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Characterization of AntB, a Promiscuous Acyltransferase Involved in Antimycin Biosynthesis

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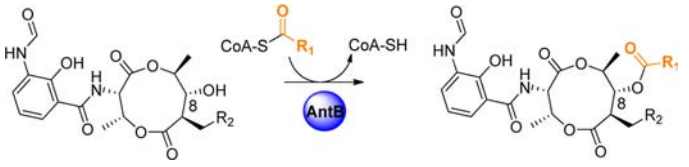
ABSTRACT



The *in vivo* and *in vitro* characterization of AntB, a dedicated acyltransferase encoded in the antimycin biosynthetic gene cluster, which catalyzes the C-8 acyloxy formation is reported. It is demonstrated that AntB has broad substrate specificity toward both the acyl substrate and the acyl carrier and produces more antimycin analogues with varying C-8 acyloxy moieties.

Antimycins are a family of natural products containing a nine-membered dilactone ring substituted with one alkyl (C-7), one acyloxy (C-8), two methyl moieties (C-4 and C-9), and an amide linkage (C-3) to a 3′-formamidosalicylic acid (Figure 1). They are well-known for their inhibitory action against the mitochondrial electron transport chain resulting in significant biological activities including antifungal, insecticidal, and nematocidal properties.[1−3] Antimycin-type compounds have also been shown to suppress the production of pro-inflammatory protein cytokines with minimal mammalian cell cytotoxicity, rendering them as drug candidates for asthma treatment.[4] In addition, antimycins have great potential as anticancer drugs due to their potent and selective inhibition of antiapoptotic proteins $Bcl_2/BclX_L$.[5−7] An antimycin derivative, 2′-methoxy antimycin A3, has been shown to bind $Bcl_2/BclX_L$ and trigger apoptosis without the general toxic effect on mitochondrial function.[5,6] Computational modeling studies predict that antimycins bind to the hydrophobic groove of $Bcl_2/BclX_L$ proteins, and the hydrophobicity of the side chains, especially the C-8 acyloxyl group on the dilactone ring, might directly affect the binding affinity and potency of antimycins.[5,6,8] Understanding the enzymatic mechanism for antimycin biosynthesis will therefore promote the production of more antimycin analogues with improved pharmaceutical properties.

We recently dissected the antimycin biosynthetic pathway by both genetic and enzymatic studies.[9] A minimum set of enzymes were identified to generate the antimycin dilactone core using a nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) assembly line from four distinct monomers: an aminobenzoate, a natural amino acid, an α-keto acid, and an acylmalonyl moiety (Figure 1). One presumable postassembly modification step, a transesterification reaction to yield a C-8 acyloxy, remains to be elucidated in order to generate the fully

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modified antimycin dilactone ring. This tailoring step is proposed to be catalyzed by AntB, a highly conserved protein encoded in all identified antimycin biosynthetic gene clusters.[10,11] AntB shows moderate sequence similarity to putative acyltransferases such as PldC[12] (Identity/Similarity = 35%/50%) and LadG (Identity/Similarity = 31%/46%) involved in the biosynthesis of pladienolide and laidlomycin, respectively.

