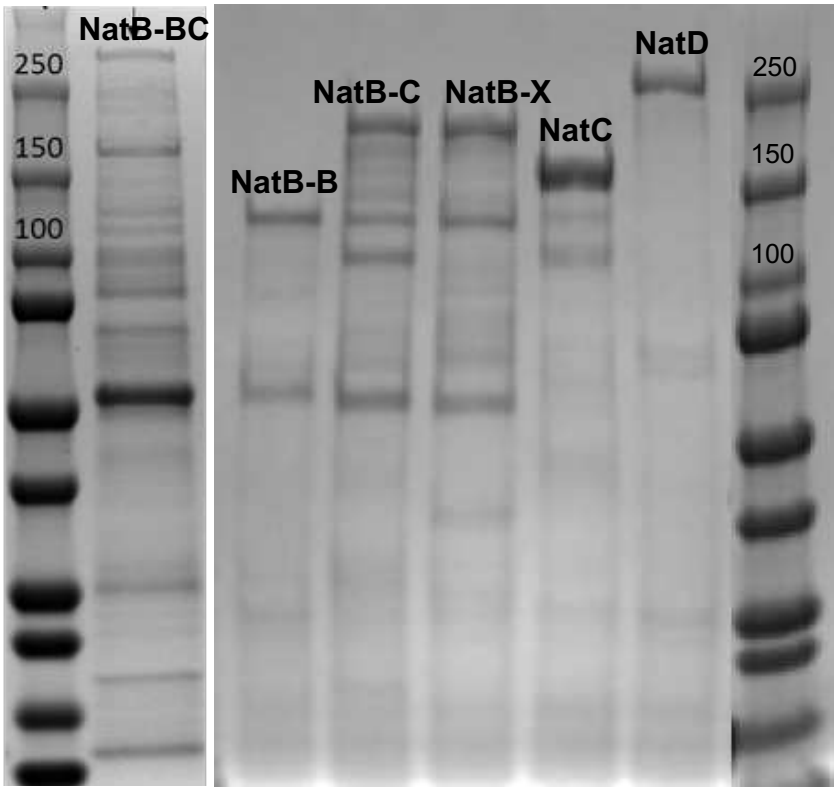


Figure S2. SDS-PAGE analysis of neoantimycin biosynthetic enzymes characterized in this study. NatB was separated into four different constructs; the dimodule NatB-BC (295 kDa); and individual modules NatB-B (120 kDa); NatB-C (185 kDa) and NatB-X (195 kDa) in order to facilitate recombinant expression in *E. coli* BAP1. NatC (156 kDa) and NatD (253 kDa) were expressed as complete constructs in *E. coli* BAP1. Protein purification yields are as follows: NatB-BC 1 mg/L, NatB-B 2 mg/L, NatB-C 2.5 mg/L, NatB-X 2.5 mg/L, NatC 3.5 mg/L, NatD 2.5 mg/L.

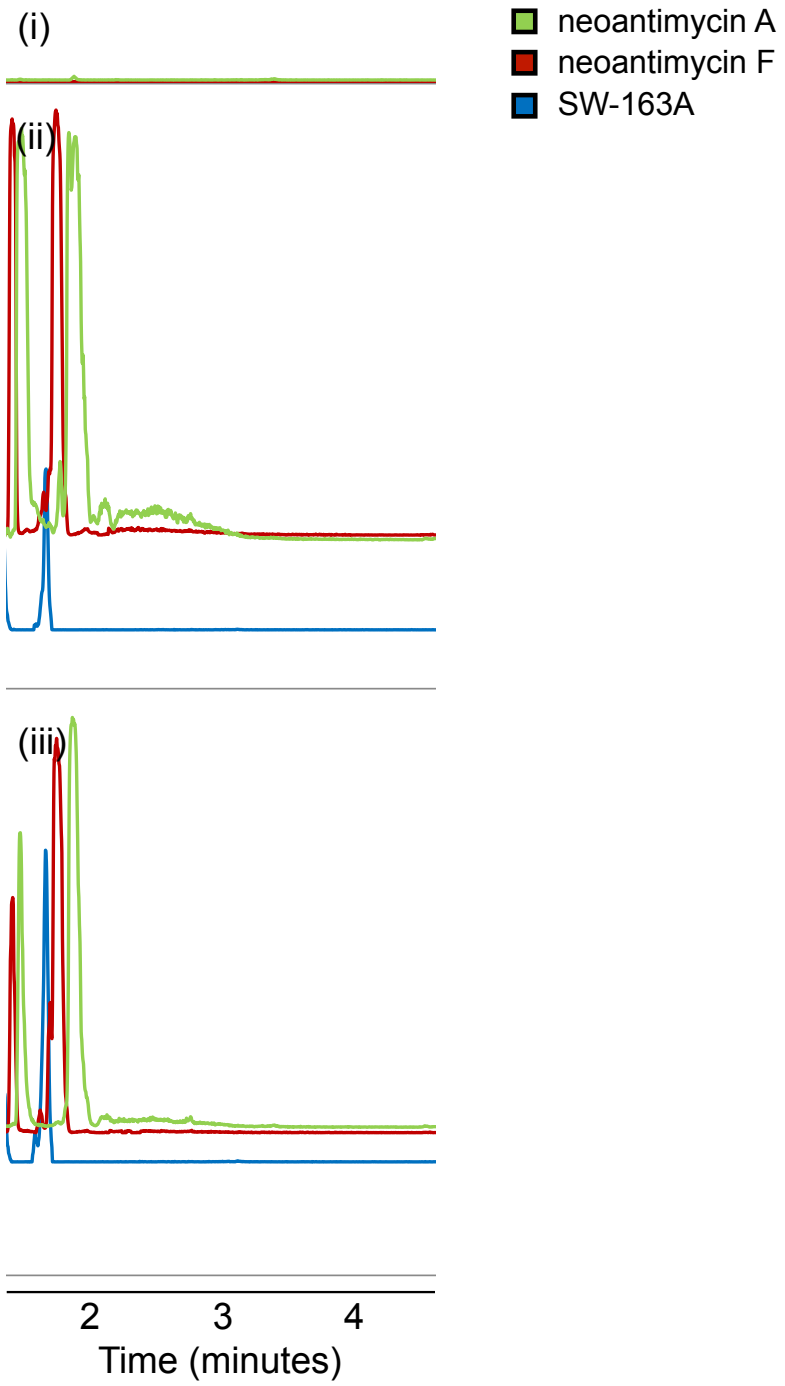
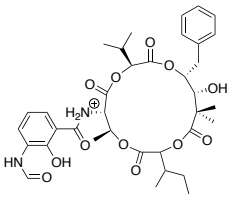
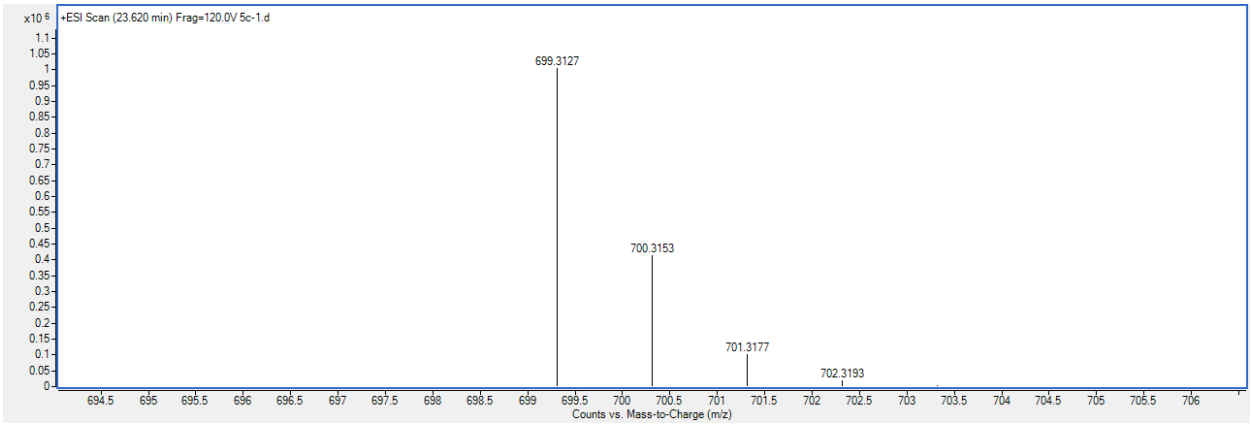


