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# Genetically Engineered Biosynthesis of Macrolide Derivatives Including 4-Amino-4,6-Dideoxy-L-Glucose from *Streptomyces venezuelae* YJ003-OTBP3

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**Two sugar biosynthetic cassette plasmids were used to direct the biosynthesis of a deoxyaminosugar. The pOTBP1 plasmid containing TDP-glucose synthase (*desIII*), TDP-glucose-4,6-dehydratase (*desIV*), and glycosyltransferase (*desVII/desVIII*) was constructed and transformed into *S. venezuelae* YJ003, a strain in which the entire gene cluster of desosamine biosynthesis is deleted. The expression plasmid pOTBP3 containing 4-aminotransferase (*gerB*) and 3,5-epimerase (*orf9*) was transformed again into *S. venezuelae* YJ003-OTBP1 to obtain *S. venezuelae* YJ003-OTBP3 for the production of 4-amino-4,6-dideoxy-L-glucose derivatives. The crude extracts obtained from *S. venezuelae* ATCC 15439, *S. venezuelae* YJ003, and *S. venezuelae* YJ003-OTBP3 were further analyzed by TLC, bioassay, HPLC, ESI/MS, LC/MS, and MS/MS. The results of our study clearly shows that *S. venezuelae* YJ003-OTBP3 constructs other new hybrid macrolide derivatives including 4-amino-4,6-dideoxy-L-glycosylated YC-17 (3, [M+ $Na^+$] m/z=464.5), methymycin (4, m/z=480.5), novamethymycin (6, m/z=496.5), and pikromycin (5, m/z=536.5) from a 12-membered ring aglycon (10-deoxymethynolide, 1) and a 14-membered ring aglycon (narbonolide, 2). These results suggest a successful engineering of a deoxysugar pathway to generate novel hybrid macrolide derivatives, including deoxyaminosugar.**

**Keywords:** 4-Amino-4,6-dideoxy-L-glucose, glycosyltransferase, heterologous expression, hybrid antibiotics, *Streptomyces venezuelae* YJ003

A number of deoxysugar moieties participate in the molecular recognition of a drug target site and often play crucial roles in determining the biological activity of the parent natural products in secondary metabolite antibiotics [21, 23]. Deoxysugars, especially deoxyaminosugars, comprise an important class of moieties synthesized by a variety of organisms, and the amino group not only mediates the hydrogen bonding interactions that are essential for the binding of antibiotic to the ribosomes [15, 19], but they are also important for the interactions based on the ability of several antibiotic molecules to organize themselves into a channel spanning the cellular membrane of the target organism [26]. Therefore, we investigated the biosynthesis and attachment of deoxyaminosugars, which are important factors for hybrid-engineered antibiotics.

The host strain must be capable of synthesizing various deoxyaminosugars and must contain flexible glycosyltransferases in order to generate novel deoxyaminosugar derivatives. TDP-D-desosamine biosynthetic gene clusters from antibiotic-producing organisms including erythromycin A [7, 20], oleandomycin [1, 16], pikromycin [25], and megalomicin [22] have been described within the last several years. It was well-known that one of these antibiotics, the pikromycin biosynthetic system in *S. venezuelae* ATCC 15439, produced two distinct groups of macrolactones, including the 12-membered ring 10-deoxymethynolide (**1**) and the 14-membered ring narbonolide (**2**) obtained from single multifunctional polyketide synthase (*pikA*). The set of genes required for the biosynthesis of a TDP-D-desosamine is clustered downstream of *pikA*. In addition, the pikromycin glycosyltransferase DesVII/DesVIII system, which replaces the native partner with a heterologous activator protein, affects the efficiency of glycosylation [9] and has been shown to be especially "flexible" in accepting different deoxysugars, which generates further structural variations [8, 13]. These unique features of *S. venezuelae* in generating structural variability can be applied to combinatorial biosynthesis of novel hybrid macrolides.

