

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

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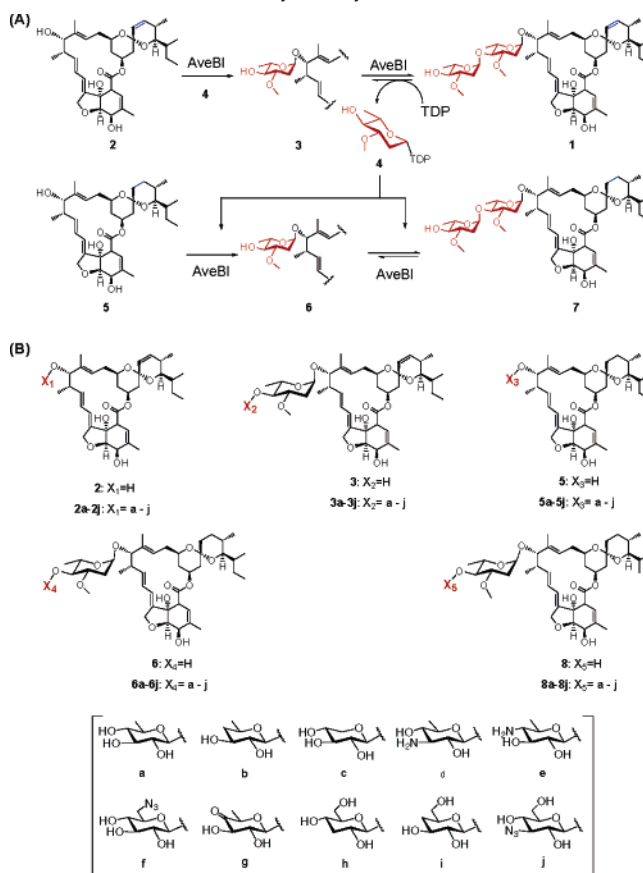
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Avermectins (AVMs, e.g., Scheme 1, **1**) are 16-membered macrocyclic lactones produced by *Streptomyces avermitilis*. The avermectins, and the related C<sub>22</sub>–C<sub>23</sub>-reduced ivermectin (IVM, e.g., Scheme 1, **7**), target the  $\gamma$ -aminobutyric acid (GABA)-related chloride ion channels unique to nematodes, insects, ticks, and arachnids, with little or no mammalian toxicity.<sup>1</sup> 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.<sup>2</sup> 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.<sup>3</sup> *S. avermitilis* crude extract studies suggestive of TDP-oleandrose (Scheme 1A, **4**) as an immediate precursor to the AVM oleandrose moiety,<sup>4</sup> and the production of a variety of glycosylated AVMs via in vivo pathway engineering.<sup>5</sup> 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,<sup>6</sup> 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<sup>5</sup> 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.<sup>7</sup> The N-His<sub>6</sub>-AveBI fusion was subsequently purified to greater than 90% purity from this recombinant strain via affinity purification (Figure S1)<sup>8</sup> 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**).<sup>9</sup> Given the lack of availability of TDP- $\beta$ -L-oleandrose,<sup>10</sup> 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.<sup>6</sup>

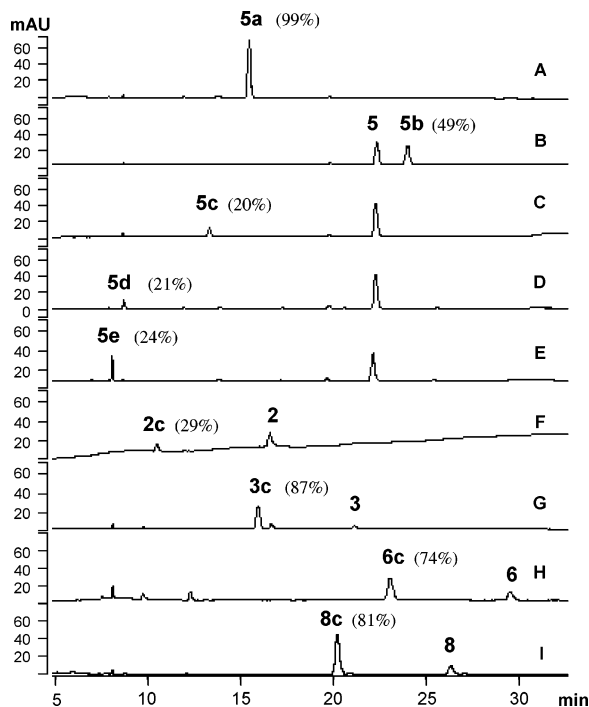
RP-HPLC analysis of an in vitro assay containing 100  $\mu$ M **1**, 2 mM TDP, and 12  $\mu$ M AveBI revealed the formation of **3** from **1** (30%, Scheme 1A and Figure S2), while **1** remained unchanged in control assays lacking either TDP or AveBI.<sup>11a</sup> This notable requirement of TDP is inconsistent with a hydrolytic event to provide free sugar and alternatively implicates a corresponding AveBI-catalyzed production of TDP- $\beta$ -L-oleandrose (**4**). To confirm this hypothesis and assess whether AveBI was capable of catalyzing an “aglycone exchange” reaction,<sup>6</sup> a reaction containing 100  $\mu$ M **1**, 100  $\mu$ M **5**, 2 mM TDP, and 12  $\mu$ M 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).<sup>11b</sup> Consistent with recent studies,<sup>6</sup> this facile TDP-dependent aglycone exchange supports the in situ intermediacy of TDP- $\beta$ -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.<sup>6</sup>

The AveBI sugar nucleotide specificity was subsequently probed with 23 NDP-sugars (generated chemically or chemoenzymatically, Figure S3, Supporting Information).<sup>12</sup> As a representative example,<sup>13</sup> IVM aglycone (**5**) with TDP-6-deoxy- $\alpha$ -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- $\alpha$ -D-glucose with UDP-6-deoxy- $\alpha$ -D-glucose in the same assay gave **5a** in only 10% yield, indicating a preference for



**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 aglycons **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,<sup>5</sup> 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,<sup>14</sup> 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,<sup>6</sup> 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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- (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<sub>2</sub>. 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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