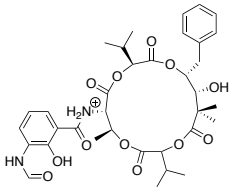
Figure S3. Heterologous production of neoantimycins by *Streptomyces coelicolor* M1146. LC-HRMS analysis of chemical extracts from the following strains: (i) M1146; (ii) M1146/pRFSUL2/pRFSUL3; (iii) *Streptomyces orinoci* NRRL-B 3379. The extracted ion chromatograms [M+H] for neoantimycin A **1**, neoantimycin F, **2,** and SW-163A, **3,** are shown for each strain. **1**, *m/z* 699.3124 $[M+H]^+$; **2**, *m/z* 685.2967 $[M+H]^+$; **3**, *m/z* 671.2811 $[M+H]^+$.

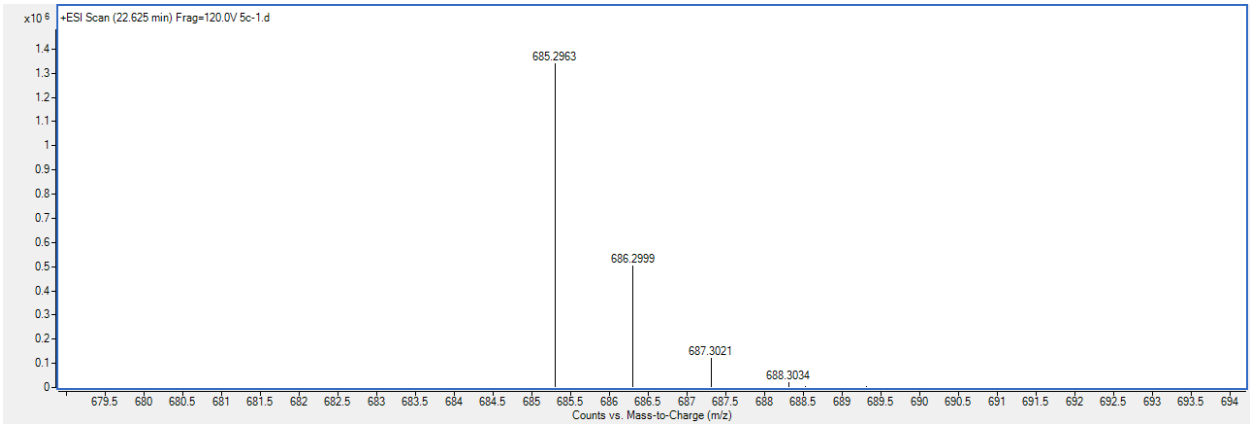
1 neoantimycin A
m/z $[M+H]^+$
obs. 699.3127
calc. 699.3124
error: 0.43 ppm



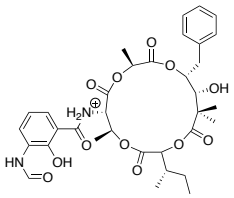


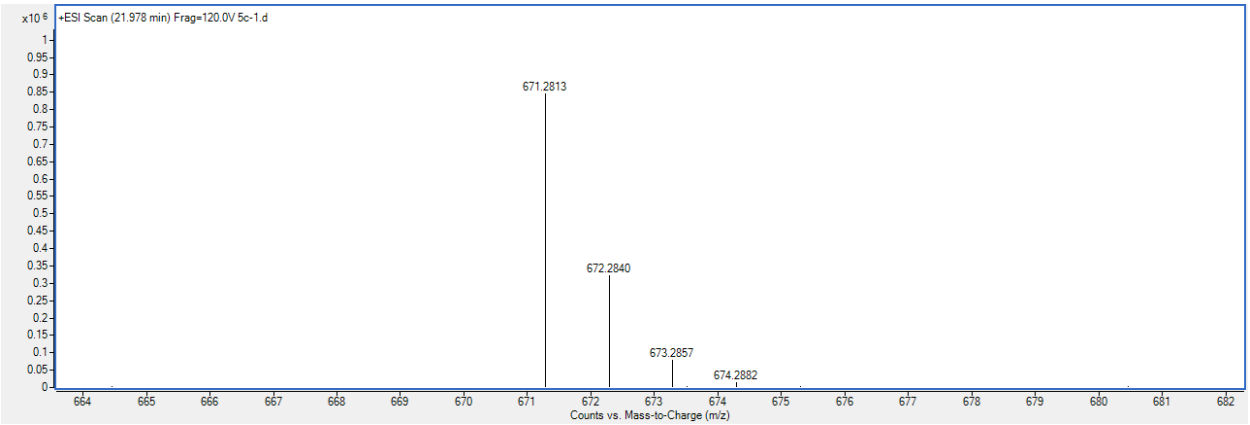
2 neoantimycin F
m/z $[M+H]^+$
obs. 685.2963
calc. 685.2967
error: 0.58 ppm



