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The Role of a Second Protein (DesVIII) in Glycosylation for the Biosynthesis of Hybrid Macrolide Antibiotics in *Streptomyces venezuelae*

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Abstract The function of the *desVIII* gene in the pikromycin producer *Streptomyces venezuelae* was characterized by gene deletion and complementation analysis. In addition to the DesVII glycosyltransferase, the *desVIII* gene that has previously been suggested to be required for the incorporation of endogenous deoxysugar, TDP-D-desosamine, into the aglycone of pikromycin is also required for the transfer of exogenous deoxysugars, TDP-D-quinovose and TDP-D-olivose.

Key words: Glycosylation, *Streptomyces venezuelae*, deoxysugar

Combinatorial biosynthesis is a promising method for modifying natural products, produced by microorganisms, for development of novel drugs. Recently, many research groups have started to manipulate polyketide biosynthetic pathways, attempting to produce hybrid or novel antibiotics [5, 7, 8, 12, 17, 19]. An important source of diversity in natural products is the range of deoxysugars attached to specific positions of an aglycone core. These deoxysugar components have been found to be involved in the molecular recognition between the antibiotic and its cellular target [16]. The flexibility of glycosyltransferases in accepting a wide variety of deoxysugar substrates has allowed attachment of non-native deoxysugars to aglycones for biosynthesis of hybrid macrolide antibiotics. However, the mechanism and requirements for the glycosylation process in macrolides is yet to be fully understood.

Methymycin (**1**) and Pikromycin (**2**) are macrolide-type antibiotics produced by *Streptomyces venezuelae* [18]. These compounds consist of a single sugar D-desosamine (**4**) attached to the C-3 and C-5 for **1** and **2**, respectively (Fig. 1A).

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The genes involved in the biosynthesis (*desI-desVI*) and transfer (*desVII*) of **4**, located in the *des* cluster, have previously been identified [20, 21, 22]. Recently, it has been demonstrated from *in vitro* studies that the transfer of **4** to the aglycones required the glycosyltransferases, DesVII, and a secondary protein, DesVIII [3] (Fig. 1C). In this study, the functions of these two proteins in attaching exogenous sugars, including TDP-D-quinovose (**5**), a modified intermediate sugar from the desosamine pathway, and TDP-D-olivose (**6**), a precursor sugar of TDP-D-oleandrose from the oleandomycin biosynthesis pathway, were investigated.

To produce a mutant strain of *S. venezuelae*, YJ003, that would produce only the aglycones of **1** and **2** (10-deoxymethynolide and narbonolide, respectively), the entire *des* cluster consisting of nine open reading frames (*desI-desVIII* and *desR*) was deleted and replaced by the kanamycin resistance gene, *aphII* [15]. A replicative plasmid-mediated homologous recombination approach was used for gene deletion/replacement in *S. venezuelae* [6]. Plasmid pYJ100 for *des* cluster deletion was constructed by using pKC1139 [6] cloned with an *aph*II gene, to replace the complete *des* cluster and DNA fragment flanking both upstream and downstream of the *des* cluster. The *des* cluster flanking DNA fragments were PCR amplified, using *Sph*I-digested *S. venezuelae* genomic DNA as template. The following nucleotides with engineered restriction sites (underlined) were used for cloning: a 1.0 kb *Hin*dIII-*Pst*I fragment containing the 5'-*des* cluster flanking region, forward 5'- GGCAAGCTTAGCGGGGCGACTGGCGTGCCCA CT-3' and reverse 5'-GGTCTGCAGTCACCGTGGGTTCTGCCATCTCTT-3'; and a 1.0 kb *Kpn*I-*Eco*RI fragment containing the 3'-*des* cluster flanking region, forward 5'-GCTGGTACCGGATGTTCCCTCCGGGCCACCGTC-3' and reverse 5'-TGAGAATTCCCT CGCCGTCCTGCCCGCGCTTGG-3'. This plasmid was introduced into *S. venezuelae* via protoplast