In this study, *S. venezuelae* YJ003 formed by the deletion of the entire gene cluster related to D-desosamine biosynthesis was used as the host bacteria [8]. The minimal components were integrated into *S. venezuelae* YJ003. The heterologous

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sugar genes encoding 3,5-epimerase (*orf9*, GenBank Accession No. AAF59933) from *S. antibioticus* Tü99 [6] and TDP-4-keto-6-deoxy-glucose 4-aminotransferase (*gerB*, GenBank Accession No. ABB52533) from *S.* sp. KCTC 0041BP [10] were also introduced into the same mutant in order to biosynthesize novel derivatives that included the deoxyaminosugar, 4-amino-4,6-dideoxy-L-glucose derivatives. We report the formation of 4-amino-4,6-dideoxy-L-glycosylated macrolide derivatives. The results presented in this study reveal the usefulness of these deoxysugar genes for the biosynthesis of hybrid macrolide compounds.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*S. venezuelae* ATCC 15439 was used to produce methymycin/pikromycin and its derivatives. *S. antibioticus* Tü99 [6] and *S.* sp. KCTC 0041BP [10] were also used to amplify *orf9* and *gerB* for the construction of the expression plasmid. Transformants were selected on R2YE plates by overlaying with aparamycin (0.5 mg/ml), kanamycin (1 mg/ml), and thiostrepton (0.5 mg/ml) [11, 18]. *S. venezuelae* mutants containing plasmids were grown on SPA medium (1 g of yeast extract, 1 g of beef extract, 2 g of tryptose, 10 g of glucose, 12.5 mg of ferrous sulfate, per 1 l distilled water) with an appropriate antibiotic or a combination of antibiotics in order to produce and analyze the products. *Escherichia coli* DH1 was used as a host for DNA manipulation and Luria-Bertani (LB) medium was used for *E. coli* propagation.

### Manipulation of DNA

DNA manipulations, restriction endonuclease digestion, and ligation were carried out according to the standard protocols [18]. The pMBE101 plasmid containing bidirectional promoters obtained from Leadlay's group was used to construct the heterologous expression plasmid [24]. Chromosomal DNA from *S. antibioticus* Tü99 and *S.* sp. KCTC 0041BP were isolated to clone *orf9* and *gerB* by lysozyme treatment and phenol-chloroform extraction, as previously described [11, 12, 17].

### Construction of Heterologous Expression Plasmid

Two primers, GerBF (5'-ATA *TTC GAA* CGC ACG CAC CGG GAA GGT-3') and GerBR 5'-GCG *TCT AGA* CCG CAG GGG ATT CCC TGA-3'), were used to amplify *gerB* (the restriction sites are indicated by italics letters). The polymerase chain reaction (PCR) product (1,218 bp) was cloned into the BstB1 and XbaI sites of pMBE101 in order to generate pOTBP2. Similarly, *orf9* was amplified from the two primers designed as *orf9*.1 (5'-AG*T TAA TTA A*TC GGC AGA ATC GGG ATC G-3') and *orf9*.2 (5'-AAC *GAG CTC* CCA GTA CGA GGG CCT TCA-3'). The PCR product (609 bp) was cloned into the PacI and SacI sites of pOTBP2 in order to generate pOTBP3 (Fig. 2A). All of the PCR products were cloned into the pGEM-T easy vector (Promega, U.S.A.) and sequenced prior to cloning into the expression vector to verify that no mutations occurred during PCR amplification. The polymerization reaction was performed in a thermocycler (Takara, Japan) under the following conditions: an initial denaturation of 7 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; an extra extension step of 7 min at 72°C was added after the 30 cycles, followed by cooling at 4°C at the end of this program.

