Mechanistic Studies of a Retaining α-Galactosyltransferase from *Neisseria meningitidis*†

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Received November 7, 2001; Revised Manuscript Received January 30, 2002

ABSTRACT: Lipopolysaccharyl α-galactosyltransferase from *Neisseria meningitidis* catalyzes the transfer of a galactosyl moiety from the activated donor UDP-Gal to glycoconjugates to yield an elongated saccharide product with net retention of anomeric configuration relative to the donor substrate. Through kinetic analyses in which the concentrations of both substrates are independently varied and through inhibition studies with dead-end analogues of both substrates and with the oligosaccharide product, we have demonstrated that this enzyme follows an ordered bi-bi kinetic mechanism. Various aspects of the chemical mechanism including the possible formation of a covalent glycosyl-enzyme intermediate were also probed using an assortment of strategies. While the results of these investigations were unable to clearly delineate the chemical mechanism of this enzyme, they provide important insights into the catalytic machinery surrounding the events involved in catalysis.

In terms of biomass, carbohydrates are by far the most abundant of all macromolecules. In addition, these complex molecules are also remarkably diverse in their structures. This diversity is due in large part to the fact that these compounds are not synthesized according to a template, as is the case with other biopolymers such as proteins and nucleic acids. Instead, it is often the substrate specificities of the individual enzymes involved in their synthesis that dictate the final structure of the carbohydrates. Despite the widely recognized importance of carbohydrates in many biological functions (*1*−*3*), very few mechanistic details are known about the enzymes responsible for their biosynthesis, namely, the glycosyltransferases.

Glycosyltransferases are enzymes that catalyze the transfer of glycosyl moieties from a donor sugar to an acceptor. In most cases the donor is a nucleoside phosphosugar and the acceptor an alcohol functionality from another sugar, a lipid, or a range of other components of glycoconjugates. In the past, the study of glycosyltransferases was greatly hindered by their limited availability and stability. However, these problems have been addressed through high-level recombinant expression of these enzymes; thus interest in the study of glycosyltransferases has increased significantly.

On the basis of sequence similarities, glycosyltransferases have been grouped into 51 distinct families, and to date, well over 2500 sequences have been included in this method of classification (*4*). In addition to these families, glycosyltransferases are also classified as either retaining or inverting, as is done with the well-studied glycosyl hydrolases (*5*, *6*), depending on the stereochemical outcome of the reaction catalyzed. Enzymes that catalyze reactions where the anomeric configuration of the product is retained relative to that of the donor sugar substrate are termed "retaining" glycosyltransferases. Conversely, those that catalyze the transfer reaction with net inversion of anomeric configuration in the product are termed "inverting" glycosyltransferases. By analogy with the glycosyl hydrolases, retaining and inverting glycosyltransferases are therefore believed to operate via two distinct chemical mechanisms (Figure 1). Unfortunately, experimental evidence for these mechanisms is scarce and limited to only a handful of enzymes (*6* and references cited therein).

Structural information on glycosyltransferases has also been scarce as it is only within the past few years that the three-dimensional structures of several of these enzymes have been solved. Of this handful of structures, the majority have been of inverting glycosyltransferases (*7*−*13*) with only two very recent examples of retaining glycosyltransferases (*14*, *15*). In the case of the inverting glycosyltransferases, the available structures are indeed supportive of a direct displacement mechanism (Figure 1b) much like that of inverting glycosyl hydrolases. On the other hand, support for the proposed double displacement mechanism as the *modus operandi* of retaining glycosyltransferases (Figure 1a) has remained elusive.

Lipopolysaccharyl α-galactosyltransferase C (EC 2.4.1.x, LgtC) from *Neisseria meningitidis* catalyzes the transfer of a galactosyl moiety from uridine diphosphogalactose (UDP-Gal) to glycoconjugates bearing a galactose at the nonreducing terminus, with an overall retention of stereochemistry at the anomeric center. The resulting Gal-α-1,4-Gal linkage that is formed by this bacterial enzyme is a common feature of human glycolipids displayed on the surface of cells lining the upper urinary tract. In particular, this linkage is an integral component of the P^k antigen for the P blood group glycolipids, which is an important receptor for the shigatoxins produced by *Shigella dysenteriae* and *Escherichia coli* 0157:H7 (*16*). As a treatment option for infections caused by these microorganisms, synthetic glycoconjugates have been suc-

† This work was supported by the Natural Sciences and Engineering Research Council of Canada and the University of British Columbia.

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10.1021/bi012031s CCC: $22.00
Published on Web 03/27/2002

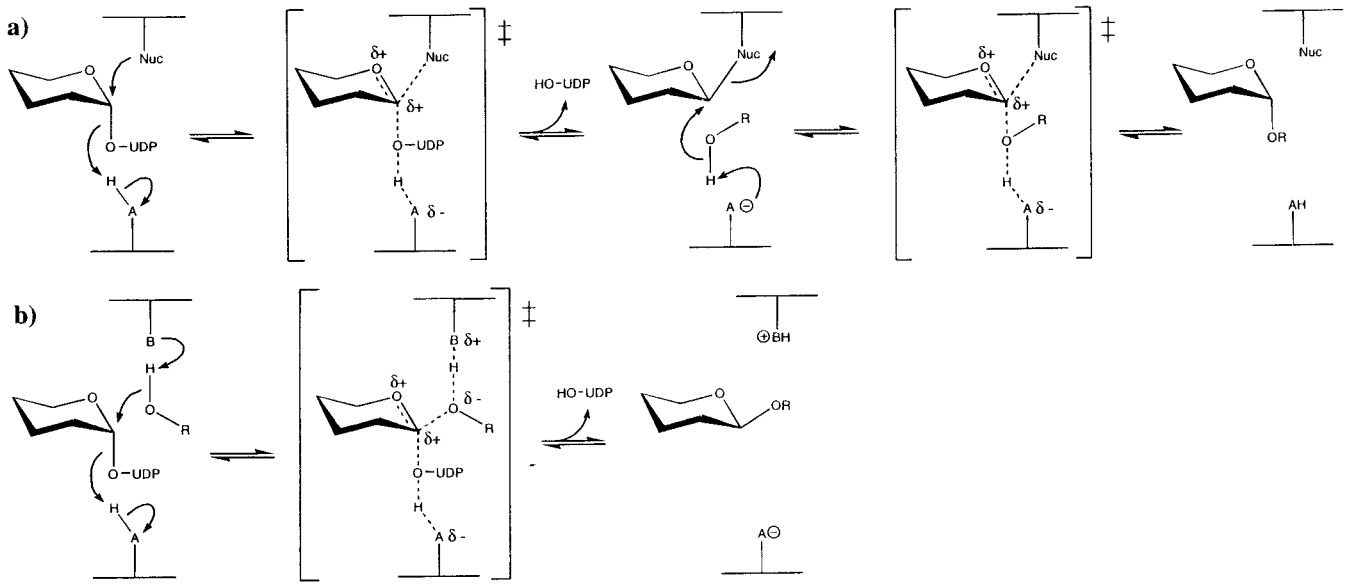

FIGURE 1: Proposed mechanisms for (a) retaining and (b) inverting glycosyltransferases.

cessfully employed as antiadhesive agents (*17*). As such, LgtC, which catalyzes the formation of a linkage that is synthetically difficult to achieve by chemical means, may be extremely useful in the rapidly growing field of oligosaccharide synthesis. A limiting factor in such applications, however, is the high cost of nucleoside phosphosugars. While attempts to circumvent this problem have been made through the development of glycosyl fluorides as a more economical alternative substrate (*18*), the reaction rates are unfortunately too low to make this process commercially viable. In the meantime, the best generally available alternative is the use of complex recycling schemes to regenerate the nucleoside phosphosugar (*19*).

On the basis of sequence similarities, LgtC is assigned to family 8 of glycosyltransferases (*4*). The gene encoding this enzyme has been cloned and overexpressed in *E. coli* as a truncated form of the enzyme that is missing the last 19 amino acids which make up a potential phospholipid binding region located at the C-terminus of the protein (*20*) (this truncated enzyme is referred to as LgtC-19). Truncation has been shown to have no effect on the in vitro activity of this enzyme (*20*). A detailed kinetic characterization of this recombinant protein as well as attempts to trap the covalent glycosyl-enzyme intermediate is reported in this work.

MATERIALS AND METHODS

General Methods. All buffer chemicals and other reagents were obtained from Sigma/Aldrich Chemical Co. unless otherwise noted. Characterizations for all final compounds are shown below. Details of their synthesis are provided in Supporting Information. All ^{1}H NMR spectra were recorded at either 200, 300, or 400 MHz using a Brüker AC-200, AV-300, or WH-400 spectrometer. All ^{19}F NMR spectra were recorded at 188 MHz using a Brüker AC-200 spectrometer with CF_3CO_2H as the reference, and all ^{31}P NMR spectra were recorded at either 81 or 121 MHz using a Brüker AC-200 with dimethyl phosphate as the reference or a Brüker AV-300 with phosphoric acid as the reference. Microanalyses were performed in-house by Mr. Peter Borda of the Department of Chemistry at the University of British Columbia. Recombinant LgtC-19 was overexpressed in *E. coli* (AS202) as described previously (*20*).

