Tandem Action of Glycosyltransferases in the Maturation of Vancomycin and Teicoplanin Aglycones: Novel Glycopeptides[†,‡]

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ABSTRACT: The glycopeptides vancomycin and teicoplanin are clinically important antibiotics. The carbohydrate portions of these molecules affect biological activity, and there is great interest in developing efficient strategies to make carbohydrate derivatives. To this end, genes encoding four glycosyltransferases, GtfB, C, D, E, were subcloned from *Amycolatopsis orientalis* strains that produce chloroeremomycin (GtfB, C) or vancomycin (GtfD, E) into *Escherichia coli*. After expression and purification, each glycosyltransferase (Gtf) was characterized for activity either with the aglycones (GtfB, E) or the glucosylated derivatives (GtfC, D) of vancomycin and teicoplanin. GtfB efficiently glucosylates vancomycin aglycone using UDP-glucose as the glycosyl donor to form desvancosaminyl-vancomycin (vancomycin pseudoaglycone), with k_{cat} of 17 min^{-1}, but has very low glucosylation activity, $\leq$ 0.3 min^{-1}, for an alternate substrate, teicoplanin aglycone. In contrast, GtfE is much more efficient at glucosylating both its natural substrate, vancomycin aglycone (k_{cat} = 60 min^{-1}), and an unnatural substrate, teicoplanin aglycone (k_{cat} = 20 min^{-1}). To test the addition of the 4-*epi*-vancosamine moiety by GtfC and GtfD, synthesis of UDP-β-L-4-*epi*-vancosamine was undertaken. This NDP-sugar served as a substrate for both GtfC and GtfD in the presence of vancomycin pseudoaglycone (GtfC and GtfD) or the glucosylated teicoplanin scaffold, **7** (GtfD). The GtfC product was the 4-*epi*-vancosaminyl form of vancomycin. Remarkably, GtfD was able to utilize both an unnatural acceptor, **7**, and an unnatural nucleotide sugar donor, UDP-4-*epi*-vancosamine, to synthesize a novel hybrid teicoplanin/vancomycin glycopeptide. These results establish the enzymatic activity of these four Gtfs, begin to probe substrate specificity, and illustrate how they can be utilized to make variant sugar forms of both the vancomycin and the teicoplanin class of glycopeptide antibiotics.

Two members of the glycopeptide antibiotic family, vancomycin, **1**, and teicoplanin, **2**, (Scheme 1) have been approved for human therapeutic use in the treatment of life-threatening bacterial infections caused by such Gram-positive pathogens as *Staphylococcus aureus* and *Enterococcus faecium* and *faecalis*. However, with the global emergence of vancomycin-resistant Enterococci (VRE)[1] over the past decade, there are few effective antibiotic treatments remaining. Researchers are now searching for vancomycin and teicoplanin analogues with increased activity against VRE (*1*−*3*).

While the heptapeptide-derived aglycone moieties of vancomycin and teicoplanin are similar, they represent two major subclasses of this antibiotic family. After synthesis of the acyclic heptapeptide by a nonribosomal peptide synthetase assembly line, the vancomycin linear heptapeptide is oxidatively cyclized at the aryl side chains of residues 2−4−6 and 5−7 to create the rigid scaffold of the mature aglycone. The teicoplanin aglycone has these cross-links and another between the aryl side chains of the first residue, 4-hydroxyphenylglycine (4-OH-Phegly$_1$), and the third residue, 3,5-dihydroxyphenylglycine (3,5-(OH)$_2$-Phegly$_3$), while vancomycin has no cross-link between its first and third residues, D-leucine and asparagine.

In addition to differences in the aglycone cores, the subsequent glycosylations by dedicated glycosyltransferases (Gtfs) also use different nucleotide sugars and transfer them to different sites on the maturing antibiotic (Scheme 1). Vancomycin is glucosylated on the phenolic hydroxyl of 4-OH-Phegly$_4$ and then converted to an α-1,2-disaccharide with the addition of the 3-amino-2,3,6-trideoxy-3-*C*-methyl-L-*lyxo*-hexose, vancosamine. Teicoplanin, bearing three

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[‡] The gene sequences encoding GtfD and GtfE from ATCC19795 have been deposited into GenBank under accession numbers AF351621 and AF351622, respectively.

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[1] Abbreviations: VRE, vancomycin-resistant Enterococci; 4-OH-Phegly, 4-hydroxyphenylglycine; 3,5-(OH)$_2$-Phegly, 3,5-dihydroxyphenylglycine; Gtf, glycosyltransferase; β-OH-Tyr, β-hydroxytyrosine; LB, Luria broth; IPTG, isopropyl-1-thio-β-D-galactoside; DTT, dithiothreitol; TCEP, tris-(2-carboxyethyl)-phosphine; BSA, bovine serum albumin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; orf, open reading frame.

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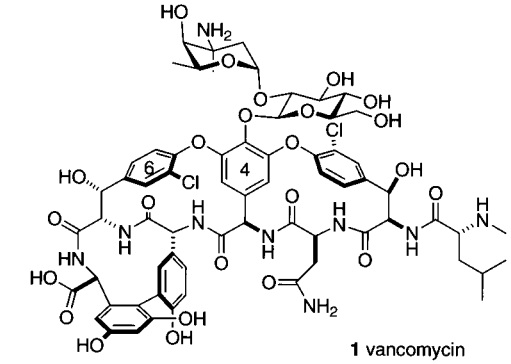
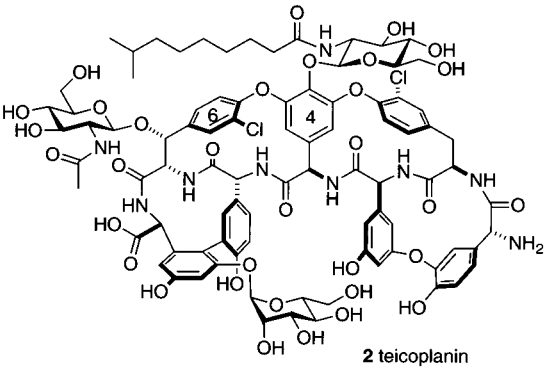
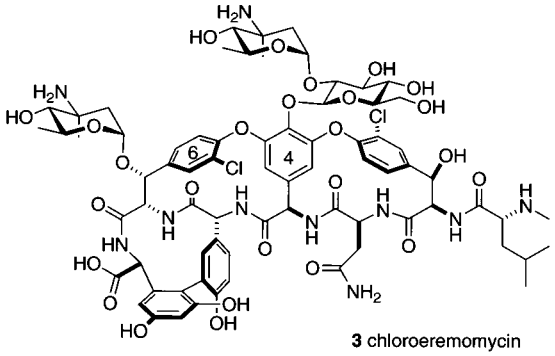
Scheme 1







sugars, has an *N*-acyl-glucosamine at 4-OH-Phegly$_4$, an *N*-acetyl-glucosamine on the beta hydroxyl of the residue 6 β-hydroxytyrosine (β-OH-Tyr$_6$), and a third sugar, mannose, on the C3-hydroxyl of 3,5-(OH)$_2$-Phegly$_7$. Chloroeremomycin, **3** in Scheme 1, is closely related to vancomycin but has three sugars: an α-1,2-disaccharide at residue 4, 4-*epi*-vancosaminyl-(1,2)-glucose, and in addition, a 4-*epi*-vancosamine at β-OH-Tyr$_6$. The sugars of the glycopeptide class of antibiotics are important for biological activity (*2*, *4*−*7*).

In this work, we report the subcloning, heterologous expression, and purification to homogeneity of two Gtfs, GtfB, and GtfC, from a chloroeremomycin-producing strain of *Amycolatopsis orientalis* (A82846) and two Gtfs, GtfD and GtfE, from a vancomycin-producing strain of *Amycolatopsis orientalis* (ATCC19795). We also report the first synthesis of an NDP-3-amino-2,3,6-trideoxy sugar substrate (UDP-4-*epi*-vancosamine) and show that it is utilized by GtfC and GtfD. Using the purified Gtfs and synthetic substrates, we have been able to produce new glycosylation patterns on the vancomycin and teicoplanin aglycones to make hybrid glycopeptide antibiotics.

MATERIAL AND METHODS

Bacterial Strains, Plasmids, and Materials. Competent cells of *E. coli* strains DH5α and BL21(DE3) were purchased from Gibco BRL. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. The pET22b expression vector was purchased from Novagen. *Pfu* DNA polymerase was purchased from Stratagene. DNA primers were obtained from Integrated DNA Technologies (Coralville, IA). UDP-glucose, UDP, and tris-(2-carboxyethyl)-phosphine (TCEP) were purchased from Sigma.