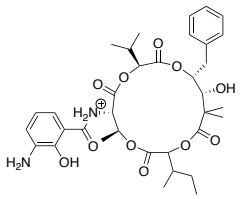


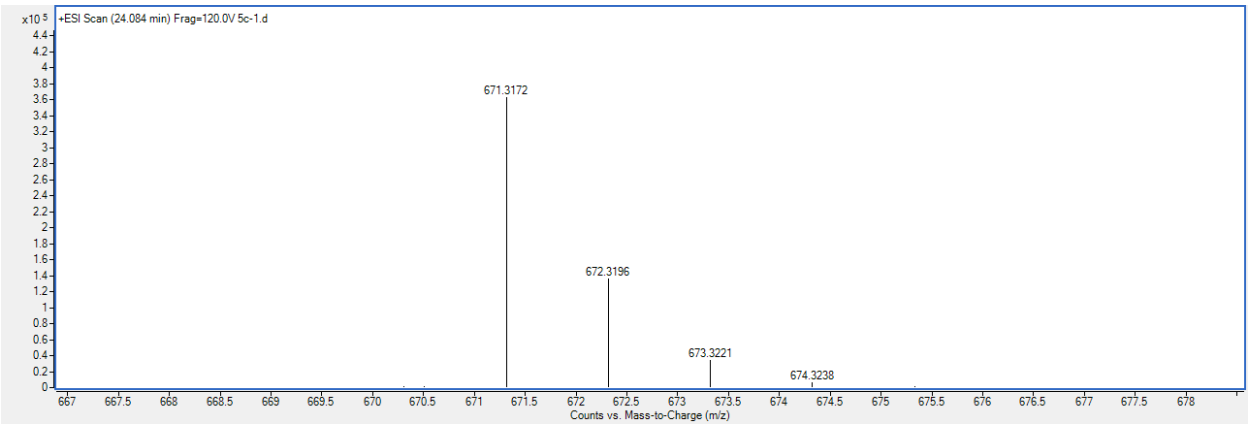
3 SW 1638
m/z $[M+H]^+$
obs. 671.2813
calc. 671.2811
error: 0.30 ppm



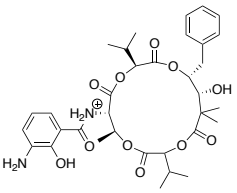


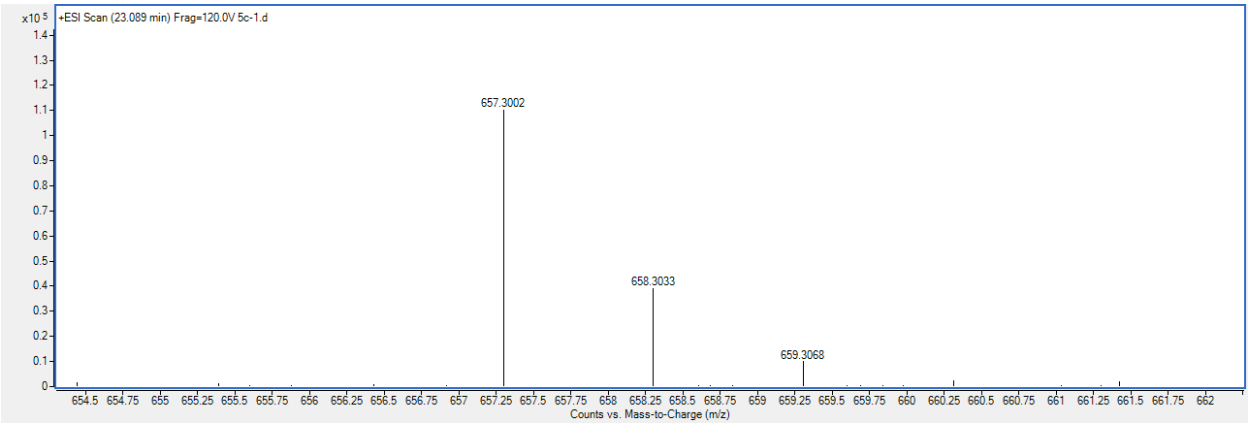
4 neoantimycin D
m/z $[M+H]^+$
obs. 671.3172
calc. 671.3180
error: 1.19 ppm



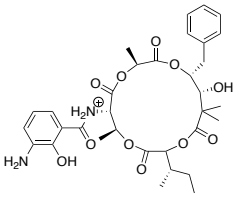


5 neoantimycin E
m/z $[M+H]^+$
obs. 657.3002
calc. 657.3018
error: 2.43 ppm





6 JBIR 05
m/z $[M+H]^+$
obs. 643.2856
calc. 643.2861
error: 0.78 ppm



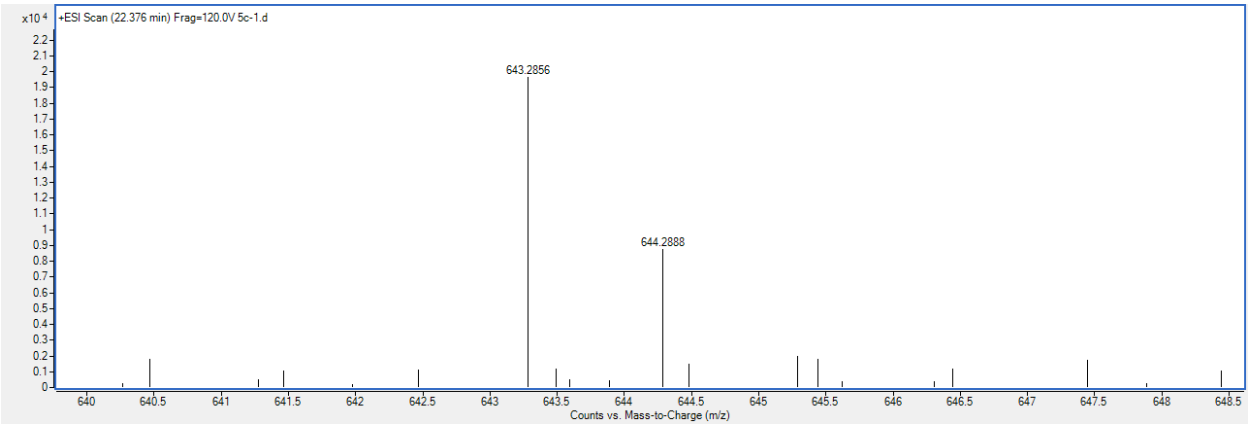


Figure S4. LC-HRMS analysis of neoantimycins **1-6** produced by heterologous expression host 5c-1.

