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Carbohydrate Research 324 (2000) 161–169

CARBOHYDRATE RESEARCH

# Enzymatic synthesis of blood group A and B trisaccharide analogues

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Received 14 May 1999; accepted 12 November 1999

**Abstract**

Glycosyltransferases A and B utilize the donor substrates UDP-GalNAc and UDP-Gal, respectively, in the biosynthesis of the human blood group A and B trisaccharide antigens from the O(H)-acceptor substrates. These enzymes were cloned as synthetic genes and expressed in *Escherichia coli*, thereby generating large quantities of enzyme for donor specificity evaluations. The amino acid sequence of glycosyltransferase A only differs from glycosyltransferase B by four amino acids, and alteration of these four amino acid residues (Arg-176 → Gly, Gly-235 → Ser, Leu-266 → Met and Gly-268 → Ala) can change the donor substrate specificity from UDP-GalNAc to UDP-Gal. Crossovers in donor substrate specificity have been observed, i.e., the A transferase can utilize UDP-Gal and B transferase can utilize UDP-GalNAc donor substrates. We now report a unique donor specificity for each enzyme type. Only A transferase can utilize UDP-GlcNAc donor substrates synthesizing the blood group A trisaccharide analog α-D-Glc*p*-NAc-(1 → 3)-[α-L-Fuc*p*-(1 → 2)]-β-D-Gal*p*-O-$(CH_2)_7CH_3$ (**4**). Recombinant blood group B was shown to use UDP-Glc donor substrates synthesizing blood group B trisaccharide analog α-D-Glc*p*-(1 → 3)-[α-L-Fuc*p*-(1 → 2)]-β-D-Gal*p*-O-$(CH_2)_7CH_3$ (**5**). In addition, a true hybrid enzyme was constructed (Gly-235 → Ser, Leu-266 → Met) that could utilize both UDP-GlcNAc and UDP-Glc. Although the rate of transfer with UDP-GlcNAc by the A enzyme was 0.4% that of UDP-GalNAc and the rate of transfer with UDP-Glc by the B enzyme was 0.01% that of UDP-Gal, these cloned enzymes could be used for the enzymatic synthesis of blood group A and B trisaccharide analogs **4** and **5**. Crown in right of Canada © 2000 Published by Elsevier Science Ltd. All rights reserved.

*Keywords:* Glycosyltransferases; Enzymes; Blood groups A and B; Trisaccharide analogs

## 1. Introduction

Glycosyltransferases are enzymes that catalyze the regio- and stereospecific transfer of monosaccharides from activated nucleotide donor substrates to oligosaccharide acceptors [1]. This transfer specificity is advantageous in oligosaccharide synthesis employing glycosyltransferases since protection and deprotection of saccharides is not necessary. Mammalian glycoconjugates are largely composed of nine monosaccharides, their corresponding sugar nucleotide donors are commercially available, and many can be generated in situ [2–4]. Over 100 different mammalian glycosyltransferases utilize the nine donors in various combinations, thus biosynthesizing arrays of natural structures [1]. A limitation in the use of glycosyltransferases for synthesis is the low abundance of the enzymes in natural sources;

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PII: S0008-6215(99)00297-9

however, cloned glycosyltransferase enzymes are increasingly available for use in small- and large-scale preparation of therapeutically significant oligosaccharides [4–6]. In principle, cloned enzymes can also be genetically engineered for broader substrate tolerance, while maintaining reaction specificity.

The human blood group A and B glycosyltransferases are responsible for the biosynthesis of the A and B blood group antigens, respectively. These antigens are found on the surface of red blood cells as well as on other cell types [7]. Blood group A individuals have glycosyltransferase A [α-(1→3)-*N*-acetylgalactosaminyltransferase (EC 2.4.1.40)], which utilizes the A donor UDP-GalNAc and the (O)H-acceptor structure α-L-Fuc*p*-(1→2)-β-D-Gal*p*-O-R to give the blood group A trisaccharide antigen α-D-Gal*p*-NAc-(1→3)-[α-D-Fuc*p*-(1→2)]-β-D-Gal*p*-O-R. Blood group B individuals have glycosyltransferase B [α-(1→3)-galactosyltransferase (EC 2.4.1.37)], which uses the same (O)H-acceptor structure but utilizes the B donor UDP-Gal to make the blood group B antigen α-D-Gal*p*-(1→3)-[α-D-Fuc*p*-(1→2)]-β-D-Gal*p*-O-R. Blood group O individuals do not express either enzyme, and AB individuals express both [8].