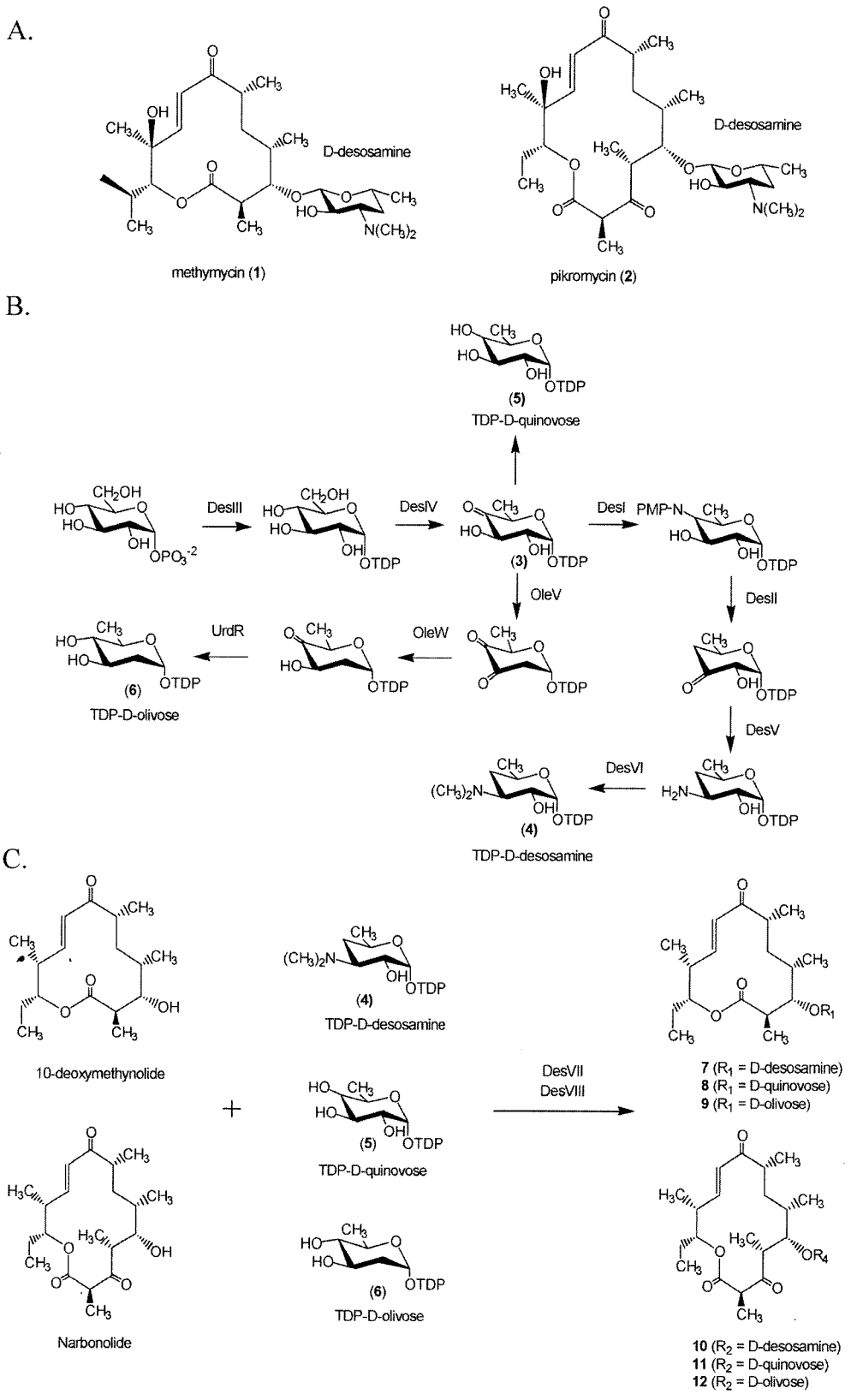

Fig. 1. (A) Structures of methymycin and pikromycin. (B) Proposed pathways for the biosynthesis of TDP-D-desosamine, TDP-D-quinovose, and TDP-D-olivose. Abbreviations are as follows: TDP, thymidine diphosphate; PMP, pyridoxamine 5'-phosphate. (C) Glycosylation of 10-deoxymethynolide and narbonolide by the deoxysugars.