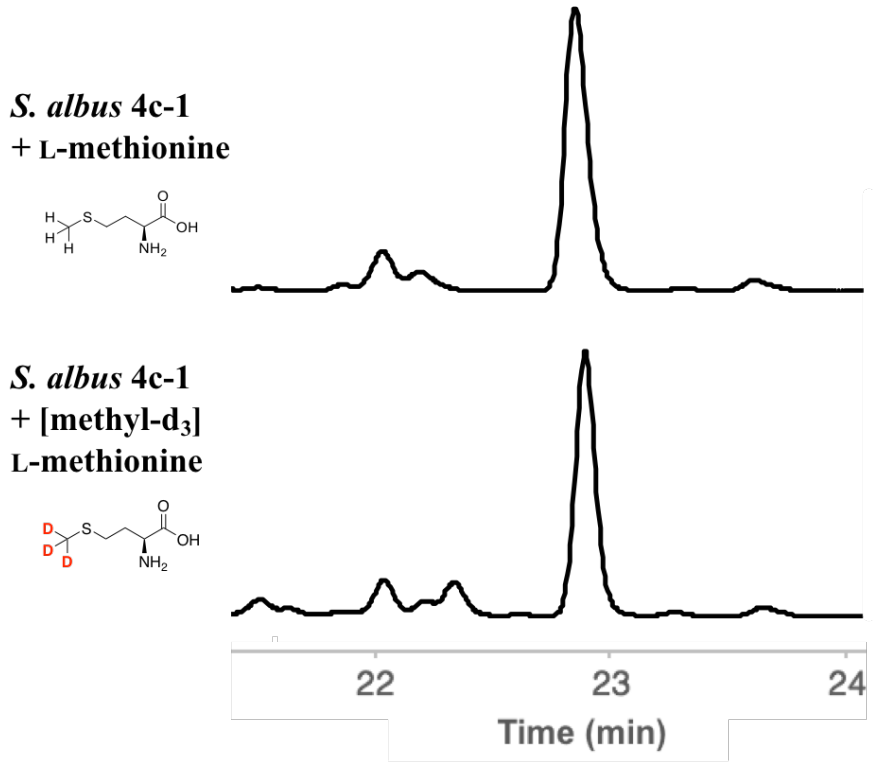
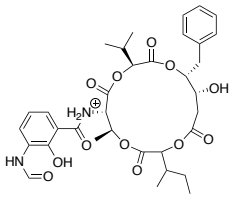
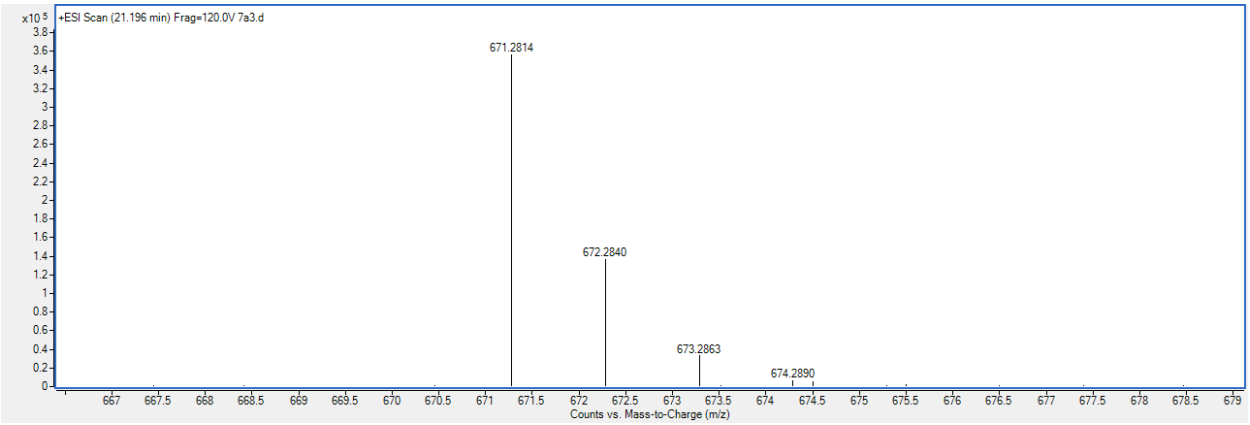


Figure S5. LC-UV analysis (320 nm) of neoantimycins produced by *S. albus* 4c-1 in the presence of L-methionine and [methyl-d_3] L-methionine.

7

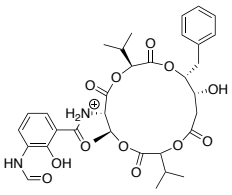
m/z $[M+H]^+$
obs. 671.2814
calc. 671.2811
error: 0.45 ppm

