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Enzymatic synthesis of UDP-(3-deoxy-3-fluoro)-D-galactose and UDP-(2-deoxy-2-fluoro)-D-galactose and substrate activity with UDP-galactopyranose mutase

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Abstract

The novel UDP-sugar uridine 5′-(3-deoxy-3-fluoro-D-galactopyranosyl diphosphate) (**1**) and UDP-(2-deoxy-2-fluoro)-D-galactose (**2**) have been prepared enzymatically and tested as substrate analogues for the enzyme UDP-galactopyranose mutase (UDP-Gal*p* mutase EC 5.4.99.9). Turnover of both **1** and **2** by UDP-Gal*p* mutase was observed by HPLC and ^{19}F NMR. The HPLC elution profile and ^{19}F chemical shift of the products are consistent with the formation of the predicted furanose forms of **1** and **2**. The K_m values for compounds **1** and **2** were similar to those of the natural substrate UDP-Gal*p* (0.26 mM for **1**, 0.2 mM for **2**, and 0.6 mM for UDP-Gal*p*), but the values for k_{cat} were substantially different (1.6/min for **1**, 0.02/min for **2**, and 1364/min for UDP-Gal*p*). A correlation was also observed between the equilibrium yield of product formed during turnover of UDP-sugar by UDP-Gal*p* mutase (UDP-Gal*p*, compound **1** or compound **2**), and the amount of furanose present for the free sugar at thermal equilibrium in aqueous solution, using ^{1}H and ^{19}F NMR spectroscopy. The implications of these results to the mechanism of the unusual enzymatic reaction are discussed.

Keywords: UDP-sugars; Fluoro-sugars; Tautomerization; UDP-galactopyranose mutase; Uridine 5′-(3-deoxy-3-fluoro-D-galactopyranosyl diphosphate); UDP-(2-deoxy-2-fluoro)-galactose

1. Introduction

UDP-galactopyranose mutase (UDP-Gal*p* mutase) catalyzes the interconversion of the α-pyranose and α-furanose forms of UDP-galactose (Scheme 1) [1–3]. In vivo, UDP-galactofuranose (UDP-Gal*f*) functions as the activated precursor of Gal*f* in the biosynthesis of a number of macromolecular structures found in prokaryotes and lower eukaryotes. These include the arabinogalactan layer of mycobacterial cell walls, and the outer leaflet lipopolysaccharide (LPS) of gram negative

Abbreviations: UDP-Gal*p*, uridine 5′-(α-D-galactopyranosyl diphosphate); HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ES-IMS, electrospray ionization mass spectrometry; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; AMPSO, (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; 3F-Gal, 3-deoxy-3-fluoro-D-galactose; 2F-Gal, 2-deoxy-2-fluoro-D-galactose.

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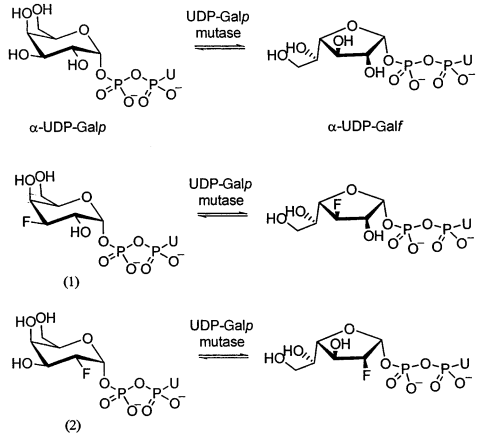
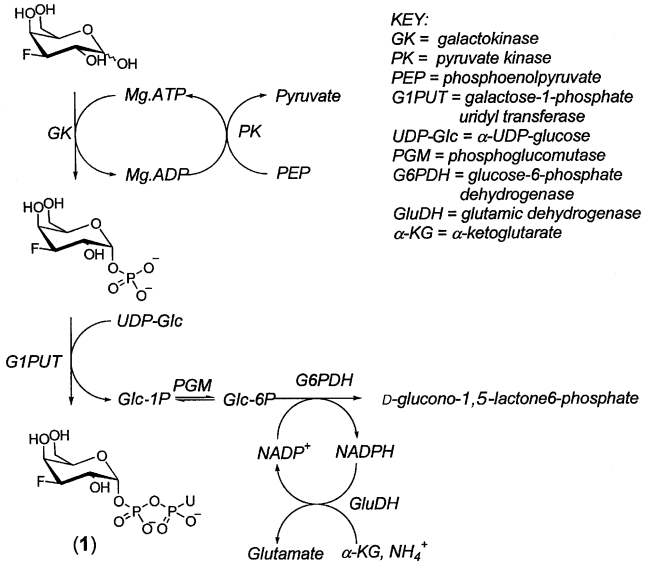
bacteria [4,5], both of which play important roles in the virulence of these organisms. This occurrence of Gal*f*, together with the absence of Gal*f* in higher eukaryotes, make UDP-Gal*p* mutase a potential drug target.

