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Incorporation of Glucose Analogs by GtfE and GtfD from the Vancomycin Biosynthetic Pathway to Generate Variant Glycopeptides

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Summary

Analogs of the glycopeptide antibiotics vancomycin and teicoplanin with alterations in one or both sugar moieties of the disaccharide have been prepared by tandem action of the vancomycin pathway glycosyltransferases GtfE and GtfD. All four regioisomers (2-, 3-, 4-, 6-) of TDP-deoxyglucoses and UDP/TDP-aminoglucoses were prepared, predominantly by action of D-glucopyranosyl-1-phosphate thymidylyltransferase, E_p. GtfE transferred the deoxyglucoses or aminoglucoses onto the 4-OH of 4-hydroxyphenylglycine of both the vancomycin and teicoplanin aglycone scaffolds. Kinetic analysis indicated the 2-, 3-, 4-, and 6-amino-glucoses were transferred by GtfE with only a 4- to 30-fold drop in k_{cat} and no effect on K_m compared to the native substrate, UDP/TDP-glucose, suggesting preparative utility. The next enzyme, GtfD, could utilize the variant glucosyl-peptides as substrates for transfer of L-4-*epi*-vancosamine. The aminosugar moieties in these variant glycopeptides introduce sites for acylation or reductive alkylation.

Introduction

The two glycopeptide antibiotics in clinical use for the treatment of life-threatening gram-positive bacterial infections are vancomycin (**1**) and teicoplanin (**2**) (Figure 1), related nonribosomal heptapeptides that are oxidatively crosslinked, differentially glycosylated, and in the case of teicoplanin, N-acylated on an aminosugar. As both vancomycin and teicoplanin have become front-line drugs for treating enterococcal infections, resistance has arisen [1]. VanA phenotypes of vancomycin-resistant enterococci (VRE) are resistant to both drugs, while VanB phenotypes are resistant to vancomycin but remain sensitive to teicoplanin. This difference arises by selective failure of teicoplanin to induce transcription of the VanHAX genes [2], which in turn remodel peptidoglycan precursors from D-Ala-D-Ala termini to D-Ala-D-lactate termini [3]. The altered termini have low affinity for the glycopeptide antibiotics [4, 5].

Efforts to modify the chemically complex glycopeptide antibiotics to create new structures that are active against VRE have led to oritavancin (**3**) [6], currently in clinical trials, suggesting modifications on and around the sugar substituents will be productive in creating novel glycopeptides active against VRE. The biosynthetic gene cluster [7] for the vancomycin analog chloroeremomycin (**4**) contains genes previously validated to encode three glycosyltransferases (GtfA, GtfB, and GtfC) [8, 9] that decorate the crosslinked aglycone scaffold with a glucosyl moiety from NDP-glucose (GtfB) or L-4-*epi*-vancosaminyl group from NDP-L-4-*epi*-vancosamine (GtfA, GtfC). Vancomycin has two sugars attached as an L-vancosaminyl-1,2-D-glucosyl disaccharide to residue four, 4-hydroxyphenylglycine, of the heptapeptide scaffold, and it has been previously established that GtfE is the D-glucosyltransferase [8, 9] and GtfD is the L-vancosaminyltransferase [9]. It has also been shown that GtfE in particular is efficient at utilizing both the vancomycin and teicoplanin aglycones [8, 9] for transfer of glucose from a TDP-glucose donor.

Multiple examples of sugar-flexible glycosyltransferases involved the biosynthesis of the polyketide class of antibiotics have been identified (as reviewed in [10]). Using these glycosyltransferases in gene-swapping and engineering experiments in antibiotic-producing organisms for in vivo combinatorial biosynthesis has generated numerous novel glycosylated compounds derived from such natural products as erythromycin, elloramycin, and urdamycin [11–14].

To evaluate the prospect of using some or all of the five Gtfs involved in vancomycin and chloroeremomycin biosynthesis in vitro for combinatorial biosynthesis of new glycopeptide antibiotics with altered sugars requires a supply of such D-glucose and L-vancosamine/4-*epi*-vancosamine analogs at the NDP-sugar level. In this study, we have synthesized some UDP-glucose analogs and also utilized the previously described promiscuity of α-D-glucopyranosyl-1-phosphate thymidylyltransferase, designated E_p [15–17], to produce a series of TDP-deoxyglucoses and TDP-aminoglucoses. These were then tested as substrates, first for GtfE, to generate the monoglycosylated peptide scaffold, and then with GtfD, to build analogs of the disaccharide chain on both the vancomycin and teicoplanin scaffolds.

Results

Chemical Synthesis of UDP-Glucose Derivatives

Six glucose-1-phosphate analogs were coupled to the nucleotide uridine monophosphate to produce three analogs replacing the position 2 hydroxyl of glucose with fluoro, azido, and amino groups (UDP-2-fluoro-glucose, UDP-2-azido-glucose, and UDP-2-amino-glucose) and three analogs replacing the position 6 hydroxyl of glu-

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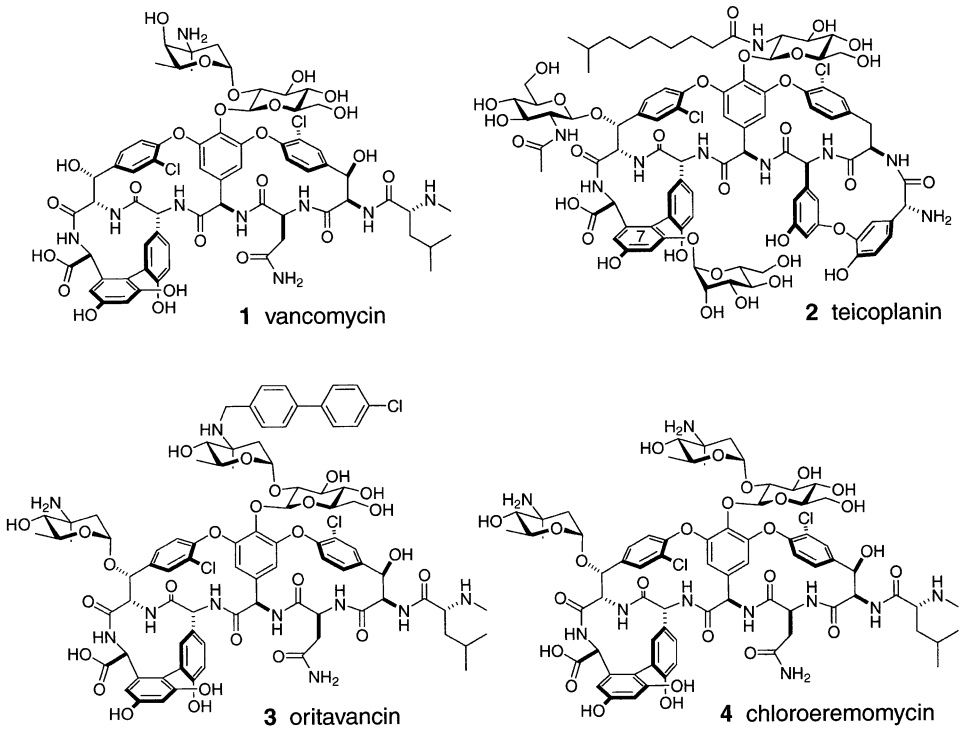