Table 1. PCR primers used in amplification of genes (restriction sites are underlined).

Gene	Sequence (5'-3')	Portion of gene	Restriction site
desIII	GGTAAGCTTAACGCGTCGGCCGCGAGGTGCCCT	5	*Hin*dIII
	GCGGCATGCTAACTCGCCACGCCGACCGTTATC	3	*Sph*I
	AAGTCTAGATGAGGTCGAGGAAGGGGACGCGGG	5	*Xba*I
desIV	GACAAGCTTACCCACACCGCGACCTACAGCGCG	3	*Hin*dIII
	GGAGGATCCGGGTGCACCAAGCAAAGAGAGAGA	5	*Bam*HI
desR	GGCGCATGCTCACCAGACGTTGACCGTGGCGCT	3	*Sph*I
	CGCGGATCCACCGGCAAGGAAGGACACGACGCC	5	*Bam*HI
desVII	GGGTCTAGATCAGTGCCGGGCGTCGGCCGGCGG	3	*Xba*I
	CGTTCTAGACCCGACCAGCCTTATCGAAGGAGC	5	*Xba*I
desVIII	CGTTCTAGAGGTCCGAGCCGTCCGGTCCGTCCG	3	*Xba*I
	CCGCCTTCTAGAGGGAATCGCGGAAGCGGCCGG	5	*Xba*I
oleV	CCTTCGCTGCAGTCAGCTCAGGCTGGATGCAGGC	3	*Pst*I
	CTTGGCCTGCAGAAGGGAACCCCATGCCCTCCC	5	*Pst*I
oleW	ACGGCCGCATGCTCAGCACCAGCGCACCCGCGC	3	*Sph*I
	TGTCACGCATGCACAGAGATCCAGGACGACGCA	5	*Sph*I
urdR	GGGCTTAAGCTTTCAGATACGGACGGCGGAGGT	3	*Hin*dIII

transformation. Spores from individual transformants were cultured on kanamycin selective agar plates, and the cycle was repeated up to three times to enhance the probability for recombination. Double crossovers, yielding targeted gene replacement with *aph*II, were selected and screened for the kanamycin-resistant, apramycin-sensitive phenotype, and the mutant genotype was confirmed by Southern blot hybridization of genomic DNA.

In a previous study, a *desI* deletion mutant led to the biosynthesis of a non-natural sugar **5** [2]. A pathway-independent reductase, encoded by an open reading frame outside the *des* cluster, has been suggested to modify **3** to **5**. Thus, reincorporation of *desIII* and *desIV* into YJ003, which encodes a glucose-1-phosphate thymidylyl transferase and glucose-4,6-dehydratase, respectively, would synthesize TDP-4-keto-6-dideoxy-D-glucose (**3**), which is converted to

