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Synthesis of the acceptor analog αFuc(1 $\rightarrow$ 2)αGal-O$(CH_2)_7$ CH_3: A probe for the kinetic mechanism of recombinant human blood group B glycosyltransferase

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We report the chemical synthesis of αFuc(1 $\rightarrow$ 2)αGal-O$(CH_2)_7CH_3$ (1) an analog of the natural blood group (O)H disaccharide αFuc(1 $\rightarrow$ 2)βGal-OR. Compound 1 was a good substrate for recombinant blood group B glycosyltransferase (GTB) and was used as a precursor for the enzymatic synthesis of the blood group B analog αGal($\rightarrow$ 3)[αFuc(1 $\rightarrow$ 2)]αGal-O$(CH_2)_7CH_3$ (2). To probe the mechanism of the GTB reaction, kinetic evaluations were carried out employing compound 1 or the natural acceptor disaccharide αFuc(1 $\rightarrow$ 2)βGal-O$(CH_2)_7CH_3$ (3) with UDP-Gal and UDP-GalNAc donors. Comparisons of the kinetic constants for alternative donor and acceptor pairs suggest that the GTB mechanism is Theorell-Chance where donor binding precedes acceptor binding. GTB operates with retention of configuration at the anomeric center of the donor. Retaining reactions are thought to occur via a double-displacement mechanism with formation of a glycosyl-enzyme intermediate consistent with the proposed Theorell-Chance mechanism.

Keywords: **oligosaccharides, blood group B trisaccharide analog, glycosyltransferase B kinetic mechanism, Theorell-Chance mechanism**

Abbreviations: **GTB, blood group B galactosyltransferase**

Introduction

The histo-blood group ABO(H) antigens are defined carbohydrate determinants found on the surface of red blood cells and are largely responsible for failure of mismatched blood transfusions. These ABO carbohydrate antigens are also found on other cell types and are important in cell development, differentiation and oncogenesis [for reviews see 1]. Blood group A glycosyltransferase (GTA, E.C. 2.4.1.40) catalyses the transfer of GalNAc from UDP-GalNAc donors to the (O)H-precursor structure αFuc(1 $\rightarrow$ 2)βGal-OR to give the A trisaccharide antigen αGalNAc(1 $\rightarrow$ 3)[αFuc(1 $\rightarrow$ 2)]βGal-OR. Blood group B individuals express glycosyltransferase B (GTB, E.C. 2.4.1.37) which uses the same (O)H-acceptor but catalyses the transfer of Gal from UDP-Gal donors to make the B antigen αGal(1 $\rightarrow$ 3)[αFuc(1 $\rightarrow$ 2)]βGal-OR [1,2]. Both enzyme reactions occur with retention of configuration of the anomeric center of monosaccharide transferred from donor.

In this paper, we report the chemical synthesis of the O(H) disaccharide analog αFuc(1 $\rightarrow$ 2)αGal-O$(CH_2)_7CH_3$ (**1**) as an alternate substrate probe for GTB reactions. Compound **1** was evaluated as a substrate for recombinant GTB in radiochemical assays. Its conversion to product was confirmed by small scale enzymatic synthesis of the blood B group trisaccharide analog αGal(1 $\rightarrow$ 3)[αFuc(1 $\rightarrow$ 2)]αGal-O$(CH_2)_7CH_3$ (**2**), (Scheme 1).

Alternative substrates are useful probes for elucidating the order of substrate addition in multisubstrate enzyme reactions. They undergo the same chemical conversions as natural substrates, however kinetic constants vary depending on the type of reaction mechanism [3,4]. Kinetic evaluations were carried out with the alternative acceptor **1** and the natural acceptor αFuc(1 $\rightarrow$ 2)βGal-O$(CH_2)_7CH_3$ (**3**) with both UDP-

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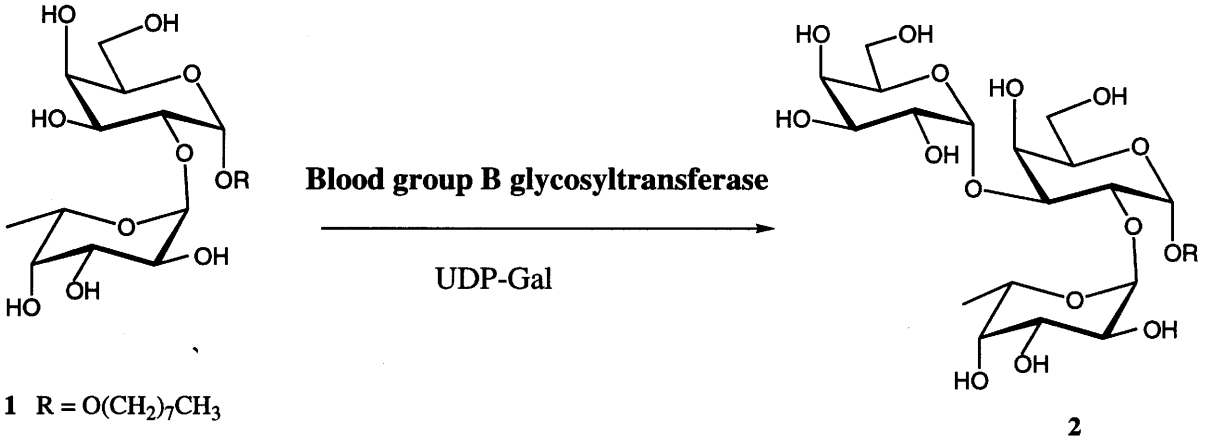


Scheme 1.