Benzyl β-Lactoside. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 7.30−7.60 (m, 5 H, Ar), 4.90 (d, 1 H, J = 11.4 Hz, PhC*H*), 4.52 (d, 1 H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.41 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1), 3.95 (dd, 1 H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.1 Hz, H-6a), 3.88 (d, 1 H, $J_{4',3'}$ = 3.3 Hz, H-4′), 3.32 (dd, 1 H, $J_{4,5}$ = 8.7 Hz, $J_{4,3}$ = 8.4 Hz, H-4).

Anal. Calcd for $C_{19}H_{28}O_{11}$: C, 52.77; H, 6.53. Found: C, 52.47; H, 6.63.

Allyl β-Lactoside. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 5.95 (m, 1 H, $OCH_2CH{=}CH_2$), 5.35 (dd, 1 H, J_{trans} = 17.3 Hz, J_{gem} = 1.4 Hz, $CH{=}CH_{trans}$), 5.26 (d, 1 H, J_{cis} = 8.4 Hz, $CH{=}CH_{cis}$), 4.50 (d, 1 H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.42 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1), 4.37 (m, 1 H, OC*H*), 4.20 (m, 1 H, OC*H*), 3.95 (dd, 1 H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 1.7 Hz, H-6a), 3.89 (d, 1 H, $J_{4',3'}$ = 3.1 Hz, H-4′), 3.52 (dd, 1 H, $J_{4,5}$ = 9.8 Hz, $J_{4,3}$ = 7.8 Hz, H-4).

Anal. Calcd for $C_{15}H_{26}O_{11}\cdot{}^1/_2H_2O$: C, 46.03; H, 6.95. Found: C, 46.52; H, 6.85.

4-Pentenyl β-Lactoside. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 5.89 (m, 1 H, $CH{=}CH_2$), 5.06 (dd, 1 H, J_{trans} = 17.3 Hz J_{gem} = 1.0 Hz, $CH{=}CH_{trans}$), 5.00 (dd, 1 H, J_{cis} = 9.3 Hz, J_{gem} = 1.0 Hz, $CH{=}CH_{cis}$), 4.45 (d, 1 H, $J_{1',2'}$ = 7.8 Hz, H-1′), 4.42 (d, 1 H, $J_{1,2}$ = 7.6 Hz, H-1), 2.07−2.17 (m, 2 H, $CH_2{-}CH{=}CH_2$), 1.65−1.75 (m, 2 H, $OCH_2{-}CH_2$).

Anal. Calcd for $C_{17}H_{30}O_{11}\cdot{}^1/_2H_2O$: C, 48.68; H, 7.45. Found: C, 48.98; H, 7.14.

2,3-Dihydroxypropyl β-Lactoside. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 4.47 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1), 4.42 (d, 1 H, $J_{1',2'}$ = 8.0 Hz, H-1′).

Anal. Calcd for $C_{15}H_{28}O_{13}$: C, 43.27; H, 6.78. Found: C, 43.30; H, 6.92.

4′-Deoxylactose. ^{1}H NMR (D_2O, 400 MHz) *selected data only for β anomer*: δ 4.63 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1), 4.40 (d, 1 H, $J_{1',2'}$ = 7.9 Hz, H-1′), 3.22−3.29 (m, 1 H, H-5), 3.19 (ddd, 1 H, $J_{5',4'ax}$ = 11.2 Hz, $J_{5',6a'}$ 8.0 Hz, $J_{5',6b'}$ = 3.2 Hz, H-5′), 1.95 (dd, 1 H, $J_{4'eq,4'ax}$ = 12.0 Hz, $J_{4'eq,3'}$ = 4.4

Hz, H-4′$_{eq}$), 1.42 (ddd, 1 H, $J_{4'ax,4'eq}$ = 12.0 Hz, $J_{4'ax,3'}$ = 11.9 Hz, $J_{4'ax,5'}$ = 11.2 Hz, H-4′$_{ax}$).

Anal. Calcd for $C_{12}H_{22}O_{10}$: C, 44.17; H, 6.80. Found: C, 43.87; H, 6.91.

Benzyl 4′-Deoxy-β-lactoside. ^{1}H NMR (CD_3OD, 400 MHz) *selected data only*: δ 7.22−7.45 (m, 5 H, Ar), 4.91 (d, 1 H, *J* = 11.8 Hz, PhC*H*), 4.66 (d, 1 H, *J* = 11.8 Hz, PhC*H*), 4.39 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1), 4.34 (d, 1 H, $J_{1',2'}$ = 7.8 Hz, H-1′), 3.93 (dd, 1 H, $J_{6a,6b}$ = 12.1 Hz, $J_{6a,5}$ = 2.4 Hz, H-6a), 3.86 (dd, 1 H, $J_{6b,6a}$ = 12.1 Hz, $J_{6b,5}$ = 4.2 Hz, H-6b), 3.13 (dd, 1 H, $J_{4,5}$ = 8.7 Hz, $J_{4,3}$ = 8.1 Hz, H-4), 1.89 (dd, 1 H, $J_{4'eq,4'ax}$ = 12.7 Hz, $J_{4'eq,3'}$ = 5.0 Hz, H-4′$_{eq}$), 1.41 (ddd, 1 H, $J_{4'ax,4'eq}$ = 12.7 Hz, $J_{4'ax,3'}$ = 11.9 Hz, $J_{4'ax,5'}$ = 11.9 Hz, H-4′$_{ax}$).

Anal. Calcd for $C_{19}H_{28}O_{10}$: C, 54.80; H, 6.78. Found: C, 54.50; H, 6.72.

Benzyl 4′-Deoxy-4′-fluoro-β-lactoside. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 7.35−7.55 (m, 5 H, Ar), 4.91 (d, 1 H, *J* = 11.6 Hz, PhC*H*), 4.54 (d, 1 H, $J_{1',2'}$ = 7.8 Hz, H-1′), 4.51 (d, 1 H, $J_{1,2}$ = 6.0 Hz, H-1), 3.98 (dd, 1 H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 1.9 Hz, H-6a), 3.33 (dd, 1 H, $J_{4,3}$ = 8.6 Hz, $J_{4,5}$ = 8.4 Hz, H-4). ^{19}F NMR (D_2O, 188 MHz): δ −141.2 (ddd, $J_{F,4'}$ = 51.0 Hz, $J_{F,3'}$ = 30.0 Hz, $J_{F,5'}$ = 30.0 Hz).

Anal. Calcd for $C_{19}H_{27}FO_{10}$: C, 52.53; H, 6.26. Found: C, 52.33; H, 6.31.

*Uridine 5′-Diphospho-2-deoxy-2-fluoro-*α*-*D*-galactopyranose Diammonium Salt*. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 7.91 (d, 1 H, $J_{6,5}$ = 6.1 Hz, H-6), 5.93 (m, 2 H, H-1′, H5), 5.76 (dd, 1 H, $J_{1'',P}$ = 7.1 Hz, $J_{1'',2}$ = 3.6 Hz, H-1″). ^{19}F NMR (D_2O, 188 MHz): δ −132.4 (dd, $J_{2'',F}$ = 49.9 Hz, $J_{3'',F}$ = 11.1 Hz). ^{31}P NMR (D_2O, 81 MHz, proton decoupled): δ −9.10 (d, $J_{P\beta,P\alpha}$ = 19.9 Hz, P$^{\beta}$), −10.8 (d, $J_{P\alpha,P\beta}$ = 19.9 Hz, P$^{\alpha}$).

Anal. Calcd for $C_{15}H_{29}FN_4O_{16}P_2$: C, 29.91; H, 4.85; N, 9.30. Found: C, 30.37; H, 5.34; N, 9.89.

β-1,6-Anhydrogalactose. ^{1}H NMR (CD_3OD, 400 MHz): δ 4.81 (s, 1 H, H-1), 4.32 (d, 1 H, $J_{6a,6b}$ = 7.0 Hz, H-6a), 4.28 (dd, 1 H, $J_{5,6b}$ = 6.0 Hz, $J_{5,4}$ = 4.5 Hz, H-5), 3.93 (dd, 1 H, $J_{4,5}$ = 4.5 Hz, $J_{4,3}$ = 4.4 Hz, H-4), 3.83 (dd, 1 H, $J_{3,4}$ = 4.4 Hz, $J_{3,2}$ = 1.1 Hz, H-3), 3.64 (d, 1 H, $J_{2,3}$ = 1.1 Hz, H-2), 3.52 (dd, 1 H, $J_{6b,6a}$ = 7.0 Hz, $J_{6b,5}$ = 6.0 Hz, H-6b).

Anal. Calcd for $C_6H_{10}O_5$: C, 44.45; H, 6.22. Found: C, 44.51; H, 6.26.

*Uridine 5′-Diphospho-[1″-^{18}O]-*α*-*D*-galactopyranose Diammonium Salt*. ^{1}H NMR (D_2O, 300 MHz): δ 7.83 (d, 1 H, $J_{6,5}$ = 8.2 Hz, H-6), 5.30−5.40 (m, 2 H, H-5, H-1′), 5.53 (dd, 1 H, $J_{1'',P}$ = 7.2 Hz, $J_{1'',2''}$ = 3.6 Hz, H-1″), 4.23−4.28 (m, 2 H, H-2′, H-3′), 4.07−4.20 (m, 3 H, H-4′, H-5a′, H-5b′), 4.06 (dd, 1 H, $J_{5'',6a''}$ = 6.2 Hz, $J_{5'',6b''}$ = 6.2 Hz, H-5″), 3.92 (d, 1 H, $J_{4'',3''}$ = 3.0 Hz, H-4″), 3.80 (dd, 1 H, $J_{3'',2''}$ = 10.2 Hz, $J_{3'',4''}$ = 3.0 Hz, H-3″), 3.68 (ddd, 1 H, $J_{2'',3''}$ = 10.2 Hz, $J_{2'',1''}$ = 3.6 Hz, $J_{2'',P}$ = 3.0 Hz, H-2″), 3.55−3.65 (m, 2 H, H-6a″, H-6b″). ^{31}P NMR (D_2O, 121 MHz, proton decoupled): δ −9.95 (d, 1 P, $J_{P\beta,P\alpha}$ = 20.1 Hz, P$^{\beta}$), −11.51 (d, 1 P, $J_{P\alpha,P\beta}$ = 20.7 Hz, P$^{\alpha}$). HR-LSIMS: Calcd for $C_{15}H_{23}N_2{}^{16}O_{16}{}^{18}OP_2^-$: 567.0513. Found: 567.0515. LR-LSIMS: ^{16}O/^{18}O = 15/85.