The amino acid sequence of glycosyltransferase A only differs from glycosyltransferase B by four amino acids, and alteration of these four amino acid residues (Arg-176→Gly, Gly-235→Ser, Leu-266→Met and Gly-268→Ala) can change the donor substrate specificity from UDP-GalNAc to UDP-Gal [9]. The A and B enzymes have been cloned as synthetic genes and expressed in *Escherichia coli* [10,11]. Recombinant hybrid A/B glycosyltransferases were produced by interchanging these four amino acids between the A and B enzymes. The glycosyltransferases were given a four-letter name to denote whether these four residues originated from glycosyltransferase A or B, with wild-type glycosyltransferase A enzyme being designated AAAA. Crossover reactions have been observed. The A transferase can use the B donor UDP-Gal at ~0.5% of the rate compared with UDP-GalNAc. Similarly, the B transferase can utilize the A donor UDP-GalNAc at approximately ~5% of the rate compared with UDP-Gal. Cloned A/B glycosyltransferases were genetically engineered to achieve broader substrate tolerance. Kinetic analysis of the hybrid A/B glycosyltransferases showed a true hybrid enzyme ABBA (Gly-235→Ser, Leu-266→Met) had been produced that efficiently utilized both UDP-GalNAc and UDP-Gal donor substrates [10–12].

In this paper, we report a distinct donor substrate specificity characteristic for A or B transferase, UDP-GlcNAc (**2**) and UDP-Glc (**3**), respectively. The hybrid A/B enzyme, ABBA, was shown to utilize both UDP-GlcNAc and UDP-Glc. In addition, the wild-type and hybrid A/B enzymes were used for the chemoenzymatic synthesis of blood group A and B trisaccharides and their corresponding analogs.

## 2. Results and discussion

*Donor substrate specificity*.—The kinetic constants of these enzymes utilizing their natural donors has been previously reported [11,12]. The availability of large quantities of recombinant blood group glycosyltransferases made it possible to determine the kinetic parameters of these enzymes with the alternate donors UDP-GlcNAc and UDP-Glc (Table 1). A unique donor specificity was observed for each enzyme type. The wild-type glycosyltransferase A and B were best able to utilize the donors UDP-GlcNAc and UDP-Glc, respectively. The reaction rate and the specificity of glycosyltransferase A utilizing UDP-GlcNAc was significantly higher than the B enzyme utilizing UDP-Glc (Table 1). Other enzymes with predominantly A activity, such as BAAA, had kinetic constants with UDP-GlcNAc similar to wild-type A enzyme (Table 1). The A enzymes (AAAA and BAAA) possess such low activity towards UDP-Glc that it was not possible to perform detailed kinetic analyses. In contrast, the hybrid A/B glycosyltransferase ABBA could utilize UDP-GlcNAc and UDP-Glc, with a higher $k_{cat}$ observed for UDP-GlcNAc (Table 1). The ability of the true hybrid ABBA to utilize both UDP-GlcNAc and UDP-Glc represents a distinct donor specificity. This further supports its classification as a true hybrid enzyme with broader donor tolerances than either wild-type A or B transferase.

Table 1
Kinetic constants of hybrid A/B glycosyltransferases using UDP-GlcNAc and UDP-Glc

| Enzyme residue 176, 235, 266, 268 | UDP-GlcNAc | | | | | UDP-Glc | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $K_A$ [a] (μM) | $K_B$ [b] (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_A$ ($mM^{-1}$ $s^{-1}$) | $k_{cat}/K_B$ ($mM^{-1}$ $s^{-1}$) | $K_A$ (μM) | $K_B$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_A$ ($mM^{-1}$ $s^{-1}$) | $k_{cat}/K_B$ ($mM^{-1}$ $s^{-1}$) |
| $A_{Arg}A_{Gly}A_{Leu}A_{Gly}$ | $144 \pm 20$ | $242 \pm 33$ | 0.021 | 0.15 | 0.087 | N/A [c] | N/A | N/A | N/A | N/A |
| BAAA | $100 \pm 4$ | $240 \pm 23$ | 0.019 | 0.19 | 0.079 | N/A [c] | N/A | N/A | N/A | N/A |
| ABBA | $316 \pm 40$ | $255 \pm 25$ | 0.003 | 0.010 | 0.012 | $321 \pm 42$ | $58 \pm 8$ | $2.4 \times 10^{-5}$ | $7.6 \times 10^{-5}$ | $4.1 \times 10^{-4}$ |
| $B_{Gly}B_{Ser}B_{Met}B_{Ala}$ | N/A [c] | N/A | N/A | N/A | N/A | $238 \pm 29$ | $116 \pm 6$ | 0.0010 | 0.004 | 0.0084 |

[a] $K_A$ is the Michaelis–Menten constant for the acceptor.
[b] $K_B$ is the Michaelis–Menten constant for the donor.
[c] N/A, values too low to measure.