The interconversion of UDP-Gal*p* and UDP-Gal*f* is unprecedented. It has recently been demonstrated that the anomeric C-1″–O-1 bond is cleaved during conversion of UDP-Gal*p* to UDP-Gal*f* [6]. This observation is consistent with glycosyltransferase-type chemistry occurring at the active site of UDP-Gal*p* mutase, in this case, intramolecular transfer of the anomeric carbon of galactose between the C-5″ and C-4″ oxygens [7]. These results are inconsistent with a mechanism involving UDP transfer to the C-2″ hydroxyl, followed by anomeric equilibration and transfer of UDP back to the anomeric hydroxyl, since this sequence would not account for C-1″–O-1 bond cleavage. Unlike other enzymes which catalyze glycosyl transferase-type chemistry, UDP-Gal*p* mutase contains bound FAD, making mechanisms invoking cryptic redox chemistry a possibility.



Scheme 1.



Scheme 2.

We report here the enzymatic synthesis of the novel compound UDP-(3-deoxy-3-fluoro-D-galactopyranose (**1**) using a variation of the method used previously to synthesize UDP-(2-deoxy-2-fluoro)-D-galactopyranose (**2**) [9]. Deoxyfluoro sugars have been widely used as probes of glycosidase mechanisms [8]. The much slower reactions of 2-deoxy-2-fluoro sugar analogues in glycosidase-catalyzed chemistry has been proposed to be due to a large inductive electronic effect of the 2-fluoro substituent that destabilizes oxocarbenium ion intermediates and transition states [8]. Compounds **1** and **2** were synthesized as mechanistic probes of the reaction catalyzed by UDP-Gal*p* mutase. We also measured the amounts of furanose present at thermal equilibrium in aqueous solutions of 3-deoxy-3-fluoro-D-galactose, 2-deoxy-2-fluoro-D-galactose and D-galactose. The results support the previously proposed mechanism for UDP-Gal*p* mutase [6] that invokes anomeric C–O bond cleavage during turnover of UDP-Gal*p*.

2. Results and discussion

The synthesis of **1** and **2** was performed in two steps using a variation of the protocol published by Hayashi et al. [9] (Scheme 2). In the first step, phosphorylation of the sugar was achieved using galactokinase [10]. The second step, uridylation to give the sugar nucleotide, was performed using galactose-1-phosphate uridyl transferase and UDP-glucose. We chose to couple this second step ($K_{eq} = 1.1$, pH 8.7, 37 °C [11]) to the phosphoglucomutase-catalyzed isomerization of D-glucose 1-phosphate to D-glucose 6-phosphate to drive the reaction forward, instead of the UDP-glucose pyrophosphorylase-catalyzed regeneration of UDP-glucose as reported previously [9]. This allowed complete removal of added UDP-glucose and thus

