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Chemoenzymatic Synthesis of Glycosylated Macrolactam Analogues of the Macrolide Antibiotic YC-17

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Abstract: YC-17 is a 12-membered ring macrolide antibiotic produced from *Streptomyces venezuelae* ATCC 15439 and is composed of the polyketide macrolactone 10-deoxymethynolide appended with D-desosamine. In order to develop structurally diverse macrolactam analogues of YC-17 with improved therapeutic potential, a combined approach involving chemical synthesis and engineered cell-based biotransformation was employed. Eight new antibacterial macrolactam analogues of YC-17 were generated by supplying a novel chemically synthesized macrolactam aglycone to *S. venezuelae* mutants harboring plasmids capable of synthesizing several unnatural sugars for subsequent glycosylation. Some YC-17 macrolactam analogues were active against erythromycin-resistant bacterial pathogens and displayed improved metabolic stability *in vitro*. The enhanced therapeutic potential demonstrated by these glycosylated macrolactam analogues reveals the unique potential of chemoenzymatic synthesis in antibiotic drug discovery and development.

Keywords: antibacterial activity; glycosyltransferase; macrolide glycosides; *Streptomyces venezuelae*; YC-17

Introduction

Macrocyclic polyketides are a highly diverse and clinically important class of natural products that include antibiotics (erythromycin),[1] immunosuppressants (FK506),[2] antiparasitics (avermectin),[3] antifungal agents (amphotericin B),[4] and antitumor agents (epothilone)[5] (Figure 1A). Their structures are characterized by a macrolactone and are often decorated by one or more deoxy and amino sugar moieties. While the macrolactone is an important pharmacophore for the observed biological activity, many of these large ring systems are susceptible to degradation by the acidic pH of the stomach, esterases in the blood plasma, various hepatic enzymes, or the macrolide esterases of resistant pathogens. While analogue generation *via* macrolactone structural modification is necessary to increase the stability or efficacy of these clinically important pharmaceuticals, many of the next-generation structures remain susceptible to hydrolysis by the macrolide esterases produced by some antibiotic-resistant microbial pathogens.[6] Consequently, the need for bioactive molecules with new or improved pharmacological properties that can tolerate these conditions has further increased interest in generating novel macrolides.[7] For example, semisynthetic derivatives of erythromycin with modified aglycone structures, such as clarithromycin,[8] azithromycin,[9] and telithromycin,[10] display increased stability under acidic conditions (Figure 1B),[11] and the recent-

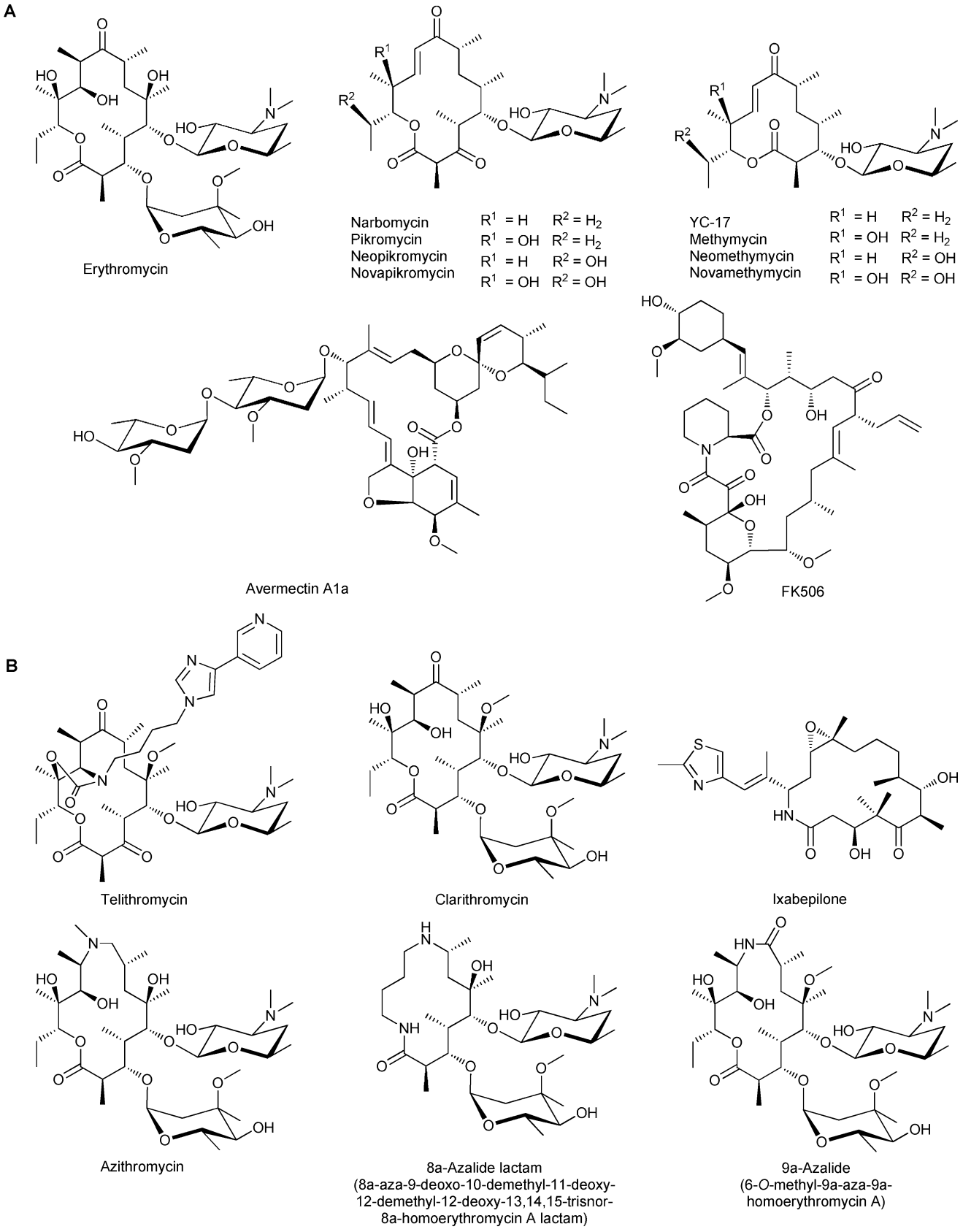


Figure 1. Representative structures of natural macrolides (**A**) and their semisynthetic derivatives (**B**).

ly approved anticancer agent ixabepilone (Figure 1B), a semisynthetic lactam analogue of epothilone B, was developed to increase metabolic stability against esterase cleavage.[12] In addition to modifying the macrolide aglycone, modification of the sugar moieties can also significantly alter biological activity profiles, and several chemical and enzymatic synthetic approaches for the diversification of macrolides have been employed.[13]

Since the synthesis of azithromycin,[9] many azalides have been reported containing nitrogen atoms attached to C-9, C-10, C-11, or C-12 (lactone carbonyl designated as C-1), and a few macrolactam analogues of erythromycin A have been synthesized (Figure 1B).[14] However, macrolactam analogues that bear structurally diverse sugar moieties have not been explored, and we were motivated to access and test the biological activity and selected pharmacological properties of this new class of molecules.

The synthesis of 12-membered macrolides bearing a single sugar group is generally more facile than the synthesis of 14-membered macrolides that contain two sugar moieties. In addition, 12-membered monoglycosylated macrolides still display significan biological activities. For example, in contrast to erythromycin, clarithromycin, and roxithromycin, methymycin possesses a 12-membered ring macrolactone, 10-deoxymethynolide (10-dml) which is appended to a single amino sugar, desosamine. Despite these chemical differences, methymycin (Figure 1A) retains antibiotic activity against Gram-positive bacteria similar to the larger polyglycosylated macrolide antibiotics.[15] Thus, YC-17 (Figure 1A) produced from *Streptomyces venezuelae* ATCC 15439 was selected for its relative ease of synthesis,[16] and a lactam analogue of this macrolide possessing diverse sugar moieties was envisioned. However, pathway engineering using a polyketide synthase (PKS) to replace the oxygen in the lactone moiety of the aglycone core with nitrogen is not available, and both the chemical synthesis of novel sugar moieties and the subsequent glycosylation with the corresponding aglycone are impractical. Therefore, we have employed a combined approach involving chemical synthesis and biotransformation to efficiently generate a series of macrolactam glycosides. First, a macrolactam analogue of the YC-17 aglycone 10-dml, aza-(10-dml) (AZDM; **1**), was chemically synthesized, and an engineered strain of *S. venezuelae* with its substrate-flexible glycosyltransferase was employed to assemble the new compounds.[7,17]

