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The in Vitro Characterization of the Iterative Avermectin Glycosyltransferase AveBI Reveals Reaction Reversibility and Sugar Nucleotide Flexibility

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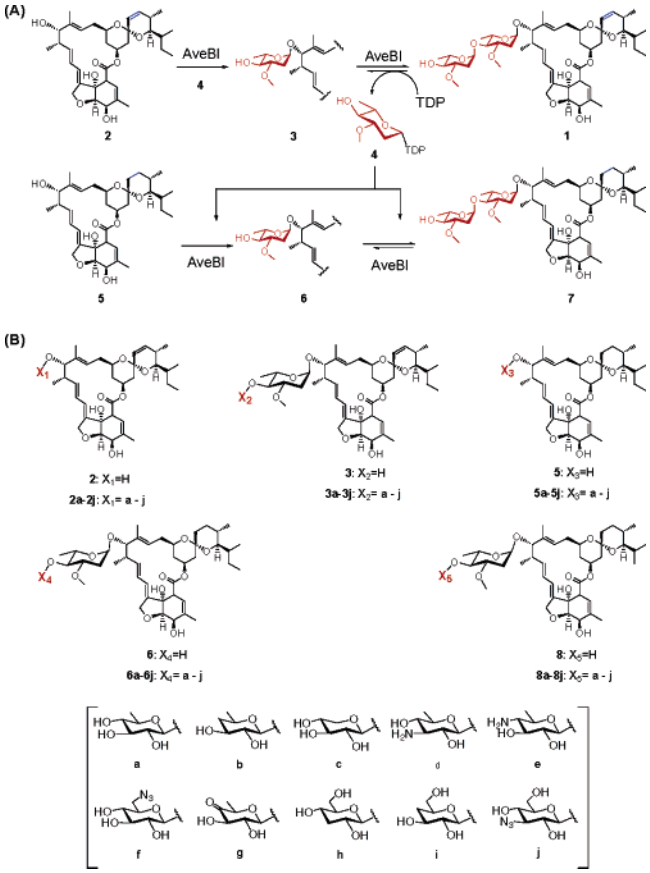
Avermectins (AVMs, e.g., Scheme 1, **1**) are 16-membered macrocyclic lactones produced by *Streptomyces avermitilis*. The avermectins, and the related $C_{22}-C_{23}$-reduced ivermectin (IVM, e.g., Scheme 1, **7**), target the γ-aminobutyric acid (GABA)-related chloride ion channels unique to nematodes, insects, ticks, and arachnids, with little or no mammalian toxicity.[1] The widespread commercial use of these remarkable anthelmintic agents began ~25 years ago as veterinary antiparasitic agents and has more recently expanded to clinical applications for the control of onchocerciasis, stongyloidiasis, and lymphatic filariasis. From a biosynthetic perspective, the AVMs are one of only a few known natural products postulated to derive from iterative glycosylation.[2] Specifically, a single glycosyltransferase (GT) is required for the attachment of the AVM oleandrosyl-disaccharide (AveBI), proposed to proceed in a stepwise, tandem manner (Scheme 1A). Evidence in support of iterative glycosylation includes the existence of a single glycosyltransferase gene (*aveBI*) within the AVM gene locus,[3] *S. avermitilis* crude extract studies suggestive of TDP-oleandrose (Scheme 1A, **4**) as an immediate precursor to the AVM oleandrose moiety,[4] and the production of a variety of glycosylated AVMs via in vivo pathway engineering.[5] Herein we describe, the first definitive in vitro biochemical verification of AveBI-catalyzed tandem glycosylation. Furthermore, consistent with the recent illumination of the reversibility of natural product GT-catalyzed reactions,[6] this study reveals the AveBI-catalyzed reaction to also be reversible, the utility of which is demonstrated by generating 50 AVM variants.