Table 2
Preparative-scale enzymatic synthesis reactions

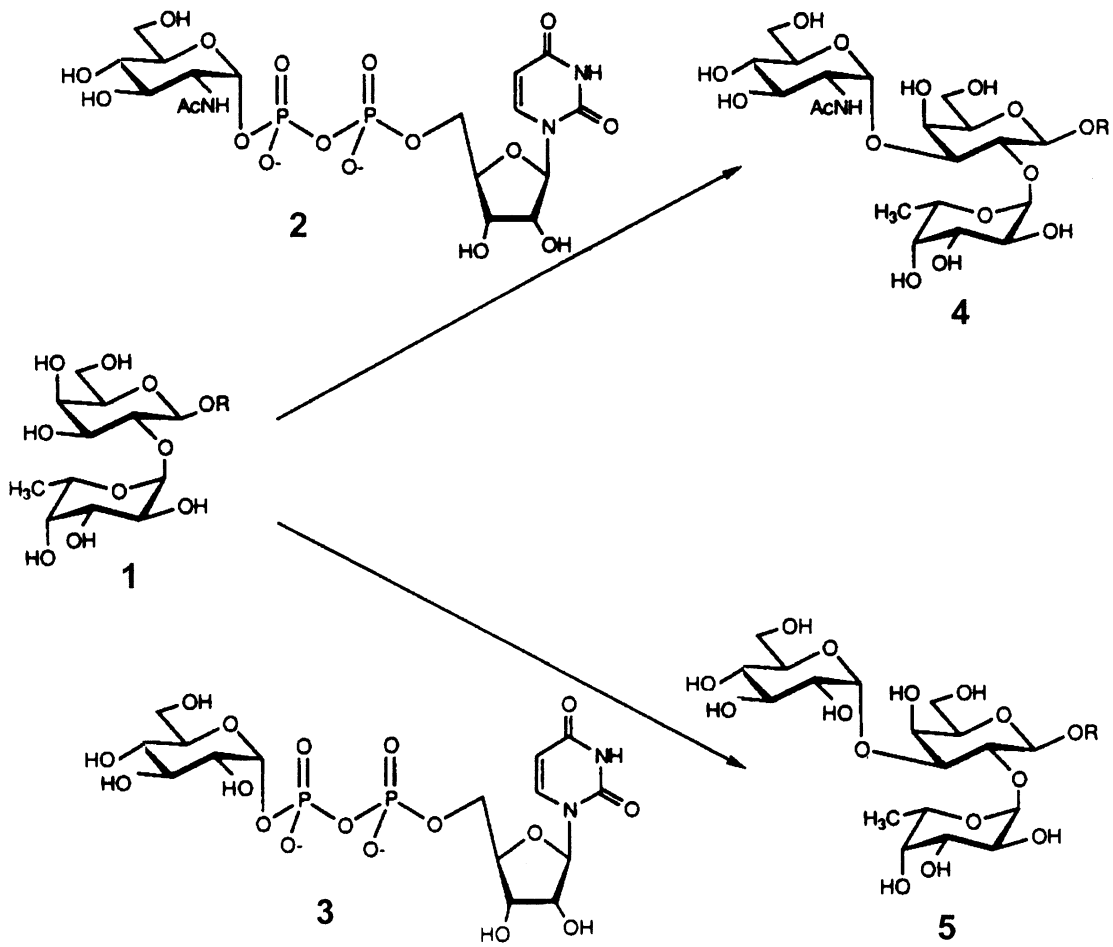
| Reaction | Enzyme | Donor [c] | Starting (mg) | Enzyme (mU) | Volume (mL) | Reaction time (days) | Yield (mg) | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | $A_{Arg}A_{Gly}A_{Leu}A_{Gly}$ | UDP-GlcNAc | 5.4 | 20,200 [a] | 3.1 | 1.25 | 6.5 | 82 |
| 2 | BAAA | UDP-GlcNAc | 0.60 | 700 [a] | 0.55 | 14 | 0.9 | 100 |
| 3 | ABBA | UDP-GlcNAc | 0.70 | 60 [a] | 1.0 | 25 | 0.6 | 60 |
| 4 | BBBA | UDP-GalNAc | 0.70 | 200 [a] | 1.0 | 11 | 1.0 | 92 |
| 5 | ABBA | UDP-GalNAc | 0.54 | 62 [a] | 0.11 | 5 | 1.0 | 100 |
| 6 | BBBA | UDP-Gal | 0.60 | 96 [b] | 0.60 | 7 | 0.9 | 100 |
| 7 | ABBA | UDP-Gal | 0.54 | 51 [b] | 0.17 | 5 | 0.6 | 60 |
| 8 | $B_{Gly}B_{Ser}B_{Met}B_{Ala}$ | UDP-Glc | 1.95 | 76,000[b] | 1.13 | 21 | N/A [d] | N/A |