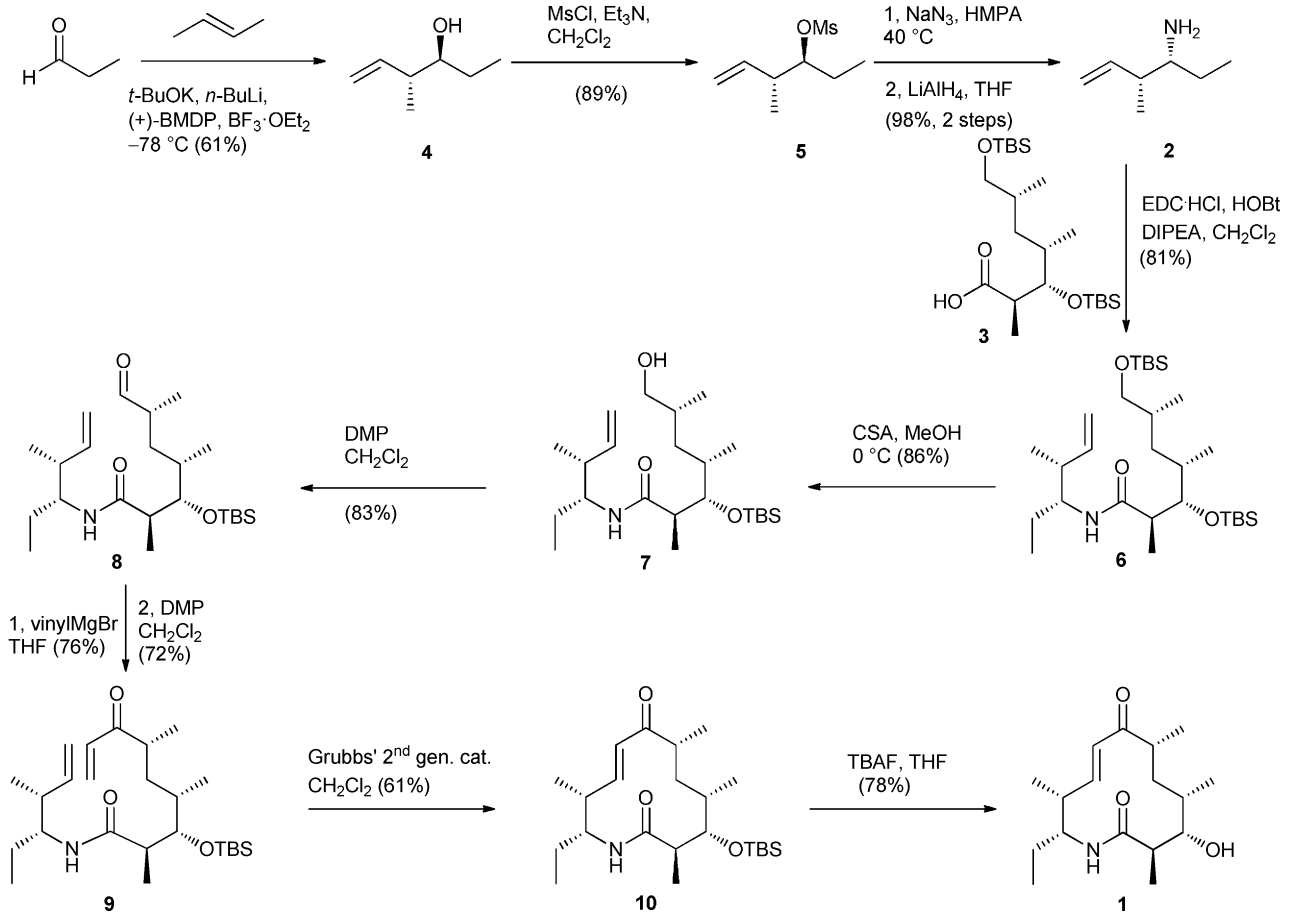
The *S. venezuelae* YJ028 mutant strain[18] in which the entire biosynthetic gene cluster encoding the pikromycin (Pik) PKS and desosamine biosynthetic enzymes were deleted was chosen as the biotransformation host, and different deoxy sugar biosynthetic gene cassettes and the genes encoding DesVII/DesVIII were expressed. In the wild-type *S. venezuelae* strain, Pik PKS generates both the 12- and 14-membered macrolactone rings of 10-dml and narbonolide, and the glycosyltransferase DesVII, along with its auxiliary partner DesVIII, transfer TDP-D-desosamine onto the aforementioned aglycones to give YC-17 and narbomycin, respectively. Following glycosylation, narbomycin is converted to pikromycin by the action of PikC cytochrome P450 monooxygenase. This P450 also converts narbomycin to neopikromycin and novapikromycin, and YC-17 to methymycin, neomethymycin, and novamethymycin.[19] Due to inherent flexibility, DesVII/DesVIII can transfer a range of structurally diverse TDP-sugars, demonstrating their potential for the structural diversification of macrolide antibiotics both *in vitro* and *in vivo*.[17]

The engineered strains of *S. venezuelae* capable of generating desosamine and a range of alternative sugar moieties produced new analogues which exhibited improved *in vitro* antibacterial activities and metabolic stabilities. To the best of our knowledge, this is the first synthesis of diversely glycosylated macrolactam analogues for a macrolide antibiotic, and the first report of DesVII/DesVIII recognizing and processing a lactam for glycosylation. By utilizing chemical synthesis to supply an unnatural functional group in combination with flexible biological machinery, novel macrolide analogues with increased therapeutic potential were generated.

Results and Discussion

Synthesis of Aza-(10-deoxymethynolide) (1)

The retrosynthetic analysis of AZDM **1** is shown in Scheme S1 (Supporting Information). Analogous to our previously reported synthesis of 10-dml, AZDM **1** could be synthesized from key amine **2** and known carboxylic acid **3**[16] using EDC coupling and ring closing metathesis (RCM) as the key steps. Our synthesis of AZDM **1** is summarized in Scheme 1. The desired amine **2** was prepared in a stereoselective manner in four steps starting from propanal. Crotylation of propanal was successfully performed to provide alcohol **4** according to the previously reported procedure.[20] Mesylation of alcohol **4** followed by successful nucleophilic substitution with sodium azide and reduction of the resultant azide with $LiAlH_4$ afforded amine **2**. This key amine was successfully coupled with carboxylic acid **3**[16] utilizing EDC to afford **6**. The primary TBS ether was selectively cleaved yielding alcohol **7**, which in turn was oxidized with DMP to yield aldehyde **8**. Grignard reaction of aldehyde **8** with vinylmagnesium bromide followed by DMP oxidation produced vinyl ketone **9**. RCM with the second generation Grubbs catalyst successfully provided protected macrolactam **10** (see Supporting In-



Scheme 1. Synthesis of AZDM **1**.

formation for detailed synthetic methods and Figure S1 to Figure S16 for ^{1}H and ^{13}C NMR spectra of the synthetic intermediates). The desired AZDM **1** was finally obtained upon cleavage of the TBS ether with TBAF. The structure of the synthesized macrolactam **1** was fully confirmed by 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC, and HMBC) NMR analysis aided by MS data (Supporting Information, Figure S17 to Figure S22).

Production and HPLC-ESI-MS/MS Analysis of AZDM Glycosides 11–18

In order to glycosylate AZDM **1**, four previously constructed expression plasmids[21] were independently introduced to the mutant strain *S. venezuelae* YJ028 to carry out both the biosynthesis of the unnatural TDP-deoxy sugars and the subsequent glycosylation of these sugars with the exogeneously supplied AZDM (**1**). These plasmids include pDDSS (expressing TDP-D-desosamine biosynthetic genes with glycosyltransferase genes *desVII*/*desVIII*), pLRHM2 (expressing TDP-L-rhamnose biosynthetic genes with *desVII*/*desVIII*), pDQNV (expressing TDP-D-quinovose biosynthetic genes with *desVII*/*desVIII*), and pLOLV2 (expressing TDP-L-olivose and TDP-L-digitoxose biosynthetic genes with *desVII*/*desVIII*) (Supporting Information, Table S1). Mutant strains were cultured in 50 mL of SCM liquid medium at 30 °C for 48 h under appropriate antibiotic selection. AZDM **1** (5 mg L^{-1}) was added into each growing medium and then incubated for an additional 48 h. Organic extracts of the culture broths were prepared and subjected to HPLC-ESI-MS analysis using a previously described method.[21] The HPLC chromatograms were monitored for common characteristic fragment ions arising from AZDM **1** at $m/z = 296$, 278, 260 and, thus, peaks for the glycosylated AZDMs were identified (Figure 2).

The mutant YJ028/pLRHM2 expressing L-rhamnose biosynthetic genes produced L-rhamnosyl-AZDM (**11**) (Figure 3A) and D-quinovosyl-AZDM (**12**) (Figure 3A) at relatively low levels. In the HPLC-ESI-MS analysis, glycosides **11** and **12** were eluted at 15.2 min and 16.5 min, respectively (Figure 2A), and both of these compounds showed similar mass spectra with their proton adduct ions appearing at $m/z = 442$. MS/MS analysis of these compounds produced the characteristic fragmentation pattern