The *aveBI* gene was amplified from pWHM473[5] and assessed in several expression systems. However, the functional expression of *aveBI* was only achieved in *S. lividans* TK64 by the use of expression vectors pPWW49 and pPWW50.[7] The *N*-His_6-AveBI fusion was subsequently purified to greater than 90% purity from this recombinant strain via affinity purification (Figure S1)[8] and used directly for these studies. Aglycons **2**, **3**, **5**, **6**, and **8** (Scheme 1B) were prepared for this study via selective acid-mediated hydrolysis of AVM B1a (**1**) and IVM (**7**).[9] Given the lack of availability of TDP-β-L-oleandrose,[10] we opted to first examine the reversibility of the AveBI reaction using commercially available **1** and TDP based upon the recent precedent of the reversibility of natural product glycosyltransferase-catalyzed reactions.[6]

RP-HPLC analysis of an in vitro assay containing 100 μM **1**, 2 mM TDP, and 12 μM AveBI revealed the formation of **3** from **1** (30%, Scheme 1A and Figure S2), while **1** remained unchanged in control assays lacking either TDP or AveBI.[11a] This notable requirement of TDP is inconsistent with a hydrolytic event to provide free sugar and alternatively implicates a corresponding AveBI-catalyzed production of TDP-β-L-oleandrose (**4**). To confirm this hypothesis and assess whether AveBI was capable of catalyzing an "aglycone exchange" reaction,[6] a reaction containing 100 μM **1**, 100 μM **5**, 2 mM TDP, and 12 μM AveBI was subsequently analyzed. Examination of this reaction revealed the production of

Scheme 1. (A) Tandem Sugar Assembly by AveBI-Catalyzed Aglycone-Exchange Reaction; (B) Library of AVM Analogues Constructed via AveBI-Catalyzed Glycorandomization



3 (63%) from **1** and the subsequent transfer of oleandrose to **5**, to provide **6** (28%) and trace amounts of **7** (7%) (Scheme 1A and Figure S2).[11b] Consistent with recent studies,[6] this facile TDP-dependent aglycone exchange supports the in situ intermediacy of TDP-β-L-oleandrose (**4**). Cumulatively, these studies unequivocally establish AveBI as the GT responsible for the stepwise tandem assembly of the AVM oleandrosyl disaccharide and reveal the AveBI-catalyzed reaction to be readily reversible and amenable to aglycone exchange strategies.[6]

The AveBI sugar nucleotide specificity was subsequently probed with 22 NDP-sugars (generated chemically or chemoenzymatically, Figure S3, Supporting Information).[12] As a representative example,[13] IVM aglycone (**5**) with TDP-6-deoxy-α-D-glucose led to a new product (99% conversion, Figure 1A), the LC−MS of which was consistent with the anticipated product **5a** (Scheme 1B). Substitution of TDP-6-deoxy-α-D-glucose with UDP-6-deoxy-α-D-glucose in the same assay gave **5a** in only 10% yield, indicating a preference for

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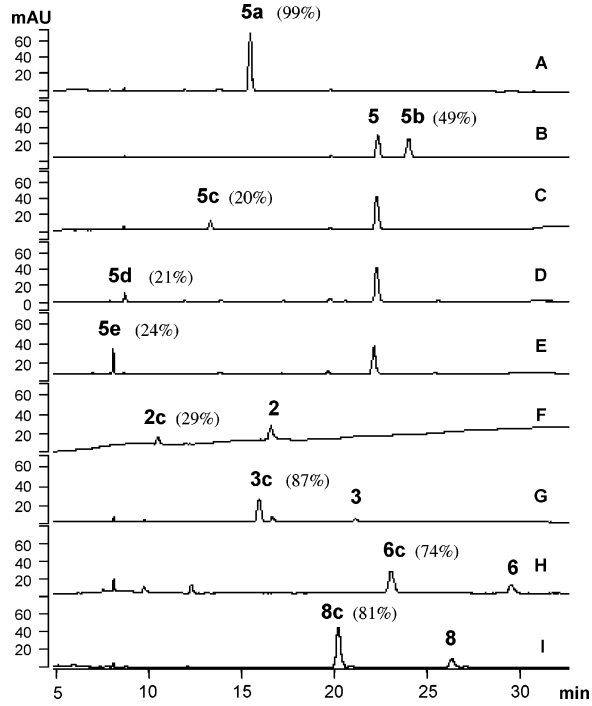

Figure 1. RP-HPLC analysis of representative AveBI reactions. Panels A−E depicted the formation of glycosides of **5a**−**5e** in AveBI reactions with **5** as an acceptor. Panels F−I represented the attachment of xylose to aglycons **2**, **3**, **5**, and **8** to form **2c**, **3c**, **5c**, and **8c** by AveBI, respectively. Conversion rates for each reaction were indicated in parentheses. Assay and HPLC conditions are available in Supporting Information.