Anal. Calcd for $C_{15}H_{22}N_2{}^{16}O_{16}{}^{18}OP_2{}^{2-}\cdot 2NH_4^+$: C, 29.86; H, 5.18; N, 9.29. Found: C, 30.21; H, 5.16; N, 8.97.

Gal-α-1,4-Gal-β-1,4-Glc. ^{1}H NMR (D_2O, 400 MHz) *selected data only*: δ 5.20 (d, 1 H, $J_{1\alpha,2}$ = 2.9 Hz, H-1α), 4.93 (d, 1 H, $J_{1'',2}$ = 2.0 Hz, H-1″), 4.64 (d, 1 H, $J_{1\beta,2}$ = 8.6 Hz, H-1β), 4.48 (d, 1 H, $J_{1',2}$ = 7.7 Hz, H-1′).

Anal. Calcd for $C_{18}H_{32}O_{16}\cdot 2^1/_2H_2O$: C, 39.34; H, 6.79. Found: C, 39.35; H, 6.83.

Enzyme Kinetics. Steady-state kinetic studies were performed using a continuous coupled assay similar to that described by Gosselin et al. (*42*) in which UDP release is coupled to the oxidation of NADH (λ = 340 nm, ε = 6.22 mM^{-1} cm^{-1}). Standard reaction conditions were as follows: 20 mM HEPES, pH 7.5, 0.1% bovine serum albumin, 50 mM KCl, 5 mM $MnCl_2$, 5 mM DTT, 0.7 mM phosphoenolpyruvate, 300 μM NADH, 375 units of pyruvate kinase, and 500 units of lactate dehydrogenase. The concentrations of substrates added depended upon the experiment. For all inhibition studies, the concentration of the fixed substrate was usually held as close to saturation levels as possible (200−300 μM for UDP-Gal and 100−120 mM for lactose) while the concentration of the variable substrate usually ranged from 0.5K_m to 10K_m for UDP-Gal and 0.1K_m to 2K_m for lactose. Reactions were initiated by the addition of LgtC-19 (0.25−1.0 μg/mL), bringing the final volume to 200 μL in the quartz reaction vessels. The change in absorbance at 30 °C was monitored by means of a UNICAM 8700 UV−vis spectrophotometer equipped with a circulating water bath.

UDP-Gal/UDP Exchange. LgtC-19 (150 μg/mL) was incubated with ^{18}O-UDP-Gal (5 mM) and excess unlabeled UDP (50 mM) in the presence of 20 mM HEPES, pH 7.5, 5 mM $MnCl_2$, and 5 mM DTT to give a total reaction volume of 600 μL. After 4 h at room temperature, the enzyme was removed by centrifugation through a Centricon filter equipped with a 10 kDa molecular mass cutoff membrane. The filtrate was then purified by anion-exchange chromatography using a Pharmacia HiTrap Q HP column (5 mL) and a Beckman Biosepra Prosys Workstation. Elution was afforded by a 50−500 mM gradient of NH_4HCO_3 at a flow rate of 1 mL/min. The peaks corresponding to UDP and UDP-Gal were difficult to resolve and were collected together. After lyophilization, the sample was then analyzed by both electrospray mass spectrometry and ^{31}P NMR. Analysis by electrospray mass spectrometry was performed on a single quadrapole instrument built in the laboratory of Professor Don Douglas in the Department of Chemistry at the University of British Columbia. An approximately 50 μM sample (in terms of UDP-Gal) was prepared by dissolving the lyophilized powder in a 10% solution of water in methanol. This sample was injected into the mass spectrometer at a flow rate of 1 μL/min. The instrument was run in negative ion mode at a voltage of 3500 V. The remainder of the lyophilized sample was dissolved in 500 μL of Chelex-treated D_2O containing approximately 10 mM EDTA, and a proton-decoupled ^{31}P NMR (121 MHz) was obtained. Acquisition parameters were as follows: sweep width = 5482 Hz, acquisition time = 2 s, delay between pulses = 2 s, and pulse width = 10.0 μs.

Positional Isotope Exchange. ^{18}O-UDP-Gal (1.8 mg) was dissolved in 510 μL of Chelex-treated D_2O, and the proton-decoupled ^{31}P NMR spectrum was recorded on a 300 MHz Brüker AV-300 spectrometer. This sample of ^{18}O-labeled UDP-Gal was then removed from the NMR tube and added to a solution of HEPES (20 mM), pD 7.5, 4dLac (10 mM), $MnCl_2$ (5 mM), DTT (5 mM), and LgtC-19 (150 μg/mL) in D_2O to bring the final volume to 600 μL. After being incubated for 7 h at room temperature, the reaction mixture

Table 1: Kinetic Parameters for the Utilization of Various Acceptor Substrates by LgtC-19

substrate	K_m^{app} (mM)	k_{cat}^{app} (s^{-1})	$(k_{cat}/K_m)^{app}$ ($mM^{-1}\,s^{-1}$)
galactose[a]			0.0011
lactose	60	34	0.57
pentenyl lactoside[b]			1.0
allyl lactoside	33	35	1.1
benzyl lactoside	22	25	1.2
2′,3′-dihydroxylpropyl lactoside	26	35	1.4

[a] No saturation was obtained up to 250 mM. [b] No saturation was obtained up to 20 mM. Problems with solubility were encountered at higher concentrations.

was centrifuged through a Centricon filter equipped with a 10 kDa molecular mass cutoff membrane to remove the enzyme. The ^{18}O-UDP-Gal was then re-isolated by anion-exchange chromatography using a Pharmacia HiTrap Q HP column (5 mL) and a Beckman Biosepra Prosys Workstation. The compound was eluted with a linear gradient of 50−500 mM NH_4HCO_3 at a flow rate of 1 mL/min. After the desired fractions were pooled and lyophilized, this compound was then redissolved in Chelex-treated D_2O (600 μL) containing EDTA (10 mM), and the proton-decoupled ^{31}P NMR spectrum was again recorded. Acquisition parameters were as follows: sweep width = 5482 Hz, acquisition time = 2 s, delay between pulses = 2 s, and pulse width = 6.8 μs.

RESULTS AND DISCUSSION

Substrate Specificity of LgtC-19. Lipopolysaccharyl α-galactosyltransferase C naturally catalyzes the transfer of a galactosyl moiety from UDP-Gal to a complex lipooligosaccharide (LOS) which is composed of a lipid A moiety linked to an outer core oligosaccharide through a 2-keto-3-deoxyoctulosonic acid residue (KDO). The outer core oligosaccharide in this strain of *N. meningitidis* terminates in a nonreducing end α-galactose residue. For quantitative assays, use of LOS is problematic because of its lack of solubility and structural heterogeneity. High-sensitivity assays with fluorescently labeled synthetic acceptors have been developed but are not suitable for detailed kinetic analysis. In an effort to find a more practical substrate, several simple analogues of the terminal sugars were synthesized and evaluated as potential acceptor substrates; the kinetic parameters are shown in Table 1. As can be seen from the high K_m values, the enzyme does not bind these acceptors very tightly. This low affinity for the acceptor suggests that there are few critical interactions with this substrate, a result that could also explain the ability of LgtC-19 to transfer galactose to such a broad range of compounds. Among the different acceptors tested, the monosaccharide galactose was found to be a much poorer substrate than the disaccharide lactose or any of its glycoside derivatives. The second-order rate constant for transfer to galactose was 500−1200-fold lower than that of the disaccharide substrates, indicating the importance of a second sugar binding site. However, there was very little variation in the kinetic parameters between lactose and its various derivatives. These results imply that the acceptor binding site is composed of at least two important subsites designed to recognize a lactose moiety at the terminus of the LOS. While the importance of other potential subsites was not explored, results from the various lactoside derivatives do show that binding was not significantly enhanced by longer nonsugar substrates.

