Mechanistic Investigation of UDP-Galactopyranose Mutase from *Escherichia coli* Using 2- and 3-Fluorinated UDP-Galactofuranose as Probes

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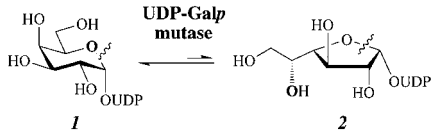
Abstract: The galactofuranose moiety found in many surface constituents of microorganisms is derived from UDP-D-galactopyranose (UDP-Gal*p*) via a unique ring contraction reaction catalyzed by UDP-Gal*p* mutase. This enzyme, which has been isolated from several bacterial sources, is a flavoprotein. To study this catalysis, the cloned *Escherichia coli* mutase was purified and two fluorinated analogues, UDP-[2-F]Gal*f* (**9**) and UDP-[3-F]Gal*f* (**10**), were chemically synthesized. These two compounds were found to be substrates for the reduced UDP-Gal*p* mutase with the K_m values determined to be 65 and 861 μM for **9** and **10**, respectively, and the corresponding k_{cat} values estimated to be 0.033 and 5.7 s^{-1}. Since the fluorine substituent is redox inert, a mechanism initiated by the oxidation of 2-OH or 3-OH on the galactose moiety can thus be firmly ruled out. Furthermore, both **9** and **10** are poorer substrates than UDP-Gal*f*, and the rate reduction for **9** is especially significant. This finding may be ascribed to the inductive effect of the 2-F substituent that is immediately adjacent to the anomeric center, and is consistent with a mechanism involving formation of oxocarbenium intermediates or transition states during turnover. Interestingly, under nonreducing conditions, compounds **9** and **10** are not substrates, but instead are inhibitors for the mutase. The inactivation by **10** is time-dependent, active-site-directed, and irreversible with a K_I of 270 μM and a k_{inact} of 0.19 min^{-1}. Since the K_I value is similar to K_m, the observed inactivation is unlikely a result of tight binding. To our surprise, the inactivated enzyme could be regenerated in the presence of dithionite, and the reduced enzyme is resistant to inactivation by these fluorinated analogues. It is possible that reduction of the enzyme-bound FAD may induce a conformational change that facilitates the breakdown of the putative covalent enzyme−inhibitor adduct to reactivate the enzyme. It is also conceivable that the reduced flavin bears a higher electron density at N-1, which may play a role in preventing the formation of the covalent adduct or facilitating its breakdown by charge stabilization of the oxocarbenium intermediates/transition states. Clearly, this study has led to the identification of a potent inactivator (**10**) for this enzyme, and study of its inactivation has also shed light on the possible mechanism of this mutase.

D-Galactose is a common sugar found ubiquitously in nature. In free solution, the majority of D-galactose exists as a pyranose sugar with the thermodynamically less favored D-galactofuranose as a minor component (<8%).[1] Similarly, the pyranose form is the prevalent form of D-galactose in naturally occurring galactoconjugates, where D-galactofuranose is a much rarer constituent. The occurrence of D-galactofuranose so far has been limited mainly to surface polymers of microorganisms, such as bacterial *O*-antigens, mycobacterial cell walls, fungal glycoconjugates, and protozoal cell membranes.[2] An exception is prymnesin-1 (PRM1), a polycyclic ether toxin from the red tide algae *Prymnesium parvum*, in which D-galactofuranose has been shown as a structural component of this hemolytic and ichthyotoxic agent.[3] The biosynthetic donor of the D-galactopyranosyl unit in galactoconjugates has been shown to be UDP-galactopyranose (UDP-Gal*p*, **1**), which is derived from UDP-glucose in a process catalyzed by UDP-galactose 4-epimerase.[4] Many bacteria can also directly activate galactose to UDP-Gal*p* via the combined actions of galactokinase and α-D-galactose-1-phosphate uridylyltransferase.[5] Unlike the formation of UDP-Gal*p* which is well-studied, the biosynthesis of galactofuranose has been puzzling for many years. Although early feeding



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experiments had revealed that the donor of the galactofuranosyl unit in microbial surface polymers is UDP-galactofuranose

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(1) (a) Wertz, P. W.; Garver, J. C.; Anderson, L. *J. Am. Chem. Soc.* **1981**, *103*, 3916−3922. (b) Angyal, S. J.; Pickles, V. A. *Aust. J. Chem.* **1972**, *25*, 1695−1710.

(2) (a) de Lederkremer, R. M.; Colli, W. *Glycobiology* **1995**, *5*, 547−552. (b) Brennan, P. J.; Nikaido, H. *Annu. Rev. Biochem.* **1995**, *64*, 29−63. (c) Whitfield, C. *Trends Microbiol.* **1995**, *3*, 178−185. (d). Previato, J. O.; Mendonca-Previato, L.; Jones, C.; Wait, R. *Glycoconjugates J.* **1993**, *10*, 340.

(3) Igarashi, T.; Satake, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1999**, *121*, 8499−8511.

(4) (a) Shibaev, V. N. *Adv. Carbohydr. Chem. Biochem.* **1986**, *44*, 277−339. (b) Frey, P. A. *FASEB* **1996**, *10*, 461−470.

(5) (a) Thomas, P.; Bessel, E. M.; Westwood, J. H. *Biochem. J.* **1974**, *139*, 661−664. (b) Kalckar, H. M. *Adv. Enzymol.* **1958**, *20*, 111.