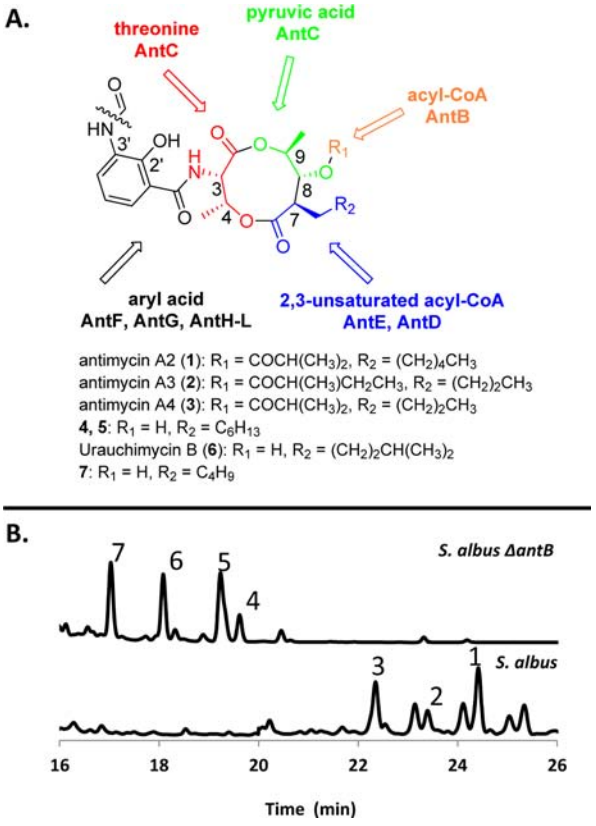

Figure 1. (A) NRPS-PKS catalyzed antimycin biosynthesis. (B) HPLC analysis (320 nm) of *S. albus* extracts showing production of antimycins from the wild-type (lower trace) and not from Δ*antB* (upper trace) and production of C8-hydroxy antimycins from Δ*antB* but not from the wild-type.

In order to identify the dedicated enzyme responsible for the transesterification on the antimycin C-8 hydroxyl group, we first performed a gene disruption experiment of *antB* in the antimycin-producing organism *Streptomyces albus* J1074. *antB* was deleted in-frame through double crossover, and the resulting mutant was confirmed by PCR (Figure S2). High Performance Liquid Chromatograpy (HPLC) analysis of culture extracts revealed that the deletion of *antB* completely abolished the production of antimycins that could be identified from the culture of wild-type strain. Instead, a new set of metabolites were detected in *S. albus* Δ*antB* supernatant extracts, with UV absorbance spectra characteristic of antimycin-type compounds, but more hydrophilic retention times compared to those of the antimycins produced by the wild-type strain (Figure 1). LC-High Resolution Mass Spectrometry (HRMS) and HRMS/MS analysis established that these new compounds are antimycin analogues with a C-8 hydroxyl moiety (Figures S8−S11). Large-scale purification of **6** was further performed for molecular structure characterization through NMR analysis (Tables S2, S3−S7). **6** was revealed to be a known antimycin-type compound, uranchimycin B, which was previously identified in marine actinomycetes.[13] The accumulation of compounds **4**−**7** suggested the role of AntB in the transesterification reaction to yield a C-8 acyloxyl substituent on the dilactone core of antimycins. Although the reduction of the C-8 β-keto to a hydroxy is catalyzed by AntM on the AntD-tethered intermediate before its release from the NRPS-PKS assembly line,[9] the comparable yields (~10 mg/L) of **4**−**7** to those of the wild-type antimycins strongly indicate that AntB catalyzes the transesterification reaction after dilactone scaffold assembly.

The activity of AntB was then reconstituted *in vitro* to further confirm the role of AntB as an acyltransferase. *antB* from *S. albus* was amplified and cloned into the expression vector pET-30 Xa/LIC, encoding an *N*-terminal His_6-tag. The corresponding protein was overproduced in *E. coli* cells and purified using Ni-NTA affinity chromatography (Figure S1). Purified **6** was used as the acyl acceptor, and the commercially available isobutyl-CoA was chosen as the acyl donor because this same acyl group occurs naturally at the C-8 position of the known antimycins A2 (**1**) and A4 (**3**) (Figure 1).[5,9,14] HPLC and LC-HRMS/MS analysis of the enzymatic reactions showed that AntB catalyzed the regioselective C-8 transesterification of **6** to form **8** (Figures 2, S12). We next examined the tolerance of AntB toward different acyl moieties. A suite of antimycin-type compounds with presumably different C-8 side chains have been found to be produced by *S. albus*, indicative of AntB possessing broad substrate specificity toward the acyl substrate (Figure 1).[9,10] Different acyl-CoAs as listed in Figure 2 were tested as possible substrates for AntB in the biochemical assays. AntB was shown to take all of the tested acyl-CoA substrates except stearoyl-CoA. These results indicate that AntB is fairly flexible toward the substituents on the acyl moiety as long as the acyl groups are small enough to fit in the AntB active site (Figures 2, S13−S17).