Cloning. The genes encoding GtfB and GtfC were amplified from *Amycolatopsis orientalis* (A82846) genomic DNA. GtfB was amplified using the following primer pairs: 5′-GAATTCCATATGCGTGTGCTGTTGGC-3′ and 5′-TGATGTCCTCGAGCGCGGAAACAGTCGGCTT-3′. GtfC was amplified using the following primer pairs: 5′-GAATTCCATATGCGTGTGTTGTTGTCGACG-3′ and 5′-CCCAAGCTTTCATCACGCGAGAACAGCCGACTT-3′. The 5′ and 3′ gene sequences that encode GtfD and GtfE in the vancomycin producing strain *A. orientalis* C329.4 (*8*) were used to design PCR primers to amplify the same genes from a cosmid clone containing a 5.7-kb *Bam*HI portion containing the two Gtf genes from *A. orientalis* ATCC19795 (gift from C. Olano and C. R. Hutchinson). GtfD was amplified using the following primer pairs: 5′-GGTAGCCATATGCGTGTGTTGTTGTCGGTG-3′ and 5′- CGCTGATCTCGAGCGCGGGAACGGCGGGCTC-3′. GtfE was amplified using the following primer pairs: 5′-AAATACCATATGCGTGTGTTGTTGTCGACC-3′ and 5′-CTGGTGACTCGAGGGCGGGAACGGCGGGCTG-3′. PCR reactions were carried out using *Pfu* DNA polymerase as described by the enzyme suppliers. In all cases, the first primer in each pair introduced a *Nde*I restriction site (underlined), and in the case of GtfB, GtfD, and GtfE, the second primer introduced a *Xho*I site into the PCR product. Cloning into the *Xho*I site of pET22b added a C-terminal hexahistidine tag to the protein, appending the amino acid sequence LEHHHHHH. In the case of GtfC, the second primer in the pair introduced a *Hind*III restriction site after a stop codon into the PCR product, which when cloned into pET22b yielded the untagged version of GtfC. Restriction-digested PCR products were cloned into digested pET22b to yield the following constructs: pET22b-gtfB-his$_6$, pET22b-gtfC, pET22b-gtfD-his$_6$, and pET22b-gtfE-his$_6$. All constructs were sequenced to ensure no introduced errors, and in the case of the genes corresponding to GtfD and GtfE, the sequences have been deposited into GenBank under accession numbers AF351621 and AF351622.

Overproduction. Cultures of *E. coli* BL21(DE3) transformants with each of the constructs described above (2L Luria broth (LB) media with 100 μg/mL ampicillin) were grown at 25 °C to an OD$_{600}$ of 0.6, at which point the cultures were induced with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) and grown an additional 4 h. Cells were harvested by centrifugation and frozen.

Purification. In the case of the hexahistidine-tagged proteins GtfB, GtfD, and GtfE, the corresponding cells were resuspended in buffer A [5 mM imidazole, 0.5 M NaCl, 25 mM Tris-Cl (pH 8)] and lysed by two passages through a French pressure cell at 15 000 psi. After clarification by centrifugation at 95000*g*, the lysates were loaded onto charged 5-mL His·Bind columns (Novagen) at a flow rate

of 1 mL/min. The columns were washed with 50 mL of buffer A followed by 30 mL of buffer A with 30 mM imidazole. The proteins were eluted with a gradient of imidazole in buffer A (5−500 mM imidazole). Fractions were analyzed by SDS−PAGE, pooled, and dialyzed twice against 3 L of 50 mM Tris-Cl (pH 8), 2 mM dithiothreitol (DTT), and 10% glycerol. Protein concentrations were determined from the calculated extinction coefficients at 280 nm of 37560 M^{-1} for GtfB, 31040 M^{-1} for GtfD, and 36280 M^{-1} for GtfE (*9*).

Cells from expression cultures of pET22b-gtfC were resuspended in buffer B [50 mM Tris-Cl (pH 8), 2 mM DTT] and then lysed and clarified in the same manner as described above. The supernatant was fractionated by ammonium sulfate precipitation. The 20−60% pellet was resuspended in buffer B, dialyzed for 3 h against buffer B, and loaded onto a 20-mL Q-Sepharose column (resin from Pharmacia). Protein was eluted with a linear gradient of buffer B to buffer C [buffer B + 500 mM KCl] over 600 mL at 2 mL/min. After analysis by SDS−PAGE, GtfC-containing fractions were pooled, and ammonium sulfate was added to 1 M. The sample was then loaded onto a 5-mL Phenyl Superose column (Pharmacia) at 1 mL/min, and eluted with a 300-mL linear gradient of buffer D [buffer B + 1 M ammonium sulfate] to buffer B. GtfC-containing fractions were pooled and dialyzed twice against 3 L of buffer B with 10% glycerol. Protein concentration was determined from the calculated extinction coefficient at 280 nm of 35920 M^{-1} for GtfC (*9*).

GtfE and GtfB Assays. In a total volume of 300 μL, UDP-glucose and heptapeptide aglycone substrates were incubated with 75 mM Tricine (pH 9), 2.5 mM tris-(2-carboxyethyl)-phosphine (TCEP), 1 mg/mL bovine serum albumin (BSA), and either GtfB or GtfE. Reactions were initiated by addition of GtfB or GtfE, incubated at 37 °C, and 50 or 100 μL aliquots were quenched at specified times with an equal volume of cold 100% MeOH. For the determination of steady-state kinetic parameters, GtfB and GtfE were added to a final concentration of 50 nM. For determination of the K_m for vancomycin aglycone, **4**, with GtfB, the concentration of UDP-glucose was fixed at 6 mM, and the concentration of **4** was varied from 0.1 to 3.2 mM. For each concentration, time points were taken at 0, 2, and 4 h. For the determination of the K_m for UDP-glucose with GtfB, the concentration of **4** was fixed at 600 μM, and the concentration of UDP-glucose was varied from 0.2 to 6.4 mM. For each UDP-glucose concentration, time points were taken at 0, 2, and 4 h. Parameters for GtfE were determined in the same manner as for GtfB, except time points for a given substrate concentration were taken at 0, 1, and 2 h. In addition, the K_m for teicoplanin aglycone was determined by varying the concentration from 0.1 to 1.5 mM.

After quenching the reactions, samples were pelleted for 10 min at 13 000 rpm in a microcentrifuge at 4 °C. Analytical HPLC was carried out on a Beckman Gold Nouveau system with a Vydac small pore C18 column (250 × 4.6 mm) using a gradient of 0−45% acetonitrile in water/0.1% TFA over 15 min at a flow rate of 1 mL/min. Samples were monitored at 285 nm and absorbance area correlated with a vancomycin pseudoaglycone standard available by selective degradation of vancomycin (*10*). Initially, new product peaks were collected, lyophilized, and analyzed by mass spectrometry to verify predicted molecular masses. MALDI-TOF mass spectrometry was carried out using a Perseptive Biosystems Voyager-DE STR mass spectrometer.

Synthesis. Aglycones **4** and **6** were synthesized as described (*11*−*13*). The synthesis of lactol **10** and spectroscopic data for the products reported in this paper can be found in the Supporting Information.

*Uridine 5′-Diphospho 3-benzyloxycarbonylamino-4-O-acetyl-2,3,6-trideoxy-3-C-methyl-α,β-L-arabino-hexopyranoside (**11**).* The coupling of the lactol to UDP followed the procedure of Hindsgaul (*14*). The lactol **10** (28 mg, 0.083 mmol) was azeotroped with toluene and dissolved in dichloromethane (1 mL). Triethylamine (35 μL, 0.249 mmol) was added and the reaction was cooled to −10 °C before TMS-Cl was added dropwise. The reaction was warmed to room temperature over 45 min and then stirred for another 10 min before the solvent was evaporated. To the residue was added 4:1 petroleum ether/EtOAc, and the mixture was filtered. The filtrate was concentrated to give 34 mg (100% crude) of the 4-*epi*-vancosamine TMS glycoside as a colorless oil: R_f = 0.41, 0.48 (25% EtOAc/petroleum ether).

UDP sodium salt (9.4 mg, 0.020 mmol) was dissolved in cold water (0−4 °C). The solution was passed through a column packed with Dowex 50WX8-200 acidic resin and eluted with cold water. The elutant (∼7 mL) was dripped directly into an aqueous solution of 4% $Bu_4N^+OH^-$ (0.3 mL). More 4% $Bu_4N^+OH^-$ aqueous solution was added until pH 5−6. The resulting solution was lyophilized to a white powder (18 mg). This UDP tetrabutylammonium salt was azeotroped with toluene and dissolved in dichloromethane (0.6 mL). DIEA (3.5 μL, 0.020 mmol) was added to this solution.