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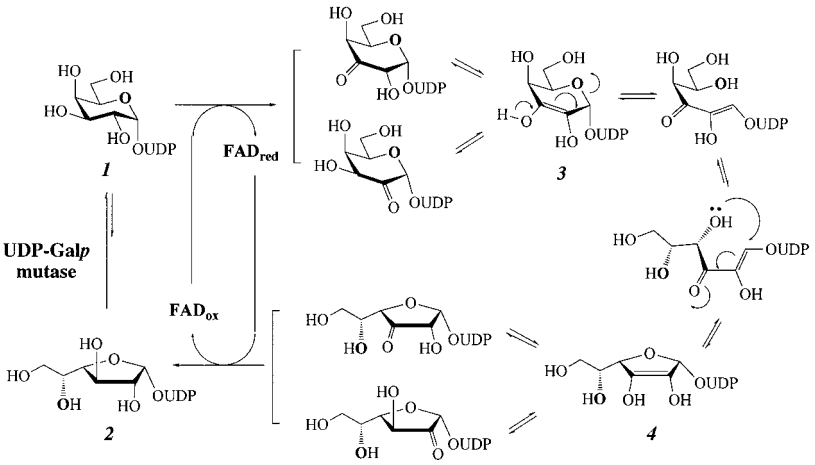
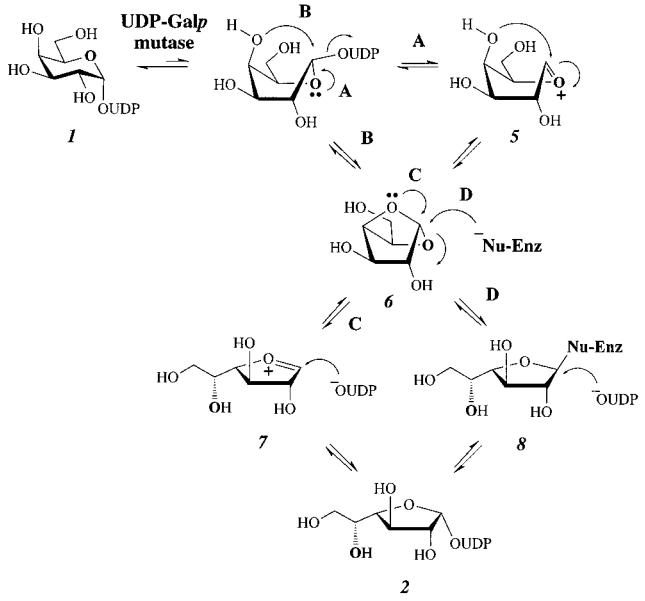
Scheme 1



(UDP-Gal*f*, **2**), the precursor of UDP-Gal*f* was only recently established to be UDP-Gal*p* (**1**), whose conversion to UDP-Gal*f* is catalyzed by UDP-galactopyranose mutase.[6] The UDP-Gal*p* mutase has been isolated from several bacterial sources[7] and has been shown to be a flavoprotein, with the flavin adenine dinucleotide (FAD) coenzyme bound noncovalently. The fact that galactofuranose units are found in many pathogens but not in human tissues makes UDP-Gal*p* mutase an attractive target for therapeutic agents.

The reaction catalyzed by UDP-Gal*p* mutase must involve ring cleavage and recyclization for the interconversion of **1** and **2**. A few possible mechanisms can be envisioned to account for this unique ring rearrangement. The mechanism depicted in Scheme 1 is an example in which the redox capability of FAD is exploited, allowing the oxidation of 2- or 3-OH to produce the enediols (**3**/**4**, or the corresponding enediolates) as possible intermediates. Subsequent ring opening, recyclization, and reduction would regenerate the flavin coenzyme and afford the expected product. A nonoxidative ring cleavage initiated by a nucleophilic attack at the α-phosphoryl atom to yield an UDP-enzyme intermediate is another plausible mechanism for the catalysis of UDP-Gal*p* mutase.[8] A reversible 1,2-migration of the UDP group from C-1 to C-2 to facilitate the requisite ring opening is also a conceivable alternative.[8] However, little mechanistic investigation of this mutase had been carried out until a recent study[9] in which a reversible cleavage of the anomeric C−O bond of **1** was elegantly demonstrated to be part of the catalysis. Clearly, mechanisms involving P_α−O bond cleavage could be ruled out on the basis of this finding.[9] As illustrated in Scheme 2, a new mechanism initiated by distortion of the ring to allow attack of O_4 on C-1 to release UDP, or by elimination of UDP first to form an oxocarbenium ion **5** followed by O_4 attack on C-1, was proposed by Blanchard and his coworkers.[9] Subsequent ring opening between C-1 and O_5

Scheme 2



of the bicyclo-acetal intermediate **6**, via either **7** or **8**, followed by the rebound of UDP at C-1 should lead to the formation of **2**. Since oxidation/reduction is not a requisite step of this mechanism and there is no net change in the redox states of the substrate or the product, whether the enzyme-bound FAD plays an active role in the reaction mechanism remains obscure.

To study this transformation, we have recently amplified the *glf* gene of *Escherichia coli*[6] by polymerase chain reaction (PCR) and expressed it in *E. coli* BL-21(DE3) host cells.[10] The encoding UDP-Gal*p* mutase was purified to near homogeneity. Using UDP-Gal*f* as the substrate, a K_m of 194 μM and a k_{cat} of 1.5 s^{-1} for the catalysis in the reverse direction (**2** → **1**) were obtained. Interestingly, when the enzyme was reduced by sodium dithionite, its catalytic efficiency was increased by more than 2 orders of magnitude (K_m = 22 μM, k_{cat} = 27 s^{-1}). While both the FAD coenzyme and the cysteine residues of this mutase were reduced upon treatment with dithionite, reoxidation of the flavin coenzyme alone was enough to abolish the increment of the activity. A comparable rate enhancement was also noted when the flavin coenzyme was selectively reduced by photoreduction in the presence of 5-deazariboflavin under anaerobic

(6) (a) Stevenson, G.; Neal, B.; Liu, D.; Hobbs, M.; Packer, N. H.; Batley, M.; Redmond, J. W.; Lindquist, L.; Reeves, P. *J. Bacteriol.* **1994**, *176*, 4144−4156. (b) Nassau, P. M.; Martin, S. L.; Brown, R. E.; Weston, A.; Monsey, D.; McNeil, M. R.; Duncan, K. *J. Bacteriol.* **1996**, *178*, 1047−1052.

(7) (a) Köplin, R.; Brisson, J.-R.; Whitfield, C. *J. Biol. Chem.* **1997**, *272*, 4121−4128. (b) Weston, A.; Stern, R. J.; Lee, R. E.; Nassau, P. M.; Monsey, D.; Martin, S. L.; Scherman, M. S.; Besra, G. S.; Duncan, K.; McNeil, M. R. *Tuberc. Lung Dis.* **1998**, *78*, 123−131. (c) Chen, P.; Bishai, W. *Infect. Immun.* **1998**, *66*, 5099−5106. (d) McMahon, S. A.; Leonard, G. A.; Buchanan, L. V.; Giraud, M.-F.; Naismith, J. H. *Acta Crystallogr.* **1999**, *D55*, 399−402.

(8) Barlow, J. N.; Blanchard, J. S. *Carbohydr. Res.* **2000**, *328*, 473−480.

(9) Barlow, J. N.; Girvin, M. E.; Blanchard, J. S. *J. Am. Chem. Soc.* **1999**, *121*, 6968−6969.