[a] mU of enzyme calculated with respect to the standard assay for glycosyltransferase A with UDP-GalNAc.
[b] mU of enzyme calculated with respect to the standard assay for glycosyltransferase B with UDP-Gal.
[c] The products formed with the donor substrates UDP-GalNAc, UDP-Gal, UDP-GlcNAc and UDP-Glc are α-D-GalNAc-(1→3)[α-L-Fuc-(1→2)]-β-D-Gal-O-$(CH_2)_7CH_3$, α-D-Gal-(1→3)[α-L-Fuc-(1→2)]-β-D-Gal-O-$(CH_2)_7CH_3$, α-D-GlcNAc-(1→3)[α-L-Fuc-(1→2)]-β-D-Gal-O-$(CH_2)_7CH_3$ (**4**), and α-D-Glc-(1→3)[α-L-Fuc-(1→2)]-β-D-Gal-O-$(CH_2)_7CH_3$ (**5**), respectively.
[d] A trace of protein in the final isolated sample precluded accurate yield determination. Conversion was complete, >98% as judged by NMR spectroscopy.

For comparison, the kinetic constants previously obtained with the natural donors for wild-type A enzyme using UDP-GalNAc had a $K_A$ of 15 μM, $K_B$ of 13 μM and a $k_{cat}$ of 4.9 $s^{-1}$. The wild-type B enzyme with UDP-Gal had a $K_A$ of 54 μM, $K_B$ of 34 μM and a $k_{cat}$ of 6.5 $s^{-1}$ [11,12]. The rate of transfer with UDP-GlcNAc by the A enzyme was 0.4% that of UDP-GalNAc, and the rate of transfer with UDP-Glc by the B enzyme was 0.01% that of UDP-Gal.

We have previously shown that residue 266 is important in determining donor substrate specificity. Glycosyltransferase A contains Leu-266, which is smaller than the corresponding Met-266 in glycosyltransferase B. Modelling shows that the difference in size between Leu and Met at position 266 correlates very well with the difference in size between the *N*-acetyl and hydroxyl groups on the A and B trisaccharide antigens, respectively [12]. In the A transferase a pocket could be formed, which is of a size and shape to accommodate the *N*-acetyl group on α-D-GalNAc or α-D-GlcNAc of the A trisaccharide, and the enzyme may be specifically interacting with this group.

*Preparative-scale synthesis of trisaccharide analogs.*—The kinetic data suggest that these cloned enzymes may be used with the donor substrates UDP-GlcNAc and UDP-Glc in the preparative-scale synthesis of blood group A and B trisaccharide analogs (Scheme 1). The glycosyltransferases shown in Table 2 were used with the donor substrates UDP-GalNAc, UDP-Gal, UDP-GlcNAc, and UDP-Glc to




Scheme 1. Enzymatic synthesis of blood group A and B trisaccharide analogs. Recombinant human glycosyltransferase A was used to synthesize the blood group A analog α-D-Glc*p*-NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-O-$(CH_2)_7CH_3$ (**4**) by transferring GlcNAc from the donor UDP-GlcNAc (**2**) to the (O)H-precursor α-L-Fuc-(1→2)-Gal-β-D-O-$(CH_2)_7CH_3$ (**1**). Recombinant human glycosyltransferase B was used to synthesize the blood group B analog α-D-Glc*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-O-$(CH_2)_7CH_3$ (**5**) by transferring Glc from the donor UDP-Glc (**3**) to the same (O)H-precursor **1**. R = $(CH_2)_7CH_3$.

make all four corresponding trisaccharide products. These products were generally made on a less than 2 mg scale in order to conserve the acceptor **1**, which was made by chemical synthesis. The wild-type A enzyme was used to synthesize blood group A trisaccharide analog **4** as a representative 5-mg scale synthesis. Conversion was complete in 30 h with $>80\%$ recovery of the product (Table 2, Reaction 1). Wild-type B enzyme was used with UDP-Glc in the preparative synthesis of the blood group B trisaccharide analog **5**. The synthesis of **5** required more enzyme and a longer incubation period compared with the other reactions listed in Table 2. Increasing the quantity of recombinant wild-type A glycosyltransferase used in the synthesis of trisaccharide **4** decreased the reaction time to 30 h. Similarly, increasing the quantity of the corresponding wild-type B enzyme should decrease the reaction time required for this reaction. The stability of the enzyme during these reactions was determined by removing an aliquot of the reaction mixture at different times and monitoring enzyme activity. Sufficient enzyme activity was found to be present for up to 25 days at 37 °C, with approximately half of the existing enzyme activity lost every 5 days. Reaction times were greater when single hybrid A/B enzymes (ABBA and BBBA) were used with either UDP-GalNAc and UDP-Gal, compared with using two different wild-type A or B enzymes to perform the same reactions [10,11]. A single hybrid A/B enzyme, ABBA, utilized UDP-GalNAc, UDP-Gal and UDP-GlcNAc to synthesize all three corresponding trisaccharide structures (Table 2). Although ABBA can utilize UDP-Glc (Table 1), wild-type B enzyme was preferred for synthesis to minimize reaction times.