The 4-*epi*-vancosamine TMS glycoside (8.3 mg, 0.020 mmol) was azeotroped with toluene and dissolved in dichloromethane (0.5 mL). The solution was cooled to −78 °C before TMS-I (2.9 μL, 0.020 mmol) was added. The reaction was stirred at −78 °C for 40 min before the previous UDP tetrabutylammonium salt solution was added. The reaction was slowly warmed to room temperature over 2 h, quenched with DIEA (10 μL) and methanol (10 μL), concentrated, and purified by reverse-phase HPLC (gradient 0−70% methanol/water with 0.1% NH_3/NH_4OAc over 50 min, with a flow rate of 7.5 mL/min and UV detection at 265 nm) to give 9.2 mg (60%) of **11** as its ammonium salt as a white solid: R_f = 0.46 (6:4:1 CH_2Cl_2:MeOH:H_2O); ^{1}H NMR (CD_3OD, 500 MHz) δ 8.06 (d, J = 8.0 Hz, α,β-U-H-6), 7.35−7.25 (m, α,β-Ph-H), 5.98 (d, J = 5.0 Hz, β-U-H-1′), 5.96 (d, J = 5.0 Hz, α-U-H-1′), 5.85 (d, J = 8.0 Hz, β-U-H-5), 5.84 (d, J = 8.0 Hz, α-U-H-5), 5.74 (br s, α-V-H-1), 5.48 (br apt, J = 8.8 Hz, β-V-H-1), 5.42 (d, J = 10.0 Hz, α-V-H-4), 5.33 (d, J = 9.5 Hz, β-V-H-4), 5.00 (m, α,β-benzylic-H), 4.37−4.10 (m, α,β-U-H-2′,H-3′,H-4′,H-5′,H-5″,α-V-H-5), 3.74 (dq, J = 9.5, 6.0 Hz, β-V-H-5), 2.95 (br d, J = 12.0 Hz, α-V-H-2), 2.75 (br apt, J = 10.5 Hz, β-V-H-2), 2.07 (s, α-Ac-H), 1.97 (s, β-Ac-H), 2.14−1.94 (m, α,β-V-H-2′), 1.51 (s, α-V-C-3-Me), 1.36 (s, β-V-C-3-Me), 1.18 (d, J = 6.0 Hz, β-V-H-6-Me), 1.15 (d, J = 6.0 Hz, α-V-H-6-Me); LRMS (ESI) calculated for $C_{26}H_{34}N_3O_{17}P_2$ (MH^-) 722, found 722. The 0.1% NH_3/NH_4OAc elutant for HPLC was prepared by dissolving 1 g of NH_4OAc and 0.5 mL of saturated aqueous NH_3 in 1 L of methanol or water.

*Uridine 5′-Diphospho 3-amino-2,3,6-trideoxy-3-C-methyl-α,β-L-arabino-hexopyranoside (**12**).* To a solution of the

protected UDP-4-*epi*-vancosamine **11** (6 mg, 0.008 mmol) in methanol (2 mL) was added Pd/C (2 mg). The reaction was hydrogenated at 1 atm at room temperature for 4 h and filtered. The filtrate was concentrated, and the residue was dissolved in methanol (1 mL) and 10 mg/mL NaOMe in MeOH (1 mL) was added. The reaction was stirred at room temperature for 1.5 h, quenched with 30 mg/mL NH_4OAc in MeOH (1.5 mL), concentrated, and purified by reverse-phase HPLC (100% water with 0.1% NH_3/NH_4OAc, prepared as before, over 20 min, with a flow rate of 7.5 mL/min and UV detection at 265 nm) to give 3.2 mg (72%) of UDP-4-*epi*-vancosamine **12** as its ammonium salt as a white solid: $R_f = 0.10$ (4:4:1 CH_2Cl_2:MeOH:H_2O); ^1H NMR (CD_3OD, 500 MHz) δ 8.09 (d, $J = 8.0$ Hz, α,β-U-H-6), 5.98 (d, $J = 5.0$ Hz, β-U-H-1′), 5.97 (d, $J = 5.0$ Hz, α-U-H-1′), 5.87 (d, $J = 8.0$ Hz, β-U-H-5), 5.85 (d, $J = 8.0$ Hz, α-U-H-5), 5.75 (br s, α-V-H-1), 5.50 (br apt, $J = 8.8$ Hz, β-V-H-1), 4.39−4.11 (m, α,β-U-H-2′,H-3′,H-4′,H-5′,H-5″), 4.03 (dq, $J = 9.5$, 6.0 Hz, α-V-H-5), 3.58 (dq, $J = 9.5$, 6.0 Hz, β-V-H-5), 3.23 (d, $J = 9.5$ Hz, α-V-H-4), 2.47 (d, $J = 14.0$ Hz, α-V-H-2), 2.43 (m, β-V-H-2), 1.95−1.84 (m, α,β-V-H-2′), 1.60 (s, α-V-C-3-Me), 1.42 (s, β-V-C-3-Me), 1.29 (d, $J = 6.0$ Hz, β-V-H-6-Me), 1.27 (d, $J = 6.0$ Hz, α-V-H-6-Me); HRMS (FAB) calculated for $C_{16}H_{26}N_3O_{14}P_2$ 546.0890, found 546.0898.

GtfC and GtfD Assays. In a total volume of 130 μL, 2 mM UDP-4-*epi*-vancosamine and 500 μM vancomycin pseudoaglycone (**5**) were incubated with 75 mM Tricine (pH 9), 2.5 mM TCEP, 2 mM $MgCl_2$, 1 mg/mL BSA, and 2 μM GtfC or GtfD to test for formation of **8**. To test activity of GtfC or GtfD with the teicoplanin scaffold, 2 mM UDP-4-*epi*-vancosamine and 500 μM teicoplanin aglycone (**6**) were incubated as above but also with 5 μM GtfE and 6 mM UDP-glucose to generate the glucosylated intermediate **7**. Reactions were initiated by addition of GtfC or GtfD, incubated at 37 °C, and 25 μL aliquots were quenched at specified times with 9 vol of cold 100% MeOH. After quenching the reactions, samples were pelleted for 60 min at 13 000 rpm in a microcentrifuge at 4 °C. Samples were evaporated and resuspended in 50 μL of water with 5% acetonitrile. Reactions were analyzed by HPLC as before, except the gradient used was 7.5−20% acetonitrile in water/0.1% TFA over 25 min at 1 mL/min. New product peaks were collected and analyzed by MALDI-TOF mass spectrometry as before. To scale-up the syntheses of **8** and **9** enzymatically, the same reaction conditions above were carried out in a reaction volume of 5 mL. The products were purified by preparative HPLC using a Vydac C18 protein and peptide column (250 × 22 mm) using the same gradient above at 10 mL/min.

*Structure Determination of **8** and **9** by NMR.* Glycopeptide samples from the enzymatic synthesis were dissolved in 600 μL of DMSO-d_6 to a concentration of ca. 1 mM. Spectra were recorded on either a Varian ^{UNITY}INOVA 500 or 600 MHz instrument at 30 °C. Through bond connectivities in the carbohydrate portion of the glycopeptides were established through gradient-selected COSY experiments. NOESY spectra were recorded to establish the through space interaction. $^3J_{H,H}$ couplings were taken directly from 1D spectra or by selective decoupling on the H-2a proton and observation of the H-1 signal of the sugar moieties. One-dimensional spectra were processed and analyzed using the Vnmr software (Varian, Palo Alto, CA).

gCOSY spectra were recorded at 500 MHz in absolute value mode using 512 t_1 increments of 48 scans and 2048 points in the t_2 dimension (5000 Hz). Processing and analysis was done using the Vnmr software where the FIDs were apodized with a combination of exponential line broadening and sine bell functions in both dimensions.

Phase sensitive NOESY spectra of **8** were acquired at 500 MHz using 300 ms mixing time, while spectra for **9** were collected at 600 MHz with 300 and 1000 ms mixing time, respectively. In all cases, States-Redfield phase incrementation (a variant of States-TPPI) (*15*) was used in t_1. At 500 MHz 16 scans of 4096* points were collected in t_2 for a 5940 Hz spectral window, while t_1 was incremented in 400* steps. Spectra at 600 MHz were acquired over an 8000 Hz window with 4448* points in t_2 and 512* increments in t_1. Sixteen scans were averaged for each increment.

The NOESY spectra were processed off-line using the NMRPipe software (*16*); visualization and analysis was accomplished in NMRView (*17*) using standard procedures. The typical processing protocol included time domain suppression of the water signal, apodization by a combination of a slightly shifted cosine function and Gaussian line broadening, zero filling, and polynomial baseline correction (*15*).