(10) (a) Zhang, Q.; Liu, H.-w. *J. Am. Chem. Soc.* **2000**, *122*, 9065−9070. (b) Zhang, Q.; Liu, H.-w. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 145−149.

conditions. These findings clearly demonstrated the involvement of FAD in regulating the catalytic efficiency of this enzyme.[10a] To gain further insight into the mechanism of this interesting transformation, we have synthesized UDP-2-deoxy-2-fluoro-galactofuranose (UDP-[2-F]Gal*f*, **9**) and UDP-3-deoxy-3-fluoro-galactofuranose (UDP-[3-F]Gal*f*, **10**) and examined the competence of these two compounds as substrates and/or inhibitors for UDP-Gal*p* mutase. Reported in this paper are the results of these experiments that not only confirmed an active role played by the enzyme-bound flavin in regulating the catalysis, but also provided preliminary evidence suggesting the possible involvement of a covalent intermediate in the mechanism of this mutase.

Experimental Section

General. Unless otherwise specified, all chemicals were purchased from Aldrich or Sigma and used without further purification. NMR spectra were recorded on Varian 200, 300, or 500 MHz spectrometers. ^{13}C NMR spectra were recorded with proton broad-band decoupling, and the reported spin couplings are from fluorine and/or phosphorus. ^{31}P NMR spectra were recorded with proton decoupling and externally referenced with 85% phosphoric acid. ^{19}F NMR spectra were recorded without proton decoupling, and the external reference was fluorotrichloromethane. The *J*-values are given in hertz (Hz). For NMR spectra recorded in D_2O, *t*-BuOH was added as an internal reference; the chemical shifts are 1.27 and 31.2 ppm for ^{1}H NMR and ^{13}C NMR, respectively.[11] All UV−visible spectra were taken on a Beckman DU650 spectrophotometer. Fast-atom bombardment (FAB), chemical ionization (CI), and electrospray ionization (ESI) mass spectra were recorded by the MS facility at the Department of Chemistry of the University of Minnesota. High-performance liquid chromatography (HPLC) analysis and/or purification were conducted with a Hewlett-Packard 1090A system equipped with a photodiode array detector. To detect nucleotide sugar, the detector wavelength was set at 262 nm. Analytical thin-layer chromatography was carried out on Merck silica gel 60 G-254 plates, and the spots were visualized either under UV light or by heating plates previously stained with solutions of vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% in EtOH).

Synthesis of UDP-[2-F]Gal*f* (9). 1,3,5,6-Tetra-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactofuranose (13α) and 1,3,5,6-Tetra-*O*-acetyl-2-deoxy-2-fluoro-*β*-D-galactofuranose (13*β*). The starting material, 2-deoxy-2-fluoro-D-galactose (**12**, 7.0 g, 38 mmol), was prepared from **11** in two steps. The fluorination step was accomplished by using SELECTFLUOR as the reagent,[12] and the product was deacetylated using NaOMe/MeOH. The product **12** was dissolved in dry pyridine (100 mL) and heated at 110 °C for 2 h. To this solution was slowly added acetic anhydride (60 mL), and the resulting mixture was stirred for an additional 1.5 h at 110 °C. The reaction was cooled to room temperature and evaporated to dryness under reduced pressure. The residual oil was chromatographed on silica gel and eluted with ethyl acetate/hexanes (1:2). The pyranose products (6.4 g, 48%) were eluted first from the column, followed by the elution of the furanose products. While the furanose products in the earlier fractions were a mixture of both α and *β* isomers (2.37 g, α:*β* = 1:2), that in the latter fractions was mainly the *β* isomer (1.4 g). A small amount of α isomer was also purified to homogeneity by repeated chromatography on silica gel for analytical purposes. The combined yield for the furanose products was 28%. Spectral data for the α isomer (**13α**): ^{1}H NMR ($CDCl_3$) *δ* 2.03 (3H, s), 2.10 (3H, s), 2.11 (3H, s), 2.14 (3H, s), 4.13 (1H, dd, *J* = 12.0, 6.0, 6-H), 4.13 (1H, t, *J* = 6.0, 4-H), 4.28 (1H, dd, *J* = 12.0, 4.2, 6-H), 5.10 (1H, ddd, *J* = 52.2, 6.0, 4.2, 2-H), 5.26 (1H, td, *J* = 6.0, 4.2, 5-H), 5.57 (1H, dt, *J* = 16.2, 6.0, 3-H), 6.27 (1H, d, *J* = 4.2, 1-H); ^{13}C NMR ($CDCl_3$) *δ* 20.66, 20.68, 20.8, 21.0, 62.0, 70.0, 73.7 (d, *J* = 24), 79.1 (d, *J* = 8), 92.1 (d, *J* = 203), 92.4 (d, *J* = 18), 169.4, 169.7, 169.8, 170.4; ^{19}F NMR ($CDCl_3$) *δ* −206.5 (dd, *J* = 52.3, 15.8); high-resolution FABMS calcd for $C_{14}H_{20}FO_9$ [M + H]$^+$ 351.1091, found *m*/*z* 351.1103. Spectral data for the *β* isomer (**13*β***): ^{1}H NMR ($CDCl_3$) *δ* 1.99 (3H, s), 2.04 (3H, s), 2.06 (3H, s), 2.07 (3H, s), 4.16 (1H, dd, *J* = 12.0, 6.9, 6-H), 4.25 (1H, dd, *J* = 12.0, 4.5, 6-H), 4.32 (1H, dd, *J* = 4.5, 3.3, 4-H), 4.91 (1H, dd, *J* = 48.9, 0.9, 2-H), 5.07 (1H, ddt, *J* = 21.6, 4.5, 0.9, 3-H), 5.34 (1H, ddd, *J* = 6.9, 4.5, 3.3, 5-H), 6.29 (1H, d, *J* = 10.8, 1-H); ^{13}C NMR ($CDCl_3$) *δ* 20.6, 20.7 (2C), 20.9, 62.4, 69.1, 76.2 (d, *J* = 31), 83.2, 97.3 (d, *J* = 185), 98.8 (d, *J* = 38), 169.0, 169.7, 170.2, 170.5; ^{19}F NMR ($CDCl_3$) *δ* −191.3 (ddd, *J* = 49.3, 21.1, 11.1); high-resolution FABMS calcd for $C_{14}H_{20}FO_9$ [M + H]$^+$ 351.1091, found *m*/*z* 351.1099.