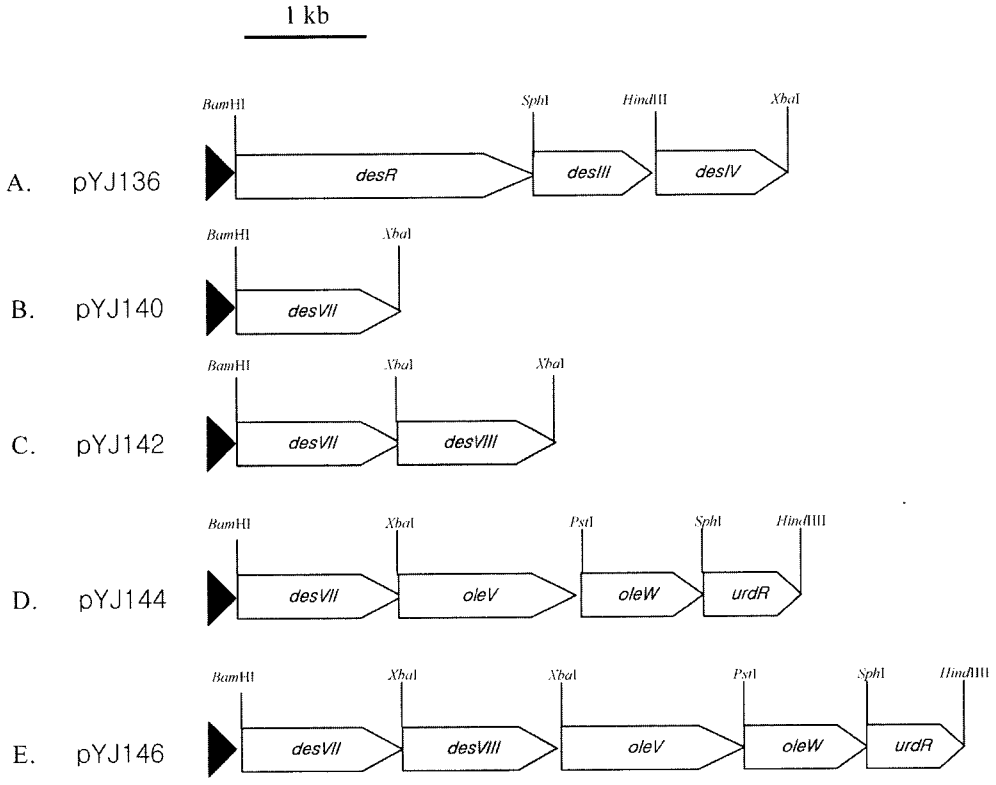

Fig. 2. Insert DNA cloned for expression in the plasmid pSET512-ermE* (A) and pSE34 (B-E) used in this study.
The black triangle represents the ermE* promoter, and the *desVIII* gene is shaded.

5 through the action of this pathway-independent reductase (Fig. 1B). DesR is known to function as a β-glucosidase, involved in a secondary self-resistance mechanism, to protect the host strain from the antibiotic it produces [23]. In the present study, the 1.0 kb *desIII*, 1.1 kb *desIV*, and 2.5 kb *desR* genes from *Sph*I-digested genomic DNA of *S. venezuelae* were amplified by polymerase chain reaction (PCR) using the primers summarized in Table 1. The three genes were then subcloned into pGEM3Zf (+) (Promega, WI, U.S.A.), and a *Bam*HI/*Xba*I fragment containing all three genes was ligated into pSET152 [6], containing an *erm*E* promoter [14]. The resulting plasmid pYJ136 (Fig. 2A) was introduced into YJ003 for integration into the genomic DNA. The integrated strain conferred resistance to thiostrepton antibiotic and was designated YJ013. To incorporate the genes involved in glycosylation (*desVII* and *desVIII*), the 1.3 kb *desVII* and 1.3 kb *desVIII* genes from *Sph*I-digested genomic DNA of *S. venezuelae* were PCR amplified by using the primers summarized in Table 1. The *Bam*HI/*Xba*I-digested *desVII* gene was cloned into pSE34 [14] that contains an *erm*E* promoter and designated plasmid pYJ140. The *Xba*I-digested *desVIII* gene was cloned into the single *Xba*I site of pYJ140 to produce plasmid pYJ142 (Figs. 2B and 2C). YJ013 was transformed by plasmids pYJ140 and pYJ142 to produce the strains, identified by their resistance to apramycin, YJ018 and YJ019, respectively. Thus, both strains YJ018 and YJ019 contain *desIII*, *desIV*, *desR*, and *desVII* genes, with the only difference being that YJ019 has an additional *desVIII* gene.