Unlike the acceptor substrate, binding of the donor substrate, UDP-Gal by LgtC-19 was found to be extremely tight, with an apparent K_m value of 18 μM. A comparison of the apparent second-order rate constant for UDP-Gal ($k_{cat}/K_m = 0.5\ \mu M^{-1}\ s^{-1}$) with that of its epimer UDP-Glc ($k_{cat}/K_m = 0.0026\ \mu M^{-1}\ s^{-1}$) also revealed the enzyme's high specificity for the donor substrate. These kinetic parameters were obtained using lactose as the acceptor. In the absence of lactose or other suitable glycosyl acceptors, however, turnover of UDP-Gal was still observed, albeit at a very low rate. The value of k_{cat}/K_m for UDP-Gal in the absence of any saccharide acceptors was found to be 0.0039 $\mu M^{-1}\ s^{-1}$, some 120 times lower than when lactose was present. Presumably, when suitable glycosyl acceptors are absent, water is capable of acting as an acceptor, resulting in hydrolysis of the nucleoside diphosphosugar to yield galactose and UDP. Evidence for the formation of galactose was obtained through TLC analysis of the reaction mixture in which UDP-Gal alone was incubated in the absence or presence of enzyme overnight. After this time, a spot with a similar R_f value to that of galactose was indeed observed, but only for the mixture in which enzyme was added. Mass spectral analysis of products resulting from the hydrolysis of ^{18}O-labeled UDP-Gal by the enzyme (with ^{18}O incorporated at the bridging position between the galactose and UDP) revealed that the ^{18}O remained with the UDP. This observation suggests that the attack by water on the UDP-Gal substrate occurs at the expected anomeric carbon of the galactosyl portion of the substrate rather than at the β-phosphorus. In this regard, the hydrolysis of UDP-Gal by LgtC-19 is unlike that of the Nudix family of enzymes, which have all been shown to hydrolyze nucleoside diphosphate derivatives via nucleophilic substitutions at phosphorus (*21*, *22*). The ability of glycosyltransferases to behave as hydrolases is not without precedent since several glucosyltransferases including toxins A and B from *Clostridium difficile* and T4 phage β-glucosyltransferase have all been shown to be capable of hydrolyzing UDP-Glc in the absence of saccharide acceptor (*23*−*25*).

Determining the Kinetic Mechanism of LgtC-19. Using UDP-Gal and lactose as the substrates, initial studies on the kinetic mechanism of this enzyme were carried out by examining the pattern of the double reciprocal plot when the two substrates were varied independently of each other. On the basis of eqs 1−3, which respectively describe an

$$\frac{1}{v} = \frac{K^A K_m^B}{V_{max}[A][B]} + \frac{K_m^A}{V_{max}[A]} + \frac{K_m^B}{V_{max}[B]} + \frac{1}{V_{max}} \quad (1)$$

$$\frac{1}{v} = \frac{\alpha K^A K^B}{V_{max}[A][B]} + \frac{\alpha K^A}{V_{max}[A]} + \frac{\alpha K^B}{V_{max}[B]} + \frac{1}{V_{max}} \quad (2)$$

$$\frac{1}{v} = \frac{K_m^A}{V_{max}[A]} + \frac{K_m^B}{V_{max}[B]} + \frac{1}{V_{max}} \quad (3)$$

ordered bi-bi, a random bi-bi, and a ping-pong bi-bi mechanism, the resulting family of seemingly parallel lines suggested a ping-pong bi-bi kinetic mechanism (Figure 2). However, an inherent difficulty with this conclusion is in

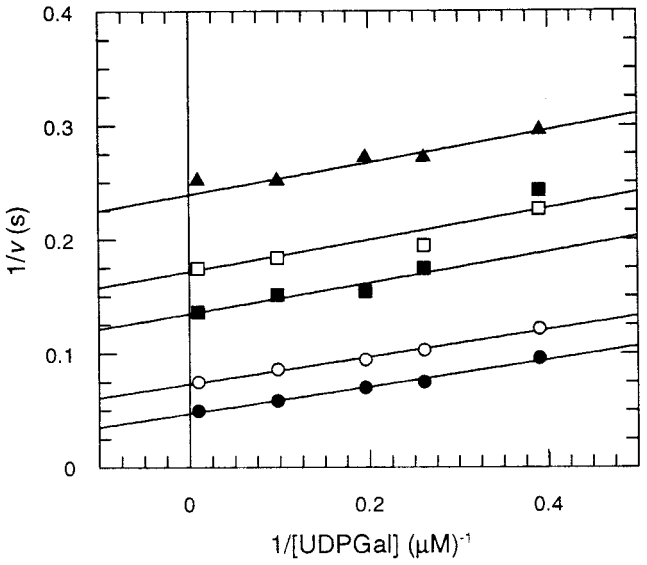

FIGURE 2: Double reciprocal plot showing the activity of LgtC-19 as a function of UDP-Gal concentration in the presence of 40 (●), 20 (○), 10 (■), 7.5 (□), and 5 mM (▲) of lactose.

deciding whether the lines are actually parallel or only appear parallel because they intersect at a point far from the origin. For instance, when the dissociation constant, K^A, is much smaller than the value of K_m^A in an ordered bi-bi system (eq 1), the resulting family of lines in the double reciprocal plot will intersect far to the left of the $1/v$ axis and far below the 1/[S] axis (*26*). As such, the "best fitting" lines through the data will appear to be parallel. This situation is also true of random bi-bi systems when the binding of one substrate strongly inhibits the binding of the other (i.e., $\alpha \gg 1$ in eq 2) (*26*). Given that UDP-Gal is bound so tightly by LgtC-19 and lactose is not, neither ordered nor random bi-bi mechanisms can be ruled out on the basis of the apparent parallel lines in the double reciprocal plot.

As a means of testing the authenticity of the parallel lines, the pattern of the double reciprocal plot when UDP-Gal and lactose were varied independently of each other was reexamined in the presence of uridine diphosphoglucose (UDP-Glc). Since the enzymatic turnover of UDP-Glc is much slower than that of the natural substrate (UDP-Gal), the presence of UDP-Glc will affect the binding constants associated with UDP-Gal much like that of an inhibitor. Therefore, if the kinetic mechanism of LgtC-19 is actually ordered, then the presence of UDP-Glc should result in a convergence of the previously "parallel" lines, as the value of K^A becomes larger. The same should be true in the case of a random kinetic mechanism, as the value of α will also be affected by the presence of UDP-Glc. When this experiment was carried out, a set of converging lines was indeed observed (data not shown). The implication of this finding is that the family of lines previously observed in the absence of UDP-Glc was in fact not parallel but rather must have intersected at a point far from either of the axes. This result is therefore the first indication that the kinetic mechanism of LgtC-19 may not be ping-pong.

As a diagnostic test of a ping-pong kinetic mechanism, a UDP/UDP-Gal exchange experiment was carried out. In this study, the enzyme was incubated with ^{18}O-labeled UDP-Gal (with the ^{18}O incorporated at the bridging position between galactose and UDP) in the presence of excess UDP but in the absence of any suitable saccharide acceptors. After 4 h of incubation, the UDP-Gal was re-isolated and analyzed by both mass spectrometry and ^{31}P NMR. The expected result for a ping-pong kinetic mechanism is a washing out of the ^{18}O heavy atom in the re-isolated UDP-Gal due to UDP exchange with the excess unlabeled species following cleavage of the C1″−^{18}O1″ bond. From the results of the mass spectrum and ^{31}P NMR, no UDP exchange was evident as only ^{18}O-labeled UDP-Gal was observed. Data from the mass spectrum revealed only the mass of the heavy atom containing compound, and the ^{31}P NMR showed no change in the chemical shift of the β-phosphate signal. As such, a ping-pong kinetic mechanism is strongly disfavored.

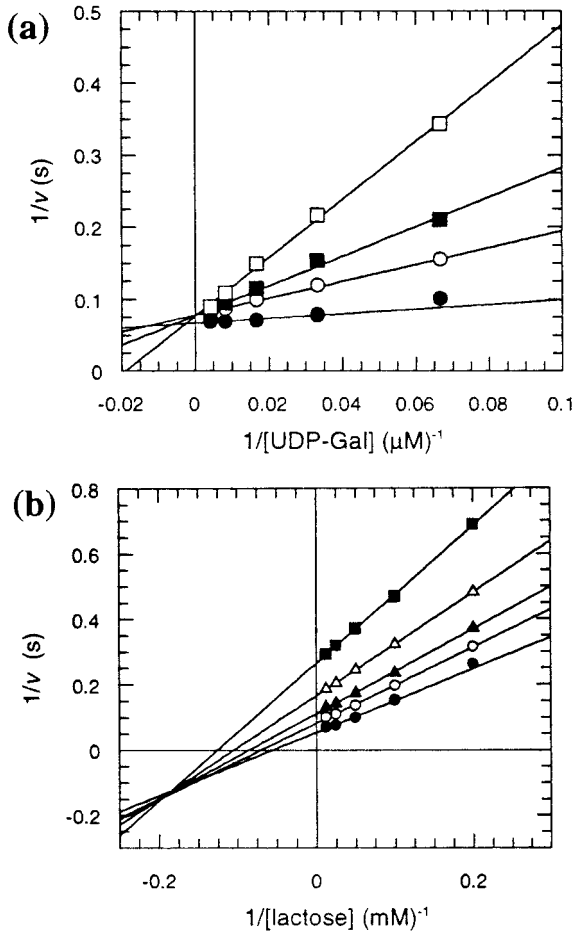

FIGURE 3: Double reciprocal plots showing the inhibition of LgtC-19 by UDP-2FGal at varying concentrations of (a) the donor substrate UDP-Gal and (b) the acceptor substrate lactose. When the UDP-Gal concentration was varied, the concentrations of UDP-2FGal were 0 (●), 6.25 (○), 12.5 (■), and 25 μM (□). When the concentration of lactose was varied, the concentrations of UDP-2FGal were 0 (●), 37.5 (○), 75 (▲), 150 (△), and 300 μM (■).