All trisaccharide products were stable to purification. The structures of the products were confirmed by their $^1$H NMR and mass spectral data (Tables 3 and 4). The blood group A trisaccharide product showed the terminal GalNAc in an α-(1→3) linkage to Gal and the B trisaccharide product shows the terminal Gal in an α-(1→3) linkage to Gal as described previously [13]. The $^1$H NMR spectrum for product **4** shows the terminal GlcNAc in an α-(1→3) linkage to Gal [α anomeric H(GlcNAc) = $\delta$ 5.14 ppm, $J_{1,2}$ = 3.7–4.0 Hz; α anomeric H (Fuc) = $\delta$ 5.35–5.36 ppm, $J_{1,2}$ = 2.7 Hz, α anomeric H (Gal) = $\delta$ 4.51–4.52 ppm, $J_{1,2}$ = 7.8–7.9 Hz]. The $^1$H NMR spectrum for product **5** shows the terminal Glc in an α-(1→3) linkage to Gal [α anomeric H(Glc)] = $\delta$ 5.22 ppm, $J_{1,2}$ = 3.9 Hz; α nomeric H (Fuc) = $\delta$ 5.33 ppm, $J_{1,2}$ = 2.0 Hz, β anomeric H (Gal) = $\delta$ 4.54 ppm, $J_{1,2}$ = 7.7 Hz]. Correct α-(1→3) linkage of the GlcNAc or Glc was confirmed by a gradient-enhanced COSY and one-dimensional T-ROESY experiments on both α anomeric protons (data not shown). The mass spectra further confirmed the product identity showing that a single GlcNAc or Glc residue was added to the acceptor (Table 4).

In summary, we observed a unique donor specificity for blood group A and B glycosyltransferases. Glycosyltransferase A uses UDP-GlcNAc and glycosyltransferase B uses UDP-Glc. Also, a true hybrid A/B enzyme was capable of using both UDP-GlcNAc and UDP-Glc. The novel donor substrate specificities observed allowed the use of the cloned glycosyltransferases in the preparative synthesis of blood group A and blood group B trisaccharide analogs **4** and **5**, respectively. The formation of novel and diverse unnatural oligosaccharide structures in addition to their biosynthetic products should find application in glycoprotein and glycolipid remodelling.

## 3. Materials and methods

*Materials.*—UDP, UDP-GalNAc, UDP-Gal, UDP-GlcNAc, UDP-Glc and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. The radioactive [6-$^3$H]-labeled analogs of UDP-GalNAc, UDP-Gal, UDP-GlcNAc, and UDP-Glc were from American Radiolabeled Chemicals. The acceptor substrate α-D-Fuc-(1→2)-β-D-Gal-O-$(CH_2)_7CH_3$ was from Dr O. Hindsgaul (University of Alberta). TLC plates used were Silca Gel $60F_{254}$ (E. Merck, Darmstadt). Calf intestinal alkaline phosphatase (Boehringer–Mannheim), SP Sepharose FF (Pharmacia), Slide-a-Lyzer Dialysis system (Pierce Chemical Co.), Millex 0.22 μm GV filters (Millipore),