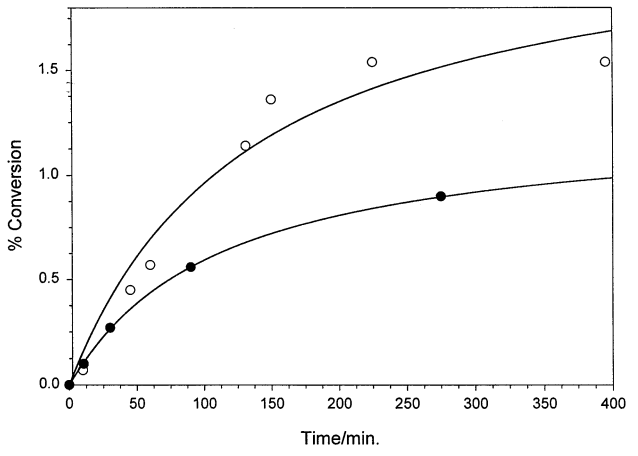


Fig. 1. Time course of product formation as detected by HPLC during turnover of **1** (closed circles) and **2** (open circles) by UDP-Gal*p* mutase.

Table 1
Steady-state kinetic parameters for UDP-Gal*p* mutase

Compound	k_{cat} (per min)	K_m (μM)
UDP-Gal*p*	1400	645
1	1.6	261
2	0.02	199

avoided problems in separating **1** and **2** from UDP-glucose. However, a sub-stoichiometric quantity of UDP-glucose must necessarily be used with resulting lower yields.

Conversion of **1** and **2** into the predicted furanose products, uridine 5′-(3-deoxy-3-fluoro-D-galactopyranosyl diphosphate) and uridine 5′-(2-deoxy-2-fluoro-D-galactopyranosyl diphosphate), respectively, was performed enzymatically using UDP-Gal*p* mutase under optimized assay conditions (Scheme 1). The substrate pyranose and product furanose forms of the natural substrate of UDP-Gal*p* mutase, UDP-galactose, are well separated by analytical PA-100 anion-exchange HPLC. We expected a similar elution profile for the pyranose and furanose forms of **1** and **2**. Indeed, analysis of the reaction of **1** and **2** with UDP-Gal*p* mutase by PA-100 HPLC revealed the time-dependent formation of peaks eluting at positions expected for the furanose forms. Integration of the peak height permitted the time courses of the reaction to be determined (Fig. 1). Surprisingly, the final amount of furanose product at enzymatic equilibrium from **1** (1.3%) or **2** (2.2%) was significantly less than that observed with UDP-Gal*p* (7.7%). One possible explanation for this result was the formation of a stable, covalent adduct between these fluoro-sugar nucleotides and the enzyme, since similar sugars have been shown to be mechanism-based inhibitors of other glycosidases [7]. Addition of UDP-Gal*p* to the equilibrium mixture resulting from incubation of **1** with UDP-Gal*p* mutase resulted in 7% conversion of UDP-Gal*p* to UDP-Gal*f*, demonstrating that the UDP-Gal*p* mutase was still active. The activity of UDP-Gal*p* mutase after incubation with **2** could not be determined in this way because of co-elution of **2** with UDP-Gal*f* under our HPLC conditions. The steady-state kinetic parameters, k_{cat} and K_m, for **1** and **2** are shown for comparison with those of UDP-Gal*p* in Table 1.

Further support for the formation of the predicted furanoses was obtained by ^{19}F NMR spectroscopy. Upon incubation of **1** with UDP-Gal*p* mutase, a new resonance at $\delta = -202.9$ ppm was observed, with an integrated intensity $\sim 1\%$ that of the resonance from **1** at $\delta = -203.77$ ppm, corresponding to the amount of furanose product determined by HPLC (1.3%).

The low amounts of furanose product produced from **1** and **2** by UDP-Gal*p* mutase prompted us to investigate whether similarly low levels of furanose forms are present in aqueous solutions of the corresponding free sugars at thermal equilibrium. The distribution of the pyranose and furanose anomers of D-galactose at equilibrium has previously been determined by gas–liquid chromatography and ^1H NMR [12,13]. At 35 °C, 7.0% of aqueous D-galactose exists in the α and β furanose forms at equilibrium [12]. We used a combination of ^1H (1D and 2D COSY) and ^{19}F NMR spectroscopies to quantitate the relative amounts of furanose at 37 °C in aqueous solutions of D-galactose, 3-deoxy-3-fluoro-D-galactose and 2-deoxy-2-fluoro-D-galactose (Table 2). Identification of the NMR resonances was made using UDP-galactose, **1**, and **2** as references for the α-pyranose tautomers, and using criteria discussed in Ref. [12] (for example, the β anomers of galactopyranose and galactofuranose are more stable than the

corresponding α anomers at 37 °C). The relative amounts of each tautomer are shown in Table 3. It is evident that the total percentage of furanose form at equilibrium for 3-deoxy-3-fluoro-D-galactose and 2-deoxy-2-fluoro-D-galactose are indeed significantly less than the