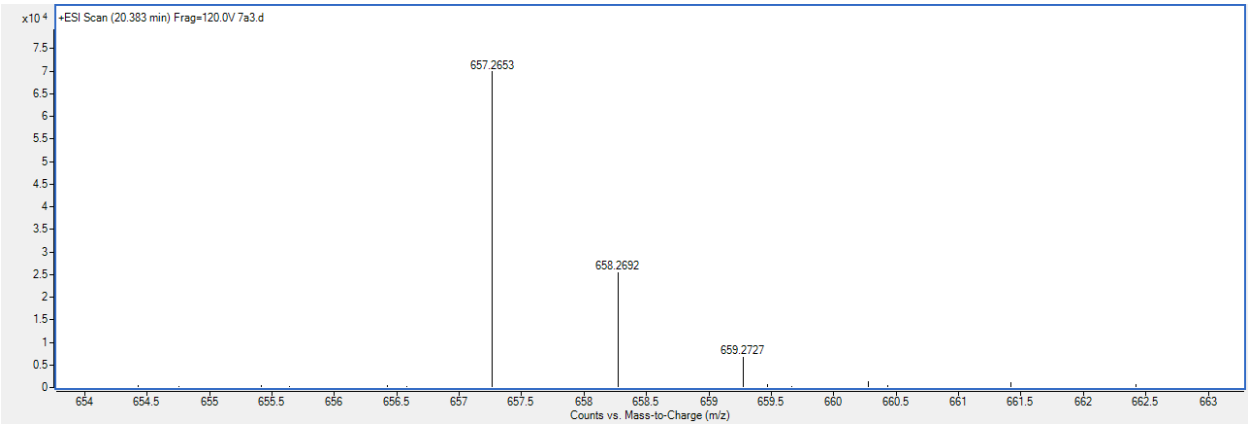




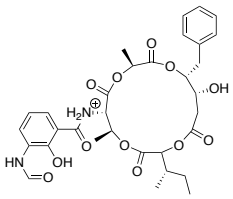
8

m/z $[M+H]^+$
obs. 657.2653
calc. 657.2654
error: 0.15 ppm





9
m/z $[M+H]^+$
obs. 643.2504
calc. 643.2498
error: 0.93ppm



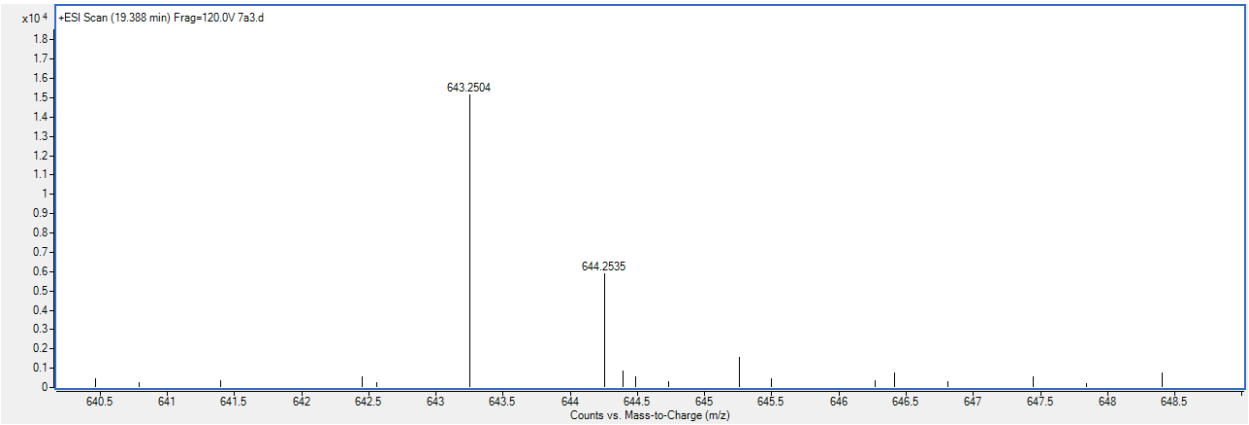


Figure S6. LC-HRMS analysis of **7-9** produced by 7a-3, the $NatC^{MT}$ deficient strain. Proposed structures are indicated on the upper right.

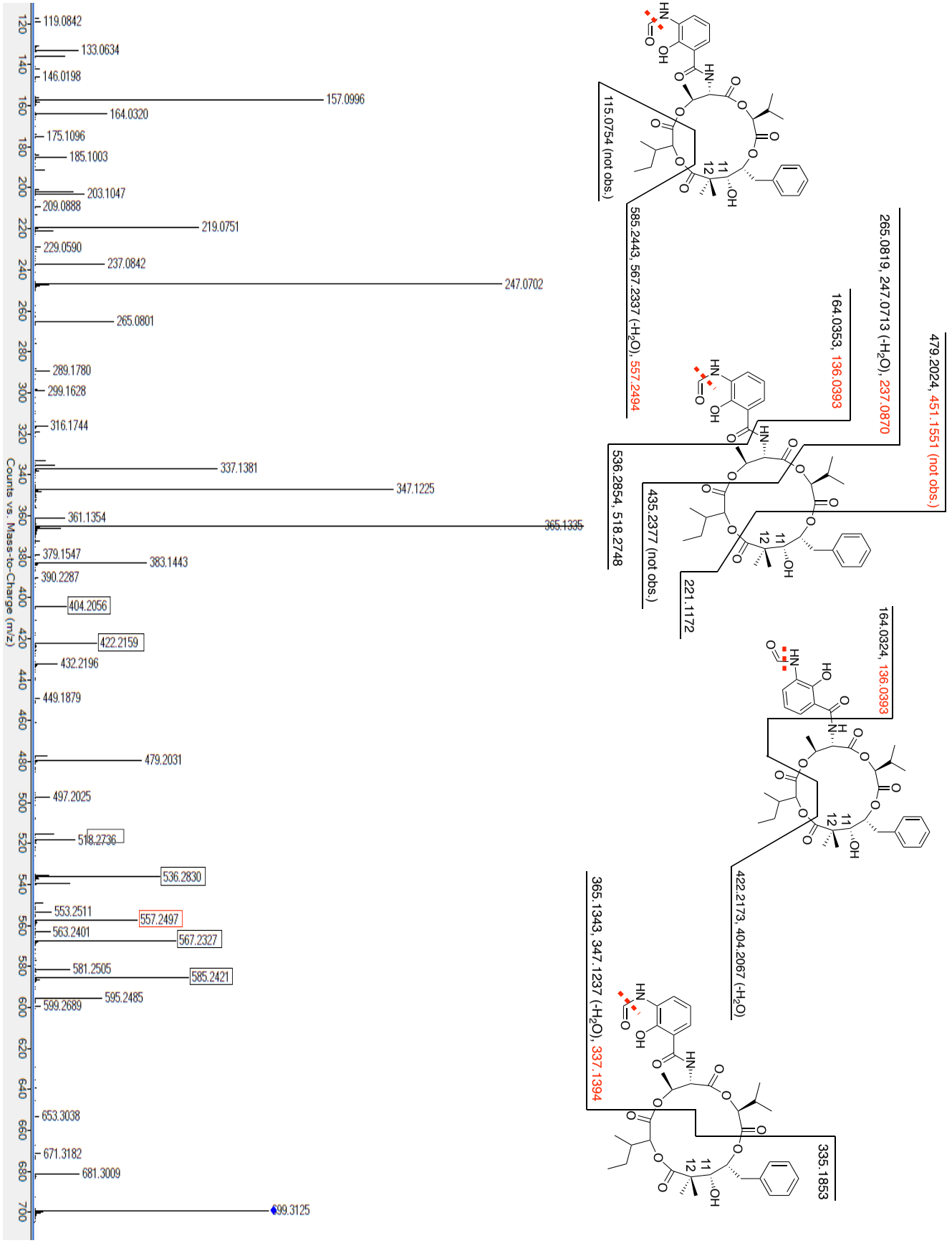


Figure S7. LC-HRMS/MS analysis of **1**. Masses in red indicated deformylated fragment masses.