Table 3
Selected $^1$H NMR [a] data for trisaccharide products

| Reaction [b] | Donor added | Donor α-H-1 | NHAc | GalNAc α-H-2 | Gal β-H-1 | Gal β-H-4 | Fuc α-H-1 | Fuc α-H-5 | Fuc α-H-6 | $(CH_2)_7CH_3$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | GlcNAc | 5.15(3.8) | 2.04 | | 4.52(7.9) | 4.20(3.1) | 5.36(2.7) | 4.48(6.6) | 1.24(6.6) | 0.87 |
| 2 | GlcNAc | 5.14(3.7) | 2.03 | | 4.51(7.8) | 4.19(3.4) | 5.35(2.7) | 4.48(6.6) | 1.23(6.6) | 0.86 |
| 3 | GlcNAc | 5.14(4.0) | 2.04 | | 4.52(7.8) | 4.20(3.2) | 5.36(2.7) | 4.48(6.6) | 1.23(6.6) | 0.86 |
| 4 | GalNAc | 5.17(4.0) | 2.04 | 4.25(3.7) | 4.52(7.8) | 4.21(3.1) | 5.32(3.8) | 4.47(6.6) | 1.23(6.5) | 0.86 |
| 5 | GalNAc | 5.17(3.8) | 2.04 | 4.24(3.7) | 4.52(7.8) | 4.21(3.1) | 5.32(3.8) | 4.47(6.6) | 1.23(6.6) | 0.86 |
| 6 [c] | Gal | 5.24(2.3) | | | 4.54(7.8) | 4.27(3.0) | 5.30(3.7) | 4.47(6.4) | 1.22(6.4) | 0.86 |
| 7 [c] | Gal | 5.24(2.1) | | | 4.54(7.8) | 4.27(2.9) | 5.30(3.7) | 4.47(6.6) | 1.22(6.6) | 0.86 |
| 8 | Glc | 5.22(3.9) | | | 4.54(7.7) | 4.25(2.8) | 5.33(2.0) | 4.48(6.4) | 1.22(6.6) | 0.87 |

[a] Chemical shifts in ppm; numbers in parentheses give coupling constants in Hz; external reference acetone = 2.225 ppm.
[b] Reaction numbers correspond to Table 2.
[c] Gal α-H-5: 4.21(6.2, 2×).

Sep-Pak $C_{18}$ (+) reversed-phase cartridges (Waters), and Ecolite (+) liquid scintillation cocktail (ICN) were purchased commercially.

*Expression and purification of glycosyltransferases.*—The recombinant glycosyltransferases have a four-letter name, which denotes whether amino acid residues 176, 235, 266 and 268 originated from the A or the B enzyme. For example, wild-type A or AAAA (Arg-176, Gly-235, Leu-266, Gly-268); BBBB (Gly-176, Ser-235, Met-266, Ala-268) and BAAA (Gly-176, Gly-235, Leu-266, Gly-268). The enzymes used in all the preparative synthesis reactions (except for wild-type A and B) were cloned, expressed and purified from the periplasm of *E. coli*, as described previously [10,11]. The preparative-scale synthesis of **5** required a larger quantity of more concentrated glycosyltransferase B (wild-type BBBB) that was devoid of UDP-Gal epimerase activity. Therefore, the following protocol was devised for the purification of recombinant glycosyltransferases from whole cell extracts. For the purification of glycosyltransferase B, *E. coli* TG-1 cells transformed with the plasmid harboring glycosyltransferase B were grown at 30 °C in TB media with vigourous shaking in 4 L baffled flasks. The cells were induced with 1 mM IPTG at $OD_{600}$ 0.6–1.0 and harvested 20–24 h after induction. Cell pellets (30 g/L culture) were resuspended in 20 mM MOPS, 1 mM EDTA, 1 mM DTT (pH 7.0) at 0.3 g cells/mL. The suspension was passed once through a French Pressure cell at 20,000 psi and centrifuged at 25,900*g* for 30 min, followed by ultracentrifugation of the supernatant at 101,300*g* for 60 min (Beckman). The supernatant was loaded onto SP Sepharose

Table 4
Selected HRMS data for the trisaccharide products

| Reaction [a] | Calcd for | *m*/*z* | Found [M+Na]$^+$ |
|---|---|---|---|
| 1 | $C_{28}H_{51}O_{15}N_1$ | 664.315640 | 664.315963 |
| 2 | $C_{28}H_{51}O_{15}N_1$ | 664.315640 | 664.316116 |
| 3 | $C_{28}H_{51}O_{15}N_1$ | 664.315640 | 664.315648 |
| 4 | $C_{28}H_{51}O_{15}N_1$ | 664.315640 | 664.315870 |
| 5 | $C_{28}H_{51}O_{15}N_1$ | 664.315640 | 664.315323 |
| 6 | $C_{26}H_{48}O_{15}$ | 623.289091 | 623.288099 |
| 7 | $C_{26}H_{48}O_{15}$ | 623.289091 | 623.289975 |
| 8 | $C_{26}H_{48}O_{15}$ | 623.289091 | 623.289940 |

[a] Reaction numbers correspond to Table 2.