Dibenzyl (3,5,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactofuranosyl)phosphate (14). A solution of **13** (3.7 g, 10 mmol, α:*β* = 1:2) in anhydrous dichloromethane (30 mL) was cooled with an ice−water bath. To this solution was added bromotrimethylsilane (9 mL), and the resulting mixture was stirred overnight at room temperature. The solution was then evaporated to dryness under reduced pressure. The residual oil was redissolved in anhydrous toluene (5 mL), and the mixture was evaporated to dryness to remove more volatile silane derivatives. The residue and dibenzyl phosphate (4.3 g, 15 mmol) were then mixed with anhydrous toluene (10 mL). To this solution was added triethylamine (2.1 mL, 15 mmol), and the mixture was stirred overnight at room temperature. After removal of solvent, the residue was chromatographed on silica gel and eluted with EtOAc/hexanes (1:1). The fast-moving unreacted starting material (2.8 g, 65%, α:*β* = 1:3.6) was recovered. The desired product was eluted next from the column and was purified again on silica gel to give pure **14** in 8% yield (540 mg). Only a small amount of *β* isomer was detected in the crude mixture by ^{19}F NMR. Spectral data for **14**: ^{1}H NMR ($CDCl_3$) *δ* 1.95 (3H, s), 2.02 (3H, s), 2.11 (3H, s), 4.11 (1H, dd, *J* = 11.7, 6.5, 6-H), 4.17 (1H, dd, *J* = 6.5, 4.8, 4-H), 4.28 (1H, dd, *J* = 11.7, 4.8, 6-H), 5.06 (1H, dddd, *J* = 52.2, 6.5, 4.2, 1.8, 2-H), 5.06−5.12 (4H, m, benzylic C*H*$_2$'s), 5.21 (1H, dt, *J* = 6.5, 4.8, 5-H), 5.58 (1H, dt, *J* = 15.6, 6.5, 3-H), 5.94 (1H, dd, *J* = 6.0, 4.2, 1-H), 7.32−7.36 (10H, m, Ar-H's); ^{13}C NMR ($CDCl_3$) *δ* 20.55, 20.61, 20.7, 61.9, 69.36 (d, *J* = 6), 69.37, 69.6 (d, *J* = 5), 72.6 (d, *J* = 23), 79.0 (d, *J* = 8), 91.9 (dd, *J* = 198, 7), 96.4 (dd, *J* = 18, 4), 127.8 (2C), 128.0 (2C), 128.6 (4C), 128.7 (2C), 135.5 (d, *J* = 7), 135.6 (d, *J* = 8), 169.6, 169.9, 170.4; ^{19}F NMR ($CDCl_3$) *δ* −205.2 (dd, *J* = 52.8, 15.0); ^{31}P NMR ($CDCl_3$) *δ* −2.4; high-resolution FABMS calcd for $C_{26}H_{31}FO_{11}P$ [M + H]$^+$ 569.1588, found *m*/*z* 569.1555.

Triethylammonium (3,5,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactofuranosyl)phosphate (15). A solution of **14** (420 mg, 0.74 mmol) in ethyl acetate (10 mL) and triethylamine (0.8 mL) was stirred in the presence of Pd/C (10%) at room temperature overnight under a hydrogen atmosphere. The mixture was filtered through a Celite pad, and the filtrate was evaporated to dryness under reduced pressure to give **15** in 97% yield (350 mg): ^{1}H NMR ($CDCl_3$) *δ* 1.28 (9H, t, *J* = 7.5), 2.01 (3H, s), 2.07 (3H, s), 2.09 (3H, s), 3.03 (6H, q, *J* = 7.5), 4.03 (1H, t, *J* = 5.7, 4-H), 4.19 (1H, dd, *J* = 12.0, 6.3, 6-H), 4.37 (1H, dd, *J* = 12.0, 4.2, 6-H), 4.92 (1H, dt, *J* = 51.6, 4.8, 2-H), 5.23 (1H, q, br, *J* = 6.0, 5-H), 5.51 (1H, dt, *J* = 16.2, 5.4, 3-H), 5.82 (1H, dt, *J* = 7.5, 3.9, 1-H); ^{13}C NMR ($CDCl_3$) *δ* 8.5, 20.76, 20.77, 20.9, 45.4, 62.2, 70.4, 74.1 (d, *J* = 25), 77.6 (d, *J* = 7), 92.4 (dd, *J* = 200, 6), 95.6 (dd, *J* = 17, 4), 169.9, 170.5, 170.8; ^{19}F NMR ($CDCl_3$) *δ* −206.1 (ddd, *J* = 52.8, 15.8, 4.5); ^{31}P NMR ($CDCl_3$) *δ* −0.1; high-resolution FABMS calcd for $C_{12}H_{18}FO_{11}PNa$ [M + Na]$^+$ 411.0468, found *m*/*z* 411.0450.

Bis(triethylammonium) (2-Deoxy-2-fluoro-α-D-galactofuranosyl)-phosphate (16). A solution of **15** (328 mg, 0.67 mmol) in a mixture (20 mL) of methanol/water/triethylamine (5:2:1) was allowed to stand at room temperature for 2 days. The reaction was monitored by TLC (ethanol:NH_4OH:H_2O = 5:3:1) and was stopped when the conversion was complete. The mixture was evaporated to dryness under reduced pressure to afford the desired product **16**. The yield was quantitative (345 mg): ^{1}H NMR (D_2O) *δ* 1.30 (18H, t, *J* = 7.5), 2.95 (12H, q, *J* = 7.5), 3.66 (1H, dd, *J* = 11.7, 7.2, 6-H), 3.73 (1H, dd, *J* = 11.7, 4.2, 6-H), 3.77−3.88 (2H, m, 4-H, 5-H), 4.57 (1H, dt, *J* = 17.7, 7.2, 3-H), 5.00 (1H, dddd, *J* = 53.1, 7.2, 4.5, 1.2, 2-H), 5.65 (1H, dd, *J* = 5.4, 4.5, 1-H); ^{13}C NMR (D_2O) *δ* 9.7, 48.1, 63.7, 73.2, 73.6 (d, *J* = 21), 82.2 (d, *J* = 10), 96.0 (dd, *J* = 197, 7), 96.1 (dd, *J* = 18, 5); ^{19}F NMR (D_2O) *δ* −206.1 (dd, *J* = 53.3, 17.5); ^{31}P NMR (D_2O) *δ* 1.4; high-

(11) Zhang, Q.; Cone, M. C.; Gould, S. J.; Zabriskie, T. M. *Tetrahedron* **2000**, *56*, 693−701.

(12) Burkart, M. D.; Zhang, Z.; Hung, S.-C.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 11743−11746.

resolution FABMS calcd for $C_6H_{12}FO_8PNa$ $[M + Na]^+$ 285.0152, found *m*/*z* 285.0152.