Gal and UDP-GalNAc donors to elucidate the kinetic mechanism of GTB.

Material and methods

General Methods

Analytical TLC was performed on Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by quenching of fluorescence and/or by charring with H_2SO_4. Mass spectra were recorded on samples suspended in a matrix of glycerol and HCl using a Kratos AEIMS9 instrument with Xe as the bombarding gas. ^{1}H NMR spectra were recorded at 360 MHz (Bruker WM 360) or 300 MHz (Bruker AM 300) with either internal $(CH_3)_4Si$ (δ 7.26, $CDCl_3$) or DOH (δ 4.80, D_2O). ^{13}C NMR spectra were recorded at 75.5 MHz (Bruker AM 300) with internal standards. ^{1}H NMR data are reported as though they were first order. All ^{13}C chemical shift assignments are tentatively assigned. Unless otherwise noted, all reactions were carried out at room temperature. Organic solutions were dried (Na_2SO_4) prior to concentration under vacuum at $<40^\circ$C (bath). Microanalyses were carried out by the analytical services of this department, and all the samples submitted for elemental analyses were dried overnight under vacuum over P_2O_5 at 56°C (refluxing Me_2CO). Optical rotations were measured with a Perkin-Elmer 241 polarimeter at $22 \pm 2^\circ$C.

General Materials

UDP, UDP-GalNAc, UDP-Gal and bovine serum albumin (BSA) were purchased from Sigma. The radioactive [6-^{3}H]-labeled analogs of UDP-GalNAc (10 Ci/mmol) and UDP-Gal (15 Ci/mmol) were from American Radiolabeled Chemicals. Millex-GV (0.22 filter units (Millipore), Iatrobeads refers to a silica gel (product 6RS-8060, Iatron Laboratories, Tokyo, Japan), calf intestinal alkaline phosphatase (Boehringer-Mannheim), Sep-Pak C_{18} (+) reverse phase cartridges (Waters) and Ecolite (+) liquid scintillation cocktail (ICN) were purchased commercially. All commercial reagents were used as supplied, except for chromatography solvents which were distilled prior to use. GTB cloned and expressed in *E.coli* [5,6] was isolated from periplasm or by disruption of the cells with a French Press followed by successive chromatography on SP Sepharose Fast Flow and UDP-hexanolamine [7].

Syntheses

*Octyl 6-*O*-tert-butyldiphenylsilyl-3,4-*O*-isopropylidene-α-*D*-galactopyranoside* (**5**)

Compound **4** [8] (0.4 g, 1.4 mmol) was dissolved in 5 ml *N*,*N*-dimethylformamide (DMF) and *p*-toluenesulfonic acid (20 mg) was added. After 1 h, 2.2-dimethoxypropane (5 ml) was added and the reaction was stirred for an additional 12 h. The reaction mixture was diluted with ethyl acetate (EtOAc) and extracted with $NaHCO_3$ and water. The organic layer was dried and concentrated under reduced pressure to a syrup (0.4 g). The crude syrup was used directly in the next step.

The syrup was dissolved in DMF (10 ml) followed by addition of imidazole (0.16 g, 2.60 mmol). *tert*-Butyldiphenylchlorosilane (0.28 g, 1.10 mmol) was added to the reaction mixture which was stirred for 16 h at RT under an inert atmosphere. The reaction mixture was diluted with EtOAc and washed with satd $NaHCO_3$ and water. The organic layer was dried and concentrated under reduced pressure. Column chromatography using 7:3 pentane–EtOAc as eluent furnished the desired–product **5** (0.42 g, 54%, 2 steps) as a clear syrup: $[\alpha]_D + 46.2^\circ$ (*c* 0.9, $CHCl_3$); R_f 0.51 (7:3 pentane–EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.20–7.80 (m, 10 H, Ph), 4.80 (d, 1 H, $J_{1.2}$ 4 Hz, H-1), 4.25 (dd, 1 H, $J_{1.2}$ 3 Hz, $J_{2,3}$ 6 Hz, H-2), 4.17 (t, 1 H, *J* 6 Hz, H-3), 2.40 (d, 1 H, OH), 1.30 & 1.50 (s each, 2×3 H, $2 \times$ Me), 1.20–1.60 (m, 12 H, octyl-CH_2), 0.88 (t, 3 H, octyl-CH_3); ^{13}C NMR ($CDCl_3$): δ 135.8, 135.7, 135.6, 135.5, 133.5, 133.4, 129.5, 127.7, 127.6, 127.5, 109.2, 97.2, 76.2, 72.8, 69.7, 68.5, 68.0, 63.0, 31.7, 29.4, 29.3, 29.1, 27.7,

26.8, 26.7, 26.0, 25.8, 22.5, 19.1, 14.0. Anal. Calcd for $C_{33}H_{50}O_6Si$: C, 69.43: H, 8.83. Found: C, 69.36; H, 8.98.