Determination of Minimum Inhibitory Concentrations. The strains used were *Enterococcus faecium* 49624, *E. faecium* CL 4931(VanA), *E. faecalis* 29212, *E. faecalis* CL 4877-(VanB), and *Staphylococcus aureus* 29213. Test compounds were dissolved in DMSO. Minimum inhibitory concentrations were determined by a standard broth microdilution assay using Brain Heart Infusion medium. Viable cells were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The minimum inhibitory concentration is defined as the lowest concentration of compound that prevented visible growth.

RESULTS

Purification of Cognate Pairs of Glycosyltransferases from Chloroeremomycin and Vancomycin producers by Heterologous Expression in E. coli. The congeneric glycopeptide antibiotics vancomycin, **1**, and chloroeremomycin, **3**, have the identical cross-linked heptapeptide aglycone scaffold and differ only in the number of appended hexoses (two for **1**, three for **3**) and the stereochemistry of the 4-OH substituent between the vancosamine and 4-*epi*-vancosamine sugars. After sequencing the biosynthetic cluster for chloroeremomycin (**3**) from the producer strain *Amycolatopsis orientalis*, three putative glycosyltransferase open reading frames (orfs) were identified, orfs 11, 12, and 13 (*18*). One would expect two Gtf genes in the corresponding vancomycin cluster. Solenberg et al. (*8*) had previously identified three predicted glycosyltransferase genes corresponding to GtfA, GtfB, and GtfC from a chloroeremomycin-producing strain of *A. orientalis*, and genes corresponding to GtfD and GtfE, the two Gtfs in the vancomycin-producing strain of *A. orientalis*. They demonstrated glucosyltransferase activity in crude extracts containing GtfB and GtfE, but detected no activity for GtfA or GtfC. We purified a C-terminal His_6-tagged version of GtfB (44 kDa) to homogeneity (lane 1 of Figure 1) to enable determination of catalytic efficiency and to allow preparative conversions of the vancomycin aglycone, **4**, to

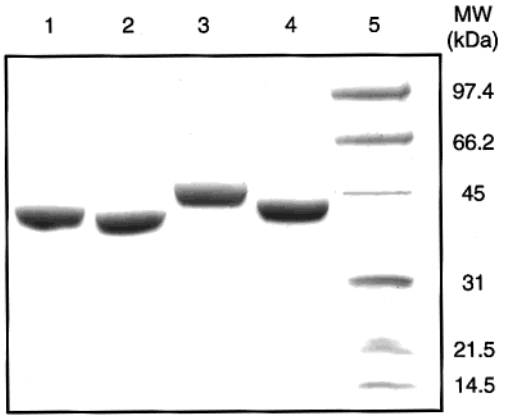


FIGURE 1: SDS−PAGE of purified glycosyltransferases GtfB, C, D, and E. GtfB (lane 1), GtfD (lane 3), and GtfE (lane 4) were purified by nickel-chelate chromatography. GtfC (lane 2) was purified by ion exchange (Q-Sepharose) followed by hydrophobic interaction (Phenyl-Superose) chromatography. Lane 5 shows the molecular weight standards. 150 pmol of each enzyme was loaded on this 4−20% SDS polyacrylamide gel.

its monoglucosyl intermediate, the vancomycin pseudoaglycone, **5** (see Figure 2). We also purified the untagged form of GtfC (43 kDa) to substantial purity (lane 2 of Figure 1). GtfB and GtfC are readily produced by *E. coli* in quantities of 12 and 5 mg/L of culture, respectively. We demonstrate below that GtfC adds the 4-*epi*-vancosaminyl residue to the 2-OH of the glucose moiety of **5** to produce the α-1,2-disaccharide chain of **8** (see Figure 4).

The genome of vancomycin-producing *A. orientalis* strains contains cognate Gtfs for the analogous two-step modification of the aglycone to the vancosaminyl-α-(1,2)-glucosyl disaccharide found attached to the 4-OH-Phegly$_4$ of vancomycin (**1** in Scheme 1). GtfE is the glucosyltransferase (*8*, *19*), and GtfD is the presumed vancosaminyltransferase. The 5′ and 3′ gene sequences which encode GtfD and GtfE in the vancomycin-producing strain *A. orientalis* C329.4 (*8*) were used to design PCR primers to amplify the same genes from *A. orientalis* ATCC19795. The genes encoding GtfD and GtfE were cloned into *E.coli* expression vectors and overproduced as his$_6$-tagged versions as described in the Materials and Methods. The predicted protein sequences of GtfD and GtfE from *A. orientalis* ATCC19795 are 89 and 91% identical, respectively, to the sequences previously published from *A. orientalis* C329.4 (*8*). GtfD (44 kDa) (lane 3, Figure 1) and GtfE (44 kDa) (lane 4, Figure 1) were readily purified by nickel affinity chromatography with final yields of 12 and 15 mg/L of culture, respectively. At this stage, the four Gtfs could be assayed for catalytic properties with nucleotide sugar donors and aglycosyl or glucosylated acceptor substrates.

Activity Profiles of the Glucosyltransferases GtfB and GtfE. The activity of pure GtfB with UDP-glucose and vancomycin aglycone (**4**) to produce UDP and vancomycin pseudoaglycone (**5**) (structures in Figure 2, panel C) was assayed by HPLC and integration of the product peak corresponding to **5**. As shown in Figure 2, panel A, the product peak **5** grows in over 4 h with 5 pmol of the GtfB

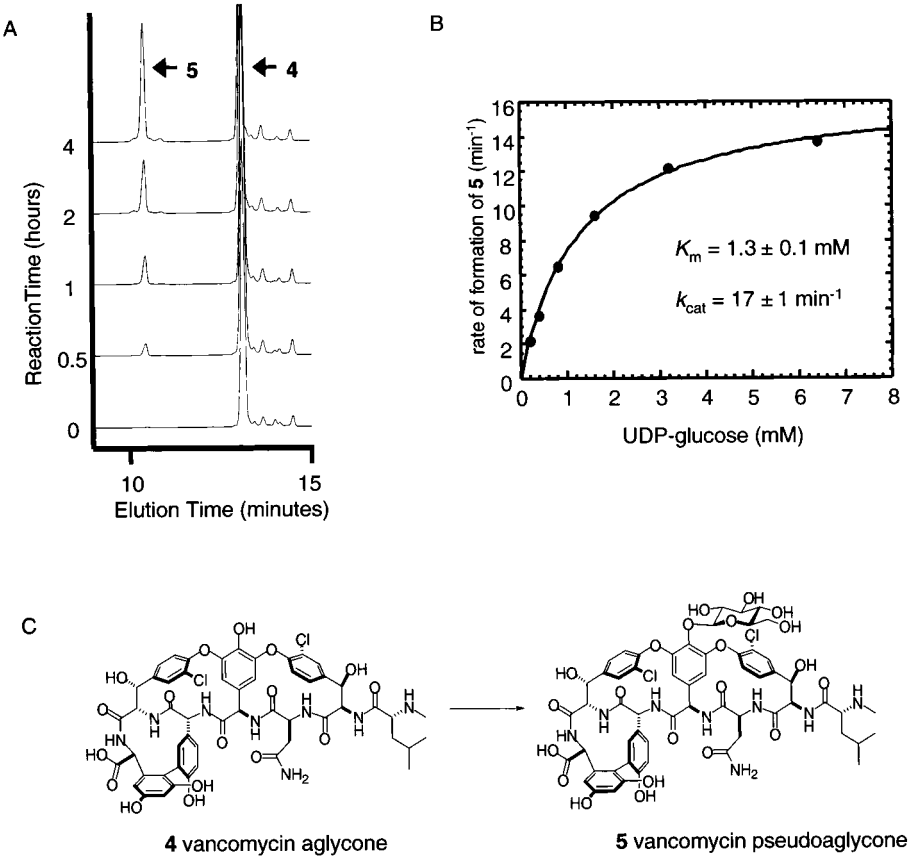


FIGURE 2: GtfB action on the vancomycin aglycone (**4**). (A) HPLC time course from 0 to 4 h showing the production of vancomycin pseudoaglycone (**5**). (B) Rate versus substrate concentration plot measuring the kinetic parameters of GtfB with varying UDP-glucose concentrations. **4** was fixed at 600 μM, and GtfB was added to a final concentration of 50 nM. Conditions of the reactions are as described in Materials and Methods. (C) Schematic representation of the conversion of **4** to **5**.