Bis(triethylammonium) UDP-[2-F]Gal*f* (9). Compound **16** (310 mg, 0.67 mmol) was dried by repetitive coevaporation with anhydrous pyridine (3 mL). To this residue was added UMP-morpholidate (1.2 g, 1.7 mmol) in anhydrous pyridine (3 mL), and the solution was evaporated again to dryness under vacuum. This was followed by the addition of 1*H*-tetrazole (155 mg, 2.0 mmol) in anhydrous pyridine (3 mL), and the resulting solution was stirred at room temperature for 34 h. Removal of the solvent under vacuum gave a solid residue which was dissolved in methanol and chromatographed on a Sephadex LH-20 column (2.5 × 120 cm) using methanol as the eluent. Fractions containing the desired product, judging by TLC analysis (EtOH:NH_4OH:H_2O = 5:3:1), were pooled and evaporated to dryness under reduced pressure. Compound **9** was further purified by HPLC using a C_{18} column (10 × 250 mm). The eluent was 1% aqueous acetonitrile in 50 mM triethylammonium acetate, pH 6.8, and the flow rate was 5 mL/min. The retention time of **9** under these conditions was 14.4 min. The yield was 43% (70 mg) after lyophilization: ^{1}H NMR (D_2O, 500 MHz) δ 1.30 (18H, t, J = 7.2, Et_3N-*Me*), 3.22 (12H, q, J = 7.2, Et_3N-CH_2), 3.66 (1H, dd, J = 12.0, 7.0, 6-H), 3.73 (1H, dd, J = 12.0, 5.0, 6-H), 3.82 (1H, dt, J = 7.0, 5.0, 5-H), 3.89 (1H, dd, J = 7.0, 5.0, 4-H), 4.20−4.26 (2H, m, 5′-H_2), 4.28−4.31 (1H, m, 4′-H), 4.36−4.41 (2H, m, 2′-H, 3′-H), 4.57 (1H, dt, J = 17.5, 7.0, 3-H), 5.05 (1H, dddd, J = 53.0, 7.0, 5.3, 1.5, 2-H), 5.79 (1H, t, 5.3, 1-H), 5.97 (1H, d, J = 8.0, 5″-H), 6.03 (1H, d, J = 4.5, 1′-H), 7.94 (1H, d, J = 8.0, 6″-H); ^{13}C NMR (D_2O) δ 9.8 (Et_3N-*Me*), 48.1 (Et_3N-CH_2), 63.5 (C-6), 66.5 (d, J = 5, C-5′), 71.2 (C-2′), 73.2 (d, J = 21, C-3), 73.3 (C-5), 75.2 (C-3′), 82.6 (d, J = 10, C-4), 84.9 (d, J = 9, C-4′), 89.8 (C-1′), 95.7 (dd, J = 198, 7, C-2), 96.8 (dd, J = 18, 5, C-1), 104.2 (C-5″), 143.3 (C-6″), 153.3 (C-2″), 167.6 (C-4″); ^{31}P NMR (D_2O) δ −11.1 (d, J = 20), −12.8 (d, J = 20); ^{19}F NMR (D_2O) δ −204.6 (dt, J = 52.8, 18.1); high-resolution FABMS calcd for $C_{15}H_{23}FN_2O_{16}P_2Na$ $[M + Na]^+$ 591.0405, found *m*/*z* 591.0441.

Synthesis of UDP-[3-F]Gal*f* (10). 1,2-*O*-Isopropylidene-3-deoxy-3-fluoro-α-D-galactofuranose (19). 1,2:5,6-Di-*O*-isopropylidene-3-deoxy-3-fluoro-α-D-galactofuranose (**18**, 4.7 g, 18 mmol), which was prepared from **17** by a literature procedure,[13] was dissolved in 80% aqueous acetic acid (40 mL) and kept at 70 °C for 25 min. The mixture was evaporated to dryness under reduced pressure, and the residual oil was subjected to chromatography on silica gel and eluted with EtOAc/hexanes (1:1). The desired product **19** was isolated in 93% yield (3.7 g): ^{1}H NMR ($CDCl_3$) δ 1.33 (3H, s), 1.52 (3H, s), 2.24 (1H, s, exch.), 2.89 (1H, s, exch.), 3.66 (1H, dd, J = 11.7, 4.5, 6-H), 3.78 (1H, dd, J = 11.7, 3.9, 6-H), 3.86 (1H, dddd, J = 7.5, 4.5, 3.9, 0.9, 5-H), 4.31 (1H, dddd, J = 24.0, 7.5, 1.8, 0.9, 4-H), 4.78 (1H, dd, J = 15.3, 4.2, 2-H), 5.00 (1H, dd, J = 51.0, 1.8, 3-H), 5.98 (1H, d, J = 4.2, 1-H); ^{13}C NMR ($CDCl_3$) δ 25.9, 26.5, 63.4, 69.9 (d, J = 9), 84.4 (d, J = 32), 85.9 (d, J = 25), 95.3 (d, J = 182), 105.6, 113.5; ^{19}F NMR ($CDCl_3$) δ −187.3 (ddd, J = 50.1, 24.1, 15.0); high-resolution CIMS calcd for $C_9H_{19}FNO_5$ $[M + NH_4]^+$ 240.1247, found *m*/*z* 240.1263.

5,6-Di-*O*-acetyl-1,2-*O*-isopropylidene-3-deoxy-3-fluoro-α-D-galactofuranose (20). A solution of **19** (3.7 g, 17 mmol) in pyridine (10 mL) and acetic anhydride (10 mL) was stirred at room temperature for 2 h. Removal of the solvent under reduced pressure afforded the acetylated product **20** in quantitative yield (5.1 g). The product was pure enough to be used in the next step without purification: ^{1}H NMR ($CDCl_3$) δ 1.34 (3H, s), 1.57 (3H, s), 2.06 (3H, s), 2.10 (3H, s), 4.17 (1H, dd, J = 12.2, 5.8, 6-H), 4.30 (1H, ddd, J = 24.8, 7.0, 3.0, 4-H), 4.36 (1H, dd, J = 12.2, 4.0, 6-H), 4.73 (1H, dd, J = 14.2, 3.8, 2-H), 4.96 (1H, dd, J = 50.8, 3.0, 3-H), 5.34 (1H, ddd, J = 7.0, 5.8, 4.0, 5-H), 5.90 (1H, d, J = 3.8, 1-H); ^{13}C NMR ($CDCl_3$) δ 20.7, 20.9, 26.3, 26.7, 62.7, 69.7 (d, J = 9), 82.6 (d, J = 28), 84.2 (d, J = 32), 94.1 (d, J = 182), 105.1, 114.0, 170.1, 170.5; ^{19}F NMR ($CDCl_3$) δ −187.7 (ddd, J = 50.8, 25.0, 14.5); high-resolution FABMS calcd for $C_{13}H_{20}FO_7$ $[M + H]^+$ 307.1193, found *m*/*z* 307.1181.