### Construction of *S. venezuelae* YJ003-OTBP1 and *S. venezuelae* YJ003-OTBP3

A plasmid was constructed for the integration of the genes essential for the TDP-4-keto-6-deoxyglucose, which is a common intermediate in deoxysugar biosynthesis and glycosylation. The pYJ136 plasmid containing *desR*, *desIII*, and *desIV* was used as the integration vector [8]. A pair of *desVII* (glycosyltransferase) and *desVIII* (auxiliary protein for glycosylation) from *S. venezuelae* was subcloned into pGEM-3zf(−) in order to generate the appropriate endonucleases. The XbaI-PstI-XbaI fragment of the *desVIII/desVII* genes was later cloned into pYJ136 by using XbaI and was designated as pOTBP1 (Fig. 2B). The plasmid pOTBP1 was integrated into the genomic DNA of *S. venezuelae* YJ003 to yield *S. venezuelae* YJ003-OTBP1 using the PEG-mediated protoplast transformation method [18]. *S. venezuelae* YJ003-OTBP3 was generated after the transformation of pOTBP3 (Fig. 2A) into *S. venezuelae* YJ003-OTBP1 using the same method.

### Production and Analysis of Macrolide Derivatives

*S. venezuelae* ATCC 15439, *S. venezuelae* YJ003, *S. venezuelae* YJ003-OTBP1, *S. venezuelae* YJ003-OTBP3, and *S. venezuelae* YJ003 harboring the pMBE101 vector were grown on SPA solid media at 30°C for 5 days with the antibiotics. The grown culture was diced and extracted with double volumes of MeOH. The extract was then pooled and concentrated under reduced pressure by a rotary evaporator. The residues were washed with water and then partitioned with an equal volume of ethyl acetate with proper shaking. The combined organic phases were concentrated once more under a vacuum and then resolved into MeOH. Prior to performing high-performance liquid chromatography (HPLC) analysis, a solid-phase extraction (SPE) cleanup column was employed not only to minimize the loss of low-level compounds of interest but also to remove unwanted impurities, such as pigments and particles, which were not readily dissolved in methanol. The column was properly washed with water, methanol, and acetonitrile until the baseline was previewed. Ten µl aliquots of the solvent extracts were analyzed using a reverse-phase $C_{18}$ column (Mytstil RP-18, 4.6× 250 mm× 5 µm) with 80% acetonitrile in 5 mM ammonium acetate with 0.05% acetic acid buffer for over 65 min using HPLC analysis. The flow rates were 1 ml/min and were detected using a UV absorbance detector, monitoring peaks at 220 nm. The products were analyzed with an electrospray ionization-mass/mass spectrometer (ESI-MS/MS). Thin-layer chromatography (TLC) was carried for the crude product of *S. venezuelae* ATCC 15439, *S. venezuelae* YJ003, and *S. venezuelae* YJ003-OYBP3 [5] (Fig. 3). Liquid chromatography/mass spectroscopy (LC/MS) was performed to obtain further structural information. *S. venezuelae* YJ003-OTBP3 crude product was purified by eluting 1−20% methanol in chloroform using silica gel 60 $GF_{254}$ (Merck, Germany). The active eluate was concentrated and subjected to mass to mass spectroscopy (MS/MS) analysis.

### Biological Activity Assay

The antibacterial activity from crude products of *S. venezuelae* YJ003, *S. venezuelae* YJ003 harboring pMBE101 vector, *S. venezuelae* YJ003-OTBP1, and *S. venezuelae* YJ003-OTBP3 was

assayed against *Bacillus subtilis* ATCC 23857 [13]. *B. subtilis* was initially grown on LB medium, and aliquots of the grown culture were dispensed on LB agar-based medium. Each compound was reconstituted in a small volume of MeOH and dispensed into paper disks. An equal volume of MeOH solvent, which served as the negative control, was also dipped onto a paper disk. The dried disks were placed onto agar plates and incubated at 37°C for 8 h.