Table 2
NMR assignments for 3-deoxy-3-fluoro-D-galactose, 2-deoxy-2-fluoro-D-galactose, and D-galactose

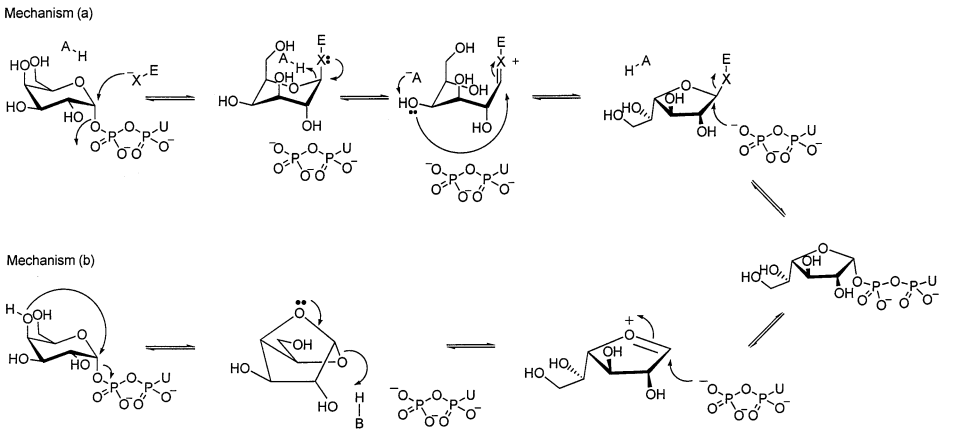
	α-Pyranose	β-Pyranose	α-Furanose	β-Furanose
3-Deoxy-3-fluoro-D-galactose [a]				
H-1	$\delta = 5.45$ (t [b], J 4.58 Hz)	4.75 (d, J 7.9 Hz)	5.50 (d, J 4.8 Hz)	5.49 (s)
H-2	4.23 (m)	3.91 (m)	4.55 (dt [b], J 21.2, 5.5 Hz)	4.44 (d, J 16.7 Hz)
H-3	4.91 (ddd, J 49.1, 10.0, 3.4 Hz)	4.71 (ddd, J 48.1, 9.5, 3.7 Hz)	5.21 (dt [b], J 56.0, 5.2 Hz)	5.12 (ddd, J 53.0, 4.0, 2.2 Hz)
H-4	4.41 (dd, J 7.5, 3.5 Hz)	4.35, (dd, J 6.2, 3.7 Hz)	4.21 (m)	4.53 (dt [b], J 24.3, 4.5 Hz)
H-5	4.23 (m)	3.84 (m)	[c]	4.02 (dd, J 24.1, 9.0 Hz)
H-6a + H-6b	3.89 (m)	3.90 (m)	[c]	3.8 (m)
2-Deoxy-2-fluoro-D-galactose				
H-1	5.60 (d, J 4.2 Hz)	4.97 (dd, J 7.8, 3.5 Hz)	5.55 (s)	5.64 (d, J 11.4 Hz)
H-2	4.81 (ddd, J 50.1, 10.1, 4.0 Hz)	4.47 (ddd, J 51.9, 9.4, 7.9 Hz)	5.08 (dt [b], J 52.4, 5.3 Hz)	5.02 (dd, J 51.1, 2 Hz)
H-3	4.24 (m)	4.07 (ddd, J 14.3, 9.5, 3.6 Hz)	4.58 (ddt [b], J 19.6, 6.0 Hz)	4.48 (d, J 22 Hz)
H-4	4.19 (t [b], J 3.7 Hz)	4.13 (t [b], J 3.3 Hz)	3.92 (s)	[c]
H-5	4.25 (m)	3.88 (m)	[c]	[c]
H-6a + H-6b	3.86 (m)	3.90 (m)	[c]	[c]
D-Galactose				
H-1	5.40 (d, J 3.9 Hz)	4.71 (d, J 8.0 Hz)	5.42 (d, J 4.7 Hz)	5.36 (d, J 3.1 Hz)
H-2	3.95 (dd, J 10.3, 3.8 Hz)	3.63 (dd, J 9.8, 7.9 Hz)	4.21 (m)	4.14 (m)
H-3	3.98 (dd, J 10.3, 3.2 Hz)	3.78 (dd, J 9.8, 3.5 Hz)	3.94 (m)	[c]
H-4	4.12 (d, J 6.0 Hz)	4.06 (d, J 3.4 Hz)	[c]	[c]
H-5	4.21 (t [b], J 6.0 Hz)	3.84 (m)	[c]	[c]
H-6a + H-6b	3.86 (m)	3.88 (m)	[c]	[c]