5,6-Di-*O*-acetyl-3-deoxy-3-fluoro-α,β-D-galactofuranose (21). A solution of **20** (5.0 g, 16 mmol) in 80% aqueous acetic acid (100 mL) was kept at 75 °C for 6 h. At the end of the incubation, solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel column which was eluted with EtOAc/hexanes (1:1). The desired product was isolated as a mixture of α and β anomers in 71% total yield (3.1 g). Spectral data for this mixture: ^{1}H NMR ($CDCl_3$) δ 2.05 (3H, s), 2.06 (3H, s), 2.10 (3H, s), 2.11 (3H, s), 4.08−4.20 (3H, m), 4.25−4.35 (4H, m), 4.48 (1H, ddd, J = 24.0, 4.5, 3.5, 4-H), 4.79 (1H, ddd, J = 52.5, 3.5, 1.5, 3-H), 4.95 (1H, dt, J = 54, 4.5, 3-H), 5.26−5.37 (4H, m); ^{13}C NMR ($CDCl_3$) δ 20.70, 20.72, 20.9, 21.0, 62.4, 62.6, 70.2 (d, J = 6), 71.6 (d, J = 7), 75.3 (d, J = 23), 78.5 (d, J = 27), 79.3 (d, J = 24), 80.9 (d, J = 28), 96.5 (d, J = 7), 96.8 (d, J = 184), 97.0 (d, J = 185), 102.4 (d, J = 4), 171.0, 171.2, 171.31, 171.33; ^{19}F NMR ($CDCl_3$) δ −194.4 (ddd, J = 53.6, 23.4, 18.9), −186.2 (ddd, J = 52.8, 24.0, 15.2); high-resolution CIMS calcd for $C_{10}H_{19}FNO_7$ $[M + NH_4]^+$ 284.1146, found *m*/*z* 284.1169.

1,2,5,6-Tetra-*O*-acetyl-3-deoxy-3-fluoro-α,β-D-galactofuranose (22). A solution of **21** (3.1 g, 12 mmol, a mixture of α and β anomers) in pyridine (10 mL) and acetic anhydride (10 mL) was stirred at room temperature for 2 h. Removal of solvent to dryness under reduced pressure gave the desired product **22** as a mixture of α and β anomers in quantitative yield (3.78 g). This mixture was pure enough to be used in the next step of synthesis without purification: ^{1}H NMR ($CDCl_3$) δ 2.08 (3H, s), 2.09 (3H, s), 2.10 (3H, s), 2.12 (3H, s), 2.13 (9H, s), 2.15 (3H, s), 4.13 (1H, dd, J = 12.5, 6.0), 4.19 (1H, dd, J = 11.5, 7.0), 4.29 (1H, ddd, J = 20.5, 7.5, 5.5), 4.34 (1H, dd, J = 12.0, 4.0), 4,35 (1H, dd, J = 12.0, 3.5), 4.52 (1H, dt, J = 23.5, 4.0), 4.93 (1H, ddt, J = 51.3, 4.2, 0.9), 5.21 (1H, ddd, J = 56.0, 6.5, 5.5), 5.25−5.40 (4H, m), 6.21 (1H, s), 6.36 (1H, d, J = 4.5); ^{13}C NMR ($CDCl_3$) δ 20.4, 20.60, 20.66, 20.69, 20.79, 20.81, 21.0 (2C), 62.0, 62.2, 69.1 (d, J = 8), 70.5 (d, J = 5), 75.6 (d, J = 23), 78.6 (d, J = 27), 80.0 (d, J = 29), 82.6 (d, J = 28), 92.9 (d, J = 10), 93.3 (d, J = 190), 94.8 (d, J = 187), 98.8 (d, J = 3), 169.0, 169.1, 169.2, 169.6, 170.0, 170.1, 170.5; ^{19}F NMR ($CDCl_3$) δ −199.4 (dt, J = 55.9, 21.2), −188.6 (ddd, J = 51.9, 24.0, 18.2); high-resolution FABMS calcd for $C_{14}H_{19}FO_9Na$ $[M + Na]^+$ 373.0911, found *m*/*z* 373.0924.

Dibenzyl (2,5,6-Tri-*O*-acetyl-3-deoxy-3-fluoro-α-D-galactofuranosyl)phosphate (23α) and Dibenzyl (2,5,6-Tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-galactofuranosyl)phosphate (23β). A solution of **22** (2.0 g, 5.7 mmol) in anhydrous dichloromethane (10 mL) was cooled with an ice−water bath. To this solution was slowly added bromotrimethylsilane (5 mL), and the mixture was stirred at room temperature for 18 h. The solution was evaporated to dryness under reduced pressure. Anhydrous toluene (5 mL) was then added and dried in vacuo to facilitate the evaporation of more volatile silane derivatives. The resulting dry residue and dibenzyl phosphate (2.4 g, 8.4 mmol) was redissolved in anhydrous toluene (10 mL). To this solution was added triethylamine (1.2 mL, 8.4 mmol), and the mixture was stirred overnight at room temperature. After removal of solvent in vacuo, the residue was chromatographed on a silica gel column which was eluted with EtOAc/hexanes (1:1). The fast-moving unreacted starting material (724 mg, 36%) was recovered. The α isomer of the desired product was eluted next from the column and was further purified on silica gel with $CHCl_3$/methanol (99:1) to give pure **23α** in 8% yield (252 mg). The next-eluted component was the β isomer of the desired product (**23β**) that was isolated in 17% yield (539 mg). Spectral data for the α isomer **23α**: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.96 (3H, s), 2.01 (3H, s), 2.05 (3H, s), 4.13 (1H, dd, J = 12.0, 6.5, 6-H), 4.32 (1H, dd, J = 12.0, 4.5, 6-H), 4.34 (1H, dt, J = 20.0, 7.0, 4-H), 5.04 (4H, d, J = 7.5, benzylic CH_2's), 5.15 (1H, dt, J = 56.5, 7.0, 3-H), 5.26 (1H, dddd, J = 21.0, 7.0, 4.5, 2.5, 2-H), 5.29 (1H, m, 5-H), 5.99 (1H, dd, J = 7.0, 4.5, 1-H), 7.34 (10H, m, Ar-H's); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.2, 20.6 (2C), 61.9, 69.4 (2C, d, J = 6), 69.8 (d, J = 3), 75.8 (dd, J = 23, 7), 78.6 (d, J = 26), 91.9 (d, J = 188), 97.0 (dd, J = 10, 5), 127.9 (2C), 128.0 (2C), 128.61 (2C), 128.63 (3C), 128.7, 135.5 (d, J = 7), 135.6 (d, J = 7), 169.8, 170.1, 170.4; ^{31}P NMR ($CDCl_3$) δ −2.5; ^{19}F NMR ($CDCl_3$) δ −203.7 (dt, J = 56.4, 20.3); high-resolution FABMS calcd for $C_{26}H_{31}FO_{11}P$ $[M + H]^+$ 569.1588, found *m*/*z* 569.1633. Spectral data for the β isomer **23β**: ^{1}H NMR ($CDCl_3$) δ 2.02 (3H, s), 2.07 (3H, s), 2.11 (3H, s), 4.10 (1H, dd, J = 12.0, 6.9, 6-H), 4.24 (1H, dd, J = 12.0, 4.0, 6-H), 4.49 (1H, dt, J = 24.2, 4.0, 4-H), 4.89 (1H, dd, J = 51.6, 4.0, 3-H), 5.07 (2H, d, J = 8.0, benzylic CH_2), 5.08 (2H,