Further evidence against a ping-pong kinetic mechanism has come from the results of our study involving the inhibition of LgtC-19 by uridine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactopyranose (UDP-2FGal). Originally, UDP-2FGal was intended to function as a probe of the chemical mechanism of LgtC-19 (vide infra). As it turned out, this compound also provided valuable insights about the kinetic mechanism of this enzyme. For example, while UDP-2FGal was found to inhibit LgtC-19 competitively as expected with respect to the donor UDP-Gal (Figure 3a), the noncompetitive pattern of inhibition obtained with this compound at varying

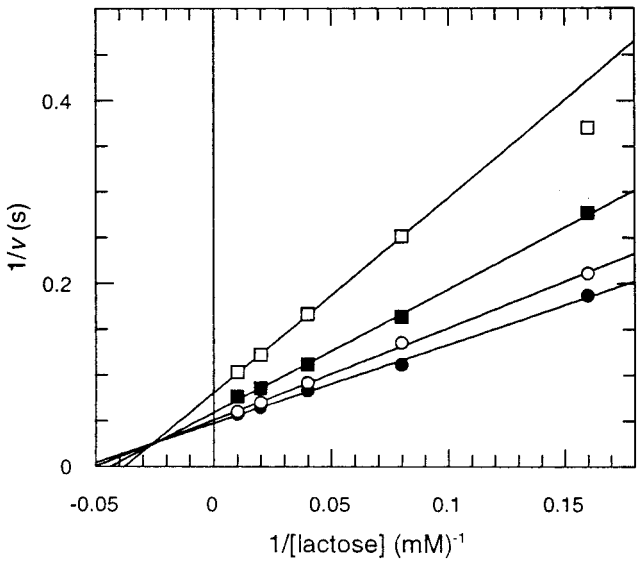

FIGURE 4: Double reciprocal plot showing the inhibition of LgtC-19 by 4dLac at varying concentrations of the acceptor substrate lactose and saturating concentrations (300 μM) of UDP-Gal. The concentrations of 4dLac were 3.75 (●), 7.5 (○), 15.0 (■), and 30.0 mM (□).

concentrations of the acceptor lactose (Figure 3b) was inconsistent with the uncompetitive pattern expected for a ping-pong bi-bi mechanism (*27*). Taken together with the results of our earlier studies, these findings strongly suggest a sequential kinetic mechanism for LgtC-19 where the donor and acceptor substrates must both be bound before any chemical catalysis can occur, though an order of binding is not defined. Moreover, in the case where substrate binding and product release are ordered as opposed to random processes, results from this particular inhibition study further dictate that UDP-Gal will bind first, followed by the acceptor lactose.

To complement the results arrived at from the analysis of the inhibition patterns produced by the donor analogue, UDP-2FGal, a suitable dead- end analogue of the acceptor is needed. A likely candidate for this purpose is the incompetent acceptor substrate 4′-deoxylactose (4dLac), which was originally synthesized with the intention of trapping the covalent glycosyl-enzyme intermediate of LgtC-19 (vide infra). Unfortunately, the binding of this compound not only to the lactose binding site but also to the enzyme−lactose complex, as seen by the noncompetitive inhibition pattern at varying lactose concentrations (Figure 4), greatly complicates the analysis. The need to eliminate this noncompetitive binding in the acceptor analogue prompted the synthesis of benzyl 4′-deoxylactoside (Bn4dLac) and benzyl 4′-deoxy-4′-fluorolactoside (Bn4FLac) in the hope that these compounds would bind specifically to the lactose subsite. While very slight improvements in specificity were observed with these compounds, based on their lower K_{ei} values (Table 2), inhibition of LgtC-19 by these compounds remained noncompetitive; thus they could not be used in further studies to delineate the kinetic mechanism of LgtC-19.

Given that the kinetic mechanism of LgtC-19 could not be fully determined through inhibition studies with dead-end substrate analogues, inhibition studies with products became necessary. In the LgtC-19-catalyzed transfer of galactose from UDP-Gal to lactose, the products of the reaction are UDP and the trisaccharide Gal-α-1,4-Lac. To

Table 2: Inhibition Constants for Various Acceptor Analogues with Respect to Varying Concentrations of Lactose and a Fixed Saturating Concentration of UDP-Gal

acceptor analogue	K_{ei}^{app} *a* (mM)	K_{eis}^{app} *b* (mM)
4′-deoxylactose (4dLac)	16	33
benzyl 4′-deoxylactoside (Bn4dLac)	6	102
benzyl 4′-deoxy-4′-fluorolactoside (Bn4FLac)	6	27

*K_{ei}^{app} refers to the apparent dissociation constant of the inhibitor from the lactose-free enzyme. **K_{eis}^{app} refers to the apparent dissociation constant of the inhibitor from the enzyme−lactose complex.

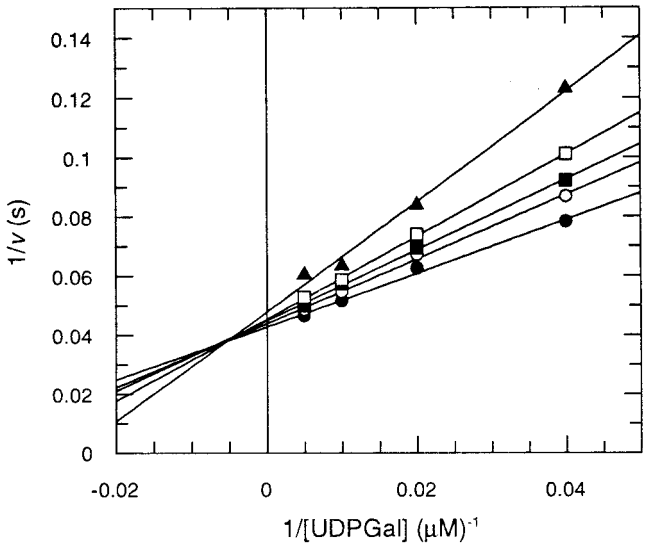

FIGURE 5: Double reciprocal plot showing the inhibition of LgtC-19 by the product Gal-α-1,4-Gal-β-1,4-Glc at varying concentrations of UDP-Gal and a fixed concentration (120 mM) of lactose. The concentrations of the product trisaccharide were as follows: 0 (●), 15 (○), 20 (■), 30 (□), and 40 mM (▲).

Table 3: Expected Patterns of Product Inhibition for Ordered and Random Bi-Bi Kinetic Mechanisms

mechanism	product inhibitor	varying A		varying B	
		unsat. B	sat. B	unsat. A	sat. A
ordered bi-bi	P	NC	UC	NC	NC
	Q	comp	comp	NC	NI
rapid equilibrium random bi-bi	P	comp	NI	comp	NI
	Q	comp	NI	comp	NI
nonrapid equilibrium random bi-bi	P	NC	NC	NC	NC
	Q	NC	NC	NC	NC

carry out the product inhibition study, the trisaccharide product Gal-α-1,4-Lac was first synthesized enzymatically from UDP-Gal and lactose using LgtC-19. The inhibition pattern exhibited by this product was found to be noncompetitive when UDP-Gal was the varied substrate while lactose was held at a fixed but nonsaturating concentration (Figure 5). This observation strongly disfavors a rapid equilibrium random bi-bi mechanism since only competitive inhibition patterns or no inhibition are expected in that case (Table 3). The data therefore pointed to either an ordered bi-bi or a nonrapid equilibrium random bi-bi kinetic mechanism. Kinetically, the differentiation of these two possibilities would require further inhibition studies either with the other product, UDP, or again with Gal-α-1,4-Lac but at saturating concentrations of lactose. Unfortunately, neither study is practical. An inhibition study with UDP is problematic because this product is used in the coupled assay to monitor

enzyme activity and as such cannot be added. With regard to an inhibition study with Gal-α-1,4-Lac at saturating levels of lactose, the combination of the limited solubility of lactose and its low affinity for the enzyme as well as its propensity to cause substrate inhibition greatly restricts the employment of this substrate at high concentrations. As a result, the unequivocal assignment of a kinetic mechanism for LgtC-19 becomes a nontrivial task. Fortunately, the X-ray crystal structure of the enzyme in complex with analogues of both the donor and acceptor substrates was able to provide some important insights into this matter (*14*). From the structural data, it is clear that UDP-Gal is bound to the enzyme in a site buried within the protein. The binding of the donor substrate appears to trigger a conformational change involving the closing of two loops over the active site, which leads to the formation of a pocket into which the acceptor substrate is then bound. This observation therefore disfavors a random kinetic mechanism since the binding site for the acceptor substrate is not formed until the donor substrate has been bound. Taken together with the fact that crystallization of the enzyme was only achieved in the presence of an analogue of the donor UDP-Gal but not in the presence of an acceptor analogue, the data from the crystal structure therefore appear to favor an ordered bi-bi kinetic mechanism. If this is indeed the case, then the order of substrate binding and product release might remain in question since there exist several scenarios whereby inhibition by a product can yield a noncompetitive pattern (Table 3). However, previous inhibition studies with UDP-2FGal had dictated that UDP-Gal must be the first substrate bound in an ordered mechanism. As such, the noncompetitive pattern arising from the inhibition of LgtC-19 by Gal-α-1,4-Gal-*β*-1,4-Glc at varying concentrations of UDP-Gal must indicate that the trisaccharide is the first product released. On the basis of kinetic and structural evidence, we therefore conclude that LgtC-19 follows an ordered bi-bi mechanism in which UDP-Gal binds first, followed by the acceptor lactose. After the chemical step, the trisaccharide is then the first to depart followed by UDP.

