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Chemical variation from the neoantimycin depsipeptide assembly line

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ABSTRACT

Here we report the biosynthetic pathway for the neoantimycin and present three novel neoantimycin analogues, neoantimycin D (**1**), E (**2**) and F (**3**), from this assembly system from *Streptoverticillium orinoci*. Identification of these novel neoantimycin variants was achieved by selective MS/MS interrogation of natural product extracts using diagnostic fragments of the known neoantimycins. Their structures, including the absolute configurations, were elucidated using a combination of NMR experiments, detailed MS/MS experiments and the advanced Marfey's method. The biosynthetic pathway of neoantimycin was dissected by genome sequencing data analysis for the first time, which includes a hybrid nonribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) assembly lines.

Depsipeptides are noted for their potent and varied activities against cancerous cells.[1,2] Structures of this class are defined by having at least one lactone/ester for amide substitution, and this alteration affords conformational flexibility which seemingly is important for their bioactivities. The antimycin family of depsipeptides is particularly noteworthy for their varied actions and chemical diversity. Antimycin A, the 9-membered dilactone, for instance exerts its apoptotic inducing effects through blocking a protein–protein interaction between Bcl-xL and BH3 proteins.[3] The 15-membered macrocyclic depsipeptides (the neoantimycin subfamily) also induce apoptosis, but through an alternative pathway, the down-regulation of GRP-78. GRP-78 is a molecular chaperone critical to the unfolded protein response, and has emerged as a new therapeutic target for drug-resistant cancer cells and cancer stem cells.[4,5]

Focused interrogation of a natural source is a key strategy in exploring and expanding the chemical space of natural product leads. Such efforts have been useful in revealing variation imparted by depsipeptide natural assembly line machineries in the cases of antimycin, beauvericin and cryptophycin.[6,7] In these instances, structure–activity relationships have been refined and sources of variation revealed. Frequently, the variation seemingly emerges from introduction of different building blocks (particularly the α-hydroxy acids) and this flexibility has been exploited for precursor-directed biosynthesis. As of yet an expanded spectrum of analogues of the GRP-78 down-regulating depsipeptide series has not been realized and the biosynthetic assembly line for neoantimycin has yet been revealed. However, given the commonality in the chemistry of neoantimycin to antimycin, and in particular the aminosalicylate starter unit one would envision a similar cluster, but with neoantimycin assembly having the modules encoding for α-hydroxy-benzenepropanoic acid incorporation.[8–10] Recognizing that depsipeptides often vary due to α-hydroxy acid substitution we devised an MS/MS strategy that would selectively identify neoantimycin variants produced from its assembly line.

To determine the neoantimycin biosynthetic pathway within *Streptoverticillium orinoci*, a shot-gun sequencing of a paired end genomic libraries was performed on a HiSeq2000 Illumina sequencer and from the ~380 Mbp of sequence data 128 large contigs were obtained. These large contigs were found to harbor several large PKS/NRPS biosynthetic clusters, however, comparing these to sequences of the antimycin clusters obtained from *Streptomyces* S4 and *Streptomyces albus* revealed only one candidate neoantimycin cluster. Recently identified antimycin biosynthetic gene cluster from *S. albus* indicates that the dilactone scaffold of antimycins is generated through a hybrid NRPS-PKS assembly line-based mechanism which starts with the activation of anthranilic acid, by an acyl-CoA ligase through adenylation, and the adenylated acid is loaded onto a carrier protein for further processing.[10] The primary amino acid sequences of antimycin biosynthetic enzymes AntHIJKL exhibited a high sequence similarity to PaaABCDE, a multicomponent oxygenase catalyzing the epoxidation of the aromatic ring of phenylacetyl-CoA.[10] Homology searches revealed a region of the *S. orinoci* genome with 71% amino acid sequence identity to the AntH and AntI and subsequent analysis of this contig provided

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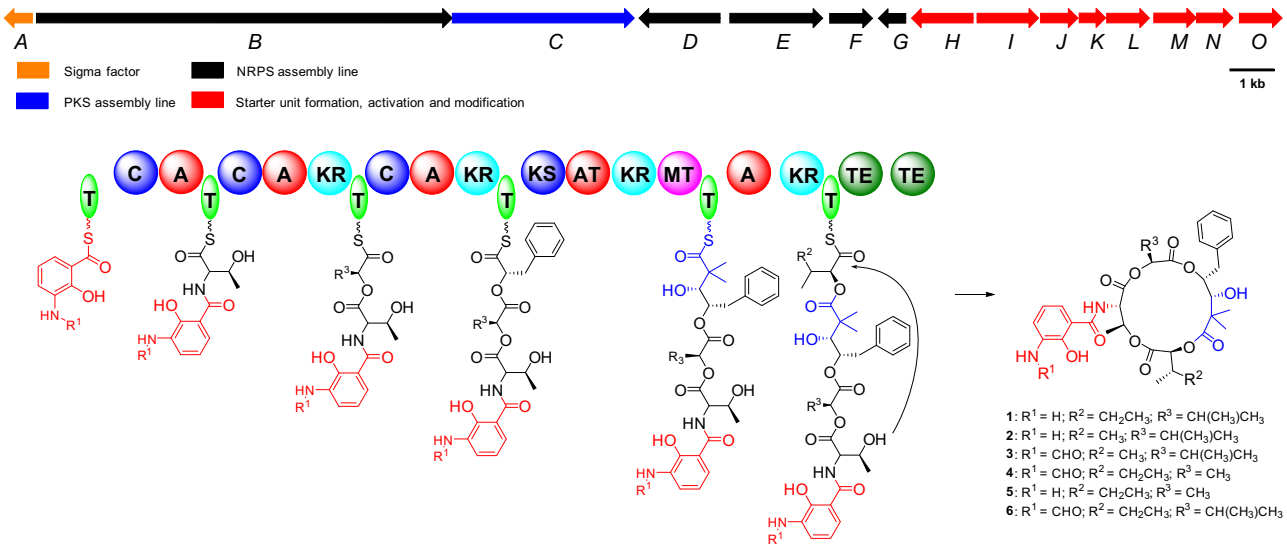

Figure 1. Proposed neoantimycin biosynthetic cluster.