A heterologous pathway for the biosynthesis of **6** has been reported in a previous study by using genes from the urdamycin and oleandomycin deoxysugar pathway [11]. For construction of strains for attaching **6** to the aglycones, heterologous deoxysugar genes, *oleV* and *oleW*, that encode a 2,3-dehydratase and 3-ketoreductase, respectively, were chosen from the strain *Streptomyces antibioticus* [1], and *urdR* encoding a 4-ketoreductase was selected from *Streptomyces fradiae* [4] (Fig. 1B). The 1.5 kb *oleV* and 1.0 kb *oleW* genes were PCR amplified by using *S. antibioticus* genomic DNA as template, and the 0.8 kb *urdR* gene was amplified by using the *S. fradiae* genomic DNA. The primers used for the PCR reaction are summarized in Table 1. The three genes were subcloned into pGEM3zf (+), and a *Hind*III/*Xba*I fragment that contained all three genes was ligated into pYJ140 and pYJ142 to produce plasmids pYJ144 and pYJ146, respectively (Figs. 2D and 2E). The strain YJ013 was transformed by plasmids pYJ144 and pYJ146 to produce the strains YJ020 and YJ012, respectively. The difference between these strains was the presence of the *desVIII* gene in YJ021.

The engineered strains, YJ018, YJ019, YJ020, and YJ012, were grown on SPA solid medium [6] at 30°C for 3 to 4 days. The agar-grown culture was diced and extracted with two volumes of methanol. The extract was filtered, evaporated by a rotary evaporator at 30°C, redissolved in one volume of ethyl acetate, and washed with distilled water. The washed extract was concentrated in methanol for subsequent analysis. The products were analyzed by liquid chromatography coupled with electrospray ionization-mass/mass spectrometry (LC/ESI-MS/MS). HPLC was performed with a Waters (Milford, MA, U.S.A.) Model 2690 separations module, using a 25 cm×4.6 mm Watchers 120 ODS-BP (5 μm) column (Daiso Watchers, Osaka, Japan). Macrolides were resolved by gradient elution from 20% (vol/vol) to 80% (vol/vol) acetonitrile in 10 mM ammonium acetate buffer (pH 8.0) for 60 min at the flow rate of 1 ml/min. The effluent was split 1:5 before ESI interface, with approximately 200 μl/min routed to the Micromass (Beverly, MA, U.S.A.) Quattro LC triple tandem quadrupole mass spectrometer. Mass spectrometric data were acquired in the positive-ion mode, while scanning the specific parent mass to be fragmented. The hybrid macrolides were identified by mass spectral fragmentation pattern corresponding to the predicted products. Under the ionization conditions used, the hybrid macrolides produced characteristic macrolide and sugar detached aglycone patterns.

To confirm the role of DesVIII as being involved in the glycosylation process, the culture extracts of the strains YJ018 and YJ019 were analyzed. The expected compounds **8** and **11** were not detected in the extract of YJ018, however; only the aglycones, 10-deoxymethynolide and narbonolide, were detected (data not shown). On the other hand, compounds **8** and **11** were detected from the extract of the strain YJ019 containing the additional *desVIII* gene. Compounds **8** and **11** were detected at a retention time of 30.4 min and 34.9 min, respectively, with a characteristic fragmentation pattern in the MS/MS spectrum. The compound **8** gave the parent peak at m/z 465.4 for the Na^+ form and daughter peak at m/z 319.3 corresponding to the aglycone, 10-deoxymethynolide (Fig. 3A). The compound **11** produced a parent peak at m/z 521.5 for the Na^+ form and a daughter peak at m/z 375.4, corresponding to the aglycone, narbonolide (Fig. 3B). The difference of m/z 146 corresponds to the loss of the neutral sugar, D-quinovose.

Similarly, the involvement of DesVIII in glycosylation of an exogenous sugar, TDP-D-olivose, was examined. The culture extract from the strains YJ020 and YJ021 were analyzed to find out whether glycosylation would take place with or without DesVIII. In accordance with the above findings, the culture extract from YJ020 did not contain the compounds **9** or **12** (data not shown), but only the extract from YJ021 was found to contain both the compounds **9** and **12**. The compound **9** was detected at a retention time of 38.4 mins and gave the parent peak (Na^+ form) at m/z 449.6 and a fragmentation peak at m/z 319 (Fig. 3C). This difference of 130 between the parent and daughter peaks corresponds to the loss of the neutral olivose residue. The compound **12** was detected at a retention time of 42.0 min