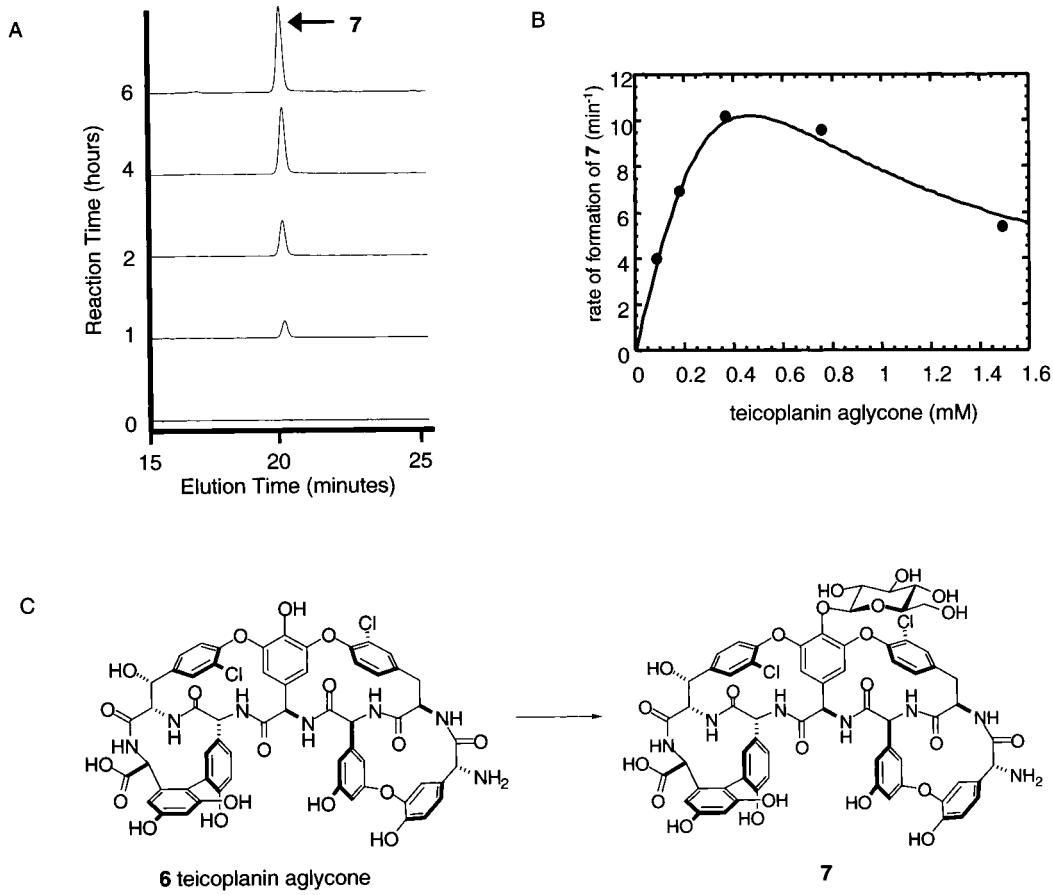


FIGURE 3: GtfE action on the teicoplanin aglycone (**6**). (A) HPLC time course from 0 to 6 h showing the production of **7**. (B) Rate versus substrate concentration plot measuring the kinetic parameters of GtfE with varying concentrations of **6**. UDP-glucose was fixed at 5 mM, and GtfE was added to a final concentration of 50 nM. Conditions of the reactions are as described in Materials and Methods. (C) Schematic representation of the conversion of **6** to **7**.

catalyst. The product was identified by coelution with authentic vancomycin pseudoaglycone, generated by selective degradation of the parent antibiotic vancomycin (*10*), and by mass spectrometry. The anticipated mass of 1304.3 Da was observed, as compared to the mass of 1142.3 for the starting substrate **4**. In panel B of Figure 2, the data show normal rectangular hyperbolic saturation with UDP-glucose as the variable substrate (**4** fixed at 600 μM), giving a nucleotide sugar K_m of 1.3 mM and a k_{cat} of 17 glucosyl transfers per minute per enzyme molecule. When **4** was examined as the variable substrate, there was severe substrate inhibition (data not shown) above 600 μM, and the maximum velocity observed was 17 min^{-1}. The concentration of **4** at half-maximal velocity was 250 μM.

As anticipated, the cognate Gtf from the vancomycin biosynthetic operon, GtfE (identity between GtfB and GtfE = 80%), also functioned efficiently as a glucosyltransferase to **4**, with a k_{cat} of 60 min^{-1} (data not shown), about 3-fold faster than GtfB under comparable assay conditions. Interestingly, there was no evidence for substrate inhibition of GtfE by **4**, with a K_m for **4** of 1.5 mM. At this point, we tested the tolerance of GtfE and GtfB for alterations in the aglycone scaffold and turned to the aglycone of teicoplanin, **6** in Figure 3, panel C. The teicoplanin aglycone, **6**, has one additional phenolic cross-link between residues 1 and 3 as compared to the vancomycin aglycone, **4**. **6** was prepared by selective degradation of the parent antibiotic teicoplanin as described (*13*).

As depicted in Figure 3, panel A, pure GtfE generates a new HPLC peak, dependent on **6** and UDP-glucose. Mass spectrometric analysis established a molecular mass of 1359.3 for the new product, an increase of 162 mass units over the starting **6** (mass = 1197.2), consistent with the formation of the glucosylated derivative of the teicoplanin scaffold, **7** (Figure 3, panel C). Formation of **7** by GtfE was previously observed in crude extracts after heterologous expression in *E. coli* (*8*). Figure 3, panel B, shows a rate versus teicoplanin aglycone (**6**) concentration plot with substrate inhibition setting in above 0.4 mM of this aglycone. An estimate of the k_{cat} for GtfE using UDP-glucose and **6** as substrates is 20 min^{-1}, about 3-fold lower than the k_{cat} with its physiologic aglycone **4** noted above. When GtfB was similarly evaluated for its ability to use **6** as the aglycone substrate, the same product was produced but at much lower rates. A k_{cat} of about 0.3 min^{-1} was estimated (data not shown), some 70-fold lower than with GtfE. To generate enough **7** for the later studies, only GtfE was useful for transfer of glucose to this heterologous substrate.

*Synthesis of UDP-β-*L*-4-epi-vancosamine to Evaluate the Enzymatic Activity of GtfC and GtfD.* Because vancomycin (**1**) has only two sugars attached, GtfD must be the vancosaminyltransferase that catalyzes the formation of the disaccharide. In chloroeremomycin (**3**), GtfA and GtfC are both 4-*epi*-vancosaminyltransferases. GtfC, which is 69% identical to GtfD, is most likely the transferase that forms the α-1,2-disaccharide on the heptapeptide scaffold, while

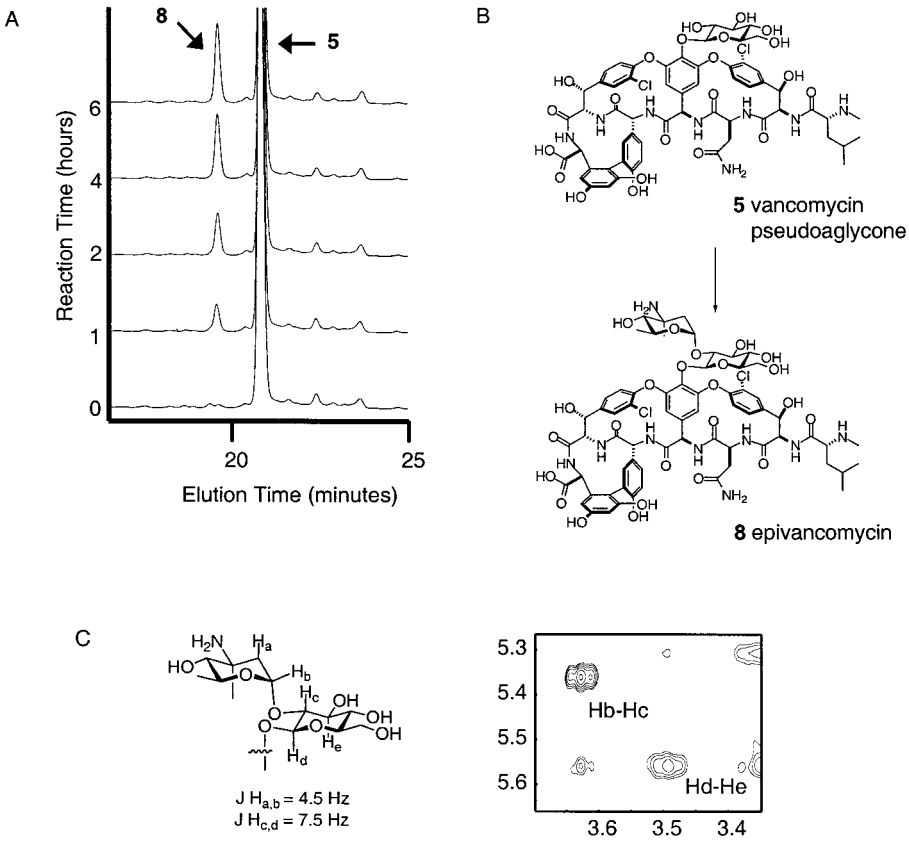


FIGURE 4: GtfC action on vancomycin pseudoaglycone (**5**). (A) HPLC time course from 0 to 6 h showing the production of **8**. Conditions of the reaction are as described in Materials and Methods. (B) Schematic representation of the conversion of **5** to **8**. (C) Schematic showing disaccharide on **8** with labeled protons and coupling constants for structure determination. A detail of the NOESY spectrum that establishes the disaccharide linkage through an interresidue NOE between 4-*epi*-vancosamine H1 and glucose H2.