Octyl 6-O-*tert-butyldiphenylsilyl-3,4*-O-*isopropylidene-2*-O-*(2,3,4-tri*-O-*benzyl-α-L-fucopyranosyl)*-α-D-*galactopyranoside* (**7**).

Compound **5** (0.14 g, 0.25 mmol) and tetrabutylammonium bromide (Bu_4NBr) (96 mg, 0.30 mmol) were dissolved in dry CH_2Cl_2 (20 ml) and DMF (5 ml) containing 0.5 g of crushed 4 Å molecular sieves. The system was purged with Ar and stirred overnight. To this slurry was added freshly prepared 2,3,4-tri-*O*-benzyl-L-fucopyranosyl bromide (**6**, 0.37 g, 0.75 mmol) in CH_2Cl_2 (2 ml) and the mixture was stirred for 2 days. Methanol (2 ml) was added and stirring continued for 30 min. The mixture was filtered and concentrated. Column chromatography of the residue furnished the desired product **7** (0.18 g, 75%): $[\alpha]_D$ +5.57 (*c* 0.7 $CHCl_3$); R_f 0.37 (4:1 pentane-EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.20–7.80 (m, 25 H, aromatic), 5.12 (d, 1 H, $J_{1',2'}$, 3.5 Hz, H-1′), 4.78 (d, 1 H, $J_{1,2}$ 4 Hz, H-1), 4.35 (dd, 1 H, *J* 5.5 Hz, *J* 8 Hz, H-3), 4.25 (dd, 1 H, *J* 2.5 Hz, *J* 5.0 Hz, H-2), 1.30 & 1.50 (s each, 2 × 3 H, 2 × Me), 1.20–1.60 (m, 12 H, octyl-CH_2), 0.88 (t, 3 H, octyl-CH_3); ^{13}C NMR ($CDCl_3$): δ 139.1, 138.8, 138.6, 135.6, 135.5, 134.7, 133.5, 133.4, 129.6, 129.5, 128.4, 128.2, 128.1, 128.0, 127.7, 127.6, 127.5, 127.4, 127.3, 127.2, 108.8, 99.5, 98.2, 77.6, 76.1, 75.0, 74.7, 73.5, 73.4, 71.9, 68.0, 67.8, 66.3, 63.0, 29.4, 29.3, 29.2, 26.7, 26.4, 26.1, 22.6, 16.7. Anal. Calcd for $C_{60}H_{78}O_{10}Si$: C, 72.99; H, 7.96 Found: C, 72.57; H, 7.79.

Octyl 2-O-*(α-L-fucopyranosyl)*-α-D-*galactopyranoside* (**1**).

Compound **7** (0.18 g, 0.18 mmol) was dissolved in 80% acetic acid, and the reaction mixture was heated at 60°C for 5 h. The solvent was evaporated and the residue was co-evaporated several times with dry toluene to afford a syrup (0.16 g). This syrup was not further characterized but was used directly in the next step.

The syrup was dissolved in dry THF (10 mL), 1.0 M Bu_4NF (1 ml) was added and the reaction was stirred overnight. The solvent was then evaporated to furnish a syrup (0.10 g) which was treated with 10% $Pd(OH)_2$-C in MeOH (4 ml) for 20 h under an atmosphere of H_2 (0.1 MPa). The catalyst was removed by filtration, washed with 95% EtOH and the solvent was evaporated. The residue was purified by chromatography on Iatrobeads using 10:4:1 $CHCl_3$–MeOH–H_2O as eluent to furnish a syrup which was dissolved in deionized water (3 ml) and loaded onto a Sep-Pak C_{18} cartridge that was then washed with H_2O (20 mL). The product was eluted with MeOH (10 ml) and concentrated. The residue was re-dissolved in deionized water (4 ml) and passed through a Millipore (0.22 μm) filter. Lyophilization of the filtrate furnished **1** as a white fluffy solid (25.8 mg, 32%, 3 steps): $[\alpha]_D$ +3.94 (*c* 0.4 H_2O); R_f 0.51 (10:4:1 CH_2Cl_2–MeOH–H_2O). ^{1}H NMR (D_2O): δ 4.96 (d, 1 H, $J_{1',2'}$ 3.5 Hz, H-1′), 4.94 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.03 (d, 1 H, *J* 3.5 Hz, H-4), 4.01 (m, 1 H, H-5′), 3.99 (dd, 1 H, *J* 3.5 Hz, *J* 10 Hz, H-2), 3.92 (t, 1 H, *J* 8 Hz, H-2′), 3.88 (dd, 1 H, *J* 3.5 Hz, *J* 10 Hz, H-3), 3.72–3.82 (m, 6 H, H-5, H-6, H-3′, H-4′, OCH*H*CH$_2$), 3.49 (m, 1 H, OCH*H*CH$_2$), 1.52–1.68 (m, 2 H, OCH$_2$*CH*$_2$), 1.22–1.30 (m, 10 H, octyl-CH_2), 0.88 (t, 3 H, *J* 7 Hz, octyl-CH_3); ^{13}C NMR (D_2O): δ 102.4 (C-1′, *J* 171 Hz), 99.7 (C-1, *J* 172 Hz), 78.7 (C-5), 72.5 (C-4′), 71.5 (C-2′), 70.3 (C-3), 70.1 (C-4), 69.3 (C-2), 69.2 (C-3′), 69.1 (O*CH*$_2$), 68.0 (C-5′), 61.9 (C-6), 31.9, 29.4, 29.3, 29.2, 26.4, 22.8 (octyl-*CH*$_2$), 16.3 (C-6′), 14.3 (octyl-*CH*$_3$). MS: Calcd for $C_{20}H_{38}O_{10}$ (M + Na): *m/z* 461.2362; found: *m/z* 461.2368.