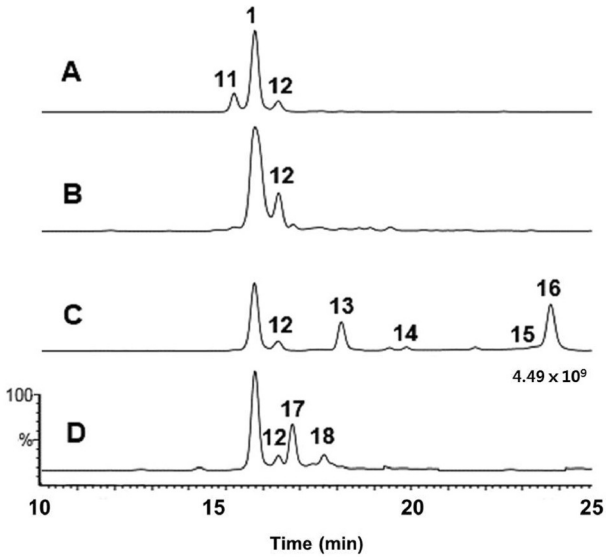


Figure 2. HPLC-ESI-MS extracted ion chromatograms ($m/z = 296$) of cultures of recombinant *S. venezuelae* strains. Compounds **11** ($[M+H]^+ = 442$ and **12** ($[M+H]^+ = 442$) detected from YJ028/pLRHM2 (**A**). Compound **12** ($[M+H]^+ = 442$) detected from YJ028/pDQNV (**B**). Compounds **12** ($[M+H]^+ = 442$), **13** ($[M+H]^+ = 426$), **14** ($[M+H]^+ = 467$), **15** ($[M+H]^+ = 453$), and **16** ($[M+H]^+ = 424$) detected from YJ028/pDDSS (**C**). Compounds **12** ($[M+H]^+ = 442$), **17** ($[M+H]^+ = 426$), and **18** ($[M+H]^+ = 426$) detected from YJ028/pLOLV2 (**D**). All chromatograms are drawn to the same scale.

with a difference between the parent and daughter peak corresponding to the loss of the neutral sugar (Supporting Information, Figure S23 and Figure S24). Macrolactam glycoside **12** may have arisen as a result of a previously observed *S. venezuelae* pathway independent 4-ketoreductase (Sv 4-KR).[22] Sv 4-KR can reduce TDP-4-keto-6-deoxy-D-glucose, a biosynthetic intermediate of TDP-L-rhamnose, to TDP-D-quinovose, which could then be appended to AZDM (**1**) by DesVII/DesVIII to afford **12** (Figure 3B). As expected, macrolactam glycoside **12** was also produced by mutant YJ028/pDQNV harboring the D-quinovose biosynthetic genes (Figure 2B). HPLC-ESI-MS analysis of the organic extract of the mutant YJ028/pDDSS possessing D-desosamine biosynthetic genes showed five peaks at retention times 16.5, 18.0, 19.7, 23.2, and 23.7 min with proton adduct ions $m/z = 442$, 426, 467, 453, and 424, respectively (Figure 2C), which were identified as macrolactam glycoside **12**, 3-*O*-demethyl-D-chalcosyl-AZDM (**13**), *N*-dedimethyl-*N*-acetyl-D-desosaminyl-AZDM (**14**), D-desosaminyl-AZDM (**15**), and 3-keto-4,6-dideoxy-D-glucosyl-AZDM (**16**), respectively (Figure 3A). In addition to the common fragment ions arising from AZDM, the proton adduct ions $m/z = 467$ and $m/z = 453$ of macrolactam glycosides **14** and **15** displayed a fragment ion at $m/z = 172$ and $m/z = 158$, respectively, confirming that TDP-*N*-dedimethyl-*N*-acetyl-D-desosamine and D-desosamine were transferred to their respective AZDM glycosides. The structures of glycosides **13** and **16** were predicted based on the fragmentation pattern according to the difference between the parent and daughter peaks corresponding to the loss of the neutral sugar (Supporting Information, Figure S25 to Figure S28). TDP-3-keto-4,6-dideoxy-D-glucose may be converted to TDP-3-*O*-demethyl-D-chalcose by the probable action of the *S. venezuelae* pathway independent 3-ketoreductase (Sv 3-KR; Figure 3B).[23] Subsequent glycosylation reaction of AZDM **1** with sugars TDP-3-keto-4,6-dideoxy-D-glucose, TDP-*N*-dedimethyl-*N*-acetyl-D-desosamine, and TDP-3-*O*-demethyl-D-chalcose yielded glycosides **16**, **14**, and **13**, respectively. The detection of a trace amount of glycoside **14** containing TDP-*N*-dedimethyl-*N*-acetyl-D-desosamine in this *S. venezuelae* YJ028/pDDSS strain supports the previously reported observation which suggests *N*-acetylation as a self-protecting reaction.[24] Mutant strain YJ028/pLOLV2 showed three peaks arising from AZDM glycosides at retention times of 16.5, 16.7, and 17.7 min (Figure 2D). These displayed proton adduct ions at $m/z = 442$, 426, and 426, respectively, and were identified as their respective macrolactam glycosides: macrolactam glycoside **12**, D-olivosyl-AZDM (**17**), and L-olivosyl-AZDM (**18**) (Figure S29 and Figure S30). Detection of glycoside **17** possessing TDP-D-olivose suggested the action of Sv 4-KR on the intermediate sugar TDP-4-keto-2,6-dideoxy-D-glucose (Figure 3B). Further hydroxylation of the AZDM glycosides catalyzed by the PikC P450 of the *S. venezuelae* mutant strains was not detected. This is most likely due to the inability of the PikC P450 hydroxylase to accept the macrolactam ring with altered sugars lacking an *N*,*N*-dimethylamino group, which is required for active site anchoring.[25]

Structural Characterization of AZDM Glycosides 11–13, 16, and 17

Glycosides **11**–**18** were obtained from their respective culture broths. These broths were processed[21] and subjected to repeated chromatographic separation affording pure compounds **11** (1.4 mg from 2.4 L culture of YJ028/pLRHM2), **12** (0.3 mg from 1.8 L culture of YJ028/pDQNV), **13** (1.2 mg from 2.4 L culture of YJ028/pDDSS), **16** (1.8 mg from 2.4 L culture of YJ028/pDDSS), and **17** (0.8 mg from 3.0 L culture of YJ028/pLOLV2). Macrolactam glycosides **14**, **15**, and **18** were only detected in trace amounts insufficient to obtain NMR spectroscopic data. The structures of the isolated glycosides **11**–**13**, **16**, and **17** were determined using 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC, HMBC, and NOESY) NMR, MS/MS, and HR-ESI-

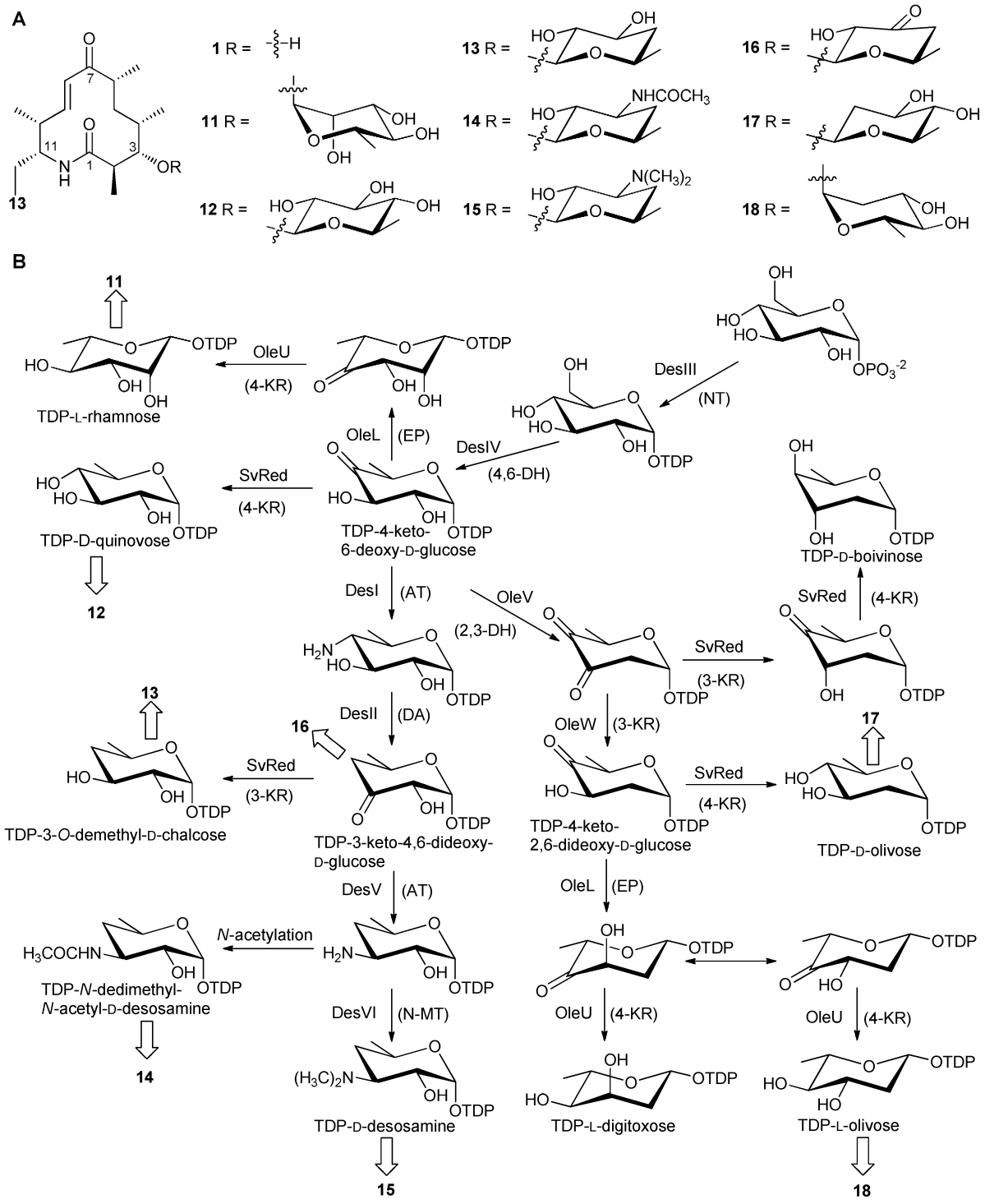


Figure 3. Structures of macrolactam glycosides **11**–**18** (**A**) and proposed biosynthesis of the different deoxy sugars directed by the plasmids (**B**). The functions of the proteins carrying out each step are shown in brackets: Nucleotidyl transferase (NT), 4,6-dehydratase (4,6-DH), 2,3-dehydratase (2,3-DH), aminotransferase (AT), deaminase (DA), epimerase (EP), 4-ketoreductase (4-KR), *N*-methyltransferase (N-MT), 3-ketoreductase (3-KR), *S. venezuelae* pathway independent reductase (SvRed).