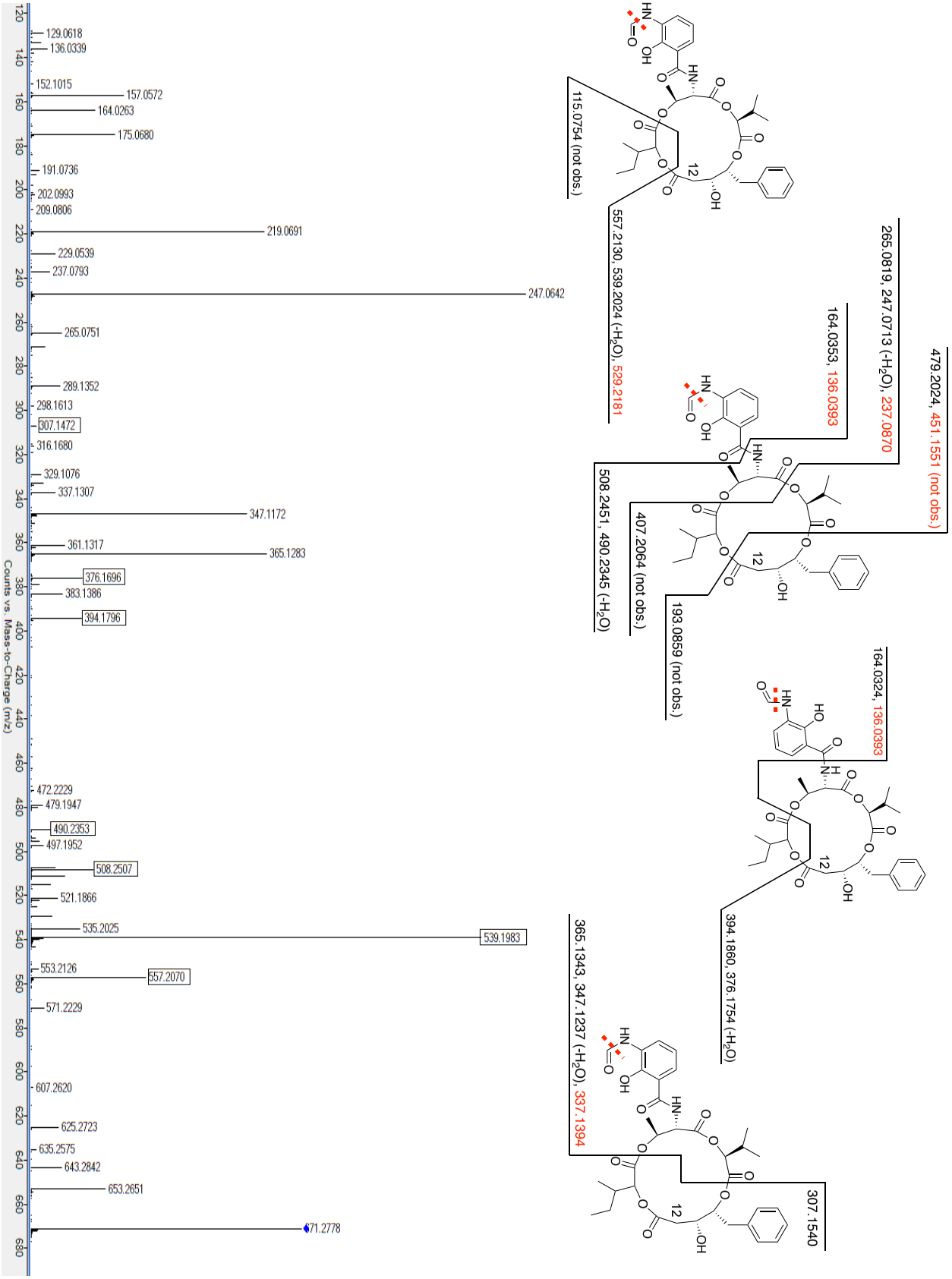


Figure S8. LC-HRMS/MS analysis of **7**. Boxed masses indicate fragments containing C12 desmethyl mass fragments. Masses in red indicate deformylated fragment masses.