Figure 1. Structures of Vancomycin, Teicoplanin, Oritavancin, and Chloroeremomycin

cose (UDP-6-chloro-glucose, UDP-6-azido-glucose, UDP-6-amino-glucose) as potential substrates for GtfE and the aglycones of teicoplanin or vancomycin. The synthetic approach to generate these compounds is shown in Figure 2. Starting with the corresponding acetylated lactols (**5a, 5b, 9a–c**), the 3,4-acetoxy sugar-1-phosphates (**6a, 6b, 10a–c**) were generated by phosphorylation, and subsequent coupling using UMP-morpholidate resulted in the six UDP-glucose derivatives (**7a, 7b, 8, 11a, 11b, 12**), with overall yields between 18%–32% (Figure 2) [18]. The purity and identity of each compound was verified by mass spectrometry (EI) and ^{1}H-, ^{13}C-, and ^{31}P-NMR (data not shown).

Chemoenzymatic Synthesis of TDP-Glucose Derivatives

In parallel to the chemical syntheses noted above, we evaluated the ability of the thymidylyl transferase E_p to take a variety of D-glucose-1-P derivatives for thymidylyl transfer catalysis (Figure 3), building on prior efforts that suggested relaxed specificity could be expected [16, 17]. In the deoxy series, the 2-, 3-, 4-, and 6-deoxyglucose-1-phosphate species were prepared as previously described [17] and were shown to be thymidylylated by E_p to the TDP-sugar products as evaluated by HPLC analysis (data not shown). Under the incubation conditions noted in the experimental section, we observed 70%–100% conversion to 4- and 6-deoxy-glucosyl-TDP species, while the TDP-2-deoxy-glucose was generated in only about 10% yield, and TDP-3-deoxy-glucose was generated in about 30% yield. In incubations for subsequent reaction with GtfE and aglycones, this resulted in 250 μM TDP-2-deoxy-glucose in the GtfE reaction, 0.75 mM TDP-3-deoxy-glucose, and approximately 2 mM solutions of TDP-4-deoxy-glucose and TDP-6-deoxy-glucose. The double-variant glucose derivative, TDP-4-amino-6-deoxy-glucose, was also made using E_p-mediated conversion of the corresponding sugar-1-phosphate in 80%–90% yield, resulting in a final concentration of 2 mM in subsequent incubations with GtfE.

Since the chemical coupling noted above gave UDP-2-amino-glucose and UDP-6-amino-glucose, we prepared the other two regiosiomers enzymatically as the TDP-sugars by E_p-mediated conversions of 3-amino-glucose-1-phospate and 4-amino-glucose-1-phosphate [17] to produce TDP-3-amino-glucose and TDP-4-amino-glucose in 80%–90% yields in the 3 hr incubations. Large-scale incubations were performed to generate 18 mg of TDP-3-amino-glucose and 3.5 mg of TDP-4-amino-glucose after purification for further studies to determine kinetic parameters with GtfE.

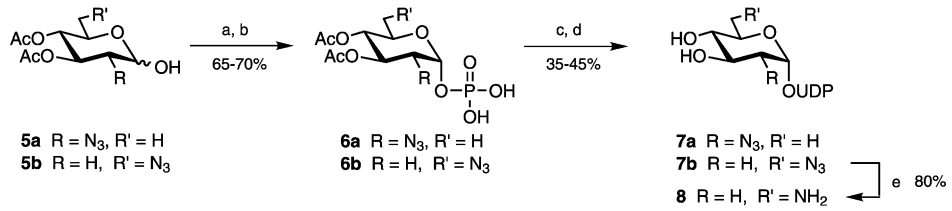
Thus, all four regioisomers of TDP-deoxyglucose and NDP-aminoglucose, as well as the doubly variant TDP-4-amino-6-deoxy-glucose and the 6-desmethyl-glucose, UDP-xylose (Figure 4), were available for testing as sugar donors for GtfE catalysis.

Transfer of Glucose Derivatives by GtfE to the Vancomycin and Teicoplanin Aglycones

The ten UDP- and TDP-glucose derivatives shown in Figure 4, as well as UDP-2- and 6-azido-glucose, UDP-2-fluoro-glucose, and UDP-6-chloro-glucose were assayed as substrates for pure GtfE with both its native aglycone heptapeptide substrate (AGV, vancomycin aglycone) and also with the teicoplanin aglycone (AGT, teicoplanin aglycone), which differs in amino acids 1 and

A

UDP-2-azido-, 6-azido- and 6-amino-glucose:



B

UDP-6-chloro-, 2-fluoro-, and 2-amino-glucose:

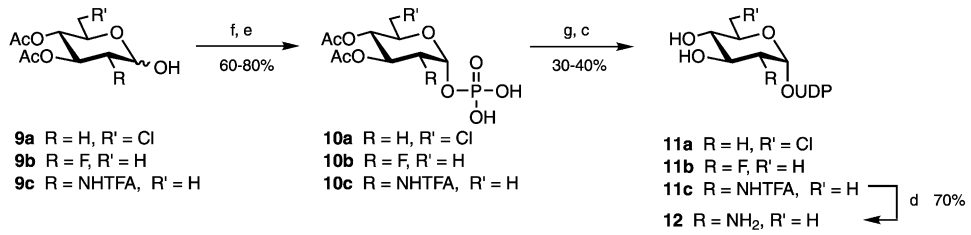


Figure 2. Synthesis of UDP-Glucose Derivatives

(A) Synthesis of UDP-2-azido-, 6-azido-, and 6-amino-glucose; (B) synthesis of UDP-6-chloro-, 2-fluoro-, and 2-amino-glucose. Reagents and conditions: (a) 1. iPr$_2$NP(OCH$_2$CH$_2$CN)$_2$, tetrazole, CH$_2$Cl$_2$, −40°–0°C; 2. mCPBA, CH$_2$Cl$_2$, −40°–0°C; (b) TMG, TMSCl, CH$_3$CN, rt, 1hr; (c) UMP-morpholidate, tetrazole, pyridine, rt, 48 hr; (d) Et$_3$N/MeOH/H$_2$O (1:2:2), 24 hr; (e) H$_2$, Pd-C, MeOH, 4 hr; (f) 1. iPr$_2$NP(OBn)$_2$, tetrazole, CH$_2$Cl$_2$, −40°–0°C; 2. mCPBA, CH$_2$Cl$_2$, −40°–0°C; (g) NaOMe, MeOH, 1 hr.

3. Glycosylated peptide products were first analyzed by HPLC, and new peaks were corroborated for molecular weight by Maldi-TOF mass spectrometry. Each of the nucleotide sugar substrates shown in Figure 4B could be recognized and the sugar moiety transferred by GtfE to the vancomycin and teicoplanin scaffolds as analyzed by HPLC and mass spectrometry, while UDP-2-azido-, UDP-2-fluoro-, and UDP-6-azido-glucose proved inadequate for transfer by GtfE. In Figure 5, representative HPLC traces are shown using the donor sugar substrates UDP-glucose, UDP-2-amino-glucose, and UDP-6-amino-glucose, showing glycosyl transfer to both the vancomycin and teicoplanin aglycone acceptor substrates. K_m and k_{cat} data for selected NDP-sugar substrates with GtfE are collected in Table 1.

First, both UDP-glucose and TDP-glucose as substrates have equal catalytic efficiency (k_{cat}/K_m), indicating that GtfE is not fastidious about the 5-methyl of thymine or the 2′-hydroxyl of ribose versus deoxyribose. This permissivity toward the NDP moiety allows cross comparison of the other UDP- and TDP-glucose analogs. The 6-deoxydesmethyl (xylose) and the 6-chloromethyl group in UDP-6-chloro-glucose are tolerated at about 9% and less than 0.1% catalytic efficiency, respectively. All K_m and k_{cat} data were gathered using the vancomycin aglycone as the acceptor substrate. The four regioisomeric TDP-deoxyglucoses also served as substrates and could be transferred not only to the vancomycin scaffold but also the teicoplanin scaffold. For example, as shown in Figure 6B, the 4-deoxy-glucosyl-AGT product (**15**) could be accumulated and detected by HPLC analysis of GtfE incubations with AGT and TDP-4-deoxy-glucose. UDP-2-fluoro-glucose was not detectably processed by GtfE, perhaps due to the electronic effects of 2-F on the reactivity at the adjacent C_1.

K_m and k_{cat} data were obtained for all four regioisomers of NDP-aminoglucose reacting with the vancomycin aglycone scaffold and are shown in Table 1. GtfE is remarkably permissive for the amino group at carbons 2, 3, 4, and 6 of the glucose ring, with all four NDP-