Table 1
ORFs of the *nat* gene cluster, closest homologues and proposed functions

ORF	Size (AA)	Proposed function	Homologous protein, species	Identity/ Similarity	Accession number
natA	217	Sigma factor	RNA polymerase sigma factor, *Streptomyces blastmyceticus*	138/173	BAM21045
natB	4580	Nonribosomal peptide synthetase	Putative peptide synthetase, *Streptomyces blastmyceticus*	1036/1526	BAM21047
natC	1411	Type I polyketide synthase	Type I polyketide synthase, *Streptomyces pyridomyceticus*	734/1422	AEF33079
natD	642	Nonribosomal peptide synthetase	Putative peptide synthetase, *Streptomyces ambofaciens*	320/596	CAJ89363
natE	842	Nonribosomal peptide synthetase	Putative peptide synthetase,*Streptomyces pyridomyceticus*	367/837	AEF33080
natF	342	Nonribosomal peptide synthetase	Putative peptide synthetase, *Streptosporangium roseum*	163/308	YP_003342246
natG	161	Thioesterase	Thioesterase, *Streptomyces cattleya*	81/160	YP_006050931
natH	495	Putative long-chain-fatty-acid CoA ligase	CoA ligase, *Streptomyces blastmyceticus*	381/475	BAM21051
natI	472	Phenylacetate-CoA oxygenase	Phenylacetate-CoA oxygenase, *Streptomyces albus*	264/315	ZP_06589159
natJ	255	Phenylacetate-CoA oxygenase	Phenylacetate-CoA oxygenase, *Streptomyces albus*	184/254	ZP_06589161
natK	140	Phenylacetate-CoA oxygenase	Phenylacetate-CoA oxygenase, *Streptomyces blastmyceticus*	116/140	BAM21056
natL	366	Phenylacetate-CoA oxidoreductase	Phenylacetate-CoA oxidoreductase, *Streptomyces blastmyceticus*	205/256	BAM21057
natM	321	Tryptophan 2,3-dioxygenase	Tryptophan 2,3-dioxygenase, *Streptomyces blastmyceticus*	209/264	BAM21059
natN	274	Lipase/esterase	Lipase-esterase, *Streptomyces hygroscopicus*	198/269	YP_006242402
natO	416	Kynureninase	Kynureninase, *Streptomyces blastmyceticus*	316/397	BAM21061

the complete pathway for the neoantimycins (Fig. 1). In Table 1 the proposed functions of encoded proteins from the neoanytimycin (nat) hybrid cluster are presented. Notably, a series of genes, *nat*IJKLMNO, that are analogous to the *ant*HIJKLMNO genes found in the antimycin biosynthetic cluster, encode for the enzymes that catalyze the creation of the common to both 3-aminosalicyloyl-tethered starter unit. Upon loading of the starter unit, natB, a tri-module NRPS with the following domains C_1-A_1-T_1-C_2-A_2-KR_1-T_2-C_3-A_3-KR_2-T_3 (C, condensation; A, adenylation; T, thiolation; KR, ketoreduction), catalyzes the extension of the molecule. The A_1 domain is predicted to activate L-threonine; A_2-KR_1-T_2 and A_3-KR_2-T_3 are typical α-hydroxy acids as described previously.[11] The PKS natC has domains organized into KS-AT-KR_3-MT-T_4 (KS, ketosynthase; AT, acyltransferase; MT, methyltransferase). natDEFG encodes an NRPS with domains organized into A_4-KR_4-T_5-TE-TE. A lipase natN presumably catalyzes the loading of 3-formamidosalicylic acid onto the C-6 position. On the basis of the proposed biosynthetic pathway for antimycins, 14 proteins (natBCDEFGHIJKLMNO) are needed for generating the fifteen-membered ring depsipeptide neoantimycin scaffold.

It is significant to note that the members of the antimycin family vary in both their ring size and in their ester forming α-hydroxy acid components.[12,13] It is evident that the majority of the variation in these analogues is derived from substitutions of the α-hydroxy acid units. The sequence similarity between the antimycin and neoantimycin clusters is relatively high throughout, with the exception of two distinct alterations observed within the assembly machinery. Each assembly line starts with a formamidosalicylic acid unit and contains a C-A-T module that has a predicted code for threonine. Upon elucidation of the cluster, it became evident that the altered chemistry of the neoantimycins is afforded by the flexibility of certain modules, C-A-KR-T modules that incorporate α-hydroxy acids and create lactone bonds.[10] This flexibility was demonstrated through the known analogues of neoantimycin that were isolated.[14–16] They vary at the 2nd and 4th unit positions, with the following different substrates, 2-hydroxy-3-methyl-butanoic acid and 2-hydroxy-3-methyl-pentanoic acid. In order to further investigate, the naturally occurring alterations of neoantimycin, we used an MS/MS technique examining ions comprising distinct sections of the neoantimycin core.

LC-MS/MS analysis of crude extract of *S. orinoci* allowed us to detect neoantimycin (**6**) as a dominant compound (Fig. 2B) and five other compounds that possess the same characteristic *m/z* ions as the reported neoantimycins (Fig. 2A), but exhibit different