Attempts at Trapping the Covalent Glycosyl-Enzyme Intermediate. By analogy with retaining glycosyl hydrolases, retaining glycosyltransferases are generally believed to follow a double displacement mechanism whereby a covalent glycosyl-enzyme intermediate is formed and subsequently broken down via oxocarbenium ion-like transition states (Figure 1a). In an effort to trap and directly observe this putative intermediate in LgtC-19, two strategies adopted from the work with retaining glycosyl hydrolases were employed. The first approach involves the use of an incompetent acceptor substrate similar to that used to trap the covalent glycosyl-enzyme intermediate of a cyclodextrin glycosyltransferase (*28*). By removing the nucleophilic hydroxyl group of the acceptor substrate that is responsible for attacking the covalent glycosyl-enzyme intermediate in the second step of the double displacement mechanism, the breakdown of this intermediate would be averted, hopefully allowing for its observation by mass spectrometry. Another approach to trapping this putative intermediate could involve the use of fluorosugar substrates. Such reagents function by forming relatively stable glycosyl-enzyme intermediates, and their use in this regard has been met with much success for numerous retaining glycosyl hydrolases (*29*−*32*).

The incompetent acceptor substrate, 4′-deoxylactose (4dLac), was synthesized in a manner similar to that reported for the preparation of 4′-deoxy-α-maltosyl fluoride (*33*) (see Supporting Information). Through kinetic analysis, 4′-deoxylactose was found to inhibit LgtC-19 in a noncompetitive manner with respect to lactose (Figure 4), yielding apparent K_{ei} and K_{eis} (the respective dissociation constants of the inhibitor from the free enzyme and enzyme−substrate complex) values of 16 and 33 mM, respectively. This type of inhibition suggests that, in addition to being able to compete with lactose for the acceptor binding site, 4dLac is also capable of binding to the enzyme−lactose complex. This result is not surprising since many potential subsites for binding to the natural LOS acceptor are left unoccupied when the much smaller lactose is used as the substrate.

In an attempt to trap and observe a covalent glycosyl-enzyme intermediate, LgtC-19 was incubated with UDP-Gal in the presence of this incompetent acceptor substrate, but unfortunately, no accumulation of such an intermediate could be detected by electrospray mass spectrometry (ESMS). One explanation for this observation is that the reaction does not proceed via such an intermediate. However, other factors may have also contributed to the inability to trap the intermediate using this approach. For instance, elimination of the nucleophilic hydroxyl group in the acceptor substrate could have disrupted important interactions needed to properly orient key active site residues for catalysis. As such, no reaction would be initiated in its absence, and no intermediate would be formed. Another possibility is that a covalent glycosyl-enzyme intermediate was formed but was then hydrolyzed under the conditions of analysis. As mentioned earlier, LgtC-19 is certainly capable of hydrolyzing UDP-Gal in the absence of suitable glycosyl acceptors, and since 4dLac is bound only weakly by the enzyme, its ability to shield the putative intermediate from attack by water would be severely limited.

Turning to the use of fluorosugars to trap the covalent glycosyl-enzyme intermediate, 5-fluoroglycosyl fluorides have been shown to function as effective inactivators of retaining α-glycosyl hydrolases by forming relatively stable covalent 5-fluoroglycosyl-enzyme intermediates (*34*−*37*). The incorporation of an electron-withdrawing fluorine substituent at the 5-position of the substrates is designed to slow both steps of the double displacement mechanism by destabilizing the oxocarbenium ion-like transition states through which each step proceeds. However, through the introduction of a good leaving group such as fluoride at the anomeric center, the rate of the first step is selectively increased over that of the second step, resulting in the accumulation of the covalent intermediate. Since retaining glycosyltransferases are believed to follow a double displacement mechanism similar to that of retaining glycosyl hydrolases, 5-fluoroglycosyl fluorides could also prove useful in the trapping of the covalent intermediate of these enzymes.

The synthesis of 5-fluoro-α-D-galactosyl fluoride (5FGalF) from galactose has been previously reported (*34*). This compound was incubated with LgtC-19, and aliquots of the reaction mixture were removed and analyzed by ESMS over the course of 3 h. Unfortunately, no accumulation of a 5-fluoroglycosyl-enzyme intermediate could be detected, even in the presence of UDP and at high concentrations (50 mM) of 5FGalF. This result was not entirely unexpected,

however, since α-D-galactosyl fluoride is itself a very poor substrate for the enzyme in terms of both binding and turnover, even in the presence of exogenous UDP (*18*). Therefore, it is very likely that 5FGalF also binds poorly to the enzyme and is not turned over at all. Indeed, no release of fluoride was observed when 5FGalF (5 mM) was incubated with high concentrations of LgtC-19 (2.5 mg/mL) nor was any inhibition of the enzyme observed in the presence of 5 mM 5FGalF.

In light of the poor binding and turnover of substrates that lack a UDP moiety, it is therefore crucial that this important recognition element be incorporated into analogues of the donor substrate. To this end, uridine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactopyranose (UDP-2FGal) was prepared through the coupling of 2-deoxy-2-fluoro-α-D-galactopyranose 1-phosphate with UMP-morpholidate according to the procedure described by Wittmann and Wong (*38*). However, without the ability to modify the reactivity of the leaving group, the trapping of the covalent glycosyl-enzyme intermediate with UDP-2FGal would rest heavily on the hope that the leaving group ability of UDP is sufficient to ensure that the first step of the reaction proceeds faster than the second step in the presence of the 2-fluoro substituent.

Kinetic evaluation of UDP-2FGal found this compound to be neither a time-dependent inactivator nor a slow substrate of LgtC-19, even at high concentrations (250 μg/mL) of enzyme. Instead, LgtC-19 was found to be reversibly inhibited by UDP-2FGal. With respect to the donor substrate UDP-Gal, inhibition by UDP-2FGal was shown to be competitive (Figure 3a) with an apparent K_i value of 2 μM. When the inhibition study was repeated using lactose as the varying substrate, the double reciprocal plot yielded a noncompetitive pattern (Figure 3b) from which the values for K_{ei} and K_{eis} were calculated to be 230 μM and 75 μM, respectively. The inability of UDP-2FGal to inactivate LgtC-19 or function as a slow substrate for this enzyme suggests that a stabilized covalent fluoroglycosyl-enzyme intermediate was not formed. Indeed, no trace of such an intermediate could be detected by ESMS when LgtC-19 was incubated with UDP-2FGal either alone or in the presence of lactose or 4dLac. This failure to trap the intermediate once again raises doubts about its possible existence. However, given that no turnover of UDP-2FGal was observed, the more likely explanation is that the reactivity of UDP as a leaving group was insufficient after all to overcome the destabilizing effects of the 2-fluoro substituent on the transition state of the first step. As such, it may not have been possible for the intermediate to even form.

While the failure of both the incompetent acceptor approach and the fluoroglycoside approach in trapping the covalent glycosyl-enzyme intermediate can be attributed to the inherent shortcomings associated with each strategy, it is also possible that the mechanism of LgtC-19 does not proceed through such an intermediate. Evidence that this may be the case comes from the work of Persson et al. and their recent solution of the three-dimensional structure of this enzyme in complex with analogues of both the donor and acceptor substrates (*14*). In this study, likely candidates to function as the catalytic nucleophile were converted to nonnucleophilic residues through site-directed mutagenesis. However, significant levels of activity were retained in each of the resulting mutants, thereby indicating that these residues are not essential for catalysis. Taken together with our inability to trap the putative intermediate, little evidence is therefore available to support the idea of a covalent glycosyl-enzyme-mediated double displacement mechanism for LgtC-19.

Although a covalent glycosyl-enzyme intermediate remains elusive in the case of LgtC-19, a claim about the existence of such an intermediate has been made for a structurally unrelated retaining α-1,3-galactosyltransferase on the basis of the recently solved three-dimensional structure of that enzyme in complex with the donor substrate UDP-Gal (*15*). From the structure, electron density extending from Glu317 of the enzyme was interpreted by the authors as being that of a galactose moiety covalently bound to the carboxylate side chain of this residue. However, in the absence of a higher resolution structure and a more defined electron density map, it is difficult to ascertain whether the galactose residue is actually bonded covalently to Glu317. In light of the possibility that this α-1,3-galactosyltransferase may also possess some hydrolytic activity, as has been demonstrated in several glycosyltransferases (vide supra), this observed electron density may simply be due to a galactose residue which has remained bound to the enzyme after its hydrolysis from UDP-Gal. Moreover, the importance of Glu317 to catalysis in this α-1,3-galactosyltransferase has also not been confirmed, as mutagenesis data for this particular residue were not presented (*43*).

Possible Nonenzymic Nucleophiles. In conjunction with our pursuit of a covalent glycosyl-enzyme intermediate, plausible alternatives to the notion of an enzymic nucleophile were also sought. Two such candidates were the 4″- and 6″-hydroxyls of the donor UDP-Gal sugar. Each of these hydroxyls on the galactosyl portion of this substrate could potentially function as the nucleophile to displace UDP in the first step of the double displacement mechanism. In the case where the nucleophile is the 4″-hydroxyl, its displacement of UDP would result in the formation of a β-1,4-anhydrogalactose intermediate (Figure 6a). Likewise, displacement of UDP by the 6″-hydroxyl would result in the formation of a β-1,6-anhydrogalactose intermediate (Figure 6b). Potentially each intermediate could then react with the acceptor substrate in the second step of the mechanism to yield a product of retained configuration.