GtfA, which is only 55% identical to GtfD, should be the 4-*epi*-vancosaminyltransferase to β-OH-Tyr$_6$ (*18*). Because of its homology to GtfD, GtfC could potentially use vancomycin pseudoaglycone (**5**) as the acceptor substrate for transfer of 4-*epi*-vancosamine. The comparison of the proposed reactions of GtfC and GtfD are shown in Scheme 2. To assay GtfC or GtfD requires the availability of the NDP-β-L-4-*epi*-vancosamine or NDP-β-L-vancosamine as a cosubstrate, in which the nucleotide is likely to be TDP or UDP.

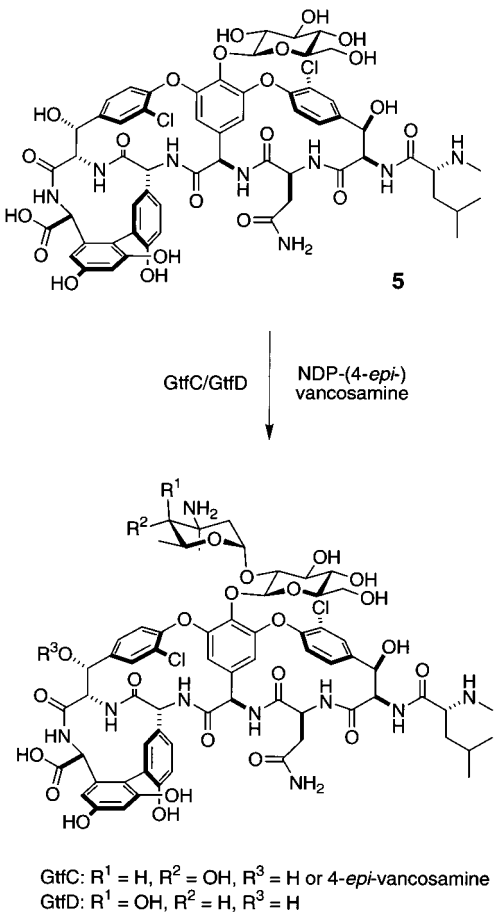
Although 3-amino-2,3,6-trideoxy sugars are biologically very important molecules, there have been no reported syntheses of their NDP derivatives. The instability of the synthetic intermediates associated with 2-deoxysugar-1-phosphates (*20*) makes the synthesis challenging, and many conventional methods to make the sugar-1-phosphate failed. The successful route to UDP-4-*epi*-vancosamine is outlined in Scheme 3.

4-*epi*-vancosamine lactol **10** was accessed through degradation from vancomycin. Briefly, the vancosamine nitrogen on vancomycin was protected as its CBZ carbamate and then the vancosaminyl sugar was cleaved from the parent compound using anhydrous HCl in methanol giving the corresponding methyl glycoside. An oxidation/reduction sequence using PCC and $NaBH_4$, respectively, gave rise to N-CBZ-4-*epi*-vancosamine. Subsequent protection of the 4-OH as the acetate and hydrolysis of the methyl glycoside delivered lactol **10** in 82% overall yield from **SI1a** (see Supporting Information).

Formation of the TMS ether of lactol **10** was followed by activation of the anomeric carbon using TMSI at −78 °C (presumably forming the glycosyl iodide) and trapping with $(Bu_4N)_2$UDP. The product of this three-step sequence was the protected UDP-4-*epi*-vancosamine **11** in a 2:1 α:β anomeric ratio. Hydrogenation and exposure to NaOMe in methanol completed the two-step deprotection sequence to give UDP-4-*epi*-vancosamine **12** as a 2:1 α:β mixture in 43% overall yield from lactol **10**. Previous work established that TDP-4-*epi*-vancosamine is the product of the biosynthetic route (*21*). Therefore, it was expected that the β-L-species of the corresponding UDP sugar would be the active anomer. UDP-L-4-*epi*-vancosamine was used as the mixture to then assay GtfC and GtfD.

*GtfC Transfers 4-epi-Vancosamine to Vancomycin Pseudoaglycone to Form **8**.* To test its activity, purified GtfC was incubated with the vancomycin pseudoaglycone, **5**, and UDP-4-*epi*-vancosamine. The reactions were analyzed by HPLC, and a new peak was detected that coelutes with authentic vancomycin. Figure 4, panel A, shows an HPLC time course of the conversion of **5** to **8**, which is respresented in Figure 4, panel B. By mass spectrometry, this compound has the same mass (1447.4) as vancomycin and is thus the 4-*epi*-vancosamine derivative of vancomycin, epivancomycin, as further verified by the following structural studies. NMR spectroscopy was used to establish the identity of the disaccharide at 4-OH-Phegly$_4$. Spin systems in each sugar were established by COSY spectroscopy. Direct inspection of the 1D spectrum gave J_{H1-H2} of glucose equal to 7.5 Hz,

Scheme 2



indicating a β-linkage at this position. The J_{H1-H2a} for 4-*epi*-vancosamine was 4.5 Hz, similar to the value already reported for the vancomycin disaccharide and consistent with an alpha linkage (*10*). An interresidue NOE between 4-*epi*-vancosamine H1 and glucose H2 secured the structure of the disaccharide (Figure 4, panel C).

*GtfD Shows 4-epi-Vancosaminyltransferase Activity to **5** and also **7**.* In the absence of UDP-vancosamine as a substrate, we tested pure GtfD with what should be the 4-epimer of its natural substrate, UDP-4-*epi*-vancosamine. GtfD was able to recognize and transfer UDP-4-*epi*-vancosamine to vancomycin pseudoaglycone (**5**) to give the same HPLC product peak as shown in Figure 4, panel A, with GtfC. Thus GtfD can be fooled into making **8** as well.

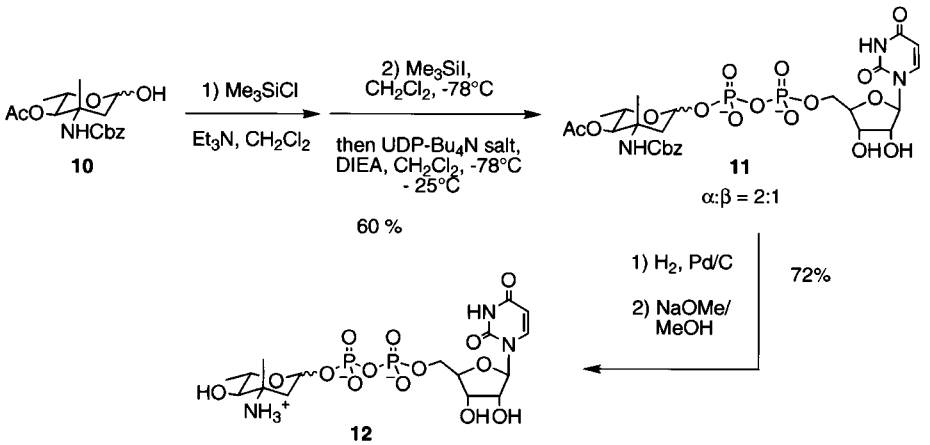
We then turned to the altered scaffold of the teicoplanin family to investigate if GtfD would in fact use **7** as a substrate for transfer of 4-*epi*-vancosamine to produce **9**, the disaccharide 4-*epi*-vancosamine-(1,2)-glucose attached to the teicoplanin scaffold (Figure 5, panel B). This could be conducted either in a one pot reaction starting from the teicoplanin aglycone (**6**) and involving the sequential action of GtfE and GtfD in the presence of both UDP-glucose and UDP-4-*epi*-vancosamine, as shown in Figure 5, panel A, or starting from **7** with pure GtfD and UDP-4-*epi*-vancosamine (data not shown). The HPLC traces in Figure 5, panel A, show first the buildup of **7** from GtfE action, then the formation of a new peak, dependent on GtfD and UDP-4-*epi*-vancosamine in the incubations. The mass spectrum of this peak gave a parent ion of mass 1502.3 Da, close to the value of 1502.4 calculated for compound **9**. In a manner analogous to epivancomycin, **8**, the disaccharide portion of glycopeptide **9** was confirmed by NMR. Coupling constants for glucose ($J_{H1-H2} = 7.5$ Hz) and 4-*epi*-vancosamine ($J_{H1-H2a} = 4.3$ Hz) as well as NOESY cross-peaks similar to those observed in **8** indicate that the disaccharide at 4-OH-Phegly$_4$ is as predicted (Figure 5, panel C). The tandem action of GtfE and GtfD produces a glycopeptide with a teicoplanin backbone and two sugars (glucose and 4-*epi*-vancosamine) not normally found in teicoplanin producer organisms.