MS data. The stereochemistry and conformation of the attached sugars in the isolated macrolactam glycosides were confirmed on the basis of coupling constant values (Supporting Information, Table S2) and NOE correlations (Supporting Information, Figure S31 to Figure S61).[21]

The sodium adduct ion observed at $m/z = 464.2614$ (calcd.: 464.2624) in the HR-ESI-MS data of AZDM

glycoside **11** suggested a molecular formula of $C_{23}H_{39}NO_7$. The ^{13}C NMR and HSQC spectra (Supporting Information, Figure S32 and Figure S34) indicated the presence of 23 carbons in the macrolactam glycoside: a ketone at $\delta_C = 207.6$ (C-7), an ester at $\delta_C = 177.4$ (C-1), two olefinic carbons at $\delta_C = 149.7$ (C-9) and $\delta_C = 126.5$ (C-8), an anomeric carbon at $\delta_C = 105.1$ (C-1′), five oxygenated methine carbons at $\delta_C = 91.2$ (C-3), 73.7 (C-4′), 72.5 (C-2′), 72.4 (C-3′), and 70.4 (C-5′), four methine carbons at $\delta_C = 51.9$ (C-11), 46.4 (C-6), 46.1 (C-2), and 39.6 (C-10), three methylene carbons at $\delta_C = 35.3$ (C-5), 34.3 (C-4), and 27.2 (C-12), and six methyl carbons at $\delta_C = 17.9$ (C-15), 17.6 (C-6′), 17.7 (C-14), 17.7 (C-16), 11.5 (C-13), and 10.0 (C-17). The ^1H NMR spectrum (Supporting Information, Figure S31) displayed the signals characteristic of these macrolide glycosides such as two olefinic protons at $\delta_H = 6.72$ (H-9) and 6.44 (H-8), an anomeric proton at $\delta_H = 4.67$ (H-1′), a methine proton at $\delta_H = 3.97$ (H-11), five oxygenated methine protons at $\delta_H = 3.92$ (H-2′), 3.71 (H-5′), 3.59 (H-3′), 3.48 (H-3), and 3.40 (H-4′), and six methyl group signals at $\delta_H = 1.24$ (H-6′), 1.24 (H-14), 1.23 (H-16), 1.07 (H-17), 1.00 (H-15), and 0.93 (H-13). The COSY spectrum (Supporting Information, Figure S33) showed the presence of three spin systems including one for the deoxy sugar (Supporting Information, Figure S61). All of these spin systems were connected with the help of HMBC correlations (Supporting Information, Figure S61). The attachment of the sugar moiety to the C-3 position of AZDM was confirmed using the HMBC spectrum (Supporting Information, Figure S35). Correlations were observed in the HMBC spectrum between the anomeric proton at $\delta_H = 4.67$ (H-1′) and the carbon at $\delta_C = 91.2$ (C-3) as well as between the oxygenated methine proton at $\delta_H = 3.48$ (H-3) and the anomeric carbon at $\delta_C = 105.1$ (C-1) confirming the point of attachment. Similarly, the positions of all methyl groups were determined on the basis of their HMBC correlation to their respective neighboring carbon centers (Supporting Information, Figure S61). The main difference between AZDM glycoside **11** and its lactone counterpart[21a] was the shift upfield for the C-11 ($\delta_C = 51.9$ for **11**, 74.6 for L-rhamnosyl-10-dml) and H-11 ($\delta_H = 3.97$ for **11**, 4.96 for L-rhamnosyl-10-dml) peaks. The sugar in **11** was confirmed as L-rhamnose on the basis of the small coupling constant values for H-1' (br s), H-2' (br s), indicating their equatorial orientation, and the large coupling constant values of H-3' (d, $J = 9.5$ Hz), H-4' (t, $J = 9.5$ Hz), H-5' (dq, $J = 9.5$, 7 Hz), indicating their axial orientation.[21] This configuration was also supported by the coupling constant between C-1' and H-1' ($^1J_{C,H} = 169$ Hz) and other literature citations reporting similar sugar moieties.[26]

The molecular formula for macrolactam glycoside **12** was deduced to be $C_{23}H_{39}NO_7$ with the help of HR-ESI-MS and ^{13}C NMR data. The HR-ESI-MS exhibited a peak at $m/z = 464.2627$ $[M+Na]^+$ (calcd.: 464.2624) and the ^{13}C NMR spectrum (Supporting Information, Figure S38) showed the presence of the 23 carbons required for the AZDM glycoside. The NMR data (Supporting Information, Table S2) for **12** were quite similar with that of **11** except for the expected difference in the chemical shift values for the sugar and C-3 position. The anomeric proton appeared as a doublet ($J = 8.1$ Hz) at $\delta_H = 4.27$ (H-1′) whereas the anomeric proton in **11** was broad singlet at $\delta_H = 4.67$ (H-1′); all other sugar protons exhibited an axial orientation as deduced from the large coupling constant values. The axial orientations were also supported by the NOE correlations (Supporting Information, Figure S42) between H-1′, H-3′, and H-5' (Supporting Information, Figure S61) indicating that the sugar in **12** was D-quinovose[21] The structure of **12** was supported by well assigned COSY, HSQC, and HMBC data (Supporting Information, Figures S39, S40, and S41).

AZDM glycoside **13** possessed a molecular formula of $C_{23}H_{39}NO_6$ assigned from the $[M+Na]^+$ ion observed at $m/z = 448.2673$ in the HR-ESI-MS. ^1H- and ^{13}C NMR data (Supporting Information, Table S2) showed it to be analogous to those of macrolactam **12** with 23 carbon centers. Careful comparison of the 1D- and 2D-NMR data of **13** with those of **12** indicated the presence of only four oxygenated carbons in the sugar moiety (including the anomeric carbon) at $\delta_C = 105.4$, 77.5, 72.5, and 68.8; and one additional methylene group at $\delta_C = 42.2$ ($\delta_H = 1.90$ and 1.27, H-4′) suggesting that the appended sugar moiety in **13** was a dideoxyhexose instead of the 6-deoxyhexose in **11** and **12**. Further analysis of the COSY and HMBC data (Supporting Information, Figure S45 and Figure S47) of **13** revealed that the additional methylene group was present at the C-4′ position and that the sugar moiety was 3-*O*-demethyl-D-chalcose due to the NOE correlations and the large coupling constant values for all the sugar protons (Supporting Information, Figure S48 and Figure S61).

The molecular formula of macrolactam glycoside **16** was deduced to be $C_{23}H_{37}NO_6$ on the basis of the sodium adduct ion observed at $m/z = 446.2510$ in the HR-ESI-MS. The NMR data (Supporting Information, Table S2) of **16** were very similar to those of **13** except for the presence of an additional ketone moiety giving rise to the signal at $\delta_C = 206.9$. The HMBC spectrum (Supporting Information, Figure S53) showed correlations between protons H-2′ ($\delta_H = 3.96$) and H-4′ ($\delta_H = 2.44$) and the carbonyl peak at $\delta_C = 206.9$ establishing it as C-3′ (Supporting Information, Figure S61). The large coupling constants of all the protons in the attached sugar moiety, supported by the NOE correlations (Supporting Information, Figure S54) between proton signals at $\delta_H = 4.39$ (d, $J = 8.0$ Hz, H-1′) and $\delta_H = 3.70$ (m, H-5′) (Supporting

Information, Figure S61), suggested that the appended sugar was 3-keto-4,6-dideoxy-glucose in the D-conformation.