Octyl α-D-*galactopyranosyl-(1 → 3)[*α-L-*fucopyranosyl-(1 → 2)]*-α-D-*galactopyranoside* (**2**)

A solution of **1** (0.87 mg, 1.8 μmol), UDP-Gal (1.4 mg, 2.5 μmol), blood group B glycosyltransferase (20 milliunits), alkaline phosphatase (0.5 U, 0.5 μl) and 1 mg/mL BSA in 0.24 ml 50 mM sodium cacodylate buffer, pH 7.0, 20 mM $MnCl_2$ was incubated at 37°C for 20 h. An additional 0.6 mg of UDP-Gal was added and the mixture was incubated for another 12 hr. The progress of the reaction was monitored by TLC (6:4:1, CH_2Cl_2–MeOH–H_2O), which indicated complete conversion of **1** to a new product (**2**) (R_f 0.78). The product was purified upon completion using two Sep-Pak C_{18} reverse phase cartridges [9,10] by dilution with water (3 ml) and application to the pre-washed cartridges which were then washed with H_2O (20 ml). The product was eluted with 5 ml HPLC grade methanol. The solvent was evaporated to give a residue which was re-dissolved in H_2O (2 ml) and passed through a Millex Millipore filter (0.22 μm). Lyophilization furnished the desired product **2** (0.87 mg, 71%) as a white powder. ^{1}H NMR (D_2O): δ 5.21 (d, 1 H, *J* 4 Hz, H-1″), 5.13 (d, 1 H, *J* 4 Hz, H-1′), 5.04 (d, 1 H, *J* 4 Hz, H-1), 4.35 (t, 1 H, H-5″), 4.32 (d, 1 H, *J* 3.5 Hz, H-4), 4.16 (dd, 1 H, *J* 3.5 Hz, *J* 8 Hz, H-3), 4.07 (m, 1 H, H-5′), 4.06 (dd, 1 H, H-2), 3.96 (d, 1 H, *J* 3.5 Hz, H-4″), 3.74-3.94 (m, 11 H, H-5, H-6, H-2′, H-3′, H-4′, H-2″, H-3″, H-6″, OC*H*H), 3.52–3.56 (m, 1 H, OCH*H*), 1.60–1.70 (m, 2 H, OCH$_2$*CH*$_2$), 1.20–1.40 (m, 10 H, octyl-CH_2), 1.25 (d, 3 H, *J* 6.5 Hz, H-6′), 0.88 (t, 3 H, octyl-CH_3). MS: Calcd for $C_{26}H_{48}O_{15}$ (M + Na): *m/z* 623.2890; found: *m/z* 623.2897.

Enzyme assay

The amount of GTB used in the enzymatic synthesis was determined by incubating 50 mM sodium cacodylate buffer, pH 7.0, 20 mM $MnCl_2$, 1 mg/ml BSA, 0.02 μCi UDP-[6-^{3}H]Gal, 600 μM UDP-Gal, 800 μM αFuc(1 → 2)*β*Gal-O-$(CH_2)_7CH_3$ (**3**) and 0.1 μl of purified enzyme at 37°C for 20 min in 33 μl total volume. Reaction mixtures were applied to Sep-Pak C_{18} cartridge, washed with water to remove unreacted labeled donor. Radiolabeled product was eluted from the cartridge with 3.5 ml methanol directly into scintillation vials. Radioactivity was quantitated in a Beckman

LS1801 scintillation counter after the addition of 10 ml of Ecolite$^+$ cocktail. One mU of activity is defined as the amount of enzyme that catalyzes the conversion of one nmol of substrate to product per min.

Enzyme kinetic analysis

Two substrate kinetic analysis was performed in 33 µl total volume at 37°C by incubation for 8–120 min. Mixtures contained enzyme in 50 mM sodium cacodylate buffer, pH 7.0, 20 mM $MnCl_2$, 1 mg/ml BSA, 0.2 µCi UDP-[6-^{3}H]Gal or UDP-[6-^{3}H]GalNAc, six different concentrations of donor UDP-Gal or UDP-GalNAc, and either αFuc(1 → 2)αGal-O-$(CH_2)_7CH_3$ (**1**) or αFuc(1 → 2)βGal-O-$(CH_2)_7CH_3$ (**3**) acceptor. The substrate concentrations ranged from 0.04–13 × Km. With the donor UDP-Gal substrate inhibition occurred at concentrations higher than 1.1 mM of acceptor **1**, therefore for this case, the highest concentration of acceptor was 1.2 × Km. A two substrate GraFit Program [10] was used to obtain V_{max}, K_A, K_B and $K_{A'}$ from which the Dalziel ϕ coefficients [11] were calculated. The kinetic analyses are elaborated in the results and discussion section.