To explore the possibility of an anhydrosugar intermediate in the mechanism of LgtC-19, β-1,4-anhydrogalactose and β-1,6-anhydrogalactose were synthesized from the corresponding β-galactosyl fluorides (*39*) and were then tested for their ability to be turned over by LgtC-19. Following incubation of each compound with LgtC-19 in the presence of UDP and the acceptor lactose, the reaction mixtures were analyzed by TLC and HPLC for the formation of the expected trisaccharide product. Unfortunately, none was detected with either anhydrosugar nor were any hydrolysis products observed. While this result does not completely eliminate the potential involvement of an anhydrosugar intermediate in the mechanism of LgtC-19, it does strongly disfavor this possibility. The recent report of turnover of both UDP-4″-deoxygalactose and UDP-6″-deoxygalactose by LgtC also argues against such an intramolecular mechanism (*40*). Furthermore, examination of the recent structure of this enzyme in complex with analogues of both the donor and acceptor substrates (*14*) reveals no obvious clues to suggest

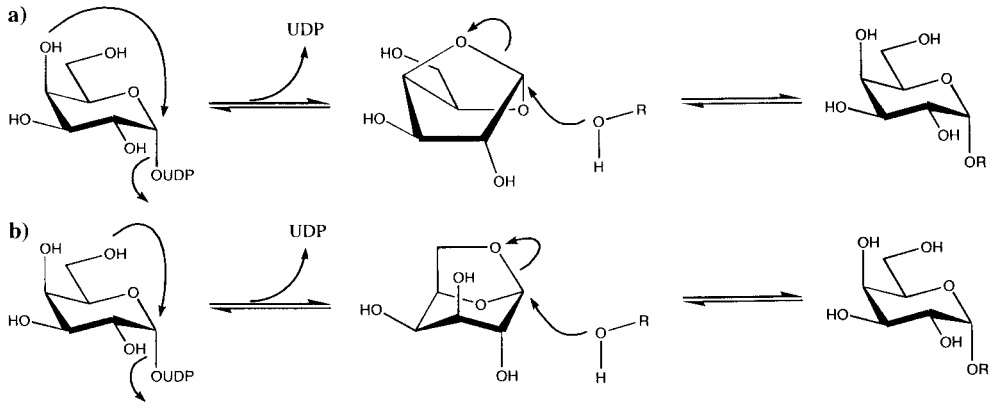

FIGURE 6: A possible anhydrosugar mediated double displacement mechanism where the role of the nucleophile in the first step of the reaction is played either by the (a) 4″-OH or the (b) 6″-OH.

the involvement of either the 4″-OH or the 6″-OH in catalysis. With both substrate analogues bound in this structure, it is not unreasonable to expect that all residues involved in catalysis be correctly positioned to carry out their task. However, neither the 4″-OH nor the 6″-OH appears to be poised to function as the catalytic nucleophile to displace UDP in the first step of the double displacement mechanism. From the structure, the 6″-OH is oriented away from the anomeric center and is held in place through a hydrogen bond with the side chain carboxylate of the conserved Asp 188 residue. As for the 4″-OH, this residue is also ill positioned for the role of the nucleophile since the $^4C_1$ conformation adopted by the galactose ring of the donor substrate places this hydroxyl group far from the reactive anomeric center. Given the constraints of the binding site, particularly the coordination of the pyrophosphate moiety by the enzyme-bound manganese ion, distortion to a boat conformation to facilitate attack by the 4″-OH is highly improbable. These structural observations therefore strongly support our chemical data, which suggest that an anhydrosugar intermediate is unlikely to be involved in the mechanism of LgtC-19.

Positional Isotope Exchange (PIX). The failure of our early attempts to directly observe and/or isolate an intermediate in the LgtC-19 catalyzed reaction therefore necessitated the use of more indirect approaches to probe the mechanism of LgtC-19 and also the nature of any likely intermediates. Since cleavage of UDP from the donor substrate UDP-Gal is expected to proceed with the breaking of the exocyclic C1″−O1″ bond, a positional isotope exchange study was carried out not only to confirm this idea but more importantly as a means of demonstrating the possible existence of a long-lived intermediate after bond cleavage. In such an experiment, ^{18}O-labeled uridine 5′-diphospho-α-D-galactopyranose (with the ^{18}O incorporated at the bridging position between galactose and UDP) would be incubated with LgtC-19 in the presence of an incompetent acceptor. Without a suitable saccharide substrate to accept the donor galactose, any bond cleavage in the donor ^{18}O-UDP-Gal substrate may be simply followed by the re-formation of the cleaved bond. However, if bond cleavage occurs between C1″ and O1″ and the UDP thus formed has a lifetime comparable to, or greater than, that required for bond rotation around the terminal phosphate, then scrambling of the isotope into the nonbridging phosphate positions would be observed in the donor substrate (Figure 7). This scrambling of the ^{18}O from a bridging position to a nonbridging position can be readily monitored by the slight

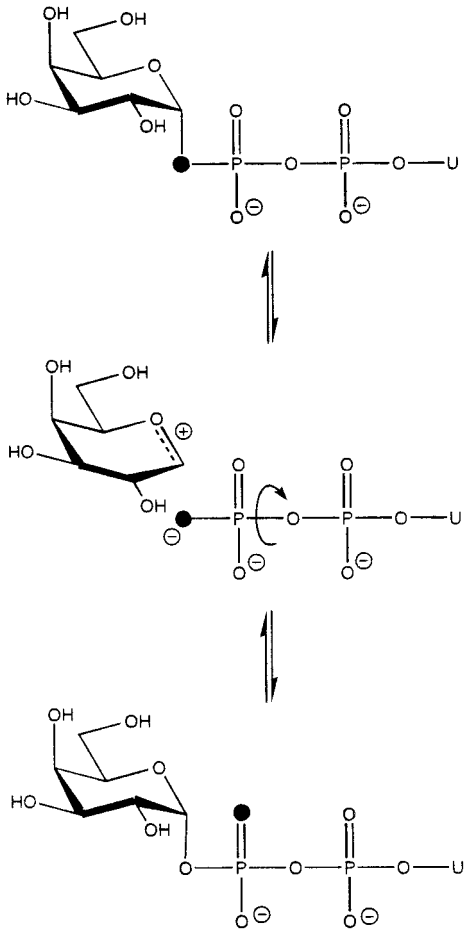

FIGURE 7: Cleavage of the C1″−O1″ bond in the substrate UDP-Gal followed by rotation of the β-phosphate and re-formation of the bond will result in the scrambling of the ^{18}O isotope (indicated by the darkened atom) from the bridging to a nonbridging position in the β-phosphate.

upfield shift in the ^{31}P NMR signal of the β-phosphate resulting from the increased bond order of the P−^{18}O bond (*41*).

The synthesis of uridine 5′-diphospho-(1″-^{18}O)-α-D-galactopyranose (^{18}O-UDP-Gal) was achieved through the coupling of α-D-galactopyranosyl-(1-^{18}O)-phosphate with UMP-morpholidate in accordance with the procedure described by Wittmann and Wong (*38*). However, 1*H*-tetrazole was excluded from the reaction in this case as the presence of this catalyst was found to increase significantly the number

of side products formed. The proton-decoupled ^{31}P NMR signal for the β-phosphorus of the desired product appears as a doublet due to coupling with the α-phosphorus, and the slight upfield shift (1.60 Hz, $\nu_{1/2} = 0.55$ Hz) as compared to the unlabeled compound is consistent with that expected for the presence of a P−^{18}O single bond. The integration of the signals also agreed well with the 85% isotopic incorporation of ^{18}O as determined by mass spectrometry.

In carrying out the positional isotope exchange (PIX) experiment, ^{18}O-UDP-Gal was first incubated with LgtC-19 along with the necessary buffer components that are required by the enzyme for activity. The incompetent acceptor 4dLac was also included in this mixture since the kinetic mechanism of this enzyme demands that the binding sites of both the donor and acceptor substrates be occupied before any catalysis can occur. After an incubation period of 7 h, the ^{18}O-UDP-Gal was then re-isolated, and its proton-decoupled ^{31}P NMR spectrum was recorded. Had scrambling of the ^{18}O isotope occurred during the incubation period, two sets of signals corresponding to the β-phosphorus of ^{18}O-UDP-Gal would have been expected. One would be the result of the ^{18}O being incorporated back into the bridging position after bond cleavage and re-formation while the other would be due to the ^{18}O being scrambled into the nonbridging positions around this β-phosphorus. Statistically, the intensities of these two signals would be in a ratio of 1:2, with the latter signal being shifted slightly upfield. Upon examination of the spectrum, it was clear that no positional isotope exchange had occurred as the signal corresponding to the β-phosphorus was unfortunately found to be unchanged when compared to the spectrum of ^{18}O-UDP-Gal prior to the addition of LgtC-19.