(13) (a) Brimacombe, J. S.; Foster, A. B.; Hem, R.; Westwood, J. H.; Hall, L. D. *Can. J. Chem.* **1970**, *48*, 3946−3952. (b) Kovac, P.; Glaudemans, C. P. J. *Carbohydr. Res.* **1983**, *123*, 326−331.

d, $J = 8.2$, benzylic CH_2), 5.22 (1H, d, $J = 15.0$, 2-H), 5.33 (1H, dt, $J = 6.9$, 4.0, 5-H), 5.84 (1H, d, $J = 5.2$, 1-H), 7.35 (10H, m, Ar-H's); ^{13}C NMR ($CDCl_3$) δ 20.6, 20.7, 20.8, 62.1, 69.0 (d, $J = 6$), 69.5 (d, $J = 6$), 69.7 (d, $J = 5$), 80.7 (dd, $J = 29$, 11), 82.8 (d, $J = 29$), 94.4 (d, $J = 187$), 102.6 (dd, $J = 5$, 3), 127.9 (2C), 128.1 (2C), 128.6 (4C), 128.7 (2C), 135.4 (d, $J = 7$), 135.5 (d, $J = 7$), 169.1, 170.0, 170.4; ^{31}P NMR ($CDCl_3$) δ −3.0; ^{19}F NMR ($CDCl_3$) δ −180.3 (ddd, $J = 51.4$, 24.3, 15.8); high-resolution FABMS calcd for $C_{26}H_{31}FO_{11}P$ $[M + H]^+$ 569.1588, found *m*/*z* 569.1613.

Triethylammonium (2,5,6-Tri-*O*-acetyl-3-deoxy-3-fluoro-α-D-galactofuranosyl)phosphate (24). A solution of **23α** (240 mg, 0.42 mmol) in ethyl acetate (5 mL) and triethylamine (0.4 mL) was stirred overnight at room temperature under a hydrogen atmosphere in the presence of Pd/C (10%). The mixture was filtered through a Celite pad, and the filtrate was evaporated to dryness under reduced pressure to give **24** in 96% yield (198 mg): ^{1}H NMR ($CDCl_3$) δ 1.20 (9H, t, $J = 7.2$), 1.94 (3H, s), 2.02 (3H, s), 2.03 (3H, s), 2.95 (6H, q, $J = 7.2$), 4.09 (1H, dd, $J = 12.0$, 6.0, H-6), 4.13 (1H, dt, $J = 20.7$, 6.0, 4-H), 4.32 (1H, dd, $J = 12.0$, 3.9, 6-H), 5.02−5.30 (3H, m, 2-, 3-, 5-H's), 5.72 (1H, dd, $J = 7.2$, 3.8, 1-H); ^{13}C NMR ($CDCl_3$) δ 8.4, 20.7 (2C), 20.9, 45.3, 62.0, 70.9 (d, $J = 3$), 75.8 (dd, $J = 21$, 6), 77.3 (d, $J = 26$), 93.0 (d, $J = 187$), 95.2 (dd, $J = 10$, 4), 170.3, 170.47, 170.49; ^{19}F NMR ($CDCl_3$) δ −201.9 (dt, $J = 57.0$, 19.2); ^{31}P NMR ($CDCl_3$) δ −0.33; high-resolution FABMS calcd for $C_{12}H_{18}FO_{11}PNa$ $[M + Na]^+$ 411.0468, found *m*/*z* 411.0472.

Bis(triethylammonium) (3-Deoxy-3-fluoro-α-D-galactofuranosyl)-phosphate (25). A solution of **24** (310 mg, 0.63 mmol) in 30 mL of a mixture of methanol/water/triethylamine (5:2:1) was stirred at room temperature for 3 days until TLC (ethanol:NH_4OH:H_2O = 5:3:1) indicated that the reaction was complete. The mixture was evaporated to dryness under reduced pressure, and the desired product was obtained in quantitative yield (290 mg): ^{1}H NMR (D_2O) δ 1.30 (18H, t, $J = 7.2$), 3.22 (12H, q, $J = 7.2$), 3.67 (1H, dd, $J = 12.0$, 6.6, 6-H), 3.75 (1H, dd, $J = 12.0$, 4.5, 6-H), 3.86 (1H, td, $J = 6.6$, 4.5, 5-H), 4.14 (1H, dt, $J = 21.0$, 6.6, 4-H), 4.51 (1H, dddd, $J = 22.2$, 6.6, 4.7, 1.2, 2-H), 5.14 (1H, dt, $J = 57.3$, 6.6, 3-H), 5.59 (1H, t, $J = 4.7$, 1-H); ^{13}C NMR (D_2O) δ 9.7, 47.8, 63.3, 73.2 (d, $J = 3$), 77.0 (dd, $J = 21$, 7), 81.3 (d, $J = 25$), 97.9 (d, $J = 182$), 98.1 (dd, $J = 12$, 6); ^{19}F NMR (D_2O) δ −202.8 (dt, $J = 57.3$, 22.6); ^{31}P NMR (D_2O) δ 0.87; high-resolution FABMS calcd for $C_{12}H_{28}FNO_8P$ $[M + Et_3N + H]^+$ 364.1537, found *m*/*z* 364.1533.