FF (80 mL column) equilibrated with 20 mM MOPS, 1 mM DTT (pH 7.0) at a flow rate of 4.5 mL/min and washed with the same buffer until the $OD_{260}$ was at the baseline ( ~ 300 mL buffer). The glycosyltransferase B was eluted with 20 mM MOPS, 0.5 M NaCl and 1 mM DTT (pH 7.0). Fractions (10 mL) were collected and assayed for enzyme activity, as described below. Fractions containing enzyme activity were pooled, 5 mM $MnCl_2$ was added to the combined fractions, and these were then loaded onto a UDP-hexanolamine Sepharose column (15–20 mL, 3–6 μmol/mL) equilibrated with loading buffer consisting of 20 mM MOPS, 0.5 M NaCl, 1 mM DTT and 5 mM $MnCl_2$ at 0.25 mL/min. The enzyme was eluted with 10 mM UDP in 20 mM MOPS, 0.5 M NaCl, 1 mM DTT and 5 mM $MnCl_2$ ($MnCl_2$ added last). The elution was at a flow rate of 0.5 mL/min. Fractions with enzymatic activity were pooled, concentrated and dialyzed using the Slide-A-Lyzer system (Pierce Chemical Co.). The activity in the crude extract could not be accurately determined due to degradation of the substrate. For glycosyltransferase B, the yield was 213 U ( ~ 42 mg protein) from 1 L of culture, which represents 5% of the total extracted protein (purification factor 20 × ). The enzyme was frozen at − 80 °C with the addition of 1 mg/mL BSA.

*Standard enzyme assays.*—We have previously reported the detailed two-substrate kinetic analysis of glycosyltransferases A and B [11]. In this study, in order to compare the activity between the native and the unnatural donors, the following standard assay conditions for the wild-type A and B glycosyltransferases were defined. The glycosyltransferase A activity assay contained 800 μM of the acceptor **1**, 600 μM UDP-GalNAc, 0.03 μCi ( ~ 70,000 dpm) UDP-[6-$^3$H]GalNAc, 20 mM $MnCl_2$, 50 mM Na cacodylate (pH 7.0), 1 mg/mL BSA and 8 microunits of purified A enzyme in a total volume of 33 μL. Reactions were incubated for 5–20 min at 37 °C. Similarly, the glycosyltransferase B activity assay contained 800 μM acceptor **1**, 600 μM UDP-Gal, 0.02 μCi ( ~ 50,000 dpm) UDP-[6-$^3$H]Gal, 20 mM $MnCl_2$, 50 mM Na cacodylate (pH 7.0), 1 mg/mL BSA and 50 microunits of purified B enzyme in a total volume of 33 μL. Enzymatic assays with UDP-GlcNAc contained 900 μM α-D-Fuc-(1 → 2)-β-D-Gal-O-$(CH_2)_7CH_3$ acceptor **1**, 500 μM UDP-GlcNAc, 0.2 μCi ( ~ 440,000 dpm) UDP-[6-$^3$H]GlcNAc, 20 mM $MnCl_2$, 50 mM Na cacodylate (pH 7.0), 1 mg/mL BSA and ~ 0.08–0.9 μg of purified enzyme in a total volume of 33 μL. Reactions were incubated for 45–120 min at 37 °C. Reactions for UDP-Glc were the same except that the UDP-GlcNAc donor substrates were substituted with a lower concentration of UDP-Glc (10 μM) and a higher-than-usual concentration of UDP-[6-$^3$H]Glc (0.4 μCi, ~ 900,000 dpm). The low cold UDP-Glc concentration was required in order to detect the low levels of enzymatic activity with this donor. Reactions were incubated for 1–4.5 h at 37 °C. All enzyme activity assays were processed using the Sep-Pak $C_{18}$ method, as described previously [14]. Counts from a control assay containing no acceptor were subtracted from all data points.

*Kinetic constants.*—$K_A$ and $K_B$ are the apparent Michaelis constants for the acceptor and the donor, respectively. The catalytic constant or turnover number, $k_{cat}$, is the maximum number of substrate molecules converted to product per active site per unit time, obtained from $v_{max}$/[E]. One milliunit (mU) of activity is defined as the amount that catalyses the conversion of one nmol of sugar transferred per minute. For UDP-GlcNAc, kinetic constants with respect to the acceptor ($K_A$ and $k_{cat}/K_A$) were determined using six different acceptor concentrations (at 0.1–8 × $K_m$) with UDP-GlcNAc (1.2 mM) at saturation, and kinetic constants with respect to the donor ($K_B$ and $k_{cat}/K_B$) were determined using six different donor concentrations (0.1–9 × $K_m$) with the acceptor (0.8–1.2 mM) at saturation. For UDP-Glc, $K_A$ and $k_{cat}/K_A$ were determined using six different acceptor concentrations at (0.2–13 × $K_m$) with UDP-Glc (0.6 mM) at saturation, and $K_B$ and $k_{cat}/K_B$ were determined using six different donor concentrations (0.07–8 × $K_m$) with the acceptor (2 mM) at saturation (Table 1). All enzyme activity assays described were processed using the Sep-Pak $C_{18}$ method, as described previously [14]. Counts from a control assay containing no acceptor were subtracted from all

data points. Kinetic parameters $v_{max}$, $K_m$ and $K_m/v_{max}$ were derived from the best fit to the Michaelis–Menten equation using unweighted non-linear regression with the SIGMAPLOT 4.1 program. Errors were within experimental limits and were 4–14% for $K_m$ values and 1.3–5% for $v_{max}$ values.