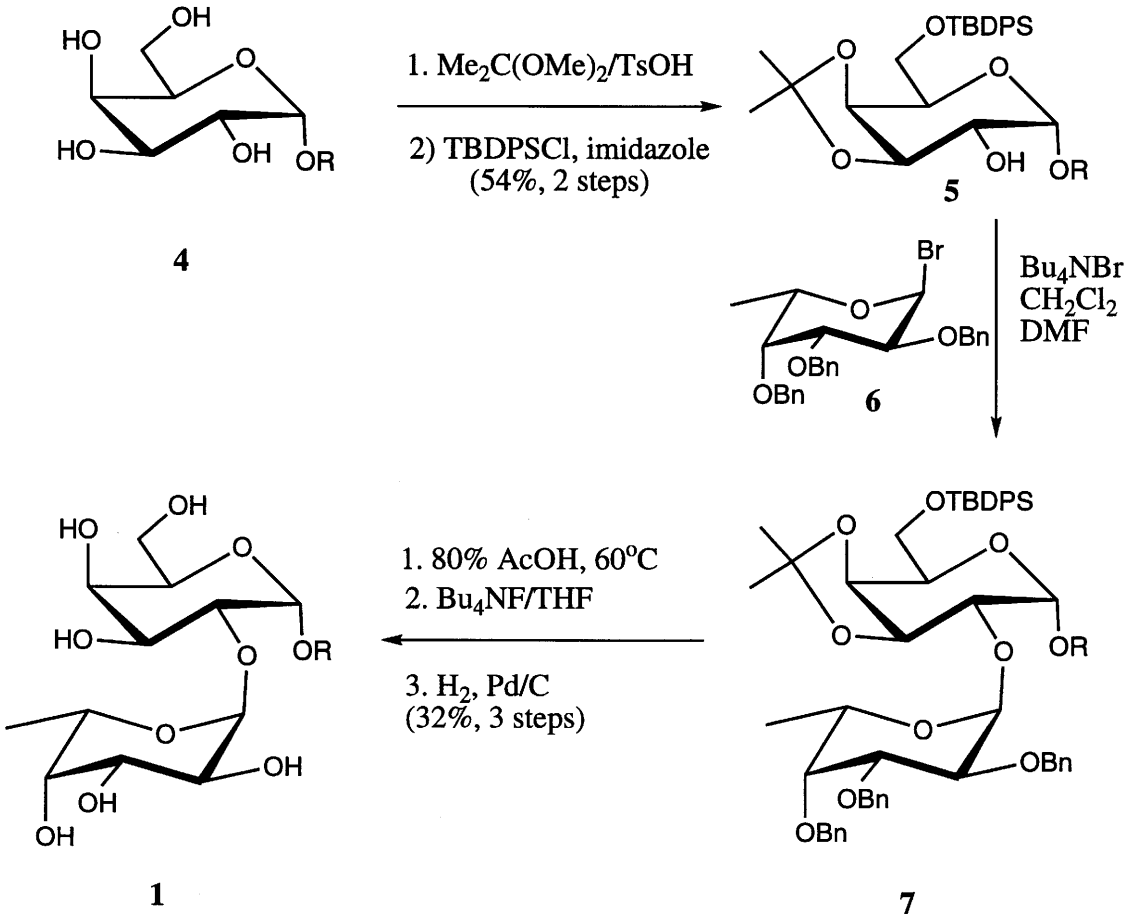
Results and Discussion

Chemical Synthesis

Treatment of octyl α-D-galactopyranoside **4** [8] (Scheme 2) with 2,2-dimethoxypropane and a catalytic amount of p-TsOH gave a syrup which was then silylated selectively at the C-6 position using tert-butyldiphenylchlorosilane to furnish compound **5** in 54% yield (2 steps). Glycosylation of 5 with freshly prepared 2,3,4-tri-*O*-benzyl-L-fucopyranosyl bromide **6** using Bu_4NBr [12] furnished the desired α-fucosylated disaccharide **7** in 75% yield (Scheme 2). Removal of the isopropylidene group was achieved using 80% acetic acid, followed by desilylation and debenzylation using 10% $Pd(OH)_2$-C to furnish the desired compound **1** in 32% yield (3 steps).

Enzymatic Synthesis

Compound **1** was initially evaluated as a substrate for GTB in standard radiochemical assays [5,6]. Since these assays are designed to quantitate the amount of radioactivity transferred from UDP-[^{3}H]Gal donor to synthetic acceptors, the reaction product identity was confirmed by isolation from a small-scale



Scheme 2.

Table 1. Kinetic constants for glycosyltransferase B using alternate acceptor and donor pairs

Acceptor substrate	k_{cat} (s^{-1})	K_A (μM)	$K_{A'}$ (μM)	K_B (μM)	$K_{B'}$ (μM)
		UDP-Gal Donor			
3	6.5 ± 0.3	61 ± 5	23 ± 8	35 ± 5	13 ± 4
1	5.9 ± 0.8	1500 ± 300	220 ± 100	60 ± 16	9 ± 4
		UDP-GalNAc Donor			
3	0.30 ± 0.02	250 ± 60	120 ± 65	340 ± 75	190 ± 100
1	0.40 ± 0.04	3220 ± 600	560 ± 390	300 ± 64	50 ± 40

reaction. Compound **1** was readily converted to trisaccharide 2 in 71% yield using recombinant human GTB expressed in *E. coli.* [5,6] and UDP-Gal as a donor.