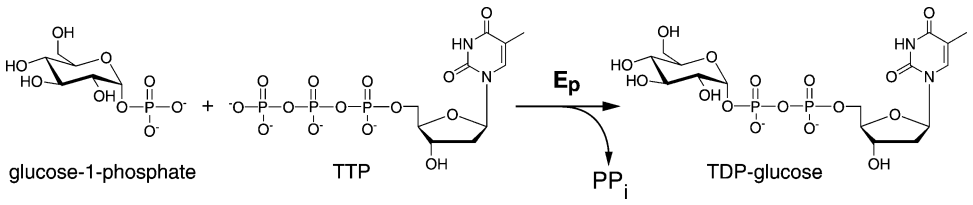


Figure 3. Scheme for E_p-Mediated Synthesis of TDP-Glucose

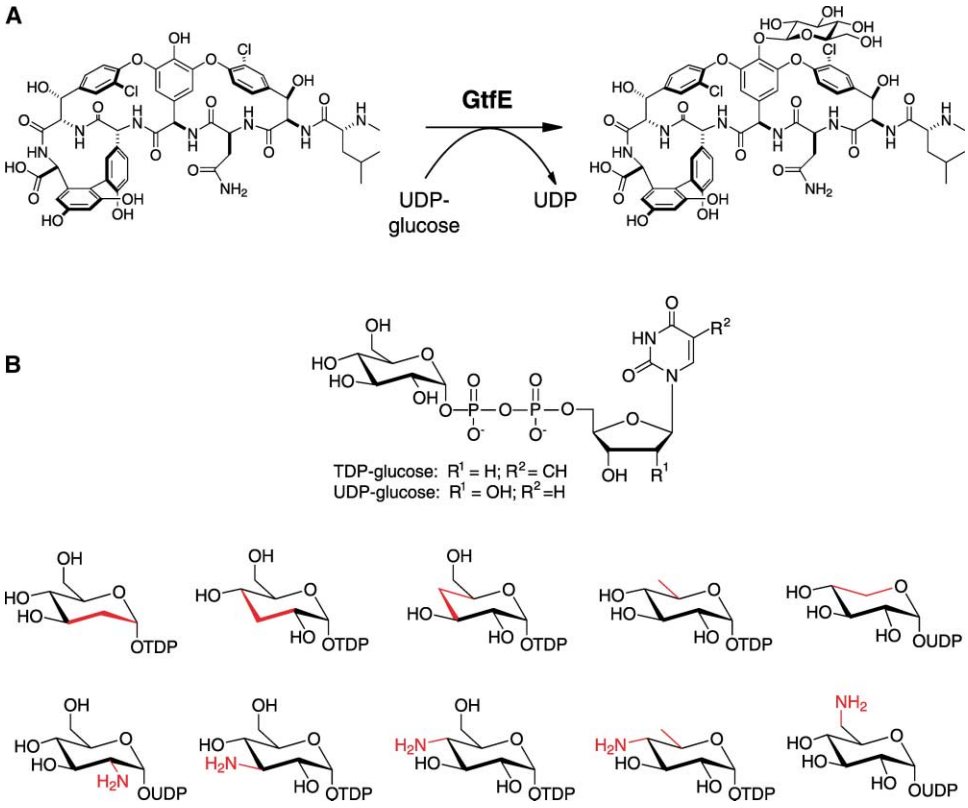


Figure 4. GtfE Recognition and Transfer of Alternate Nucleotide-Sugar Donors onto the Heptapeptide Acceptor Substrate
(A) Reaction pathway of GtfE; (B) deoxy- and amino-derivatives of NDP-glucose tested.

aminoglucoses having equivalent K_m values (0.7–1.2 mM) and also a narrow range of k_{cat} values (1.3–7.1 min^{-1}). Compared to the authentic glucosyl donor substrate, TDP- or UDP-glucose, the 4- to 30-fold reduction in catalytic efficiency with the four regioisomeric NDP-aminoglucoses is almost entirely a k_{cat} effect. The pK_a of the amine of the aminoglucoses has been reported to be between 7.75–9 [19, 20]. Since the reactions are all carried out at pH 9, which is at or above the pK_a of the aminoglucose substrates, it is not yet known whether GtfE transfers the aminoglucose moiety with a protonated amine or as the free base. Regardless, the ability of GtfE to transfer sugars with an amine displayed at many points of the hexose ring periphery indicated that this combination of enzyme and nucleotide sugars would be a useful platform for evaluation of the next glycosyltransferase, GtfD.

Activity of the Second Glycosyltransferase GtfD: Transfer of L-4-*epi*-Vancosaminyl Groups to the Glucosylpeptide Variants

The deoxyglucosyl- and aminoglucosyl-heptapeptide products from GtfE incubations could thus be evaluated as substrates for the next enzyme, GtfD, the L-vancosaminyltransferase, involved in the maturation of late-stage biosynthetic intermediates of the vancomycin pathway. The natural sugar donor is presumed to be TDP-L-β-vancosamine, where vancosamine is a 3-deoxy-3-methyl-3-amino-hexose. This TDP sugar is not available for study. However, we have reported both the biosynthesis of the 4-*epi* form, TDP-L-β-4-*epi*-vancosamine, by action of five purified enzymes EvaA-E, from the biosynthetic operon in the chloroeremomycin producer [21], and also the chemical synthesis of the α/β anomeric mixture of UDP-L-4-*epi*-vancosamine [9]. The latter material was available for use in tandem incubations of GtfE and GtfD.

The tandem action of GtfE and GtfD was attempted using TDP-4-amino-glucose and GtfE in the first stage and UDP-L-4-*epi*-vancosamine and GtfD in the second stage (Figure 6A). The heptapeptide scaffold used in this case was the vancomycin aglycone, and GtfE action resulted in transfer of 4-amino-glucose (**13**). The product of GtfE and GtfD action was 4-*epi*-vancosaminyl-(1,2)-4-amino-glucosyl-AGV (**14**), containing a 1,2-disaccharide moiety with two amino sugars (Figure 6A). The tandem incubation of GtfE and GtfD combines to yield a derivative of vancomycin with an alteration in each of the two sugars: an amine derivative of glucose and a position 4 epimer of vancosamine.

Tandem incubations of GtfE and GtfD were also carried out on the teicoplanin aglycone scaffold. As exemplified in the right-hand column of Figure 6B, incubation commenced with GtfE and TDP-4-deoxy-glucose to yield 4-deoxy-glucosyl-AGT (**15**). The second stage involved UDP-L-4-*epi*-vancosamine as the donor sub-

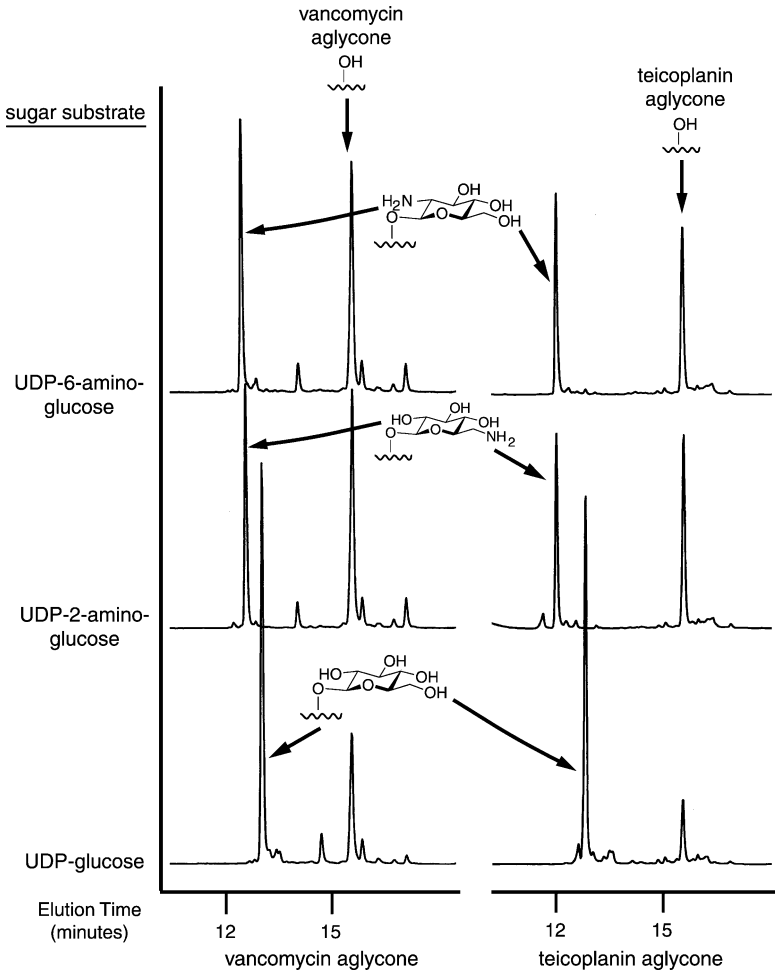


Figure 5. Transfer of Aminoglucoses by GtfE

(A) HPLC traces of glucose, 2-amino-glucose, and 6-amino-glucose being transferred to vancomycin aglycone. Mass spectrometry results for 2-amino-glucosyl-AGV, calculated [M + H^+] = 1304.4, observed [M + H^+] = 1304.5; for 6-amino-glucosyl-AGV, calculated = 1304.4, observed = 1304.5.