[a] Assignments made from ^{1}H, ^{1}H{^{1}H} COSY and ^{19}F NMR.
[b] Apparent triplet.
[c] Not assigned because peaks overlapping.

Table 3
Anomeric distributions (%) at thermal equilibrium in aqueous solution [a]

	α-Pyranose	β-Pyranose	α-Furanose	β-Furanose	% Furanose
3F-Gal [b]	40 (40) [c]	58 (58)	0.7 (0.8)	1.6 (1.8)	2.4 (2.6)
2F-Gal	41.0 (97) [d]	55.7	1.0 (0.8)	2.2 (2.3)	3.2 (3.1)
D-Galactose	31.8	60.5	3.1	4.6	7.7

[a] Anomeric distribution calculated as follows: (2F-Gal)% α-pyranose and β-pyranose calculated from H-1″ resonance areas, % β-furanose and α-furanose calculated from mean of H-1″ and H-2″ resonance areas. Area of H-1″ and H-2″ of α-furanose are less accurate because they were not fully resolved (H-2″ overlaps with isotopic sideband of H-1″ of β-pyranose) and calculated value represents upper estimate; (3F-Gal)% α-pyranose and β-pyranose calculated from H-4″ resonance areas, % β-furanose and α-furanose calculated from mean of H-1″ and H-3″ resonance areas. The H-3″ resonances were not fully resolved and the calculated values represent upper estimates; (Galactose)% α-pyranose and β-pyranose calculated from H-4″ resonance areas, % β-furanose and α-furanose calculated from H-1″ resonance areas.
[b] 3F-Gal, 3-deoxy-3-fluoro-D-galactose; 2F-Gal, 2-deoxy-2-fluoro-D-galactose.
[c] Figures in parentheses determined from ^{19}F NMR spectra (without ^{1}H decoupling).
[d] Total% area of pyranose determined by ^{19}F NMR (α-pyranose and β-pyranose resonances were partially overlapping).



Scheme 3.

percentage of furanose form observed for D-galactose. In addition, the percentage of furanose forms observed for 3-deoxy-3-fluoro-D-galactose and 2-deoxy-2-fluoro-D-galactose is similar to the percentage of furanose product observed after enzymatic turnover of **1** and **2** by UDP-Gal*p* mutase.

The substitution of an alcohol group with fluorine in a substrate has been shown to alter the nature of an enzyme-catalyzed reaction in several ways, for example disruption of hydrogen bonds, suppression of aldol chemistry and re-routing of the reaction to give net HF elimination [8]. In the case of UDP-Gal*p* mutase, no fluoride release was observed by ^{19}F NMR for either **1** or **2**. The similar K_m values for UDP-Gal*p* and compounds **1** and **2** also suggests that interactions in the Michaelis complexes are not significantly affected. The reason for the $\sim$1000-fold decrease in the observed k_{cat} exhibited by **1** is not apparent. Interestingly, a $>$100-fold decrease in k_{cat} has been reported for the β-(1→4)-galactosyltransferase with 2-acetamido-2,3-dideoxy-3-fluoro-glucopyranose as acceptor, although no explanation for this was reported [14]. Any possible mechanisms involving redox or aldol chemistry at the C-3″ position of UDP-Gal*p* during turnover by UDP-Gal*p* mutase are ruled out by the demonstration of turnover of **1** at a significant rate.