Kinetic Analysis

Two-substrate kinetic studies were carried out for GTB. UDP-Gal and UDP-GalNAc donors were individually evaluated with acceptors **1** or **3** in 6 × 6 concentration grids. The kinetic parameters V_{max}, K_A, K_B and $K_{A'}$ were obtained using a GraFit Program [10] for two substrate systems (Equation 1)

$$v = \frac{V_{max}[A][B]}{K_{A'}K_B + K_B[A] + K_A[B] + [A][B]} \qquad \text{Eq. 1}$$

where v is the initial velocity, V_{max} is the maximum velocity, K_A is the Michaelis constant for acceptor, K_B is the Michaelis constant for donor and $K_{A'}$ is the dissociation constant for acceptor. The catalytic constant or turnover number, k_{cal} was obtained from $V_{max}/[E]$. The extent to which donor affects the Michaelis constant for the acceptor is the same as the effect of acceptor on the Michaelis constant for donor, that is $K_AK_{B'} = K_BK_{A'}$.

The values for k_{cat}, K_A, $K_{A'}$, K_B and $K_{B'}$ for glycosyltransferase B with the two donors and both acceptor substrates are listed in Table 1. The k_{cat} value for GTB with the alternative acceptor **1** and UDP-Gal as a donor was 5.9 s^{-1} which is comparable to the value of 6.5 s^{-1} obtained with the natural acceptor αFuc(1 → 2)βGal-O(CH$_2$)$_7$CH$_3$ (**3**) [5,6] and UDP-Gal. For UDP-GalNAc as a donor the k_{cat} values were much lower, 0.3 and 0.4 s^{-1} for **3** and **1**, respectively. This is in accordance with our previous studies that showed the donor specificity is largely due to a difference in k_{cat} rather than K_M values [5,13].

Kinetic Mechanism from Alternative Substrate Comparisons

To elucidate the kinetic mechanism for the enzymes, Equation 1 was expressed in reciprocal form (Equation 2) in terms of ϕ's as suggested by Dalziel [11].

$$\frac{E}{v} = \phi_o + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} \qquad \text{Eq. 2}$$

The relationships between the constants in Equation 1 and 2 are:

$$\phi_o = \frac{1}{k_{cat}};\ \phi_B = \frac{K_B}{k_{cat}};\ \phi_A = \frac{K_A}{k_{cat}};\ \frac{\phi_{AB}}{\phi_A} = K_{B'};\ \frac{\phi_{AB}}{\phi_B} = K_{A'}$$

Using these relationships, the Dalziel coefficient's ϕ_o, ϕ_A, ϕ_B, ϕ_{AB}/ϕ_A, ϕ_{AB}/ϕ_B in Table 2 were calculated for GTB.

The most common two-substrate kinetic mechanisms are random addition of substrates, compulsory ordered addition, Theorell-Chance and ping-pong mechanisms. For random mechanisms either substrate can bind to free enzyme. In contrast, for compulsory ordered, Theorell-Chance and ping-

Table 2. Dalziel ϕ coefficients for glycosyltransferase B

Acceptor substrate	ϕ_O (s)	ϕ_A (μM s)	ϕ_B (μM s)	ϕ_{AB}/ϕ_A (μM)	ϕ_{AB}/ϕ_A (μM)
		UDP-Gal Donor			
3	0.15 ± 0.01	9.4 ± 0.8	5.4 ± 0.8	13 ± 4	23 ± 8
1	0.17 ± 0.02	250 ± 50	10 ± 3	9 ± 4	220 ± 100
		UDP-GalNAc Donor			
3	3.3 ± 0.2	830 ± 250	1130 ± 250	190 ± 100	140 ± 65
1	2.5 ± 0.3	8050 ± 1600	750 ± 50	50 ± 40	560 ± 390

pong mechanisms one substrate (A) must bind to the enzyme before the second substrate (B) can bind. These mechanisms can be distinguished by comparing the effects of the alternative substrates A′ and B′ on ϕ values [3,4].

Briefly, when the alternate substrate B′ is used instead of B, the invariant ϕ coefficients for the four mechanisms are: for Theorell-Chance ϕ_o, ϕ_A, ϕ_{AB}/ϕ_B, for ordered mechanisms ϕ_A, ϕ_{AB}/ϕ_B, for rapid equilibrium random mechanisms ϕ_{AB}/ϕ_B, and for ping pong mechanisms ϕ_A [3,4]. The kinetic constants that are represented by the Dalziel ϕ coefficients (Eq. 2) are shown in Equation 3.

$$\frac{E}{v} = \frac{1}{k_3} + \frac{1}{k_1 A} + \frac{1}{k_2 B} + \frac{k_{-1}}{k_1 k_2 AB} \qquad \text{Eq. 3}$$