## Results and Discussion

### Sequence Analysis of Orf9 and GerB

The Orf9 (GenBank Accession No. AAF59933) from *S. antibioticus* Tü99 exhibits the closest homology with OleL (GenBank Accession No. AAD55452) of *S. antibioticus* ATCC 11891, having an 85% identity according to BLAST from the NCBI. It also exhibits 57% and 58% homology with RfbC (GenBank Accession No. CAJ64614) from *Frankia alni* ACN14a, and StrM (GenBank Accession No. CAA07389) from *S. glaucescens*, respectively (data not shown). Similarly, GerB (GenBank Accession No. ABB52533) from *S.* sp. KCTC 0041BP shows the closest homology, an identity of 93%, with ChmCIV (GenBank Accession No. AAC79442) from *S. bikiniensis* NRRL 2737. It also has 66%, 63%, and 62% identities with OleN1 (GenBank Accession No. AAD55456) of *S. antibioticus* ATCC 11891, EryCIV (GenBank Accession No. AAB84075) of *Saccharopolyspora erythrae* NRRL 2338, and DesI (GenBank Accession No. AAC68684) of *S. venezuelae* ATCC 15439, respectively (data not shown).

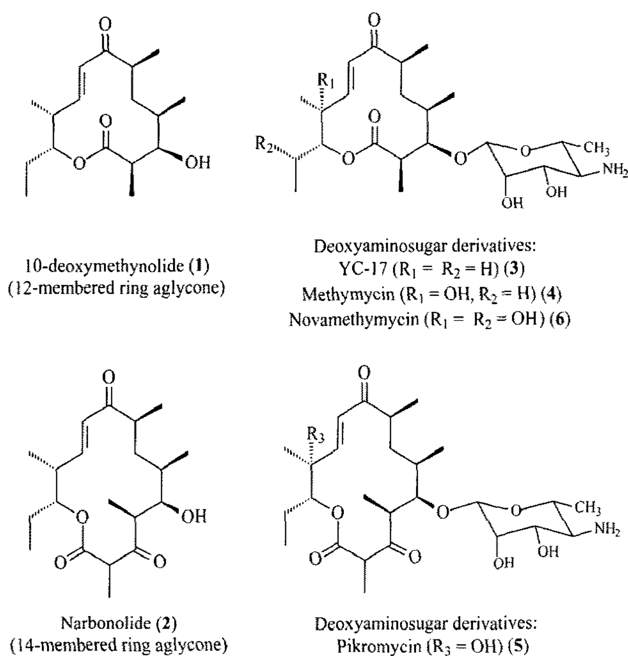


**Fig. 1.** Structures of two aglycons, 10-deoxymethynolide (**1**) and narbonolide (**2**), obtained from *S. venezuelae* and the proposed structures of deoxyaminosugar-including derivatives from *S. venezuelae* YJ003-OTBP3. **3**, YC-17 derivatives; **4**, methymycin derivatives; **5**, pikromycin derivatives; **6**, novamethymycin derivatives.

### Construction of *S. venezuelae* YJ003-OTBP1 and *S. venezuelae* YJ003-OTBP3

The substrate flexibility of DesVII has also been demonstrated to accept TDP-D-olivose (TDP-2,6-dideoxyhexose) during the glycosylation to the aglycon, such as **1** and **2** (Fig. 1) [3, 8]. In a recent *in vitro* study, the glycosylation of TDP-D-desosamine to **1** required the presence of an additional protein, DesVIII [2]. We constructed *S. venezuelae* YJ003-OTBP1, which included *desVII*/*desVIII* with the biosynthetic genes of TDP-4-keto-6-deoxy-D-glucose, in order to validate the flexibility of *desVII* and *desVIII* towards deoxyaminosugars. The integration of the glycosyltransferase and biosynthetic genes in this strain was confirmed by Southern blot and PCR (data not shown). In addition, the pOTBP3 was constructed to include TDP-glucose 3,5-epimerase (*orf9*), TDP-4-keto-6-deoxyglucose aminotransferase (*gerB*), and the SCP2* origin of replication with the bidirectional promoters *pactI* and *pactIII* [14]. Transformation of pOTBP3 into *S. venezuelae* YJ003-OTBP1 was also confirmed by the preparation of the plasmid DNA from *S. venezuelae* YJ003-OTBP3 and PCR (data not shown).

### Metabolite Analysis of *S. venezuelae* YJ003-OTBP3

*S. venezuelae* ATCC 15439, *S. venezuelae* YJ003, and *S. venezuelae* YJ003-OTBP3 were grown on SPA solid media to produce and analyze the macrolide derivatives. The crude extract was obtained and subjected to further analyses using TLC, biological activity assay, HPLC, ESI/MS, LC/MS, and MS/MS.