(B) HPLC traces of glucose, 2-amino-glucose, and 6-amino-glucose being transferred to teicoplanin aglycone. Mass spectrometry results for 2-amino-glucosyl-AGT, calculated [M + H^+] = 1359.4, observed [M + Na^+] = 1381.4; for 6-amino-glucosyl-AGV, calculated [M + H^+] = 1359.4, observed [M + Na^+] = 1381.5.

strate and GtfD as the catalyst to produce the novel teicoplanin analog 4-*epi*-vancosaminyl-(1,2)-4-deoxyglucosyl-AGT (**16**), two sugars which are not normally found appended to the teicoplanin heptapeptide scaffold. Both the heptapeptide scaffold and the two sugars in the disaccharide moiety differ in the two glycopeptide variants shown in Figure 6.

Analogous incubations demonstrated that we were able to generate derivatives of the vancomycin disaccharide containing 3-, 4-, and 6-deoxyglucoses and 3-, 4-, and 6-aminoglucoses, as well as 4-amino-6-deoxyglucose attached to both the vancomycin and teicoplanin heptapeptide scaffolds (data not shown). Each new product detected by HPLC was subsequently analyzed by Maldi-TOF mass spectrometry to verify mass.

Discussion

In the naturally occurring glycopeptide antibiotics, the sugars matter: to some extent in determining potency, to a large extent in increasing solubility, to increase dimerization constants, and to restrict conformational

Table 1. Steady-State Kinetic Parameters for Purified NDP-Glucose Derivatives

Substrate[a]	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)	Relative k_{cat}/K_m
UDP-glucose	0.72 ± 0.11	31 ± 2	43	1
TDP-glucose	0.62 ± 0.09	29 ± 2	47	1/0.91
UDP-2-NH_2-glucose	0.79 ± 0.08	2.7 ± 0.2	3.4	1/13
TDP-3-NH_2-glucose	0.72 ± 0.06	7.1 ± 0.2	9.9	1/4.3
TDP-4-NH_2-glucose	1.1 ± 0.1	2.6 ± 0.1	2.4	1/18
UDP-6-NH_2-glucose	1.2 ± 0.2	1.5 ± 0.2	1.3	1/33
UDP-xylose	1.8 ± 0.2	8.4 ± 0.6	4.7	1/9.1
UDP-6-Cl-glucose	21 ± 6	0.3 ± 0.1	0.014	1/3100

[a] The acceptor substrate, the vancomycin aglycone, was held constant at 1 mM in each experiment.