For the alternate substrate B′ the kinetic constants are given by Equation 4:

$$\frac{E}{v} = \frac{1}{k_3} + \frac{1}{k_1 A} + \frac{1}{k_2' B'} + \frac{k_{-1}}{k_1 k_2' AB'} \qquad \text{Eq. 4}$$

Figure 1a shows a minimal kinetic scheme with kinetic constants for a Theorell-Chance mechanism. In this mechanism, the release of product Q from EQ is governed by k_3 which corresponds to $1/\phi_o$; when the second substrate is replaced by B′ (1b), k_3 is unchanged. The formation of EA is also not affected by the replacement of B with B′, and since ϕ_A reflects

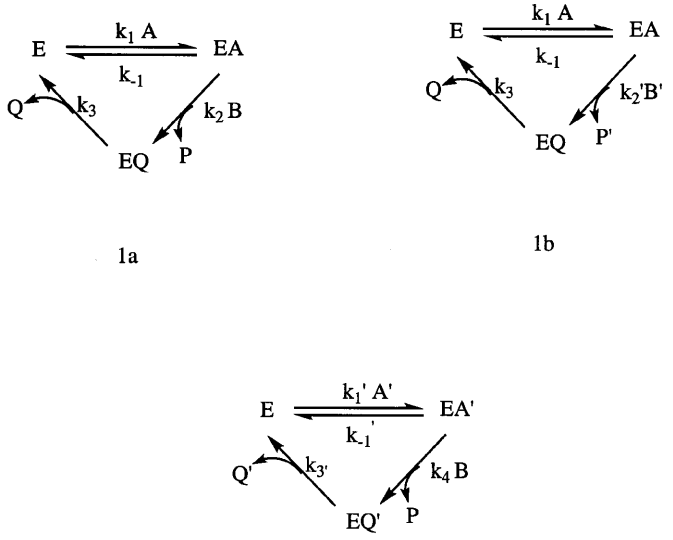


Figure 1. Two-substrate kinetic mechanism for glycosyltransferase B. The Theorell-Chance two-substrate mechanism is shown. In 1a the enzyme (E) binds the donor substrate (A) first to form EA. This is followed by the addition of the acceptor substrate (B) to form EQ (not reversible) followed by the release of the final product (Q) by the enzyme (E). The same mechanism is shown in 1b for the alternate acceptor B′ and 1c for the alternate donor A′.

$k_1 A$ and ϕ_{AB}/ϕ_B reflect k_{-1}/k_1 these values are invariant. The substitution of B with B′ will have an effect on the conversion of EA to EQ and both ϕ_B and ϕ_{AB}/ϕ_A containing k_2 (or k_2') will change. When the alternate substrate A′ is used instead of A (Equation 5) none of the ϕ coefficients are invariant for the Theorell-Chance (1c) and ordered mechanisms, for rapid equilibrium ordered ϕ_{AB}/ϕ_A is invariant and for ping pong mechanisms ϕ_B is invariant.

$$\frac{E}{v} = \frac{1}{k_3'} + \frac{1}{k_1' A'} + \frac{1}{k_4 B} + \frac{k_{-1}'}{k_1'} k_4 A' B \qquad \text{Eq. 5}$$

For GTB reactions with UDP-Gal as a donor, the use of the alternate acceptor **3** instead of acceptor **1** gives invariant ϕ_o, ϕ_B and ϕ_{AB}/ϕ_A parameters (Table 2). We consider ϕ_o values of 0.15 ± 0.01 and 0.17 ± 0.02 s, ϕ_B values of 5.4 ± 0.8 and 10 ± 3 μM s and ϕ_{AB}/ϕ_A values of 13 ± 4 and 9 ± 4 μM to be invariant. The ϕ_A (9.4 ± 0.8 and 250 ± 50 μM s) and the ϕ_{AB}/ϕ_B coefficients (23 ± 8 and 220 ± 100 μM) show 10–25 fold differences. This pattern is consistent with a Theorell-Chance mechanism where the donor UDP-Gal binds to the enzyme before the acceptor (Fig. 1). This pattern is also seen when UDP-GalNAc is used as a donor with the alternate acceptor **3** compared to acceptor **1**. In this case values of ϕ_o (3.3 ± 0.2 and 2.5 ± 0.3) s), ϕ_B (1130 ± 250 and 750 ± 150 μM s^{-1}), ϕ_{AB}/ϕ_A (190 ± 100 and 50 ± 40 μM) and ϕ_{AB}/ϕ_B (140 ± 65 and 560 ± 390 μM) are invariant compared to the ten fold difference in ϕ_A (830 ± 250 and 8050 ± 1600 μM s^{-1}). As predicted for a Theorell-Chance mechanism, when UDP-GalNAc was employed instead of UDP-Gal with the same acceptor (**3**) all ϕ coefficients change. When UDP-GalNAc was employed instead of UDP-Gal with acceptor **1** ϕ_{AB}/ϕ_B was invariant. The only discrepancies are seen for the ϕ_{AB}/ϕ_B term that was invariant with UDP-GalNAc as an alternate donor; we consider this to be coincidental.