In addition to acyl groups, the substrate tolerance of AntB toward an alternative acyl carrier *N*-acetylcysteamine (SNAC) was also tested. Using SNAC as an acyl carrier increases the number of acyl substrates that can be assayed, facilitated by the ease of chemical synthesis of

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acyl-SNACs compared to their CoA derivatives. Furthermore, compared to acyl-CoAs, acyl-SNACs are cell-permeable and therefore have potential use for precursor feeding experiments *in vivo*.[15,16] Several acyl-SNACs were synthesized according to standard protocols and tested as substrates for AntB.[16] All of the acyl-SNACs were utilized by AntB as shown in Figure 2 yielding the expected products, which were confirmed by HPLC and HRMS/MS analysis (Figures S12, S16, S18, S19). To date, no antimycin-type compound containing an alkyne moiety has been identified. We therefore carried out a large-scale biocatalytic reaction with AntB, **6**, and 4-pentynoyl-SNAC. The resulting product, **15**, was purified by HPLC, and its molecular structure was confirmed by NMR analysis (Figures S21−S24, Table S3).

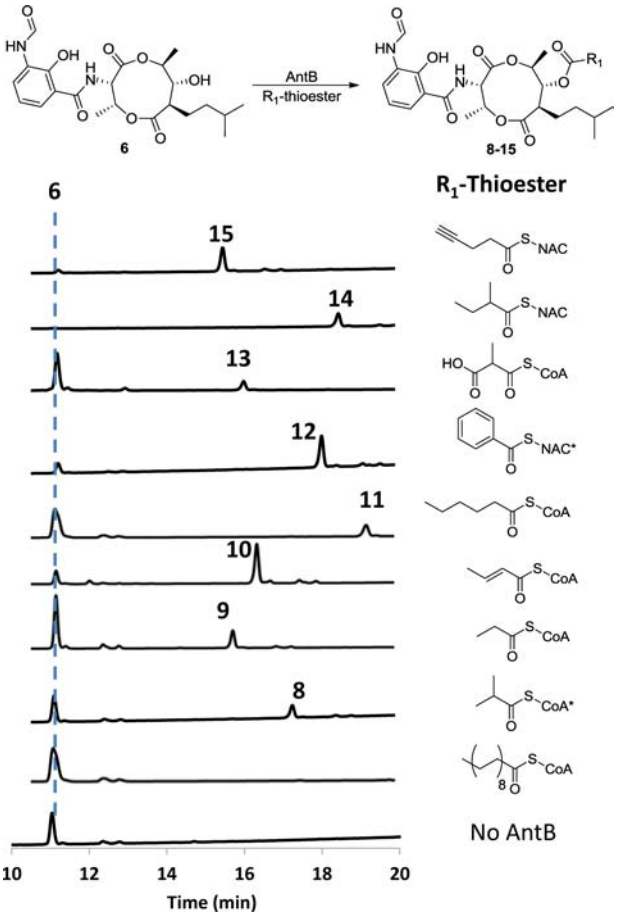

Figure 2. HPLC analysis (320 nm) showing *in vitro* production of antimycin analogues with variations at the C8 position catalyzed by AntB. R_1 groups in **8**, **12**, and **14** are found in naturally produced antimycins.[10] Reaction conditions: 50 mM HEPES, pH 8.0, 1 mM **6**, 1 mM acyl-CoA or acyl-SNAC, 10 μM AntB, 25 °C, 2 h. *The corresponding acyl-CoA or acyl-SNAC was also tested and was shown to yield the same product.