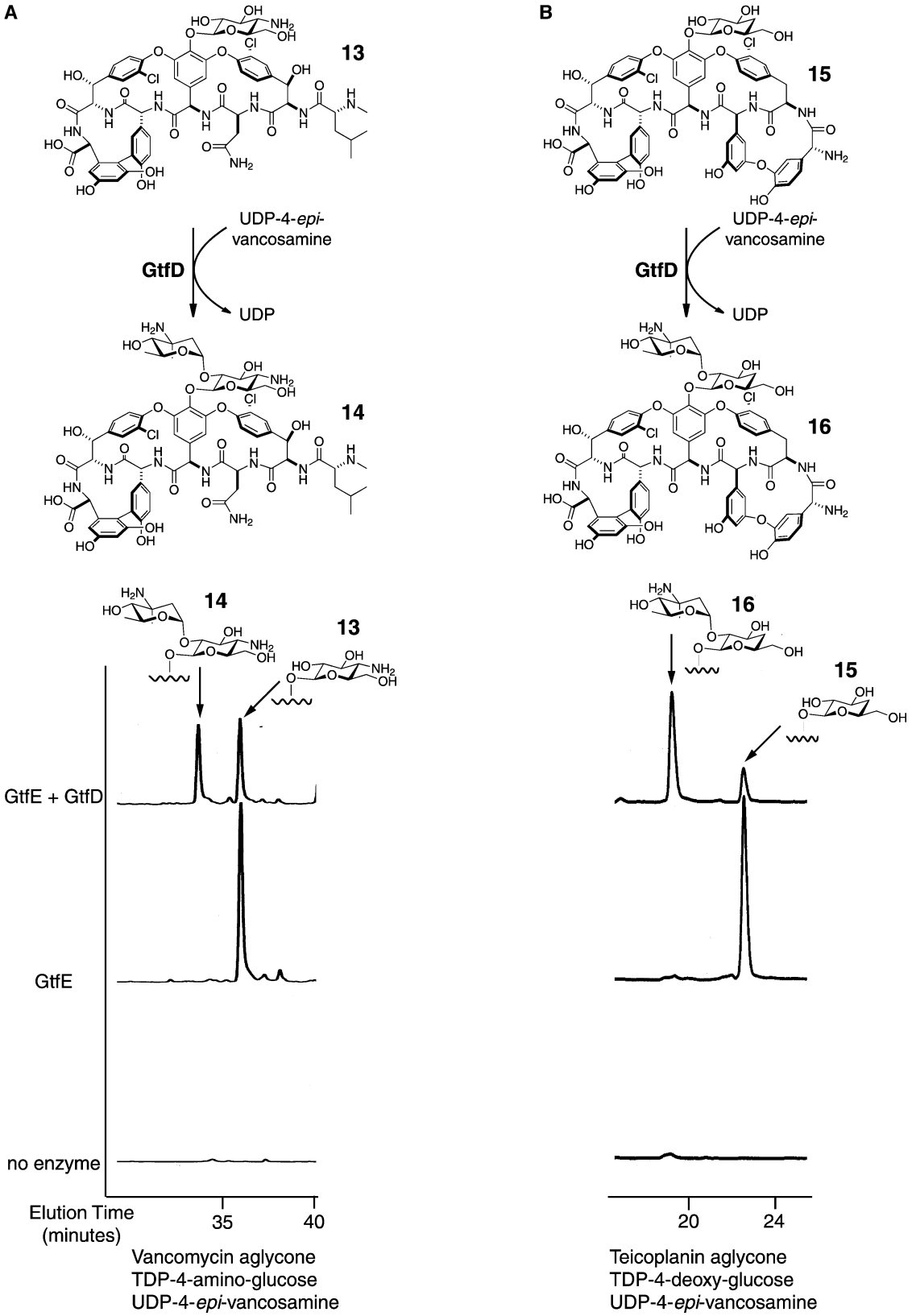


Figure 6. Recognition of Modified Scaffolds by GtfD

(A) Reaction pathway of 4-*epi*-vancosaminylation by GtfD of the vancomycin scaffold with 4-amino-glucose attached and the corresponding HPLC traces with no enzyme, GtfE alone (**13**, calculated [M + H^+] = 1304.4, observed = 1304.4), or GtfE+D (**14**, calculated [M + H^+] = 1447.4, observed [M + Na^+] = 1469.5).

(B) Reaction pathway of 4-*epi*-vancosaminylation by GtfD of the teicoplanin scaffold with 4-deoxy-glucose attached and the corresponding HPLC traces with no enzyme, GtfE alone (**15**, calculated [M + H^+] = 1344.3, observed [M + Na^+] = 1366.5), or GtfE+D (**16**, calculated [M + H^+] = 1487.4, observed [M + Na^+] = 1509.2).

flexibility of the aglycone scaffold [22–24]. The amino groups in the aminohexose moieties of both teicoplanin (glucosamine), **2**, and oritavancin (4-*epi*-vancosamine), **3**, are sites for either natural acylation (teicoplanin) or semisynthetic alkylation (oritavancin), modifications that improve activity against VRE. The ability to generate additional aminosugar variants of glycopeptides would be of particular interest.

As is typical in many glycopeptide and glycosylated polyketide biosynthetic clusters, the genes for producing the dedicated aminodeoxysugars and the glycosyltransferases that use the TDP-aminodeoxysugars as donors are integral ORFs in those gene clusters [25–27]. Our prior efforts have validated the function of eight such ORFs in chloroeremomycin biosynthetic cluster [9, 21], encoding five enzymes for conversion of TDP-4-keto-6-deoxy-glucose to TDP-L-4-*epi*-vancosamine and three enzymes that attach TDP-glucose or TDP-L-4-*epi*-vancosamine at three sites to complete antibiotic maturation. Likewise, we have established that GtfD and GtfE, cloned from the vancomycin producer and purified after heterologous expression in *Escherichia coli*, act in tandem to generate the disaccharyl chain of vancomycin [9].

In this study, we began with assay of the glucosyltransferase, GtfE, that adds the first sugar, a D-glucosyl unit, to the phenolic oxygen of the 4-hydroxyphenylglycine residue at position four of the crosslinked heptapeptide aglycone scaffold of vancomycin [9]. We have focused initially on the four regioisomeric deoxyglucoses and the four corresponding aminoglucose isomers, given the occurrence of deoxy and amino substitutions in the natural sugar chains of glycopeptide antibiotics [28]. We also evaluated the 6-chloro and 2- and 6-azido versions of UDP-glucose because of their potential for subsequent chemical elaboration to additional derivatives after GtfE or GtfE/D tandem action. None of the latter three UDP-sugars were robust substrates for GtfE. Likewise, UDP-2-fluoro-glucose was not recognized, probably due to inductive deactivation at the adjacent C_1 position.

The 2-, 3-, 4-, and 6-deoxy-glucosyl-AGV products from GtfE action could provide differential elements of hydrophobic surface patches on the sugar and might be of use in combinations to modulate solubility properties of such glycopeptide variants. Of these four isomers, the 2-deoxy-glucosyl-AGV would be a dead-end product vis a vis further elongation since the 2-OH of the glucose moiety is the nucleophile in the next reaction, the vancosaminyl/4-*epi*-vancosaminyl transfer by GtfD or GtfC to create the (4-*epi*)-vancosaminyl-(1,2)-glucosyl linkage. We have demonstrated, as shown in Figure 6, that the subsequent enzyme GtfD is sufficiently promiscuous that it can indeed transfer 4-*epi*-vancosamine to a teicoplanin scaffold with a 4-deoxy-glucose moiety in lieu of glucose.

The regioisomeric aminoglucoses attached to both the vancomycin and teicoplanin scaffolds are particularly interesting since the amino groups are chemical handles for both natural acylation and synthetic reductive alkylation to produce lipoglycopeptides. In teicoplanin, **2**, it is the 2-amino-glucose moiety that is enzymatically acylated. In oritavancin, **3**, the only amino group in the disaccharide moiety is in the distal sugar, the 4-*epi*-vancosamine, in which the amine is at position 3 of the hexose ring, which is the site of chlorobiphenyl alkylation to gain activity against VRE. The ability to move the amino group around the glucose ring to all four available positions and append to the vancomycin or teicoplanin scaffold will allow subsequent acylation to probe any differences in efficacy from differential placement of the fatty acyl chain [29]. On the vancomycin scaffold, the 2-amino-glucose is likely to be incompetent for subsequent chain elongation by GtfE since that would generate a bridging –NH group to the terminal vancosamine sugar, and no activity was detected with GtfD. The other three sites, 3-amino, 4-amino, and 6-amino, are permissive for elongation and should allow site-specific alkylation/acylation for subsequent evaluation of potency and spectrum against both sensitive and resistant enterococci, as well as a broader range of gram-positive bacterial pathogens.