To account for the absence of any scrambling of the ^{18}O isotope, three important facets of the PIX experiment are considered. First and foremost is the cleavage of the C1″−O1″ bond, which may not have occurred in the absence of an appropriate acceptor substrate. While the incompetent acceptor 4dLac can occupy the acceptor binding site and meet the substrate binding requirements of an ordered bi-bi kinetic mechanism, the correct orientation of key residues for catalysis may not have occurred in the absence of the nucleophilic 4′-hydroxyl group. As such, no bond cleavage would have been initiated. Even had bond cleavage taken place, the observation of isotopic scrambling is further dependent on the ability of the β-phosphate in the resulting UDP to rotate freely in the active site. However, from the crystal structure, the mobility of this phosphate appears to be limited as it is tightly bound by the enzyme via hydrogen bonds to the amide backbone of Gly 247 and the side chain of His 78 as well as through coordination to the enzyme-bound Mn^{2+} (*14*). Such restrictions in the motion of this β-phosphate would limit the likelihood of scrambling of the ^{18}O isotope from a bridging to a nonbridging position. Finally, in the case where cleavage of the C1″−O1″ bond has occurred and rotation of the β-phosphate has followed, the last requirement for the observation of positional isotope exchange is the re-formation of the C1″−O1″ bond. In this regard, the ability of LgtC-19 to hydrolyze UDP-Gal is problematic, as the liberated UDP would have to compete with water for reaction with the galactosyl-enzyme intermediate in order to re-form UDP-Gal. Failure to do so would result in the hydrolysis of the intermediate rather than scrambling of the isotope. Unfortunately, hydrolysis of UDP-Gal was indeed observed, as peaks corresponding to those of ^{18}O-UDP did slowly appear in the proton-decoupled ^{31}P NMR spectra taken of the reaction mixture. However, substantial amounts (>60% by NMR) of the UDP-Gal were re-isolated such that had any scrambling of the ^{18}O isotope occurred, it would have been detected.

CONCLUSION

The recombinant form of a lipopolysaccharyl α-galactosyltransferase from *N. meningitidis* has been shown to follow an ordered bi-bi kinetic mechanism, in which the donor substrate UDP-Gal binds first, followed by the acceptor substrate lactose (a derivative of the natural substrate). Following the chemical step of glycosyl transfer, the products are then released in the order of oligosaccharide followed by UDP. In terms of the chemical mechanism, the net retention of anomeric configuration in the product relative to that of the donor substrate UDP-Gal is highly suggestive of a double displacement mechanism. However, attempts to trap a covalent glycosyl-enzyme intermediate and identify the catalytic nucleophile using a number of strategies were unsuccessful. At the same time, the possibility of participation by nonenzymic nucleophiles in the double displacement mechanism was also explored but was found to be unlikely. To broaden our search for a reaction intermediate, a positional isotope exchange experiment was carried out in an effort to detect the presence of any such species. Unfortunately, none could be found. Therefore, while these experiments have provided some valuable insights about the catalytic machinery of this enzyme, a full understanding of the chemical mechanism remains elusive.

NOTE ADDED IN PROOF

A recent redetermination of the structure of this 1,3-galactosyltransferase casts serious doubts upon the findings of this paper. See Boix et al. (*43*).

ACKNOWLEDGMENT

The authors thank Professor Martin E. Tanner of the University of British Columbia and Professor Monica M. Palcic of the University of Alberta for the gifts of β-1,4-anhydrogalactose and UDP-Gal, respectively.

SUPPORTING INFORMATION AVAILABLE

Synthesis and experimental data of alternate acceptor substrates, UDP-2FGal, incompetent acceptor substrates, and ^{18}O-UDP-Gal. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Varki, A. (1993) *Glycobiology 3*, 97.
2. Kobata, A. (1993) *Acc. Chem. Res. 26*, 319.
3. Dwek, R. A. (1996) *Chem. Rev. 96*, 683−720.
4. Campbell, J. A., Davies, G. J., Bulone, V., and Henrissat, B. (1997) *Biochem. J. 326*, 929−942.
5. Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171−1202.
6. Davies, G., Withers, S. G., and Sinnott, M. L. (1997) in *Comprehensive Biological Catalysis* (Sinnott, M. L., Ed.) pp 119−208, Academic Press, London.
7. Vrielink, A., Ruger, W., Driessen, H. P. C., and Freemont, P. S. (1994) *EMBO J. 13*, 3413−3422.

8. Gastinel, L. N., Cambillau, C., and Bourne, Y. (1999) *EMBO J. 18*, 3546−3557.
9. Charnock, S. J., and Davies, G. J. (1999) *Biochemistry 38*, 6380−6385.
10. Ha, S., Walker, D., Shi, Y., and Walker, S. (2000) *Protein Sci. 9*, 1045−1052.
11. Pedersen, L. C., Tsuchida, K., Kitagawa, H., Sugahara, K., Darden, T. A., and Negishi, M. (2000) *J. Biol. Chem. 275*, 34580−34585.
12. Ünligil, U. M., Zhou, S., Yuwaraj, S., Sarkar, M., Schachter, H., and Rini, J. M. (2000) *EMBO J. 19*, 5269−5280.
13. Mulichak, A. M., Losey, H. C., Walsh, C. T., and Garavito, R. M. (2001) *Structure 9*, 547−557.
14. Persson, K., Ly, H. D., Dieckelmann, M., Wakarchuk, W. W., Withers, S. G., and Strynadka, N. C. J. (2001) *Nat. Struct. Biol. 8*, 166−175.
15. Gastinel, L. N., Bignon, C., Misra, A. K., Hindsgaul, O., Shaper, J. H., and Joziasse, D. H. (2001) *EMBO J. 20*, 638−649.
16. Sandvig, K. (2001) *Toxicon 39*, 1629−1635.
17. Mulvey, G., Kitov, P. I., Marcato, P., Bundle, D. R., and Armstrong, G. D. (2001) *Biochimie 83*, 841−847.
18. Lougheed, B., Ly, H. D., Wakarchuk, W. W., and Withers, S. G. (1999) *J. Biol. Chem. 274*, 37717−37722.
19. Ichikawa, Y., Lin, Y. C., Dumas, D. P., Shen, G. J., Garcia-Junceda, E., Williams, M. A., Bayer, R., Ketcham, C., Walker, L. E., Paulson, J. C., and Wong, C. H. (1992) *J. Am. Chem. Soc. 114*, 9283−9298.
20. Wakarchuk, W. W., Cunningham, A., Watson, D. C., and Young, N. M. (1998) *Protein Eng. 11*, 295−302.
21. Weber, D. J., Bhatnagar, S. K., Bullions, L. C., Bessman, M. J., and Mildvan, A. S. (1992) *J. Biol. Chem. 267*, 16939−16942.
22. O'Handley, S. F., Frick, D. N., Bullions, L. C., Mildvan, A. S., and Bessman, M. J. (1996) *J. Biol. Chem. 271*, 24649−24654.
23. Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) *J. Biol. Chem. 270*, 13932−13936.
24. Ciesla, W. P. J., and Bobak, D. A. (1998) *J. Biol. Chem. 273*, 16021−16026.
25. Moréra, S., Imberty, A., Aschke-Sonnenborn, U., Rüger, W., and Freemont, P. S. (1999) *J. Mol. Biol. 292*, 717−730.
26. Segel, I. H. (1993) *Enzyme Kinetics−Behaviour and analysis of rapid equilibrium and steady-state enzyme systems*, John Wiley & Sons, New York.
27. Piszkiewicz, D. (1977) *Kinetics of chemical and enzyme-catalyzed reactions*, Oxford University Press, New York.
28. Mosi, R., He, S., Uitdehaag, J., Dijkstra, B. W., and Withers, S. G. (1997) *Biochemistry 36*, 9927−9934.
29. Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B., and Aebersold, R. (1990) *J. Am. Chem. Soc. 112*, 5887−5889.
30. Tull, D., Withers, S. G., Gilkes, N. R., Kilburn, D. G., Warren, R. A. J., and Aebersold, R. (1991) *J. Biol. Chem. 266*, 15621−15625.
31. Gebler, J. C., Aebersold, R., and Withers, S. G. (1992) *J. Biol. Chem. 267*, 11126−11130.
32. Mackenzie, L. F., Brooke, G. S., Cutfield, J. F., Sullivan, P. A., and Withers, S. G. (1997) *J. Biol. Chem. 272*, 3161−3167.
33. Lindhorst, T. K., Braun, C., and Withers, S. G. (1995) *Carbohydr. Res. 268*, 93−106.
34. Ly, H. D., Howard, S., Shum, K., He, S., Zhu, A., and Withers, S. G. (2000) *Carbohydr. Res. 329*, 539−547.
35. McCarter, J. D., and Withers, S. G. (1996) *J. Am. Chem. Soc. 118*, 241−242.
36. McCarter, J. D., and Withers, S. G. (1996) *J. Biol. Chem. 271*, 6889−6894.
37. Howard, S., He, S., and Withers, S. G. (1998) *J. Biol. Chem. 273*, 2067−2072.
38. Wittmann, V., and Wong, C. H. (1997) *J. Org. Chem. 62*, 2144−2147.
39. Micheel, F., Klemer, A., Baum, G., Ristic, P., and Zumbulte, F. (1955) *Chem. Ber. 88*, 475.
40. Sujino, K., Uchiyama, T., Hindsgaul, O., Seto, N. O. L., Wakarchuk, W. W., and Palcic, M. M. (2000) *J. Am. Chem. Soc. 122*, 1261−1269.
41. Villafranca, J. J. (1989) *Methods Enzymol. 177*, 390−403.
42. Gosselin, S., Alhussaini, M., Streiff, M. B., Takabayashi, K., and Palcic, M. M. (1994) *Anal. Biochem. 220*, 92−97.
43. Boix, E., Swaminathan, G. J., Zhang, Y. N., Natesh, R., Brew, K., and Acharya, K. R. (2001) *J. Biol. Chem. 276*, 48608−48614.

BI012031S