*Antibiotic Activity of Glycopeptides **8** and **9**.* Table 1 reports the MIC values in μg/mL for the new glycopeptide antibiotics, including the parent molecules for comparison. Endpoints for the glycopeptide derivatives were not reached in the resistant (VanA/VanB) strains. Epivancomycin, **8**, displays nearly the same activity as vancomycin. The teicoplanin/vancomycin hybrid **9** showed 8- to 16-fold reduced activity versus sensitive strains in comparison to either parent glycopeptide. Teicoplanin is the only member in this collection of glycopeptides that is active against VanB resistant *E. faecalis*.

DISCUSSION

The glycosylation of several major classes of antibiotics is a crucial enzymatic step in the attainment or maintenance of pharmacologically useful biological activity (*22*). In addition to the glycopeptide antibiotics of the vancomycin and teicoplanin families that are the subject of this study, the macrolide antibiotics of the erythromycin and tylosin classes require decoration of the aglycone with deoxysugars

Scheme 3



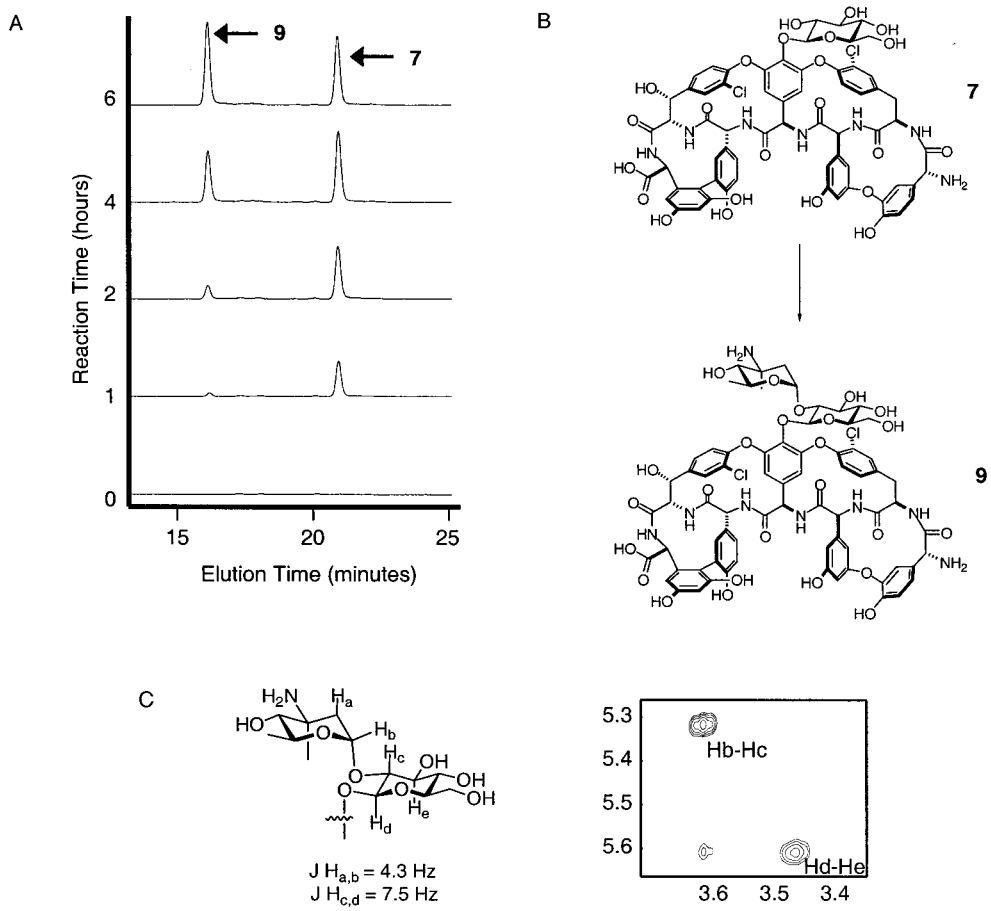


FIGURE 5: GtfD action on **7**. (A) HPLC time course from 0 to 6 h showing the production of **9**. Conditions of the reaction are as described in Materials and Methods. (B) Schematic representation of the conversion of **7** to **9**. (C) Schematic showing disaccharide on **9** with labeled protons and coupling constants for structure determination. A detail of the NOESY spectrum that establishes the disaccharide linkage through an interresidue NOE between 4-*epi*-vancosamine H1 and glucose H2.

Table 1: Minimum Inhibitory Concentrations against Vancomycin-Sensitive and Resistant Gram-Positive Pathogenic Strains[a]

	S. aureus	*E. faecium*		*E. faecalis*	
		sensitive	VanA	sensitive	VanB
vancomycin **1**	4	2	>500	16	>500
epivancomycin **8**	4	4	>130	16	>130
vancomycin pseudoaglycone **5**	4	4	>500	16	>500
vancomycin aglycone **4**	4	4	>500	16	>500
teicoplanin **2**	2	1	>500	0.5	0.5
9	32	16	>130	8	>130
7	4	4	>500	8	>500
teicoplanin aglycone **6**	0.5	1	>500	2	>500

[a] MIC values are in μg/mL.

for potent antimicrobial activity (*23*, *24*). In the assembly of the macrolide antibiotics by polyketide synthase assembly lines (*25*) and of the glycopeptide antibiotics by cognate nonribosomal peptide synthetase assembly lines (*26*), the aglycone moiety is generated by the multimodular PKS and NRPS catalysts and the free aglycone released. The addition of deoxysugar moieties by dedicated, companion Gtfs occurs late in the maturation process of antibiotic biosynthesis. There have been studies in the erythromycin system on the timing of addition of the two sugars (*27*−*29*) and also the specificity constraints of these and other macrolide Gtfs (*30*). The ability to decorate the macrolactone aglycone with a wider range of sugars would be of real value in combinatorial biosynthesis (*22*, *28*), but limited NDP-sugar availability and undetermined specificity of the Gtfs are major constraints (*30*).

Analogously, very little is known about the specificity or promiscuity of the glycosyltransferases that decorate the cross-linked nonribosomal peptides. The availability of the gene sequence for three Gtf orfs embedded in the chloroeremoycin biosynthetic cluster (*18*) and the prior study of Solenberg et al. (*8*) that these three orfs could be expressed in *E.coli* and that GtfB had glucosyltransferase activity set the stage for this study. Our goals were to purify the relevant Gtfs from both chloroeremomycin and vancomycin *Amycolatopsis orientalis* producer strains to homogeneity after heterologous expression in *E. coli*, characterize them for activity, and begin to address selectivity/promiscuity questions with regard to both NDP-sugar and cross-linked-heptapeptide substrates. We have purified two of the three Gtfs (GtfB and GtfC) from the chloroeremomycin-producing strain and both Gtfs (GtfD and GtfE) from the vancomycin-producing strain. GtfA, the presumed 4-*epi*-vancosaminyltransferase to the β-hydroxyl of residue 6 β-OH-Tyr in chloroeremomycin is yet to be studied, but GtfB, C, D, and E are now pure. A separate study has allowed crystallization of GtfB and determination of its X-ray crystal structure, which will be reported elsewhere (Mulichak, A., Losey, H. C., Walsh, C. T., and Garavito, R. M., unpublished data).

After repeating the findings of Solenberg et al. (*8*) that GtfB was likely to act first in the maturation of chloroeremomycin (**3**) via transfer of glucose to vancomycin aglycone (**4**), we were then able to determine kinetic characteristics of pure GtfB. We also validated the same activity for GtfE from the vancomycin system. While mechanistic studies remain to be conducted, the inversion of stereochemistry at C1 of the transferred glucose moiety from α in the UDP-glucose substrate to β in the glucosylated antibiotics **1** and **3** suggests that direct transfer to the phenolic hydroxyl of the 4-OH-Phegly$_4$ residue at the top of the dome-shaped aglycone substrate is the most likely route for this enzymatic group transfer.