Compound **2** was a much poorer substrate, exhibiting a k_{cat} reduced $\sim$71,000-fold compared to UDP-Gal*p*. We propose that this decrease in k_{cat} for **2** results from the suppression of anomeric C–O bond cleavage. This reduction in catalytic turnover has been well documented in the glycosidase-mediated hydrolysis of pyranosides containing fluorine at C-2 or C-5 [7]. The presence of the electronegative fluorine atom adjacent to the anomeric carbon has been proposed to destabilize oxocarbenium type intermediates or transition states. In, addition, strong stabilizing interactions between the C-2 hydroxyl group of sugars and enzyme residues have been observed for retaining glycosidases [15].

A number of mechanisms for the unprecedented reaction of UDP-Gal*p* mutase can be ruled out from these studies and our previously reported studies [6]. Cleavage of the C-1″–O-1 bond must occur during catalysis, ruling out mechanisms involving enzyme-UDP intermediates or UDP transfer from C-1″ to C-2″. Mechanisms involving dehydration or oxidation at C-2″ or C-3″ are also ruled out by the present studies, since HF elimination was not observed for either **1** or **2**, and both **1** and **2** are shown here to be substrates for the mutase. Two mechanisms that are consistent with the data presented here are shown in Scheme 3. Both invoke anomeric C–O bond cleavage in the first step, as required from previous studies [6]. In pathway (*a*), C–O bond cleavage takes place during nucleophilic attack by an enzyme-based nucleophile, reminiscent of a retaining-type glycosidase. In the second step, ring opening takes place to give an acyclic intermediate which closes in a third step to give a galactofuranosyl–enzyme adduct. In the final step, nucleophilic substitution of the enzyme-based nucleophile at the

anomeric carbon by UDP gives the product α-UDP-Gal*f*. In pathway (*b*), anomeric C–O bond cleavage takes place during nucleophilic attack by the C-4″ oxygen to give 1,4-anhydrogalactose and UDP. This intermediate subsequently breaks down to give an enzyme-stabilized oxocarbenium intermediate, which reacts with UDP to give product.

3. Experimental

General methods.—All chemicals and enzymes were purchased from Sigma–Aldrich, unless otherwise specified. 3-Deoxy-3-fluoro-D-galactose was purchased from Omicron Biochemicals, Inc. (IN, USA). 2-Deoxy-2-fluoro-D-galactose was purchased from Toronto Research Chemicals Inc. NMR spectra were run on a Bruker DRX300 spectrometer and Bruker DRX600 in 5-mm symmetrical microtubes (D_2O matched, Shigemi Co., Ltd.). Recombinant UDP-Gal*p* mutase from *Klebsiella pneumoniae* was purified from *E. coli*. Details of the cloning, expression, and purification of the enzyme will be published separately.

Uridine 5′-(3-deoxy-3-fluoro-D-galactopyranosyl diphosphate) (**1**).—To a solution (1 mL) containing 3-deoxy-3-fluoro-D-galactose (100 μmol, 18 mg), ATP (5 μmol, 2.7 mg), phosphoenolpyruvate (20 μmol, 4.1 mg), $MgCl_2$ (10 μmol, 2.0 mg), triethanolamine (100 mM, pH 7.5) and 9 units pyruvate kinase was added 4 units of galactokinase. The mixture was incubated at 37 °C under nitrogen, and conversion into 3-deoxy-3-fluoro-D-galactose 1-phosphate monitored by the appearance of a resonance in the ^1H NMR (D_2O) spectrum at $\delta = 5.56$ (dt, *J* 4.5, 6.8 Hz), which was assigned to the anomeric hydrogen of 3-deoxy-3-fluoro-D-galactose 1-phosphate. The area of this resonance was calibrated against that of the anomeric hydrogen of the α anomer of 3-deoxy-3-fluoro-D-galactose ($\delta =$ 5.45 ppm). After 72 h, 72% conversion was achieved.