The TLC spots of *S. venezuelae* YJ003-OTBP3 have $R_f$ values that are similar to the spots of the glycosylated products of *S. venezuelae*, as shown in Fig. 3. The bands on the silica TLC were monitored by development with vanillin stain (0.75% vanillin, 1.5% $H_2SO_4$, MeOH) [5]. Methymycin had the higher $R_f$ (0.24) and stained dark brown with vanillin, whereas novamethymycin had the lower $R_f$ (0.18) and stained orange with vanillin from the *S. venezuelae* ATCC 15439. The orange spots in the TLC revealed that our target 4-amino-4,6-dideoxy-L-glucosylated macrolide derivatives were produced by *S. venezuelae* YJ003-OTBP3 (Fig. 3).

The attachment of the sugar moiety plays an essential role in the antibacterial activities. The antibacterial activity from crude products of *S. venezuelae* YJ003, *S. venezuelae* YJ003 harboring pMBE101 vector, *S. venezuelae* YJ003-OTBP1, and *S. venezuelae* YJ003-OTBP3 was tested against *B. subtilis* (Fig. 4). Whereas *S. venezuelae* YJ003 and *S. venezuelae* YJ003 harboring pMBE101 vector showed no inhibition zone (Figs. 4C and 4D), the diameter of the inhibition zone showing no growth from *S. venezuelae* YJ003-OTBP3 was an indication of the antibacterial