The kinetic parameters of AntB toward several acyl-CoAs and acyl-SNACs were then determined using a 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB) spectrophotometric assay to monitor the formation of free thiol in solution (Figure 3, Table 1). AntB showed comparable K_m values toward both acyl carriers, while demonstrating significantly lower k_{cat} numbers for acyl-SNACs compared to acyl-CoAs, suggesting that the shortening of the acyl carrier does not significantly affect the substrate binding to the enzyme, albeit the turnover rate is lowered by ∼100-fold. It is notable that various acyl moieties tested in our assays have little effect on the kinetic parameters of AntB, as the specificity constants (k_{cat}/K_m) of AntB toward different acyl-SNACs were on the same order of magnitude (Table 1). Even though kinetic analysis of AntB demonstrated an obvious preference for the CoA carrier, AntB can still effectively utilize acyl-SNAC substrates *in vitro* with high overall percent conversions (Table 1). In fact, both benzoyl-SNAC and isobutyl-SNAC have higher percent conversions (95%, 42.3%) than their CoA derivatives (81.1%, 17.3%) (Table 1), possibly due to the instability of acyl-CoAs, or the hydrolysis of products in the enzymatic reaction mixture. We further performed precursor-directed biosynthesis by feeding 4-pentynoyl-SNAC to wild-type cultures of *S. albus*. As expected, the corresponding antimycin analogue was produced by *S. albus* (Figure S25), demonstrating the feasibility of generating new antimycin analogues using acyl-SNACs *in vivo*.

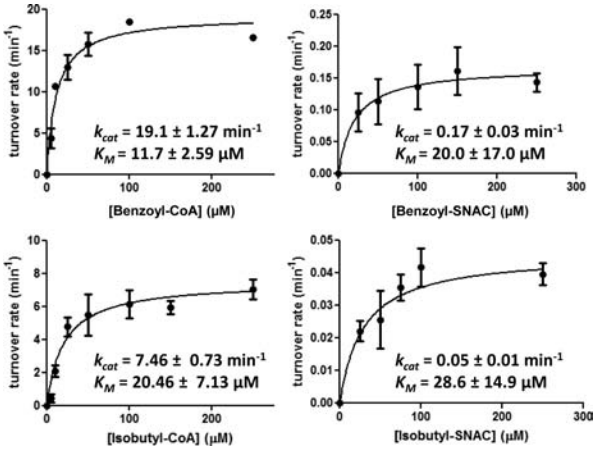

Figure 3. Determination of AntB kinetic parameters by the DTNB assay. Error bars represent standard deviations from at least three independently performed experiments. Each assay was performed with a no-enzyme control, and the rate of spontaneous thioester hydrolysis was subtracted in each case.

In summary, we have established AntB as an acyltransferase responsible for tailoring the antimycin dilactone scaffold at the C-8 hydroxy by transesterification. We have demonstrated that AntB has relaxed substrate specificity, and *in vitro* assays with AntB and a variety of acyl substrates resulted in the production of several new antimycin

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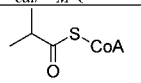
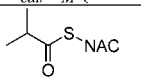
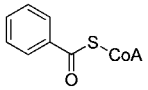
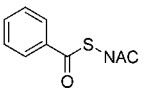
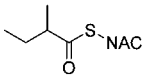
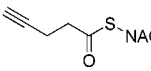
Table 1. Acylthioester Substrates of AntB[a]

substrate; conversion (%)[b]/ k_{cat}/K_M (min^{-1} μM^{-1})		substrate; conversion (%)[b]/ k_{cat}/K_M (min^{-1} μM^{-1})	
	17.3/ 0.365		42.3/ $1.75*10^{-3}$
	81.1/ 1.63		95/ $8.50*10^{-3}$
	100/ $6.60*10^{-3}$		100/ $4.78*10^{-3}$

[a] Reactions were performed in 50 mM HEPES pH 8 using 10 μM AntB, 1 mM **6**, 4 mM acyl-thioester, 10 h. The products of the reactions were verified by HPLC, HRMS, and HRMS/MS. [b] HPLC (320 nm) was used to calculate the percent of **6** converted to the corresponding antimycin analogues. The data were the averages from three independently performed experiments.

analogues with varying C-8 acyloxyl groups. The incorporation of a 4-pentynoyl moiety in **15** is particularly interesting, presenting a unique method to install a chemical handle for further derivatizations.[17] Our characterization of AntB, along with our previous characterization of the other enzymes required for antimycin dilactone scaffold assembly, provides a superior platform for priming the antimycin enzymatic machinery to generate new antimycin analogues for drug development.

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Supporting Information Available. Experimental Methods, SDS-PAGE analysis of purified AntB, NMR characterization of **6** and **15**, LCMS and LCMS/MS analysis of compounds **4**–**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.