The HR-ESI-MS of AZDM glycoside **17** displayed a sodium adduct ion peak at $m/z = 448.2672$ (calcd.: 448.2675) indicating $C_{23}H_{39}NO_6$ as its molecular formula. Analysis of the ^{1}H- and ^{13}C NMR data (Supporting Information, Table S2) suggested **17** was also an AZDM glycoside having a dideoxyhexose moiety. In the COSY spectrum (Supporting Information, Figure S57), the anomeric proton signal at $\delta_H = 4.47$ (d, $J = 9.5$, 1.5 Hz, H-1′) was coupled to the methylene protons at $\delta_H = 2.17$ (ddd, $J = 12.0$, 5.5, 1.5 Hz, H-2′a) and $\delta_H = 1.43$ (m, H-2′b), which showed further coupling to the oxygenated methine proton signal at $\delta_H = 3.48$ (ddd, $J = 12.0$, 9.0, 5.5 Hz, H-3′). This indicated that the sugar was 2,4-dideoxyhexose (Supporting Information, Figure S61). The large coupling constant values of all the sugar protons identified it as D-olivose and the NOE correlations (Supporting Information, Figure S60) between H-1′ ($\delta_H = 4.47$), H-3′ ($\delta_H = 3.48$), and H-5′ ($\delta_H = 3.17$) (Supporting Information, Figure S61) further supported this identification.[21]

Through the production of the macrolactam glycosides and analysis of the DesVII/DesVIII-catalyzed glycosylations in this study, a few novel observations were made. First, although previous work demonstrated that DesVII/DesVIII catalyzes the transfer of various sugars to unnatural macrolactones and even acyclic aglycones,[17,27] these results demonstrate for the first time that the macrolide glycosyltransferase DesVII/DesVIII can recognize and process a macrolactam aglycone providing a set of new glycosylated macrolactams. Second, even though L-digitoxosyl-narbonolide, D-boivinosyl-narbonolide, and D-boivinosyl-10-dml were previously isolated from a mutant strain carrying plasmid pLOLV2,[21] AZDM glycosides containing L-digitoxose and D-boivinose were not detected in this present study. Thirdly, to the best of our knowledge, this is the first report of the isolation of a glycoside containing TDP-3-keto-4,6-dideoxy-D-glucose, confirming it as a biosynthetic intermediate of TDP-D-desosamine. Additionally, in a previous study, a *desV* deletion *S. venezuelae* mutant was found to produce 3-*O*-demethyl D-chalcosylmethymycin instead of the expected methymycin analogue possessing TDP-3-keto-4,6-dideoxy-D-glucose.[28] These final two observations suggest that the glycosylation efficiency of DesVII/DesVIII towards different sugar donors varies with the structure of the aglycone (Supporting Information, Table S3). However, the intracellular concentration of each sugar donor and the exact glycosylation efficiency towards different sugar donors and acceptors cannot be measured in this *in vivo* system. Nevertheless, because the same expression plasmid and cloning strategy were used to express each sugar gene cassette, the level of expression for each gene cassette is expected to be similar. Therefore, the difference in the rate of production for macrolactam glycosides is likely due to the varying catalytic efficiency of the glycosyltransferase with respect to each substrate rather than a difference in the mutant strain growth behavior or difference in sugar gene cassette expression levels.

Assessment of *in vitro* Antibacterial Activity and Stability against Macrolide Esterase

Isolated compounds were evaluated for *in vitro* antibacterial activities against erythromycin-susceptible *Enterococcus faecium* ATCC 19434 and *Staphylococcus aureus* ATCC 25923 as well as against erythromycin-resistant clinical isolates *E. faecium* P00558 and *S. aureus* P00740 (Supporting Information, Table S4). Although the activities of most of the AZDM glycosides against the tested strains were similar to those of their macrolactone counterparts (Supporting Information, Table S5),[21a] AZDM glycosides **11** and **12** exhibited four times higher activity against erythromycin-susceptible pathogens when compared with erythromycin and were active against erythromycin-resistant pathogenic strains. AZDM glycosides **11** (MIC, 7.5–30 μmol) and **12** (7.5–30 μmol) containing 6-deoxyhexose L-rhamnose and D-quinovose, respectively, displayed better activities against all strains tested than those glycosides containing dideoxyhexose moieties (**13**, **16**, and **17**) (30–120 μmol) supporting the previous observation that the additional hydroxy group of **11** and **12** may positively influence their antibacterial activities.[21]

Because the resistance mechanisms of *E. faecium* P00558 and *S. aureus* P00740 are not known, we tested the susceptibility of erythromycin, L-rhamnosyl-10-dml, and AZDM glycoside **11** to the well-known macrolide esterase EreB[29] isolated from the erythromycin-resistant *Escherichia coli* to investigate the potential advantage of the macrolactam pharmacophore for the treatment of macrolide-resistant pathogens. The *ereB* gene (GenBank Accession No. P05789) was synthesized, overexpressed, and purified from *E. coli* BL21(DE3)pLysS. During HPLC-ESI-MS analysis (Figure 4A) of the reaction mixture containing erythromycin and EreB, it was observed that erythromycin (retention time 29.5 min) was initially converted partially to its linear form (retention time 22 min).[6] Additionally, at 1 h incubation, erythromycin was completely converted to the inactivation product (Figure 4D and E). The MS of both the substrate and product were similar to the previously reported spectra.[6] When L-rhamnosyl-10-dml and AZDM glycoside **11** were separately incubated with EreB and subjected to subsequent HPLC-ESI-MS analysis (Figure 4B and C), we observed that both

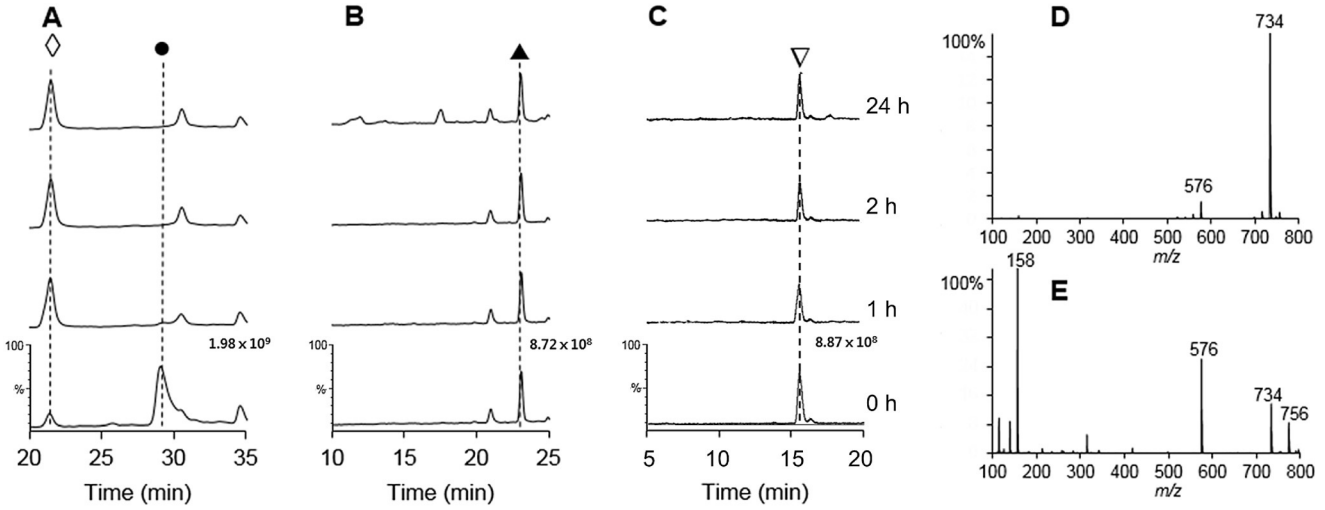


Figure 4. Erythromycin esterase assay. HPLC-ESI-MS total ion chromatograms of erythromycin (**A**), L-rhamnosyl-10-dml (**B**), and L-rhamnosyl-AZDM (**11**) (**C**) after incubation with esterase. Each chromatogram in (**A**), (**B**), and (**C**) is drawn to the same scale: linear erythromycin (◇), erythromycin (●), L-rhamnosyl-10-dml (▲), and L-rhamnosyl-AZDM (**11**) (▽). Mass spectra of erythromycin (**D**), and linear erythromycin (**E**).

compounds were not hydrolyzed by EreB even after 24 h incubation. The *m/z* values corresponding to the hydrolyzed products of L-rhamnosyl-10-dml (*m/z* = 297 and 443) and AZDM glycoside **11** (*m/z* = 296 and 442) were not observed on the extracted ion chromatogram (EIC) (Supporting Information, Figure S62). It is plausible that EreB may be an esterase specific to 14-membered macrolides and may not be able to recognize 12-membered macrolides (L-rhamnosyl-10-dml and AZDM glycoside **11**) as substrates. Nevertheless, these results demonstrate that macrolactam glycosides such as AZDM glycoside **11** are not susceptible to the microbial esterases that can degrade erythromycin, and, although the resistance mechanisms of *E. faecium* P00558 and *S. aureus* P00740 are not known, these macrolactam glycosides are still active against erythromycin-resistant pathogens as displayed by their antibacterial activity (Supporting Information, Table S4).