Perhaps the most intriguing result with GtfB and GtfE is that they will accept an alternate aglycone scaffold derived from teicoplanin, which has an additional cross-link that slightly alters substrate architecture. GtfB and GtfE show nearly a two log difference in k_{cat} for glucose transfer to this altered scaffold, suggesting GtfE but not GtfB could make this unnatural glucosylation in vivo. In teicoplanin, **2**, the comparable sugar is an *N*-acyl-glucosamine. UDP-*N*-acetyl-glucosamine is not a substrate for these two Gtfs, and the corresponding Gtf from the teicoplanin producer has not yet been cloned. The ability of these enzymes to incorporate a glucose moiety onto the teicoplanin aglycone sets the stage for building variants of glycopeptide antibiotics on the teicoplanin scaffold.

To address the activity and potential promiscuity of the second stage Gtfs, GtfC and GtfD not only require a supply of glucosylated acceptor susbtrates, such as vancomycin pseudoaglycone (**5**) or **7**, but also more problematically NDP-vancosamine or NDP-4-*epi*-vancosamine. These NDP-hexoses are the 4-axial and equatorial isomers of the 3-amino-2,3,6-trideoxy-3*C*-methyl-hexoses found in vancomycin (**1**) and chloroeremomycin (**3**), hitherto unavailable in quantities sufficient for these studies. In this study, we chose to make UDP-4-*epi*-vancosamine as the first example, based on availability and ease of synthetic manipulation, even though the TDP-sugar is the most likely biological reagent (*21*, *24*). Sufficient UDP-β-L-4-*epi*-vancosamine (tested as a 2:1 α/β anomeric mixture) was produced for initial tests of GtfC and GtfD activity. In passing, we noted that UDP-α-4-*epi*-vancosamine was not a substrate for GtfC (data not shown).

The results establish that GtfC is active as an 4-*epi*-vancosaminyltransferase, producing the 4-*epi*-vancosamine-(1,2)-glucose disaccharide chain characteristic of **3**. The product from these incubations with **5** as the substrate is **8**, an epimer of vancomycin (**1**) and a novel glycopeptide antibiotic that may be an intermediate in production of **3** if GtfA acts last in the glycosylation sequence. Since GtfD can also utilize UDP-4-*epi*-vancosamine, the epimer of its normal vancosamine sugar substrate, to give **8**, it is likely that altered NDP-aminodeoxyhexoses can be made for enzymatic transfer by these Gtfs. In a preliminary evaluation of **8** as an antibiotic (Table 1), its activity parallels the behavior of **1**.

The combined actions of GtfE (first stage) and GtfD (second stage) have been utilized to glycosylate the teicoplanin aglycone to form a novel hybrid glycopeptide, **9**. This compound has lost approximately one log of activity as compared to teicoplanin (**2**) in sensitive bacteria, but more interestingly, has lost greater than 260-fold activity against VanB-type resistant bacteria. These results substantiate a role for the lipid moiety of lipoglycopeptide antibiotics against VanB strains (*5*, *7*, *31*) while illustrating that both GtfD and GtfE can utilize unnatural substrates to make novel variants of glycopeptide antibiotics. GtfE makes use of an unnatural glycosyl acceptor, teicoplanin aglycone (**6**), and the native donor, UDP-glucose, while GtfD utilizes both the glucosylated teicoplanin scaffold (**7**) and UDP-β-4-*epi*-vancosamine. GtfD is from the vancomycin producer, and its natural substrates are the vancomycin pseudoaglycone (**5**) and TDP-β-vancosamine. These experiments demonstrate that it should be possible to combine a variety of different aglycones, NDP-aminodeoxyhexose variants, and isoforms of Gtfs to make directed libraries of new glycopeptides. In particular, the ability to alter sugar substituents of glycopeptides using the combination of enzymatic and chemical synthesis will allow dissection of mechanism of action and selective activity toward drug-resistant Enterococci.

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SUPPORTING INFORMATION AVAILABLE

Details regarding the syntheses of compounds as well as spectroscopic analyses of products not included in this paper are available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Cooper, R. D., Snyder, N. J., Zweifel, M. J., Staszak, M. A., Wilkie, S. C., Nicas, T. I., Mullen, D. L., Butler, T. F., Rodriguez, M. J., Huff, B. E., and Thompson, R. C. (1996) *J. Antibiot. (Tokyo) 49*, 575−81.
2. Ge, M., Chen, Z., Onishi, H. R., Kohler, J., Silver, L. L., Kerns, R., Fukuzawa, S., Thompson, C., and Kahne, D. (1999) *Science 284*, 507−11.
3. Rodriguez, M. J., Snyder, N. J., Zweifel, M. J., Wilkie, S. C., Stack, D. R., Cooper, R. D., Nicas, T. I., Mullen, D. L., Butler, T. F., and Thompson, R. C. (1998) *J. Antibiot. (Tokyo) 51*, 560−9.
4. Beauregard, D. A., Williams, D. H., Gwynn, M. N., and Knowles, D. J. (1995) *Antimicrob. Agents. Chemother. 39*, 781−5.
5. Cooper, M. A., and Williams, D. H. (1999) *Chem. Biol. 6*, 891−9.
6. Williams, D. H., Searle, M. S., Mackay, J. P., Gerhard, U., and Maplestone, R. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1172−8.
7. Kerns, R., Dong, S. D., Fukuzawa, S., Carbeck, J., Kohler, J., Silver, L., and Kahne, D. (2000) *J. Am. Chem. Soc. 122*, 12608−9.
8. Solenberg, P. J., Matsushima, P., Stack, D. R., Wilkie, S. C., Thompson, R. C., and Baltz, R. H. (1997) *Chem. Biol. 4*, 195−202.
9. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−26.
10. Ge, M. (1998) Princeton University, Princeton.
11. Thompson, C. (1998) Princeton University, Princeton.
12. Marshall, F. J. (1965) *J. Med. Chem. 8*, 18.
13. Malabarba, A., Ferrari, P., G., G. G., Kettenring, J., and Cavalleri, B. (1986) *J. Antibiot. 39*, 1430−1442.
14. Uchiyama, T., and Hindsgaul, O. (1998) *J. Carbohydr. Chem. 18*, 1181−1190.
15. Pelczer, I., and Carter, B. G. (1997) in *Protein NMR Techniques* (Reid, D. G., Ed.) pp 71−156, Humana Press, Totowa, NJ.

16. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J. Biomol. NMR 6*, 277.
17. Johnson, B. A., and Blevins, R. A. (1994) *J. Biomol. NMR 4*, 603.
18. van Wageningen, A. M., Kirkpatrick, P. N., Williams, D. H., Harris, B. R., Kershaw, J. K., Lennard, N. J., Jones, M., Jones, S. J., and Solenberg, P. J. (1998) *Chem. Biol. 5*, 155−62.
19. Zmijewski, M. J., and Briggs, B. (1989) *FEMS Microbio. Lett. 59*, 129−134.
20. Withers, S. G., Percival, M. D., and Street, I. P. (1989) *Carbohydr. Res. 187*, 43−66.
21. Chen, H., Thomas, M. G., Hubbard, B. L., Losey, H. C., Walsh, C. T., and Burkart, M. D. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 11942−7.
22. Thorson, J. S., Hosted, T. J., Jiang, J., Biggins, J. B., Ahlert, J., and Ruppen, M. (2001) *Curr. Org. Chem. 5*, 89−111.
23. Kirschning, A., Bechthold, A. F.-W., and Rohr, J. (1997) *Top. Curr. Chem. 188*, 1−84.
24. Liu, H. W., and Thorson, J. S. (1994) *Annu. Rev. Microbiol. 48*, 223−56.
25. Khosla, C. (1997) *Chem. Rev. 97*, 2577−2590.
26. Mootz, H. D., and Marahiel, M. A. (1997) *Curr. Opin. Chem. Biol. 1*, 543−51.
27. Lambalot, R. H., Cane, D. E., Aparicio, J. J., and Katz, L. (1995) *Biochemistry 34*, 1858−66.
28. Katz, L. (1997) *Chem. Rev. 97*, 2557−2575.
29. Gaisser, S., Bohm, G. A., Cortes, J., and Leadlay, P. F. (1997) *Mol. Gen. Genet. 256*, 239−51.
30. Quiros, L. M., Carbajo, R. J., Brana, A. F., and Salas, J. A. (2000) *J. Biol. Chem. 275*, 11713−20.
31. Mackay, J. P., Gerhard, U., Beauregard, D. A., Westwell, M. S., Searle, M. S., and Williams, D. H. (1994) *J. Am. Chem. Soc. 116*, 4581.

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