For uridylation, the mixture was diluted to 2 mL with triethanolamine (100 mM, pH 7.5) and the following components added: UDP-D-glucose (6.6 μmol, 4 mg), $NADP^+$ (2 μmol, 1.5 mg), DTT (2 μmol, 0.3 mg), α-ketoglutarate (20 μmol, 3.8 mg), D-glucose 1,6-biphosphate (0.04 μmol, 0.03 mg), 10 units galactose-1-phosphate uridyltransferase, 1.6 units phosphoglucomutase, 3 units glucose-6-phosphate dehydrogenase and 1.4 units L-glutamic dehydrogenase. Uridylation was monitored by ion exchange HPLC and by ^1H NMR spectroscopy (described below). Conversion (100%) of UDP-glucose into **1** was observed after 48 h at 37 °C. The sample was then filtered through a YM-10 membrane (10 kDa cut-off, Millipore-Amicon), diluted in several volumes of water and applied to a Mono-Q 16/10 anion-exchange column (Pharmacia), washed previously with 1 M KCl and then pre-equilibrated in water. A linear 0–300 mM KCl gradient over 1 h was run with elution of **1** at ~75 mM KCl, with optical detection at 262 nm. Fractions containing **1** were pooled, lyophilized, resuspended in a small volume of water and desalted on a Biogel P-2 gel (Bio-Rad) filtration column. Fractions containing **1** were then pooled, lyophilized and stored at −20 °C. ^1H NMR (D_2O, 600 MHz): δ 8.07 (d, 1 H, *J* 8.1 Hz, H-6), 6.12 (d, 1 H, *J* 3.7 Hz, H-1′), 6.11 (d, 1 H, *J* 8.1 Hz, H-5), 5.83 (dt, 1 H, *J* 6.8, 4.2 Hz, H-1″), 4.95 (ddd, 1 H, *J* 49.0, 9.7, 3.1 Hz, H-3″), 4.51 (d, 2 H, *J* 3.5 Hz, H-2′ and H-3′), 4.45 (dd, 1 H, *J* 7.3, 3.1 Hz, H-4″), 4.42 (m, 1 H, H-4′), 4.40–4.30 (m, 3 H, H-5a′, H-5b′ and H-5″), 3.91 (m, H-6a″ and H-6b″); ^{19}F (D_2O, 300 MHz): δ −203.95 (m). ESIMS: calculated for $C_{15}H_{23}FN_2O_{16}P_2$ ($M - H^+$) 567.1, observed = 567.2 *m*/*z*.

Uridine 5′-(2-deoxy-2-fluoro-D-galactopyranosyl diphosphate) (**2**).—The synthesis of **2** was carried out using the same procedure as that used for the synthesis of **1**. To a solution (1 mL) containing 2-deoxy-2-fluoro-D-galactose (30 μmol, 5.5 mg), ATP (1.8 μmol, 1 mg), phosphoenolpyruvate (53 μmol, 11 mg), $MgCl_2$ (10 μmol, 2 mg), Tris (100 μmol, 12.1 mg), pH adjusted to 8.0 with NaOH, was added galactokinase (1 unit) and pyruvate kinase (50 units). After 2 h at 37 °C, the components of the uridylation reaction were added: UDP-glucose (23 μmol, 14.2 mg), $NADP^+$ (1 μmol, 0.8 mg), DTT (2 μmol, 0.3 mg), α-ketoglutarate (40 μmol, 7.6 mg), D-glucose 1,6-