Assessment of *in vitro* Stability in Simulated Gastric Fluid and Liver Microsomes

Since AZDM glycoside **11** and its macrolactone derivative (L-rhamnosyl-10-dml)[21a] displayed stronger antibacterial activity than erythromycin, they were subjected to simulated gastric fluid (SGF) and liver microsomal metabolic stability assays with the aim of finding the differences in gastric and metabolic stabilities among macrolactam glycosides, macrolactone glycosides, and erythromycin. When erythromycin was incubated with SGF, HPLC-ESI-MS analysis of the reaction mixture indicated partial degradation of erythromycin immediately after incubation, with the drug almost completely disappearing after a 2 h incubation, and giving rise to its degradation product 5-desosaminyl-erythronolide A lacking the cladinose sugar (Figure 5A).[30] The erythromycin peak eluted at 29.5 min and showed the $[M+H]^+$ ion at *m/z* = 734 and the peak due to desosaminyl-erythronolide A eluted at 33 min and displayed $[M+Na]^+$ and $[M+H]^+$ ions at *m/z* = 562 and 540, respectively (Figure 5D and E). The difference between the proton adduct ions of erythromycin and 5-desosaminyl-erythronolide A was accounted for by the loss of the mass due to cladinose as expected. Macrolactam **11** and L-rhamnosyl-10-dml, however, were intact after 2 h incubation in SGF (Figure 5B and C), and aglycone ion peaks resulted from the loss of the rhamnose moiety from the L-rhamnosyl-10-dml (*m/z* = 297 for the 10-dml $[M+H]^+$) and AZDM glycoside **11** (*m/z* = 296 for the AZDM $[M+H]^+$) were not found on the EIC (Supporting Information, Figure S63). Cladinose is known to be cleaved directly from erythromycin under acidic conditions giving rise to its degradation products[30] and the 6-hydroxy group also plays an important role in the acid degradation of erythromycin by forming a spiroketal ring. Hence, clarithromycin, the 6-methoxy derivative of erythromycin, was found to be stable in acidic conditions.[31] In contrast, the glycosidic linkages of AZDM glycoside **11** and L-rhamnosyl-10-dml seem to be more stable in SGF, and should allow both AZDM glycoside **11** and L-rhamnosyl-10-dml to survive the acidic environment of the stomach. Since a β-glycosidic bond is comparatively more stable than an α-glycosidic bond,[32] the better stability of **11** and L-rhamnosyl-10-dml over erythromycin might have resulted from the combination of the presence of a β-glycosidic linkage and the absence of cor-

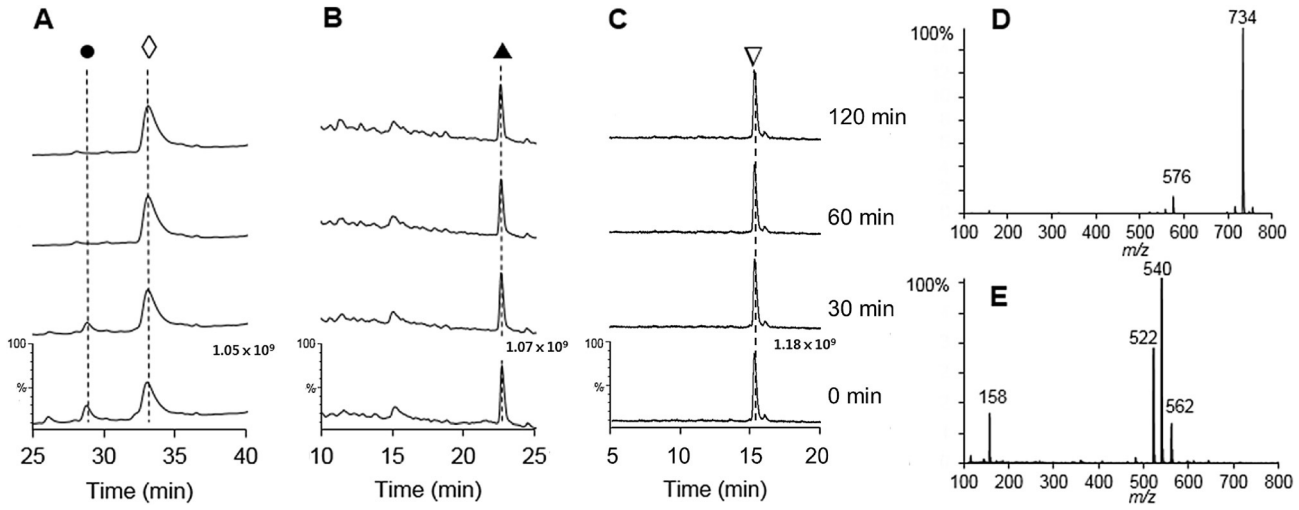


Figure 5. Simulated gastric fluid (SGF) assay. HPLC-ESI-MS total ion chromatograms of erythromycin (**A**), L-rhamnosyl-10-dml (**B**), and L-rhamnosyl-AZDM (**11**) (**C**) after incubation with SGF. Each chromatogram in (**A**), (**B**), and (**C**) is drawn to the same scale: erythromycin (●), 5-desosaminyl-erythronolide A (◇), L-rhamnosyl-10-dml (▲), and L-rhamnosyl-AZDM (**11**) (▽). Mass spectra of erythromycin (**D**), and 5-desosaminyl-erythronolide A (**E**).

responding 6-hydroxy group. Erythromycin has a short elimination half-life as it undergoes rapid metabolism, primarily in the liver, to the inactive *N*-desmethyl-erythromycin, which is immediately eliminated through bile.[33] Other macrolide antibiotics are also metabolized to descladinosyl, *N*-desmethyl, and hydroxyl derivatives by various hepatic hydrolytic and oxidative enzymes.[34] To ascertain the metabolic pattern, erythromycin, L-rhamnosyl-10-dml, and AZDM glycoside **11** were incubated with rat liver microsomes and NADPH. Consequently, erythromycin and L-rhamnosyl-10-dml were metabolized to *N*-desmethyl-erythromycin and hydroxylated L-rhamnosyl-10-dml, respectively (Figure 6A, B, D, and E) after incubation for 2 h. HPLC-ESI-MS analysis of the reaction mixture containing erythromycin exhibited two major peaks (Figure 6A): the peak eluted at 29.5 min was identified as erythromycin from its $[M+H]^+$ ion at $m/z = 734$, and the other peak, which eluted at 27 min, displayed an $[M+Na]^+$ ion at $m/z = 742$ and an $[M+H]^+$ ion at $m/z = 720$ (Figure 6D). This new metabolite was identified as *N*-desmethyl-erythromycin on the basis of the 14 Da observed difference to that of erythromycin. This is further supported by the presence

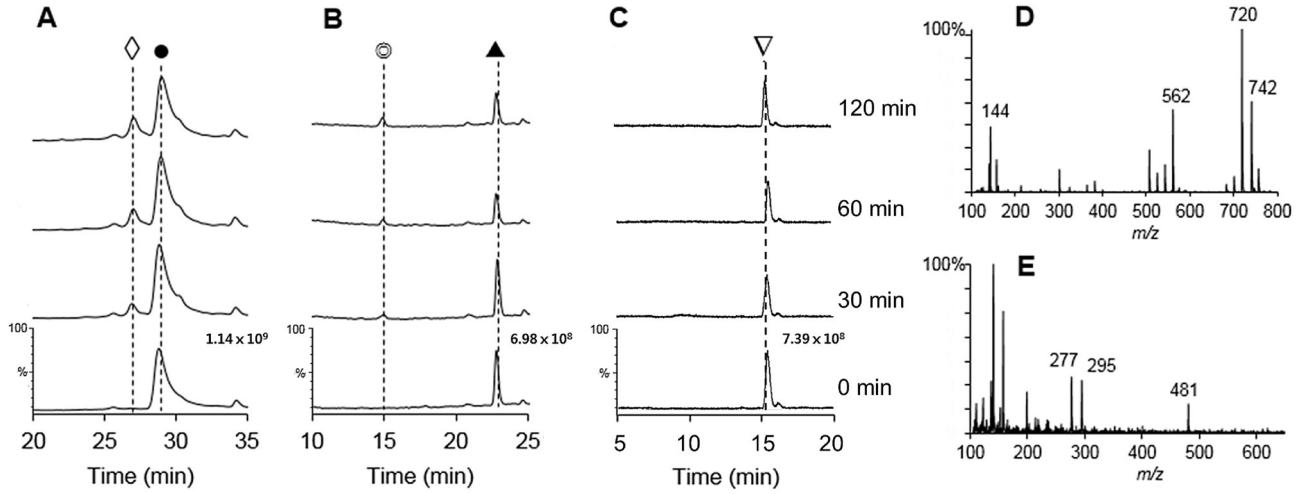


Figure 6. Liver microsomal assay. HPLC-ESI-MS total ion chromatograms of erythromycin (**A**), L-rhamnosyl-10-dml (**B**), and L-rhamnosyl-AZDM (**11**) (**C**) after incubation with liver microsomes. Each chromatogram in (**A**), (**B**), and (**C**) is drawn to the same scale: *N*-desmethyl-erythromycin (◇), erythromycin (●), hydroxylated L-rhamnosyl-10-dml (◎), L-rhamnosyl-10-dml (▲), and L-rhamnosyl-AZDM (**11**) (▽). Mass spectra of *N*-desmethyl-erythromycin (**D**) and hydroxylated L-rhamnosyl-10-dml (**E**).