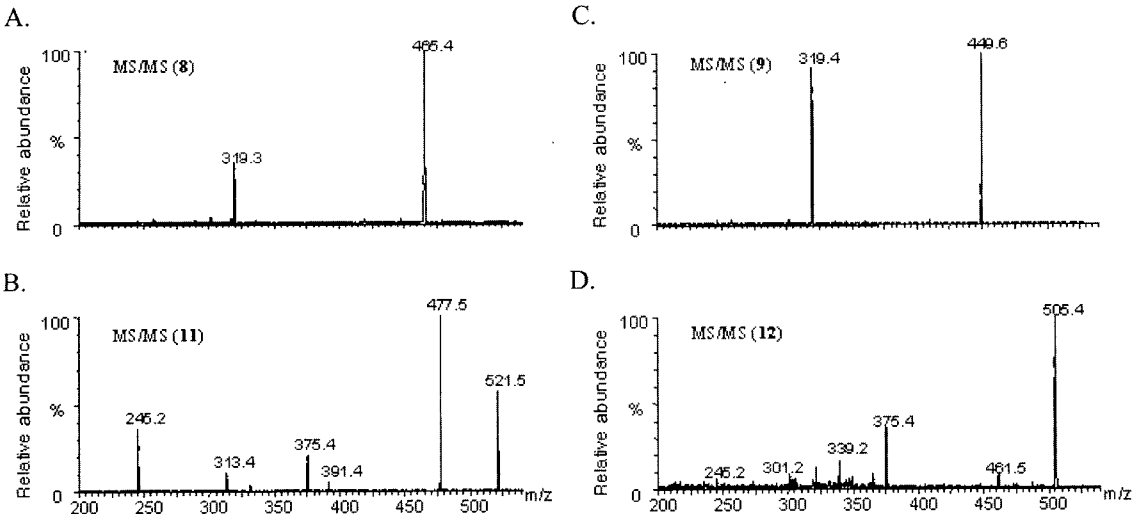

Fig. 3. MS/MS analysis of D-quinovose glycosylated (A, B) and D-olivose glycosylated (C, D) compounds.

and gave the parent peak (Na^+ form) at m/z 505.4 and a fragmentation peak at m/z 375.4 (Fig. 3D). The difference of 130 (compared to 505.4) again confirms the loss of the olivose residue.

Computation analysis of the DesVIII protein, using the BLAST software, identified the highest similarity to NbmC (70%) of *Streptomyces narbonensis* that has been suggested to be a putative NDP-deoxyhexose 3,4-isomerase. Close matches were also found with cytochrome P-450 enzymes, but DesVIII lacks the rigorously conserved cysteine residue that normally coordinates the heme iron. Other closely related proteins include the *dnrQ* gene product (35%) from *Streptomyces peucetius* [9], aknT (33%) from *Streptomyces galilaeus* [10], and eryCII (31%) from *Saccharopolyspora erythraea* [13]. The functions of these proteins have not yet been clarified, and further investigation is needed to ascertain whether these enzymes are involved in the biosynthesis of corresponding deoxysugar or in the glycosylation process.

Recently, *in vitro* experiments showed that the glycosylation of aglycones by the native deoxysugar **4** requires both DesVII and DesVIII [3]. This study revealed that other deoxysugars, including **5** and **6**, also requires the presence of this second protein DesVIII (Fig. 1B). This is a preliminary study to suggest a role of DesVIII in glycosylation along with the glycosyltransferases DesVII. A similar observation on the requirement of DesVIII in glycosylation was also made with the *dnrQ* gene product, without which glycosylation did not proceed [9]. Therefore, DesVIII, DnrQ, AknT, and EryCII enzymes may be functionally related in a role played in the glycosylation process.

This is the first report on the requirement of a second protein component for the *in vivo* transfer of exogenous deoxysugars to the macrolactone. The role of DesVIII in glycosylation is not limited to the amino sugar **4**, but also extends to transfer of other sugars that do not contain an amine group, such as **5** and **6**. Therefore, the requirement of DesVIII in glycosylation gives implications for future applications involving the generation of novel macrolide derivatives, attached with unnatural deoxysugar appendages by combinatorial biosynthesis. Further work remains to elucidate the exact mechanism by which DesVIII functions in the glycosylation process.

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