*Preparative synthesis using UDP-GlcNAc.*—The disaccharide acceptor **1** (5.4 mg, 12.3 μmol) was combined with 1 molar equivalent of UDP-GlcNAc to synthesize the trisaccharide analog **4**. The solution contained 3.0 mL of glycosyltransferase, 50 mM NaCacodylate (pH 6.8), 1 mg/mL BSA, 20 mM $MnCl_2$, and 10 U of alkaline phosphatase. The reaction mixture was incubated at 37 °C with constant rotation. An additional aliquot of donor (0.5–1 molar equivalents) was added as required. The pH of the reaction was monitored and maintained at pH 6.5–7.0 by the addition of 500 mM NaCacodylate (pH 7.2). Thin-layer chromatography (TLC) was used to monitor the progress of the reaction using the 65:35:6:1 $CH_2Cl_2$–MeOH–water–$C_5H_5N$ solvent. The reaction was complete in 30 h. The reaction product was purified using a $C_{18}$ Sep-Pak( + ) cartridge, which was pre-equilibrated with 10 mL of MeOH, followed by 10 mL of water. The cartridge was washed with 60 mL of water and eluted with 30 mL of HPLC grade MeOH. The MeOH was evaporated and the product was resuspended in water and passed through a 0.22-μm Millex-GV filter. The filtrate was lyophilized to dryness forming a white powder. The reaction times, quantity of enzymes used and yields for each synthesis are shown in Table 2. $^1$H NMR spectroscopy was performed on a Varian UNITY 500 (500 MHz) instrument for all syntheses, unless stated otherwise. HRMS spectra were recorded on a Micromass ZabSpec Hybrid Sector-TOF, by positive-ion mode electrospray. Selected NMR and HRMS data are shown in Tables 3 and 4.

*Preparative synthesis using UDP-Glc.*—The disaccharide acceptor **1** (1.95 mg) was combined with 1.5 molar equivalents of UDP-Glc in a solution containing 76 U (970 μL) glycosyltransferase B, 50 mM NaCacodylate (pH 7.0), 1 mg/mL BSA, 20 mM $MnCl_2$, and 10 U (10 μL) alkaline phosphatase in a total volume of 1.13 mL. The reaction mixture was incubated at 37 °C for 21 days, with the addition of 0.25 and 0.1 equivalents of UDP-Glc after 9 and 10 days, respectively. The progress of the reaction was monitored by TLC using 4:5:2:1 $CH_3Cl_3$–MeOH–water–AcOH and observed under ultraviolet light. The TLC showed that UDP-Glc had degraded to uridine after an incubation time of 9 days and additional UDP-Glc was added. The trisaccharide product **5** was purified as described above. The 600 MHz NMR spectrum (Varian INOVA 600) of the product indicated 2% of the starting material **1** was present after the incubation period. Selected NMR and HRMS data are shown in Tables 3 and 4, respectively.

*Representative preparative synthesis using UDP-GalNAc and UDP-Gal.*—The disaccharide acceptor **1** (0.6–0.7 mg, 1.4–1.6 mmol) was combined with 1 molar equivalent of UDP-GalNAc or UDP-Gal to synthesize the blood group A (α-D-Gal*p*-NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-O-$(CH_2)_7CH_3$) or blood group B (α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-O-$(CH_2)_7CH_3$) trisaccharides, respectively. The solution contained 150–400 μL of glycosyltransferase, 40 mM NaCacodylate (pH 6.8), 1 mg/mL BSA, 20 mM $MnCl_2$, and 10 U alkaline phosphatase. The reaction mixture was incubated at 37 °C with constant rotation. An additional aliquot of donor (0.5–1 molar equivalents) was added as required. The reaction was monitored and purified as described above. The reaction times, quantity of enzymes used and yields for each synthesis are shown in Table 2. Selected NMR and HRMS data are shown in Tables 3 and 4.

## Acknowledgements

We thank Dr O. Hindsgaul for the acceptor substrate, and Dr H. Li and M. Deshiffart for assistance with enzyme kinetic analyses, Dr A. Otter for NMR spectra and Dr A. Morales for HRMS. This work was supported in part by a collaborative grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and a NSERC/NRC Part

nership grant sponsored by Synsorb Biotech Inc., awarded to M.M.P. NRCC Publication No. 42413.

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