of the [*N*-desmethyl-desosamine]$^+$ ion peak at $m/z=144$ since the [desosamine]$^+$ ion peak appears at $m/z=158$ – a 14 Da difference. Similarly, HPLC-ESI-MS analysis of the assay with L-rhamnsoyl-10-dml also showed two peaks after incubation with rat liver microsomes (Figure 6B). The peak eluted at 22.7 min exhibited the characteristic ions $[M+Na]^+$, $[M+NH_4]^+$, and $[M+H-rhamnose-H_2O]^+$ at $m/z=465$, 460, and 279, respectively, confirming L-rhamnosyl-10-dml as the structure (Supporting Information, Figure S64). In contrast, the peak eluted at 15 min showed $[M+Na]^+$, $[M+H-rhamnose-H_2O]^+$ and $[M+H-rhamnose-2H_2O]^+$ ions at $m/z=481$, 295, and 277 (Figure 6E). The difference of 16 Da between the corresponding ions with those of L-rhamnsoyl-10-dml indicated that the metabolite at 15 min is its hydroxylated analogue. Careful comparison of both spectra from L-rhamnosyl-10-dml and its hydroxylated analogue revealed the shift in fragment ions $[M+H-rhamnose-H_2O]^+$ and $[M+H-rhamnose-2H_2O]^+$ from $m/z=279$ to $m/z=295$ and $m/z=261$ to $m/z=277$, respectively, suggesting that hydroxylation occurred on the aglycone core and not on the rhamnose sugar. Since it has been reported that clarithromycin is metabolized to 16-hydroxy-clarithromycin,[35] the obtained hydroxylated metabolite of L-rhamnosyl-10-dml is likely functionalized at the C-12 position. However, AZDM glycoside **11** was found to be stable for the duration of the 2 h incubation with rat liver microsomes (Figure 6C). The stability of AZDM glycoside **11** was also supported by the absence of desmethylated ($m/z=428$ and 281) or hydroxylated ($m/z=480$, 294, and 276) product ion peaks on the EIC (Supporting Information, Figure S65). This result suggests that AZDM glycosides, such as compound **11**, would ideally survive first-pass metabolism and remain in the body longer than erythromycin and related macrolactone glycosides. Thus, the AZDM glycosides may exert a prolonged antibiotic action similar to azithromycin.[34] Overall it can be concluded that AZDM glycosides are more stable in gastric acid than erythromycin, are more metabolically stable than both erythromycin and macrolactone glycosides, and cannot be hydrolyzed by erythromycin esterase. These results with the low cytotoxicity of compounds **11** and **12** against human cell line HEK 293 ($IC_{50}>50$ μmol, both compounds, Supporting Information) suggest the potential usability of these compounds.

Conclusions

The development of a macrolactam aglycone based on YC-17 by synthetic chemistry combined with the cell-based generation of unnatural deoxyhexose sugars and aglycone biotransformation has enabled the production of a diverse set of biologically active macrolactam glycosides. To the best of our knowledge, this is the first example of a set of diversely glycosylated macrolactam analogues of a macrolide antibiotic, and to the best of our knowledge, this is also the first example of DesVII/DesVIII desosaminyl glycosyltransferase accepting and glycosylating a macrolactam to generate an array of new macrolactam glycosides. Some of the novel AZDM glycosides display improved antibacterial activities compared to erythromycin against both erythromycin-susceptible and erythromycin-resistant pathogens. Moreover, these new antibiotics are resistant to degradation by the bacterial macrolide esterases that degrade erythromycin. The AZDM glycosides described here also show improved *in vitro* stability in SGF relative to erythromycin and show greater stability than both erythromycin and the corresponding macrolactone glycosides in rat liver microsome assays. These results demonstrate the successful application combining chemical synthesis with inherently flexible biosynthetic enzymes to generate a range of novel glycosylated macrolactam analogues of YC-17. A series of assays demonstrated that some of these new molecules have enhanced pharmacological properties and support this approach as a powerful strategy for structural diversification to create novel macrolactam glycosides with potent antibiotic activity for drug discovery and development programs.

Experimental Section

General Procedures

NMR spectra were recorded on Bruker Avance 900 MHz, Varian INOVA 500 MHz, and Bruker Avance III 400 MHz spectrometers. Chemical shifts are given in ppm using tetramethylsilane (TMS) as an internal reference. CD_3OD and $CDCl_3$ were used as NMR solvent and the chemical shift values were corrected to 3.31/49.1 ppm and 7.27/77.0 ppm for $^1H/^{13}C$ nuclei, respectively. Mnova software from Mestrelab Research S.L. was employed for NMR data processing. HPLC-ESI-MS/MS using an analytical reversed-phase Nova-Pak C18 column (Waters, Milford, MA, USA; 150×3.9 mm, 4.0 μm) were recorded on a Waters/Micromass Quattro micro MS interface consisting of a Waters 2695 separation module connected directly to a Micromass Quattro micro MS. The HR-ESI-MS data were obtained using a Waters SYNAPT G2-S mass spectrometer coupled with UPLC. HPLC purification was done using preparative Spherisorb S5 ODS2 (Waters, 250×20 mm, 5 μm) and semi-preparative Watchers 120 ODS-BP (250×10 mm, 5 μm) columns on an Acme 9000 HPLC system (YL Instrument Co. Ltd., Korea) consisting of an SP930D gradient pump coupled with a UV730D UV detector set to 220 nm.

Synthesis of Aza-(10-deoxymethynolide) (1)

The chemical synthesis of the synthetic intermediates as well as compound **1** are described in the Supporting Information.

Bacterial Strains, Culture Conditions, and Genetic Manipulation

S. venezuelae mutant strains were propagated on SPA agar (1 g yeast extract, 1 g beef extract, 2 g tryptose, 10 g glucose, trace amount of ferrous sulfate, and 15 g agar per L).[36] Standard protocols were followed for protoplast preparation and transformation of *S. venezuelae*.[36] To select the transformants of *S. venezuelae*, R2YE agar plates[36] supplemented with thiostrepton were employed. *S. venezuelae* ATCC 15439[37] and *Streptomyces antibioticus* ATCC 11891[38] were used for obtaining genomic DNA. Routine sub-cloning was done using plasmid Litmus28 (New England Biolabs) and *Escherichia coli* DH5α (Invitrogen). For expressing the genes involved in the biosynthesis of deoxy sugars and their transfer, a high-copy-number *E. coli-Streptomyces* shuttle vector, pSE34,[39] containing the constitutive *ermE** promoter[40] and a thiostrepton resistance marker,[41] was used.

Construction of Plasmids and Mutants

Construction of pDDSS, pDQNV, pLRAM2, and pLOLV2 was previously described.[21] These plasmids were separately introduced into *S. venezuelae* YJ028[18] and the resulting recombinants were used for *in vivo* glycosylation of the synthesized AZDM **1**. Details regarding the construction of plasmids are described in the Supporting Information (see also Table S1 for combined gene organizations).

Production and HPLC-ESI-MS/MS Analysis of AZDM Glycosides

The recombinants YJ028/pDDSS, YJ028/pDQNV, YJ028/pLRAM2, and YJ028/pLOLV2 were cultured in SGGP liquid medium (Tryptone 4 g, yeast extract 4 g, $MgSO_4$ 0.5 g, glucose 10 g, glycine 2 g, and potassium phosphate buffer 0.01 mol per 1 L)[36] containing the antibiotic thiostrepton in a concentration of 25 $\mu g\,mL^{-1}$ at 30 °C for 24 h. This seed culture (5 mL) was used to inoculate 50 mL of SCM medium (1.5% soluble starch, 2.0% soytone, 0.01% $CaCl_2$, 0.15% yeast extract, and 1.05% MOPS, pH 7.2)[26] in 250-mL baffled Erlenmeyer flasks to which antibiotic thiostrepton (25 $\mu g\,mL^{-1}$) was added. This was initially incubated at 30 °C for 48 h followed by feeding of the synthesized AZDM **1** in a concentration of 5 $\mu g\,mL^{-1}$ and then further incubated for 48 h. Each grown culture was centrifuged (5,000 × *g* for 10 min) and the supernatant was passed through an OASIS HLB (Waters, Milford, MA) SPE column previously conditioned with 5 mL methanol and 5 mL water. The column was washed with 5 mL 20% (vol/vol) methanol and then air-dried for *ca.* 30 s. The column was eluted three times with 1 mL 0.5% (vol/vol) methanolic acetic acid and the organic extracts were concentrated under vacuum. HPLC-ESI-MS analysis was performed with a flow rate of 250 $\mu L\,min^{-1}$ using a gradient of 5 mmol ammonium acetate–0.05% (vol/vol) acetic acid in water (solution A) and 80% (vol/vol) aqueous acetonitrile with the same additive concentrations (solution B) at 20 to 70% solution B for 25 min, to 90% solution B for 15 min, maintained at 90% solution B for 9 min, and then to 20% solution B for another 11 min for column re-equilibration.

Isolation and Identification of AZDM Glycosides

The whole culture broth (2.4 L) of strain *S. venezuelae* YJ028/pLRHM2 was centrifuged, and the supernatant was subjected to solvent-solvent partition with ethyl acetate. The obtained extract was evaporated, and the resultant brown residue was fractionated by reversed-phase semipreparative HPLC employing gradient elution (40% aqueous methanol to 60 min, to 100% aqueous methanol for 61–72 min, to 40% aqueous methanol for 72–82 min) with a flow rate of 2 $mL\,min^{-1}$. The fraction containing AZDM glycoside **11** was purified by reversed-phase semipreparative HPLC employing 18% aqueous acetonitrile as mobile phase with a flow rate of 2 $mL\,min^{-1}$ to afford the pure compound as an amorphous white solid (yield: 1.4 mg).

The residue obtained from the whole culture broth (1.8 L) of strain *S. venezuelae* YJ028/pDQNV was fractionated by reversed-phase semipreparative HPLC employing gradient elution (44% aqueous methanol to 37 min, to 100% aqueous methanol for 38–48 min, to 44% aqueous methanol for 49–59 min) with a flow rate of 2 $mL\,min^{-1}$. The fraction containing AZDM glycoside **12** was further purified by reversed-phase semipreparative HPLC using 20% aqueous acetonitrile as mobile phase with a flow rate of 2 $mL\,min^{-1}$ to yield the pure compound (0.3 mg). The whole culture broth (2.4 L) of strain *S. venezuelae* YJ028/pDDSS was processed similarly to obtain a brown extract. This brown residue was fractionated by reversed-phase semipreparative HPLC employing gradient elution (50% aqueous methanol to 25 min, to 60% aqueous methanol for 35–45 min, to 50% aqueous methanol for 46–55 min) with a flow rate of 2 $mL\,min^{-1}$. The fraction containing AZDM glycoside **16** was further purified by reversed-phase semipreparative HPLC employing 28% aqueous acetonitrile as mobile phase with a flow rate of 2 $mL\,min^{-1}$ to yield the pure compound as an amorphous white solid (yield: 1.8 mg).