TDP-sugars. Further AveBI−IVM assays revealed that nine additional TDP-sugar substrates were converted to their corresponding IVM glycosides **5b**−**5j** (Scheme 1B). In a similar fashion, the same set sugars were transferred to aglycones **2**, **3**, **6**, and **8**, producing glycosides **2a**−**2j**, **3a**−**3j**, **6a**−**6j**, and **8a**−**8j** (Scheme 1, Figure 1), respectively. The conversion rates for **a**−**e** glycosides ranged from 18% to 99% while only trace production (1−10%) of **f**−**j** glycosides was observed, with the exception of **6h** (25%) and **6g** (19%). All products were confirmed by LC−MS (Supporting Information, Tables S1 and S2), and controls lacking AveBI or sugar nucleotide gave no reaction. Consistent with the previous in vivo studies,[5] tandem additions of D-configured sugars to aglycone **2** and **5**, or trisaccharide AVM derivatives, were not observed in this study. While this study suggests AveBI to be particularly tolerant of C-6 and/or C-4 sugar modifications, the attachment of unnatural sugar appendages appears to inhibit subsequent disaccharide formation.

In summary, this study is noteworthy for a number of reasons. First, this work provides direct biochemical evidence of the AveBI-catalyzed tandem sugar addition within AVM biosynthesis. Second, this study greatly extends the repertoire of known AveBI D-sugar nucleotide substrates and provides a rapid one-pot strategy for the generation of 50 differentially glycosylated AVMs. Third, in contrast to the in vitro macrolide GT studies to date,[14] this study reveals AveBI does not require an "auxiliary/activator" protein for activity. Finally, this study demonstrates the recently established "sugar/aglycone exchange" strategies, based upon the reversibility of GT-catalyzed reactions,[6] are also applicable to macrolides.

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Supporting Information Available: Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) The sequence-confirmed *aveBI* PCR product was inserted into vector pPWW50 to give expression plasmid pCAM4.10, which was introduced into *Streptomyces lividans* TK64. The cells expressing *N*-$(His)_6$-AveBI were resuspended in 30 ml of buffer A (20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 10 mM imidazole) supplemented with 1 mg/ml of lysozyme. Cells were lysed by three rounds of French-press (1200 psi). The supernatant was loaded onto a HisTrap HT column (1 ml) and the *N*-$(His)_6$-tagged AveBI eluted with a linear gradient of imidazole (10−500 mM) in buffer A via FPLC. After desalting through PD-10 column the purified AveBI was stored in the buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 % glycerol. This purified recombinant AveBI was 50−80% active in the absence of exogenous Mg^{+2}.

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(10) The synthesis of TDP-β-L-oleandrose has not been reported, and the chemoenzymatic synthesis of the closest related sugar nucleotide (TDP-β-L-olivose, which lacks the sugar 3′-OMe) required six linear steps with an overall reported yield of 20% (Amann, S.; Drager, G.; Rupprath, C.; Kirschning, A.; Elling, L. *Carbohydr. Res.* **2001**, *335*, 23−32.).

(11) (a) Generally, AveBI assays were performed in a total volume of 100 μL in Tris-HCl buffer (50 mM, pH 8.0) containing 2 mM $MgCl_2$. Reversibility of AveBI reaction was assayed by co-incubation of 100 μM avermectin B1a (**1**) or ivermectin (**7**) and 2 mM TDP with 12 μM AveBI at 30 °C overnight. (b) The AveBI-catalyzed aglycone exchange reaction was assayed by co-incubation of 100 μM **1**, 100 μM **5**, and 2 mM TDP with 12 μM AveBI at 30 °C overnight.

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(13) A typical reaction to assess sugar nucleotide specificity contained 50 μM algycon (**1**−**3**, **5**−**8.**), approximately 300 μM TDP-sugar and 12 μM AveBI incubated at 30 °C overnight.

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