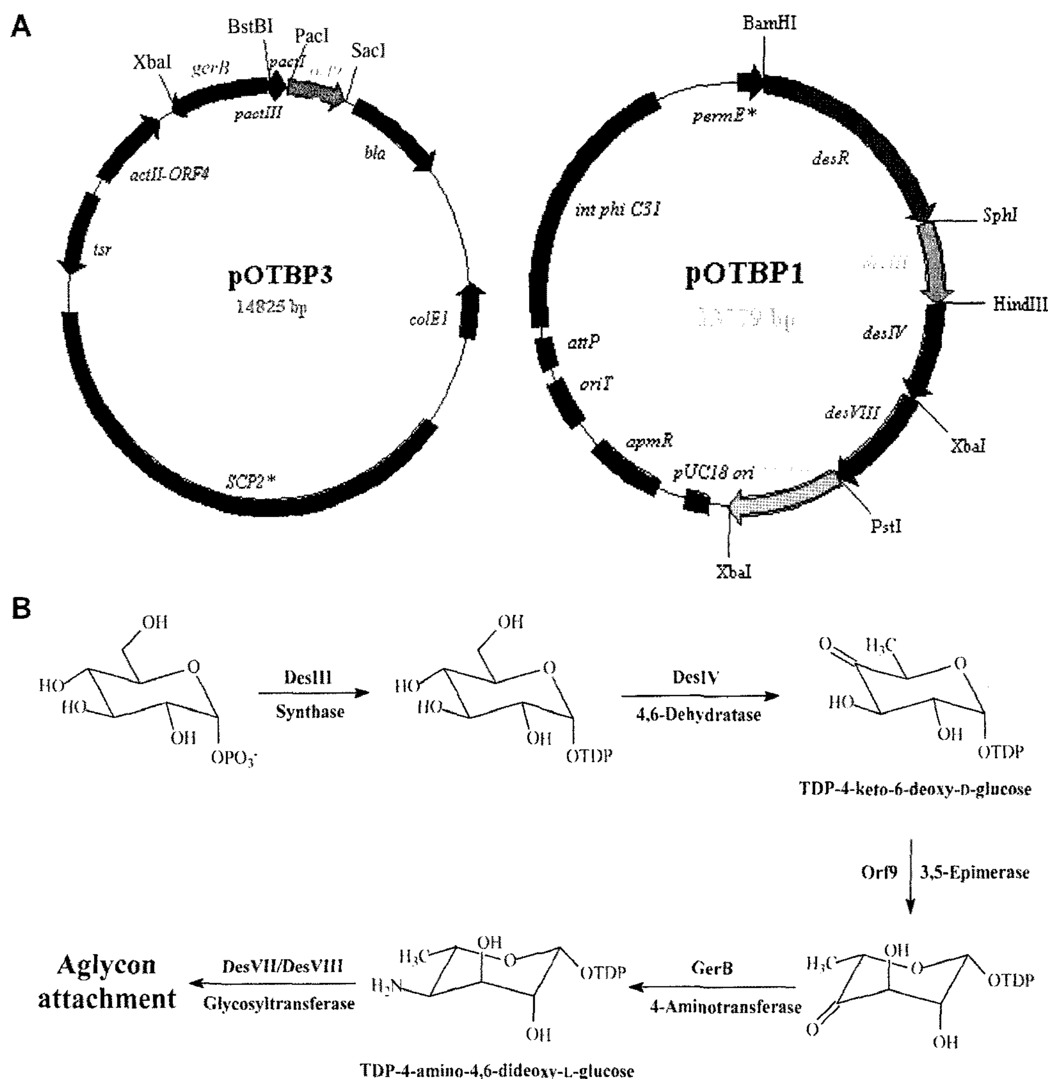


**Fig. 2.** Maps of the expression plasmid (**A**) and the integration plasmid (**B**). The restriction sites flanking the different genes were used for subcloning as indicated. **C.** Proposed biosynthetic pathway leading toward 4-amino-4,6-dideoxy-L-glucose.
*desIII*, TDP-glucose synthase from *S. venezuelae*; *desIV*, TDP-glucose-4,6-dehydratase from *S. venezuelae*; *orf9*, TDP-glucose 3,5-epimerase from *S. antibioticus* Tü99; *gerB*, TDP-4-keto-6-deoxy-glucose aminotransferase from *S.* sp. KCTC 0041BP; *desVII*/*desVIII*, glycosyltransferase from *S. venezuelae*.

activity that results from 4-amino-4,6-dideoxy-L-glycosylated macrolide derivatives (Fig. 4A). Moreover, the antibacterial activity from *S. venezuelae* YJ003-OTBP1 was due to the 4-keto-6-deoxy-D-glycosylated macrolide derivatives (Fig. 4B).

The HPLC profile from *S. venezuelae* YJ003-OTBP3 was compared with *S. venezuelae* ATCC 15439 and *S. venezuelae* YJ003. The comparison data showed the glycosylated product peaks from *S. venezuelae* YJ003-OTBP3, which was observed at the same retention times as the wild type and was not shown from *S. venezuelae* YJ003 (Fig. 5).

The LC/MS analysis revealed that the molecular ions $[M+Na^+]$ for **2** and hydroxylated **2** were detected at m/z=375 and m/z=391, respectively, with high relative abundance. The culture extracts of *S. venezuelae* ATCC 15439 were analyzed in aglycon and glycosylated derivatives,

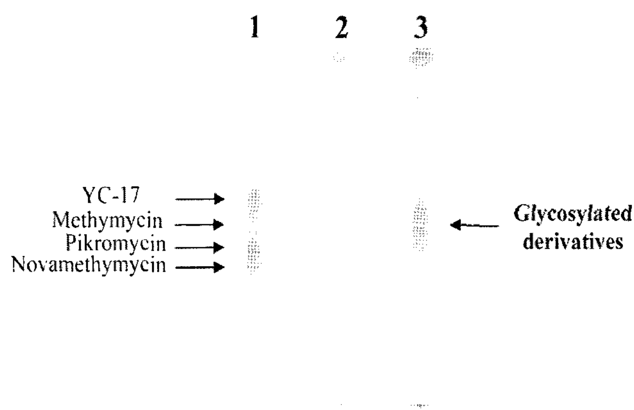


**Fig. 3.** TLC of the crude products from *S. venezuelae* ATCC 15439 (**1**), *S. venezuelae* YJ003 (**2**), and *S. venezuelae* YJ003-OTBP3 (**3**).