biphosphate (0.07 μmol, 0.05 mg), galactose-1-phosphate uridyltransferase (2 units), phosphoglucomutase (1 unit), glucose-6-phosphate dehydrogenase (1 unit) and L-glutamic dehydrogenase (1 unit, from an ammonium sulfate suspension). The reaction was left at 37 °C for 48 h during which time greater than 95% conversion of UDP-glucose into **1** was observed. Purification and storage of **2** was carried out as already described for compound **1**. ^{1}H NMR (D_2O, 300 MHz): δ 8.08 (d, 1 H, *J* 8.1 Hz, H-6), 6.11 (d, 1 H, *J* 3.8 Hz, H-1′), 6.09 (d, 1 H, *J* 8.1 Hz, H-5), 5.93 (dd, 1 H, *J* 7.4, 3.5 Hz, H-1″), 4.82 (ddt, 1 H, *J* 50.1, 9.9, 3.0 Hz, H-2″), 4.49 (d, 2 H, *J* 3.5 Hz, H-2′ and H-3′), 4.40 (m, 1 H, H-4′), 4.37–4.25 (m, 4H, H-5a′, H-5b′, H-3″ and H-5″), 4.21 (t, 1 H, *J* 3.8 Hz, H-4″), 3.85 (m, 2H, H-6a″ and H-6b″); ^{19}F NMR (D_2O, 300 MHz): δ −208.575 (ddd, F-2, *J* 49.6, 12.1, 3.9 Hz). ESIMS: calculated for $C_{15}H_{23}FN_2O_{16}P_2$ ($M-H^+$) 567.1, observed = 567.2 *m*/*z*.

Incubation of **1** *and* **2** *with UDP-Gal*p *mutase*.—Compounds **1** and **2** were tested as substrates of UDP-Gal*p* mutase using assay conditions described next. UDP-sugar (0.5 mM) was incubated anaerobically under N_2 at 37 °C in 5-mL glass Thunberg tubes (with side arm) sealed with rubber septa in a volume of 0.5 mL. The assay mixture contained FAD (0.1 mM), DTT (1 mM) and 50 mM AMPSO, pH 8.3. UDP-Gal*p* mutase was contained in the side arm (40 μL of 0.3 μM for **1**, 37.5 μM for **2**). The sample was made anaerobic by passing nitrogen gas over the surface of the assay mixture for 5 min, followed by bubbling with N_2 for 5 min. The assay mixture was then mixed with the UDP-Gal*p* mutase in the side arm and the sample incubated at 37 °C. Small aliquots were removed at various intervals, quenched by boiling for 1 min, and the high molecular weight material removed by centrifugal filtration (Ultrafree-MC tubes, 10,000 nominal molecular weight limit, Millipore). The sample was then diluted in several volumes of 75 mM KH_2PO_4, pH 4.5, and injected onto a 4 × 250 mm Carbopac PA-100 anion-exchange HPLC column (Dionex), pre-equilibrated in 75 mM KH_2PO_4, pH 4.5. The sample was eluted isocratically at 1 mL/min, with optical monitoring at 262 nm. Kinetic parameters, k_{cat} and k_{cat}/K_m, were determined as described above, with 10-min incubation times at various concentrations of **1** and **2**. Both compounds exhibited saturation kinetics, and the kinetic parameters were determined from fits of the data to the Michaelis–Menten equation.

Turnover of **1** for analysis by ^{19}F NMR was performed aerobically at 37 °C. To 300 mL of **1** in D_2O was added (to final concentrations) FAD (0.2 mM), DTT (5 mM), potassium phosphate (20 mM, pH 7.5) and UDP-Gal*p* mutase (2.1 μM). Samples were incubated for 12 h at 37 °C prior to NMR spectroscopic analysis.

Measuring pyranose/furanose distribution of D-*galactose*, *3-deoxy-3-fluoro*-D-*galactose*, *and 2-deoxy-2-fluoro*-D-*galactose*.—Solutions (100 mM) of D-galactose, 3-deoxy-3-fluoro-D-galactose and 2-deoxy-2-fluoro-D-galactose were made up in D_2O and incubated for greater than 12 h at 37 °C, prior to spectral analysis at 37 °C by a combination of ^{1}H NMR (1D, 2D ^{1}H{^{1}H} COSY) at 600 MHz, and ^{19}F NMR (with and without ^{1}H decoupling) at 300 MHz.

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