Similarly AZDM glycoside **13** was obtained by purifying the fraction containing the target compound on reversed-phase semipreparative HPLC employing 28% aqueous acetonitrile as mobile phase with a flow rate of 2 $mL\,min^{-1}$ to furnish the pure compound (yield: 1.2 mg).

The residue obtained from the whole culture broth (3.0 L) of strain YJ028/pLOLV2 was grossly separated by reversed-phase open column chromatography using gradient elution starting from 50% aqueous methanol to 100% aqueous methanol. Obtained fractions were analyzed by HPLC-ESI-MS/MS for the presence of the macrolactam glycosides and the fraction containing AZDM glycoside **17** was further separated by reversed-phase semipreparative HPLC employing 23% aqueous acetonitrile as mobile phase with a flow rate of 2 $mL\,min^{-1}$ to obtain the pure compound (yield: 0.8 mg).

L-Rhamnosyl-AZDM (11): amorphous white powder; ^1H- and ^{13}C NMR, see Table S2 (Supporting Information); (+)-ESI-MS: $m/z = 459$ $[M+NH_4]^+$, 442 $[M+H]^+$, 406 $[M+H-2H_2O]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 260 $[M+H-sugar-2H_2O]^+$; HR-ESI-MS: $m/z =$ 464.2614 $[M+Na]^+$ (calcd. for $C_{23}H_{39}NO_7Na$: 464.2624).

D-Quinovosyl-AZDM (12): amorphous white powder; ^{1}H- and ^{13}C NMR, see Table S2 (Supporting Information); (+)-ESI-MS: $m/z = 459$ $[M+NH_4]^+$, 442 $[M+H]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 260 $[M+H-sugar-2H_2O]^+$; HR-ESI-MS: $m/z = 464.2627$ $[M+Na]^+$ (calcd. for $C_{23}H_{39}NO_7Na$: 464.2624).

3-*O*-Demethyl-D-chalcosyl-AZDM (13): amorphous white powder; ^{1}H- and ^{13}C NMR, see Table S2 (Supporting Information); (+)-ESI-MS: *m/z* 448 $[M+Na]^+$, 443.0 $[M+NH_4]^+$, 426 $[M+H]^+$, 408 $[M+H-H_2O]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 260 $[M+H-sugar-2H_2O]^+$; (+)-HR-ESI-MS: $m/z = 448.2673$ $[M+Na]^+$ (calcd. for $C_{23}H_{39}NO_6Na$: 448.2675).

***N*-Dedimethyl-*N*-acetyl-D-desosaminyl-AZDM (14):** (+)-ESI-MS: $m/z = 489$ $[M+Na]^+$, 467 $[M+H]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 172 $[sugar]^+$.

D-Desosaminyl-AZDM (15): (+)-ESI-MS: $m/z = 475$ $[M+Na]^+$, 453 $[M+H]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 158 $[sugar]^+$.

3-Keto-4,6-dideoxy-D-glucosyl-AZDM (16): amorphous white powder; ^{1}H- and ^{13}C NMR, see Table S2 (Supporting Information); (+)-ESI-MS: $m/z = 446$ $[M+Na]^+$, 441 $[M+NH_4]^+$, 424 $[M+H]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 260 $[M+H-sugar-2H_2O]^+$; (+)-HR-ESI-MS: $m/z = 446.2510$ $[M+Na]^+$ (calcd. for $C_{23}H_{37}NO_6Na$: 446.2519).

D-Olivosyl-AZDM (17): amorphous white powder; ^{1}H- and ^{13}C NMR, see Table S2 (Supporting Information); (+)-ESI-MS: $m/z = 448$ $[M+Na]^+$, 443 $[M+NH_4]^+$, 426 $[M+H]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 260 $[M-H-sugar-2H_2O]^+$; HR-ESI-MS: $m/z = 448.2672$ $[M+Na]^+$ (calcd. for $C_{23}H_{39}NO_6Na$: 448.2675).

L-Olivosyl-AZDM (18): amorphous white powder; (+)-ESI-MS: $m/z = 448$ $[M+Na]^+$, 426 $[M+H]^+$, 296 $[M+H-sugar]^+$, 278 $[M+H-sugar-H_2O]^+$, 260 $[M+H-sugar-2H_2O]^+$.

Antibacterial Activity Assay

The erythromycin-resistant strains *Enterococcus faecium* P00558 and *Staphylococcus aureus* P00740 were clinically isolated as previously described.[21] The erythromycin-susceptible strains *E. faecium* ATCC 19434 and *S. aureus* ATCC 25923 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The details of the antibacterial assay, performed in triplicate, have been described previously.[21]

Stability Study with Erythromycin Esterase

DNA encoding *ereB* from *E. coli* was synthesized by Bioneer (Republic of Korea). This DNA fragment was digested with *Nde*I and *Bam*HI and then cloned into pET15b (Novagen, Germany) containing an N-terminal His_6-tag. The expression plasmid was introduced into *E. coli* BL21(DE3)-pLysS (Novagen) for overexpression under the T7 promoter. Cells were grown in LB medium supplemented with 50 $\mu g\,mL^{-1}$ ampicillin and 25 $\mu g\,mL^{-1}$ chloramphenicol. Each liter of culture was inoculated with 10 mL of overnight starter culture. The culture was grown at 37 °C to an optical density (OD_{600}) of 0.6, and then expression was induced for 3 h with 0.1 mmol isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation (6,000 × *g* for 10 min), resuspended in lysis buffer (50 mmol sodium phosphate buffer, pH 8.0, containing 300 mmol NaCl and 10 mmol imidazole), and then lysed by sonication. Clarified cell lysate was passed through a Ni-NTA superflow column (Qiagen). After washing the column with washing buffer (50 mmol sodium phosphate buffer, pH 8.0, containing 300 mmol NaCl and 20 mmol imidazole), N-His_6-tagged recombinant proteins were eluted with elution buffer (50 mmol sodium phosphate buffer, pH 8.0, containing 300 mmol NaCl and 500 mmol imidazole). The purified protein was subjected to 13% SDS-PAGE and visualized with Coomassie blue stain. The resultant protein was dialyzed against 50 mmol HEPES (pH 7.5) containing 150 mmol NaCl and 2% (v/v) glycerol, and then stored at −80 °C before use in the *in vitro* reaction. 8 μg of erythromycin, L-rhamnosyl-10-dml, and AZDM glycoside **11** were transferred to separate 2-mL glass vials. 100 μL phosphate buffer were added into each glass vial, and the reaction was started by adding 2 μmol of EreB. These reaction mixtures were incubated at 37 °C in a shaking water bath (150 rpm) for 0, 1, 2, and 24 h. The reactions were stopped by adding 150 μL cold acetonitrile. The reaction mixtures were centrifuged (3,000 × *g* for 10 min) at 4 °C, the supernatant was separated, and then HPLC-ESI-MS/MS analysis was performed. This experiment was carried out in triplicate form.

Stability Study in Simulated Gastric Fluid (SGF)

AZDM glycoside **11** and L-rhamnosyl-10-dml were selected for determining the stability in SGF. Erythromycin was also subjected to stability assay as a control. The SGF was prepared following the specifications in the United States Pharmacopeia (NaCl 2 g, pepsin 3.2 g, and HCl 7 mL, per 1 L). Three stock solutions were prepared by dissolving 0.25 mg of each compound in 1 mL of methanol. 200 μL aliquots of each compound were transferred to 2-mL vials and after evaporating methanol, 1 mL SGF was added into each vial. Aliquots (100 μL) from these solutions were incubated in six glass vials of 2-mL capacity at 37 °C in a shaking water bath (120 rpm) for 0, 30, 60, and 120 min.[42] The whole experiment was performed in triplicate and reaction mixtures were analyzed by HPLC-ESI-MS/MS using above mentioned conditions to see degradation profiles.

Metabolic Stability Study with Rat Liver Microsomes

Similarly, stock solutions of 2 $\mu g\,\mu L^{-1}$ were prepared by separately dissolving AZDM glycoside **11**, L-rhamnosyl-10-dml, and erythromycin in DMSO. 20 μL DMSO solutions of each compound were transferred to 2-mL glass vials and 655 μL phosphate buffer was added followed by the addition of 500 μg rat liver microsomal proteins (Sigma–Aldrich, USA). 100 μL aliquots of each reaction mixture were transferred into six glass vials of 2-mL capacity and were preincubated at 37 °C in a shaking water bath (150 rpm) for 5 min. 500 μg of NADPH were added into each vial and the reaction mixtures were further incubated for 0, 30, 60, and 120 min. The reactions were terminated by adding 150 μL cold acetonitrile.[43] The reaction mixtures were centrifuged (3,000 × g for 10 min) at 4 °C for 10 min and the supernatant was analyzed by HPLC-ESI-MS/MS. This experiment was carried out in triplicate.

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