where the major products are methymycin/pikromycin-glycosylated products and low levels of aglycon (**2** and hydroxylated **2**). The masses 470, 486, 526, and 542 obtained from the LC/MS correspond to methymycin, novamethymycin, pikromycin, and novapikromycin, respectively, $[M+H^+]$ (data not shown). ESI/MS datum (m/z= 480.5) from the crude product of *S. venezuelae* YJ003-OTBP3 was also in support of a generation of new aminodeoxysugar derivatives of methymycin (data not shown). The MS/MS trace corresponding to pikromycin derivatives including 4-amino-4,6-dideoxy-L-glucose produced from *S. venezuelae* YJ003-OTBP3 revealed the formation of a new aminodeoxysugar moiety (m/z=146) (Fig. 6).

The masses 464.5, 480.5, 496.5, and 536.5 (LC/MS) obtained from the products of *S. venezuelae* YJ003-OTBP3 correspond to the $[M+Na^+]$ for YC-17 (**3**), methymycin (**4**), novamethymycin (**6**), and pikromycin (**5**) containing 4-amino-4,6-dideoxy-L-glucose (Figs. 7A–7D). MS/MS analysis corresponding to the YC-17 (**3**), methymycin (**4**), novamethymycin (**6**), and pikromycin (**5**) containing 4-amino-4,6-dideoxy-L-glucose from *S. venezuelae* YJ003-OTBP3 was showing in inset as their parent masses 442.4, 458.4, 474.3, and 514.4, respectively (Figs. 7A–7D). These data revealed that DesVII/DesVIII could accept 4-amino-4,6-dideoxy-L-glucose as a substrate donor for the glycosylation in a 12-membered macrolide (**1**) and a 14-membered macrolide (**2**).

Although the pikromycin biosynthetic system in *S. venezuelae* ATCC 15439 encodes a single set of genes for

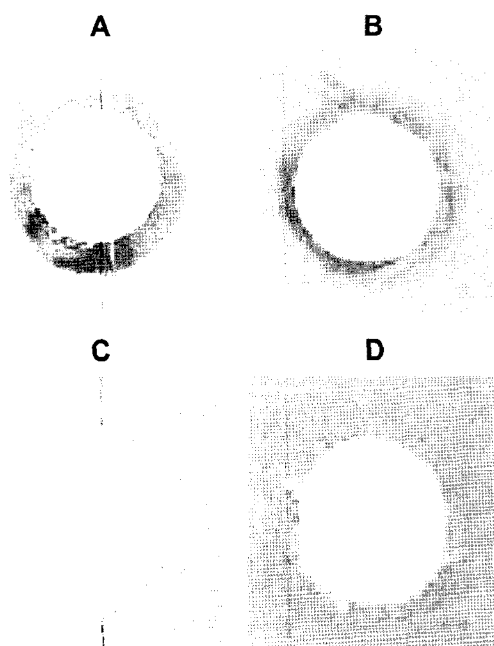


**Fig. 4.** Antibacterial activity assay of the crude products from *S. venezuelae* YJ003-OTBP3 (**A**), *S. venezuelae* YJ003-OTBP1 (**B**), *S. venezuelae* YJ003 harboring pMBE101 vector (**C**), and *S. venezuelae* YJ003 (**D**).