Experiments investigating tandem action of GtfE, then GtfD, were successful, indicating that amino- or deoxy-hybrid disaccharide chains can be built on both aglycone scaffolds. Figure 6 shows that both the 4-amino-glucosyl-AGV and the 4-deoxy-glucosyl-AGT could be elongated by transfer of 4-*epi*-vancosamine by action of GtfD. Preparatively useful amounts should be obtainable for subsequent acylation and/or reductive alkylation to test for improved antibacterial properties. The 4-*epi*-vancosaminyl-4-amino-glucosyl-AGV (**14**) is representative of a new subclass of vancomycin/teicoplanin hybrids with two amino groups in the disaccharide, and selective alkylation/acylation chemistry should be possible to evaluate the utility of double substitution against both antibiotic-sensitive and resistant bacteria.

Analogous experiments showed that heptapeptide scaffolds with 3-, 4-, and 6-deoxyglucose and 3-, 4-, and 6-amino-glucose attached could be further elongated with 4-*epi*-vancosamine by GtfD. In addition, the doubly variant 4-amino-6-deoxy-glucose could also be elongated by GtfD, suggesting that both GtfE and GtfD can tolerate more than a single change. These experiments suggest that the vancomycin glycosyltransferases are very good candidates for use in combinatorial biosynthesis of glycopeptide antibiotics.

The Gtfs that carry out the last stages of glycopeptide antibiotic maturation are promising reagents for introduction of structural versatility on complex aglycone scaffolds. To implement this approach further, four things are required. First is an expanded library of TDP-D-glucose analogs, where the E_p thymidylyltransferase with its engineered relaxation of specificity toward glucose-1-phosphate derivatives should be a particularly useful reagent [15, 30, 31]. Second will be a library of TDP-L-vancosamine analogs. Because the sugar is of the L-configuration and because the biosynthesis of TDP-L-sugars are invariably multiple enzymatic steps from TDP-D-glucose [21, 32], a chemical approach to libraries of TDP-L-hexoses may be the shorter path. The three most common biochemically generated substituents that tailor the hexose backbones are deoxy, amino, and C- and N-methyl groups, and these would be good starting elements in synthetic TDP-L-hexose libraries. The third requirement would be for additional variants

of the crosslinked aglycone scaffolds as starting substrates for glycosylation. These could include backbone alterations such as those found in the complestatin aglycone [33], O-sulfation found in the naturally occurring A47934 antibiotic of the teicoplanin family [8, 34], and selective semisynthetic alterations at the N and C termini of the heptapeptide scaffold, such as found in dalbavancin [35]. The fourth requirement would be for a broader set of Gtfs. These could come from as yet uncloned glycopeptide biosynthetic clusters that would put in the other aminodeoxy sugars found in this class of natural products, such as L-acosamine or L-ristosamine [28]. Alternatively, recent advances in structure determination of GtfB [36] and the related MurG [37] might suggest productive reengineering approaches to broaden glycosyl transfer capacity.

Finally, libraries of TDP-D-hexoses and TDP-L-hexoses would also be useful substrate collections for Gtfs that transfer glycosyl groups to nonpeptidic aglycones such as the 14- and 16-membered polyketide macrolactones, deoxyerythronolactone and tylactone on the way to erythromycin and tylosin, as well as the polycyclic polyketide aglycones, e.g., in the mithramycin and daunomycin families [25, 38–40]. Many of these glycosyltransferases have been shown to have sugar substrate flexibility (as reviewed in [10]), but available sugar substrates are limited to those that are made in these organisms by large sets of biosynthetic enzymes.

Significance

We have used a combination of chemical and chemoenzymatic approaches to generate a library of nucleotide sugar substrates for glycosyltransferases from the vancomycin biosynthetic operon in order to evaluate the potential for combinatorial biosynthesis within the glycopeptide class of antibiotics. We have demonstrated the ability to incorporate numerous analogs of NDP-glucose onto the heptapeptide scaffolds of both vancomycin and teicoplanin, including all four regioisomeric deoxy- and aminoglucoses, using the glucosyltransferase GtfE. In addition, subsequent elaboration of the modified glucosylpeptides with 4-*epi*-vancosamine by the vancosaminyltransferase, GtfD, generated vancomycin and teicoplanin derivatives with variant disaccharides. The ability to incorporate all four regioisomeric aminoglucoses allows for subsequent selective modification of the amine by chemical acylation or reductive alkylation, which has been shown to increase activity against vancomycin-resistant enterococci, as exemplified by the semisynthetic glycopeptide oritavancin. The ability to append disaccharides with two aminosugars onto the vancomycin and teicoplanin scaffolds, for example the vancomycin heptapeptide with the disaccharide 4-*epi*-vancosamine-(1,2)-4-amino-glucose attached, provides two sites for subsequent chemical modification and increases the potential to discover derivatives that are active against vancomycin-resistant enterococci. The observed relaxed specificity of the vancomycin glycosyltransferases GtfE and GtfD demonstrates the potential for combinatorial biosynthesis within the glycopeptide class of antibiotics.

Experimental Procedures

Synthesis of UDP-2-Amino-Glucose, UDP-2-Azido-Glucose, UDP-2-Fluoro-Glucose, UDP-6-Amino-Glucose, UDP-6-Azido-Glucose, and UDP-6-Chloro-Glucose

The different UDP-glucose derivatives were prepared starting from the corresponding acetylated lactols (Figure 2) which were obtained from the anomeric acetates [41–45] by cleavage with hydrazine acetate in DMF. The 2-azido and 6-azido lactols (**5a** and **5b**) were coupled with dicyanoethyl diisopropylphosphoramidite with tetrazole in dichloromethane (CH_2Cl_2) and oxidized to the protected 1-phosphates with 3-chloroperbenzoic acid (*m*CPBA) in CH_2Cl_2 ($-40°$–$0°C$). Removal of the cyanoethyl groups was achieved by treatment with tetramethyl guanidine (TMG) and chlorotrimethylsilane (TMSCl) in acetonitrile (CH_3CN) at room temperature for 1 hr [46]. The 3,4-acetoxy sugar-1-phosphates (**6a** and **6b**) were coupled with UMP-morpholidate using tetrazole as a catalyst in pyridine at room temperature for 48 hr. Deacetylation (Et_3N/MeOH/H_2O, 1:2:2 for 24 hr) gave UDP-2-azido-glucose (**7a**) and UDP-6-azido-glucose (**7b**), respectively. Hydrogenation (H_2, Pd-C) for 4 hr in methanol of the **7b** yielded UDP-6-amino-glucose (**8**) (Figure 2A).