***S. albus* 4c-3**
natABCDEG

***S. albus* 4c-1**
natABCDEFG

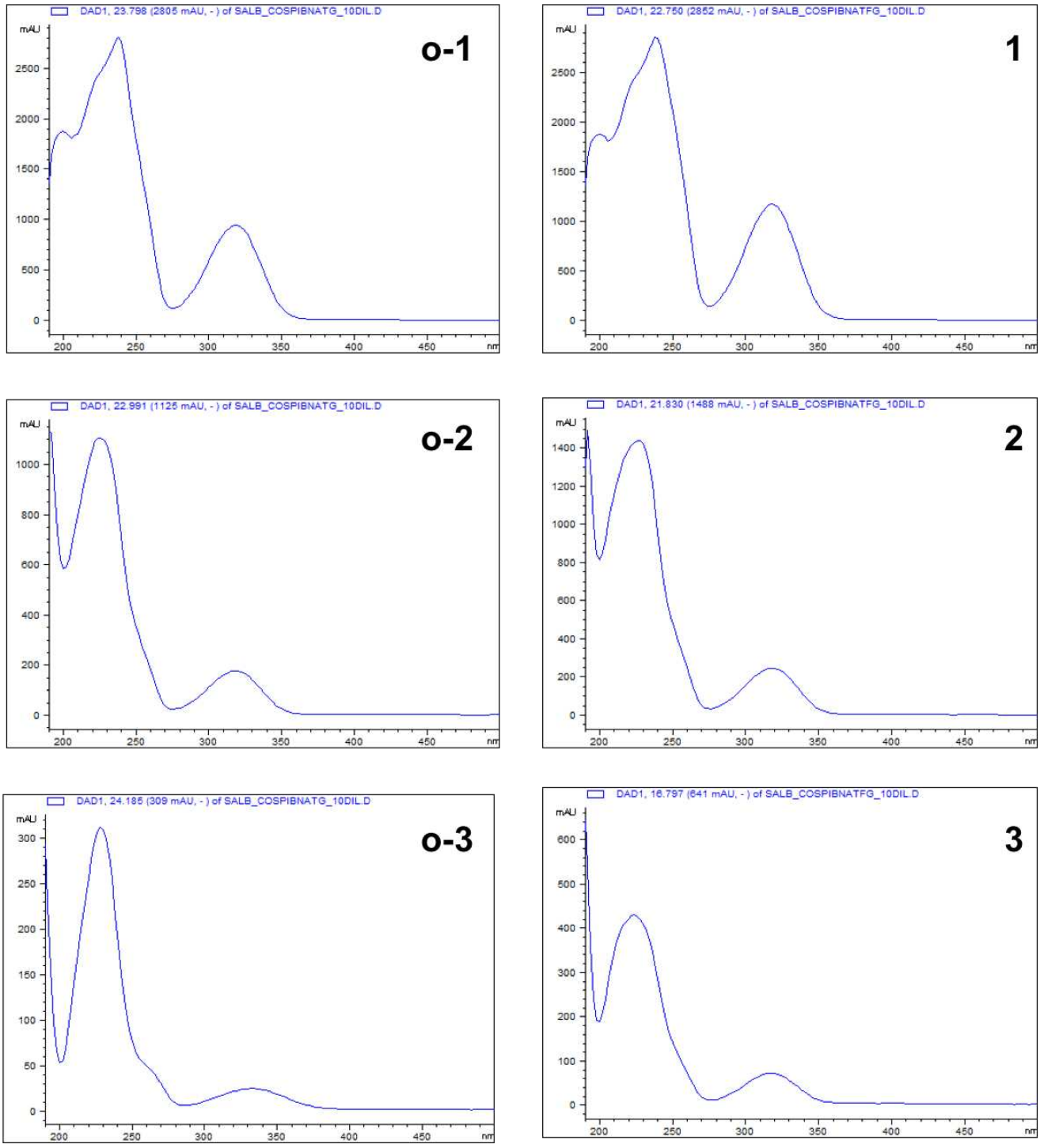
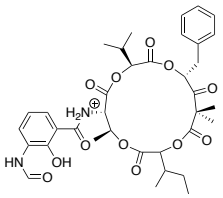
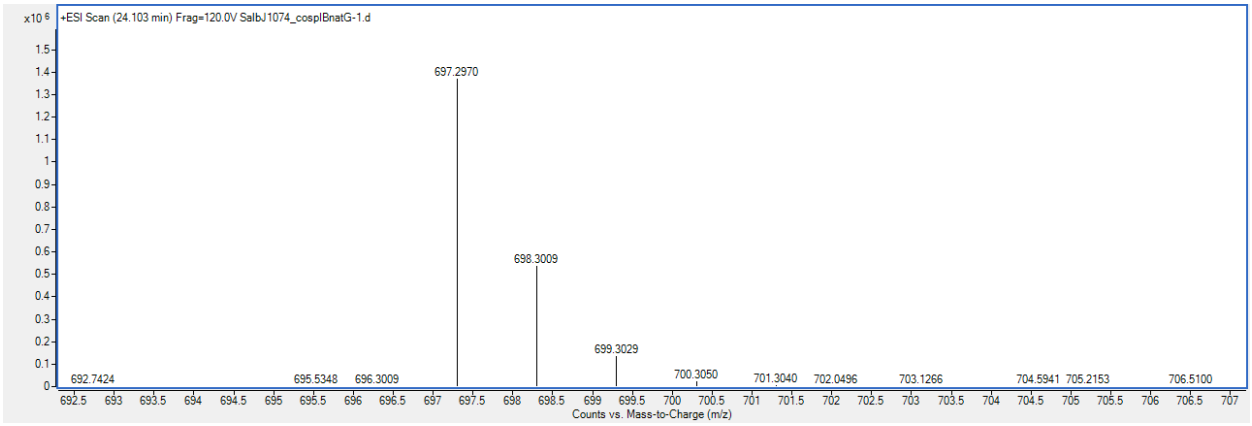


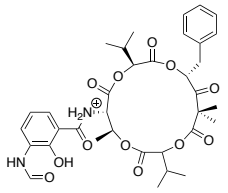
Figure S9. UV spectra of **o-1-3** produced by *natF* deficient strain *S. albus* 4c-3 compared to **1-3** produced by heterologous expression host 4c-1.

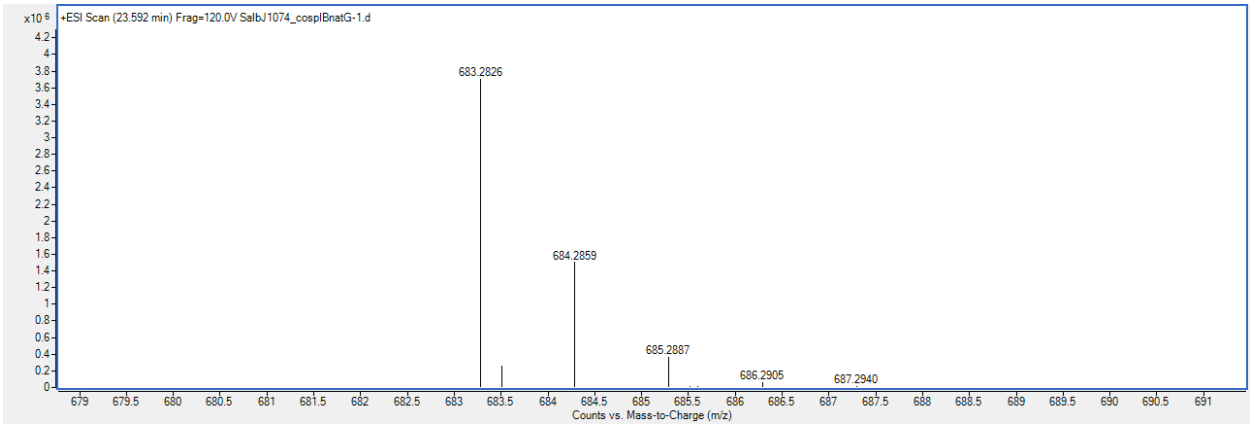
o-1
m/z $[M+H]^+$
obs. 697.2970
calc. 697.2967
error: 0.43 ppm