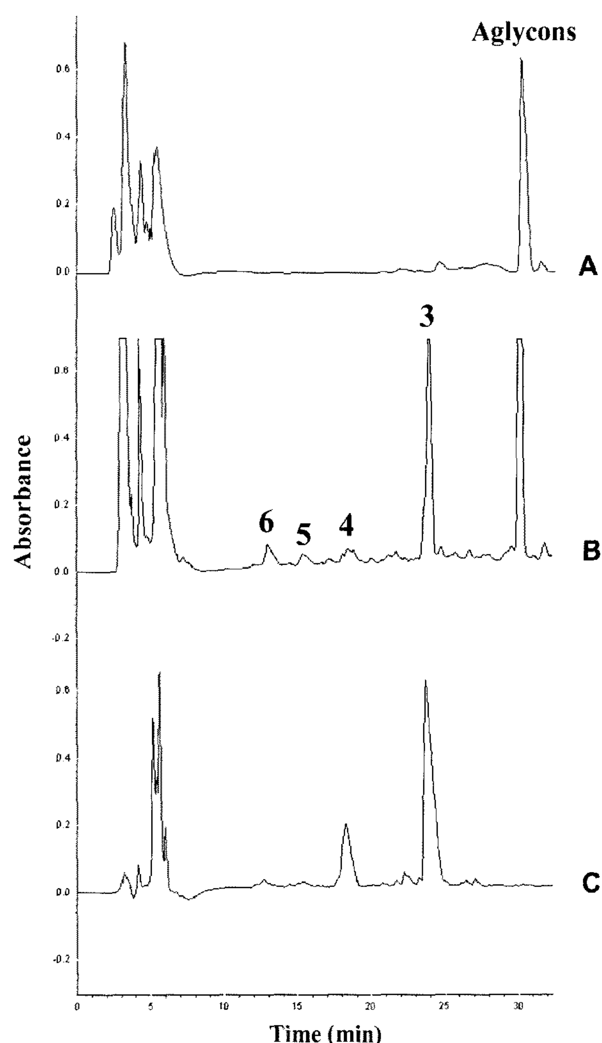


**Fig. 5.** Typical HPLC traces for the deoxyaminosugar derivatives. *S. venezuelae* YJ003 (**A**), *S. venezuelae* YJ003-OTBP3 (**B**), and *S. venezuelae* ATCC 15439 (**C**).

the biosynthesis of a TDP-D-desosamine, which contains C-3 amination, we successfully showed the incorporation of TDP-L-desosamine, including C-4 amination, onto the alternate (12- and 14-membered ring) polyketide aglycons. Therefore, we expressed the different sugar plasmids including *orf9* (3,5-epimerase) and *gerB* (4-aminotransferase) into *S. venezuelae* YJ003. In addition, we found that our glycosyltransferase system apparently catalyzes the transfer of TDP-L-desosamine onto the 12- and 14-membered ring aglycons. Based on our studies, this plasmid system demonstrated the pathway-engineered drug design of novel derivatives of glycosylated natural products. The successful manipulation of the hybrid macrolides including 4-amino-4,6-dideoxy-L-glucose in *S. venezuelae* has further expanded the usefulness of unnatural biosynthetic sugars as a tool for combinatorial biosynthesis. Therefore, it is expected that this research will aid in the development of novel deoxyaminosugar macrolides.

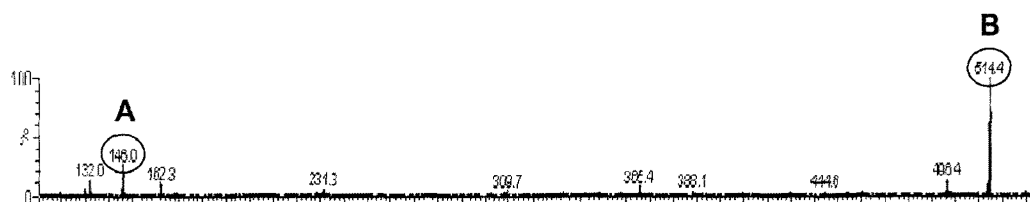


**Fig. 6.** MS/MS trace corresponding to pikromycin derivatives including 4-amino-4,6-dideoxy-L-glucose produced from *S. venezuelae* YJ003-OTBP3. Sugar moiety (m/z=146) (**A**) and glycosylated parent ion (m/z=514) (**B**).

**Fig. 7.** LC-MS analysis of the 4-amino-4,6-dideoxy-L-glycosylated derivatives produced from *S. venezuelae* YJ003-OTBP3. **A–D**, the deoxyaminosugar derivatives of YC-17 (m/z=464.5), methymycin (m/z=480.5), pikromycin (m/z=536.5), and novamethymycin (m/z=496.5), respectively. MS/MS analysis of parent ion corresponding to the compounds is shown in inset.

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