For synthesis of the 6-chloro, 2-fluoro, and 2-amino UDP-glucose derivatives, a slightly modified reaction scheme was followed (Figure 2). Coupling of the 6-chloro and 2-fluoro lactols (**9a** and **9b**), 2-*N*-trifluoroacetyl lactol (**9c**) with dibenzyl diisopropylphosphoramidite with tetrazole in CH_2Cl_2, oxidation with *m*CPBA in CH_2Cl_2 ($-40°$–$0°C$), and hydrogenation of the benzyl groups (H_2, Pd-C) for 4 hr in methanol afforded the 3,4-acetoxy sugar-1-phosphates (**10a**, **10b**, and **10c**). Deacetylation with NaOMe in methanol for 1 hr gave an anomeric mixture of phosphates, which was coupled with UMP-morpholidate [18] using tetrazole as a catalyst in pyridine at room temperature for 48 hr to give UDP-6-chloro-glucose (**11a**), UDP-2-fluoro-glucose (**11b**), and UDP-2-*N*-TFA-glucose (**11c**). UDP-2-amino-glucose (**12**) was obtained from **11c** following removal of the trifluoroacetyl protecting group with Et_3N/MeOH/H_2O (1:2:2) for 24 hr (Figure 2B). For all compounds, identity and purity were verified by ^{1}H-, ^{13}C-, and ^{31}P-NMR and mass spectrometry (EI).

Chemoenzymatic Synthesis of TDP-Deoxy- and TDP-Aminoglucose Derivatives by E_p

The procedure for the enzymatic conversion of glucose-1-phosphate to TDP-glucose by the E_p enzyme has been described previously, as has the ability of E_p to accept many derivatives of glucose-1-phosphate for catalysis [16, 17]. E_p was purified as previously described, and 2-, 3-, 4-, and 6-deoxy- and amino-glucose-1-phosphates as well as 4-amino-6-deoxy-glucose-1-phosphate were synthesized as before [16, 17]. The in vitro reactions were carried out as previously described [17], except glucose-1-phosphate derivative concentrations were 5 mM, TTP concentration was 5 mM, and 5 units of inorganic pyrophosphatase were used in each reaction. Reaction progress was monitored by HPLC (A_{260}) using an Alltech Strong Anion Exchange (SAX) column (250 $\times$ 4.6mm) and a linear gradient of 50–250 mM potassium phosphate (pH 5) over 20 min.

Large-scale incubations were carried out in order to make and purify TDP-3- and 4-amino-glucose. A 25 ml enzymatic reaction containing 25 mg 3-amino-glucose-1-phosphate at a final concentration of 3.8 mM was incubated for 3 hr at 37°C. Once the reaction reached 75% completion as monitored by SAX HPLC (see above), it was quenched by addition of 25 ml cold methanol. After centrifugation, the supernatant was concentrated and then lyophilized. After resuspension in 0.5 ml water, the mixture was chromatographed on a 10 g silica gel column with 1:1 methanol:ammonium hydroxide as the eluent. Fractions were analyzed by silica gel thin layer chromatography using the same eluent and detected by UV. Fractions containing TDP-3-amino-glucose were pooled and concentrated. SAX HPLC and ^{31}P-NMR analysis were used to verify the purity of the sample.

A 3.2 ml E_p reaction containing 3 mg 4-amino-glucose-1-phosphate at a final concentration of 3.5 mM was incubated for 2 hr at 37°C and quenched with an equal volume of cold methanol. After centrifugation, the supernatant was concentrated and purified by HPLC using a Alltech semiprep SAX column (10 $\times$ 250 mm) with a linear gradient of 50–175 mM ammonium acetate (pH 5.5) over 20

min. TDP-4-amino-glucose-containing fractions were pooled and lyophilized, and purity was analyzed by ^{31}P-NMR.

In Vitro Glycosylation Reactions

Glycosyltransferases GtfE and GtfD were overproduced and purified as previously described [9]. Reactions were carried out and analyzed by HPLC as previously described [9]. In brief, synthetic UDP-glucose derivatives were added to a final concentration of 5 mM in a 100 μl reaction, and 50 μl of the E_p reaction mixture to make each TDP-glucose derivative was added to make a final reaction volume of 100 μL. Each 100 μl reaction contained either 1 mM vancomycin aglycone or 0.5 mM teicoplanin aglycone. The final concentration of GtfE and GtfD in each reaction was 5 μM. Reactions with GtfD also contained 2 mM UDP-L-4-*epi*-vancosamine. Fifty microliter time points were taken at 0 and 20 hr and quenched with 9 vol methanol. After centrifugation, the supernatants were dried and resuspended in water for HPLC analysis. All glycosylation reactions were monitored by UV (285 nm) using a Vydac C18 small-pore column with a linear gradient of 0%–20% acetonitrile in water with 0.1% trifluoracetic acid. The molecular weight of new products was analyzed by Maldi-TOF mass spectrometry using a Perseptive Biosystems Voyager-DE STR mass spectrometer.

In order to determine steady-state kinetic parameters of GtfE, the concentrations of each sugar listed in Table 1 were varied. All reactions were carried out with 1 mM vancomycin aglycone as the acceptor substrate. For UDP- and TDP-glucose, [GtfE] was 50 nM, and the sugar substrates were varied from 0.25–8 mM; for UDP-xylose [GtfE] was 200 nM, and UDP-xylose was varied from 0.5–24 mM; for UDP-6-chloro-glucose [GtfE] was 5 μM, and the sugar substrates were varied from 1–32 mM; for UDP-2-amino-glucose [GtfE] was 150 nM, and UDP-2-amino-glucose was varied from 0.125–8 mM; for TDP-3-amino-glucose [GtfE] was 200 nM, and TDP-3-amino-glucose was varied from 0.15–6 mM; for TDP-4-amino-glucose [GtfE] was 250 nM, and TDP-4-amino-glucose was varied from 0.25–8 mM; and for UDP-6-amino-glucose [GtfE] was 200 nM, and UDP-6-amino-glucose was varied from 0.25–12 mM. Each experiment was performed in duplicate or triplicate.

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