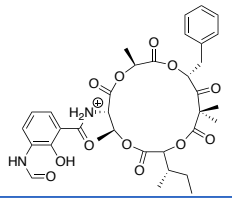


o-2
m/z $[M+H]^+$
obs. 683.2826
calc. 683.2811
error: 2.20 ppm





o-3
m/z $[M+H]^+$
obs. 669.2710
calc. 669.2654
error: 2.54 ppm



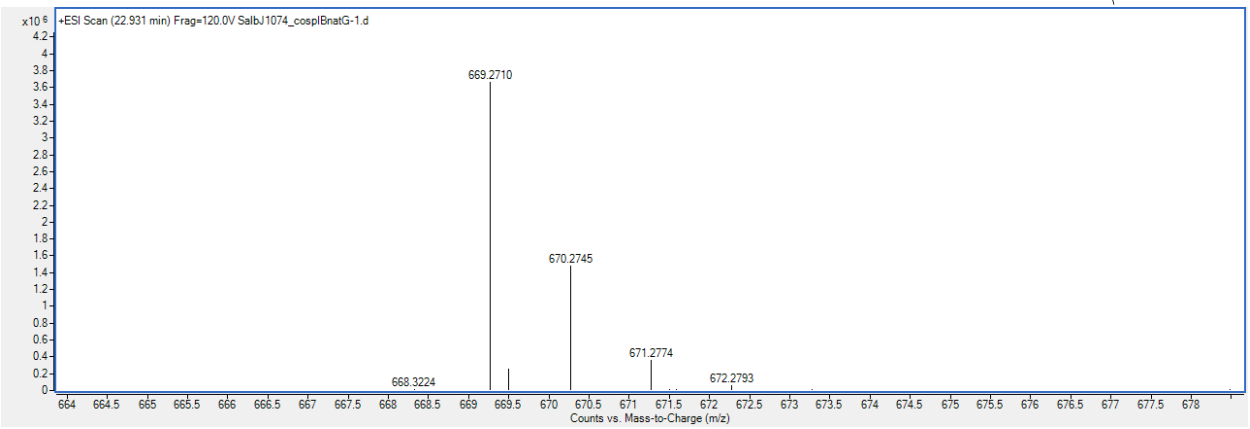


Figure S10. LC-HRMS analysis of **o-1-3** produced by expression host 4c-3 lacking *natF,* the gene encoding the ketoreductase responsible for reduction of the C11 ketone of neoantimycins. Proposed structures are indicated on the upper right.

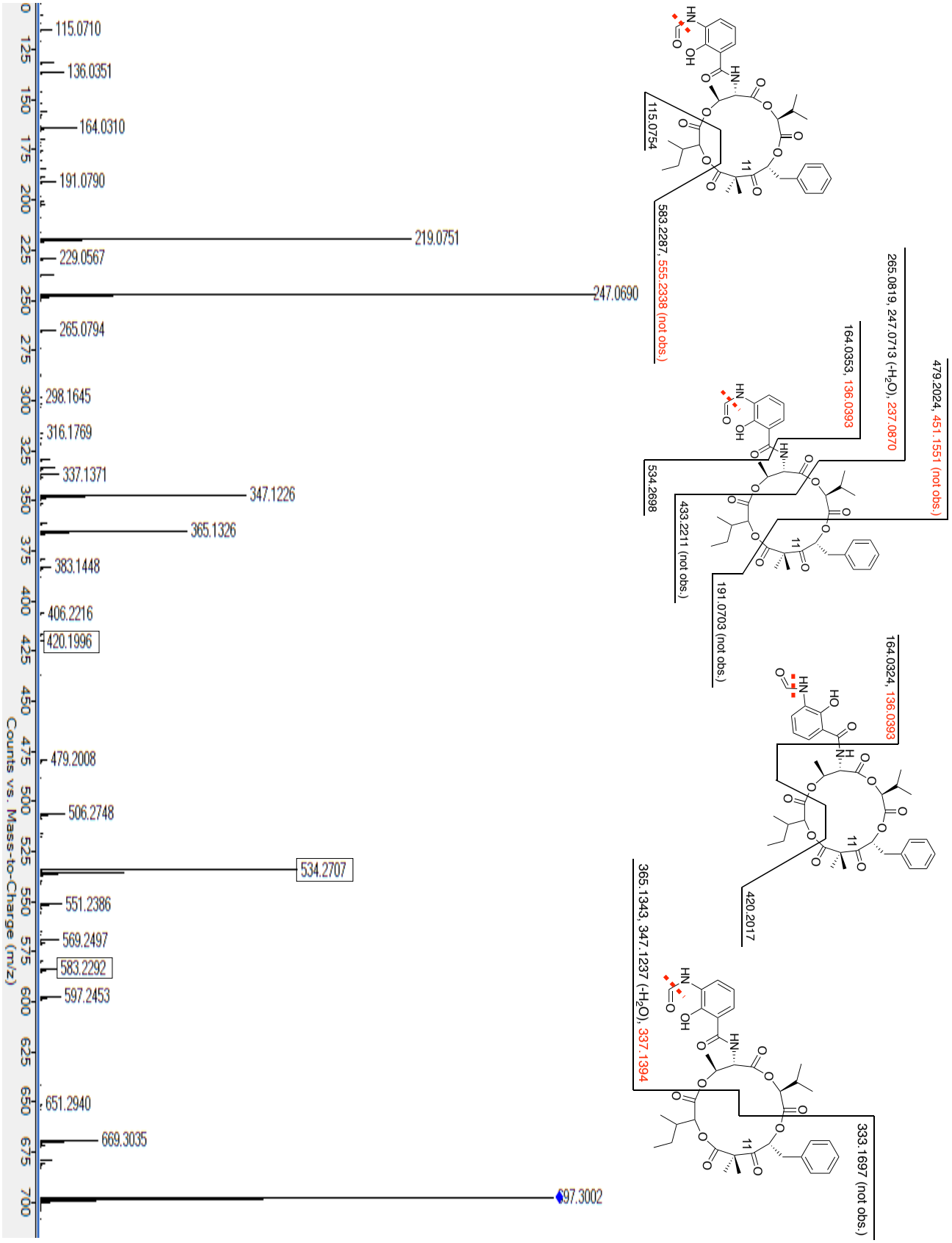


Figure S11. LC-HRMS/MS analysis of **o-1.** Boxed masses indicate fragments containing an unreduced ketone at C11. Masses in red indicate deformylated fragment masses.

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