Mechanistic Conclusions

Retaining enzymes are thought to operate via double-displacement mechanisms where a glycosyl-enzyme intermediate forms prior to reaction with the second substrate. A potential chemical scheme for retaining glycosyltransferases involves the binding of nucleotide donor substrate to free enzyme followed by formation of a glycosyl-enzyme intermediate (Figure 2). This intermediate can be a tight complex or a covalent adduct as shown in Figure 2. Subsequent nucleophilic attack by acceptor substrate gives a saccharide product where the anomeric configuration of the product is the same as the donor (ping-pong: UDP-Gal/UDP/acceptor/trisaccharide product). The Theorell-Chance kinetic mechanism predicts formation of only binary enzyme-substrate complexes. Therefore, UDP would be released from the enzyme before oligosaccharide product forms. The chemical scheme (UDP-Gal/acceptor/UDP/trisaccharide product) is

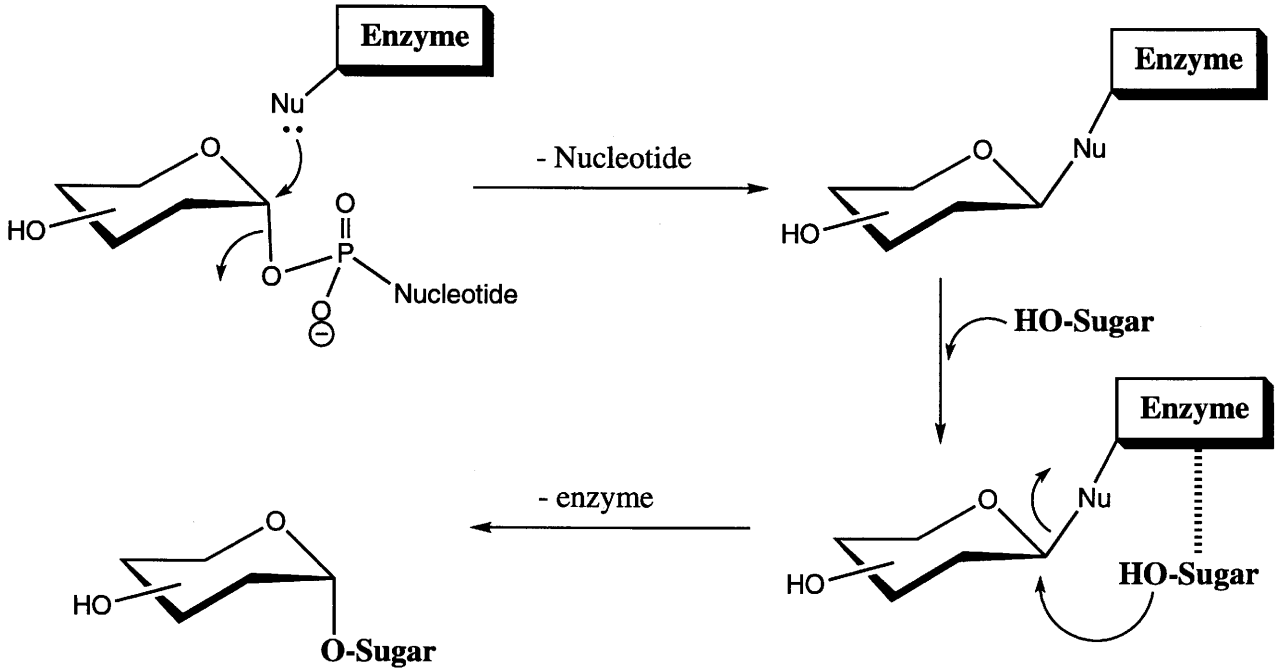


Figure 2. Hypothetical two-step, double displacement mechanism for retaining glycosyltransferase enzymes.

compatible with a Theorell-Chance kinetic mechanism. We consider ordered mechanisms which predict formation of enzyme-donor-acceptor ternary complex (EAB) as less probable; however, they cannot be discounted if $\phi_o = \phi_o'$. While our alternate substrate kinetic data and chemical mechanisms are compatible with a Theorell-Chance scheme, additional confirmatory evaluations with inhibitors are in progress.

Few detailed kinetic studies have been carried out on mammalian glycosyltransferase enzymes. For inverting glycosyltransferases ordered (or largely ordered) mechanisms with addition of donor prior to binding of acceptor have been suggested for β-1,4-galactosyltransferase [14], *N*-acetylglucosaminyltransferase II [15], α-1,3-fucosyltransferase [16] and *N*-acetylglucosaminyltransferase I [17]. Random substrate addition mechanisms have been proposed for α-1,2-fucosyltransferase [18], α-2,6-sialyltransferase [19] and glucuronosyltransferase [20]. For retaining glycosyltransferases ordered addition of donor substrate prior to acceptor has been reported for protein xylosyltransferase [21] while random substrate addition mechanisms have been proposed for polypeptide *N*-acetylgalactosaminyltransferase [22] and blood group A *N*-acetylgalactosaminyltransferase [23]. However, competitive substrate inhibition data reported in the latter are more compatible with a mechanism where donor substrate precedes acceptor substrate binding [3]. Ordered substrate addition for both blood group A and B glycosyltransferases is also consistent with the observations that nucleotide is required for binding these enzymes to immobilized acceptors in affinity chromatography isolations [24,25] while the enzymes can be bound to nucleotide resins in the absence of acceptor.

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