Bis(triethylammonium) UDP-[3-F]Gal*f* (10). Preparation of **10** followed a procedure analogous to that described for the synthesis of **9**. The reaction mixture contained **25** (270 mg, 0.58 mmol), UMP-morpholidate (1.0 g, 1.45 mmol), and 1*H*-tetrazole (135 mg, 1.89 mmol) in 9 mL of anhydrous pyridine. The resulting solution was stirred at room temperature for 30 h. Removal of the solvent under vacuum gave a solid residue which was chromatographed on a Sephadex LH-20 column (2.5 5 120 cm) using methanol as the eluent. The crude **10** was further purified by HPLC (C_{18} column, eluted with 1% acetonitrile in 50 mM triethylammonium acetate buffer, pH 6.8, flow rate 5.0 mL/min). The retention time of **10** under these conditions was 19.5 min, and the yield was 15% (32 mg): ^{1}H NMR (D_2O) δ 1.31 (18H, t, $J = 7.2$, Et_3N-*Me*), 3.24 (12H, q, $J = 7.2$, Et_3N-CH_2), 3.68 (1H, dd, $J = 12.0$, 6.5, 6-H), 3.76 (1H, dd, $J = 12.0$, 4.0, 6-H), 3.87 (1H, td, $J = 6.5$, 4.0, 5-H), 4.17 (1H, dt, $J = 20.5$, 6.5, 4-H), 4.22−4.29 (2H, m, 5′-H_2), 4.31 (1H, s, br, 4′-H), 4.38−4.42 (2H, m, 2′-H, 3′-H), 4.56 (1H, dm, $J = 22.0$, 2-H), 5.14 (1H, dt, $J = 57$, 6.5, 3-H), 5.72 (1H, t, 5.0, 1-H), 5.99 (1H, d, $J = 8.0$, 5″-H), 6.01 (1H, d, $J = 5.0$, 1′-H), 7.99 (1H, d, $J = 8.0$, 6″-H); ^{13}C NMR (D_2O) δ 9.8 (Et_3N-*Me*), 48.2 (Et_3N-*C*H_2), 63.3 (C-6), 66.5 (d, $J = 5$, C-5′), 71.2 (C-2′), 73.7 (d, $J = 4$, C-5), 75.3 (C-3′), 76.9 (dd, $J = 21$, 7, C-2), 81.9 (d, $J = 25$, C-4), 84.8 (d, $J = 9$, C-4′), 89.9 (C-1′), 97.4 (d, $J = 182$, C-3), 99.4 (dd, $J = 12$, 6, C-1), 104.2 (C-5″), 143.2 (C-6″), 153.4 (C-2″), 167.8 (C-4″); ^{31}P NMR (D_2O) δ −10.9 (d, $J = 20$), −12.4 (d, $J = 20$); ^{19}F NMR (D_2O) δ −201.5 (dt, $J = 57.4$, 20.5); high-resolution negative ion FABMS calcd for $C_{15}H_{22}FN_2O_{16}P_2$ $[M - H]^-$ 567.0429, found *m*/*z* 567.0465.

Enzyme. UDP-galactopyranose mutase used in this study was purified from a recombinant strain of *Escherichia coli* BL21 (DE3)[10a] which contained an expression plasmid (pQZ1) harboring the encoding gene (*glf*)[6] for the mutase derived from *E. coli* C600 (ATCC 23724). The *E. coli* BL21 (DE3)/pQZ-1 was grown in Luria-Bertani (LB) broth supplemented with kanamycin (50 μg/mL) at 37 °C for 18 h with vigorous agitation. The cells were harvested, and the *C*-terminal His_6-tagged mutase was purified to near homogeneity using Ni-NTA resin (Qiagen) according to the protocols recommended by the manufacturer. Details of the cloning, expression, and purification of the enzyme had been described in an earlier report.[10a] The purified mutase (Glf) was found to be stable in phosphate buffer containing 15% glycerol. The yield was about 100 mg of the pure protein from 6 L of culture. The enzyme exhibits a typical unresolved flavin spectrum with absorbance maxima at 450 and 376 nm. A stoichiometry of 0.52 bound FAD per protein monomer was estimated by quantitation of FAD released from an enzyme sample of known concentration.

Protein Assay. Protein concentrations were routinely determined by the Bradford method[14] using bovine serum albumin as the standard. This assay was calibrated by comparing the results from quantitative amino acid analysis performed on aliquots of the same sample by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota.

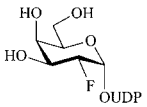
Activity Assay. Whether the mutase could catalyze the turnover of a substrate or be inhibited by an inhibitor was determined by incubating the substrate/inhibitor sample (1 mM) and an appropriate amount of the mutase in 30 μL of 100 mM potassium phosphate buffer (pH 7.6). For assays conducted under reducing conditions, 20 mM freshly prepared sodium dithionite was included in the mixture. The reaction was usually carried out at 37 °C for 2 min, and the resulting mixture was immediately frozen with liquid nitrogen to terminate the incubation. The reaction mixture was analyzed by HPLC using a C_{18} column (Microsorb-MV, Varian, 4.6 × 250 mm) which was eluted with 1.5% acetonitrile in 50 mM triethylammonium acetate buffer, pH 6.8. The detector was set at 262 nm, and the flow rate was 1.0 mL/min. The extent of conversion was determined by comparing the integration of the substrate and product peaks. The conversion was routinely controlled to be within 15% by properly adjusting the enzyme concentration.

Determination of Kinetic Parameters. The kinetic parameters of those compounds that could serve as substrates were determined by preparing a series of samples containing the purified mutase and the specific compound being tested in a total volume of 30 μL of 100 mM potassium phosphate (pH 7.5). The incubation mixture also contained 20 mM freshly prepared sodium dithionite. Each sample was incubated at 37 °C for 5 min and frozen with liquid nitrogen to terminate the reaction. The contents of these samples were analyzed by HPLC as described above, and the ratio of product and substrate peaks was used to determine the activity. The kinetic parameters were deduced by fitting these data to the Michaelis−Menten equation. For UDP-[2-F]Gal*f* (**9**), the incubation mixture contained 217 nM enzyme and one of the following concentrations of **9**: 0.083, 0.10, 0.125, 0.167, 0.25, 0.50, 1.0, and 2.0 mM. For UDP-[3-F]Gal*f* (**10**), the incubation mixtures contained 7.2 μM enzyme and one of the following concentrations of **10**: 0.20, 0.25, 0.30, 0.50, 0.70, 1.0, 1.5, 2.0, 3.0, 4.0, and 8.0 mM.

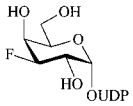
Production and Characterization of UDP-[2-F]Gal*p* (26). A mixture of UDP-[2-F]Gal*f* (**9**, 15 mg) and the purified mutase (6.3 mg) in 1 mL of 100 mM potassium phosphate buffer containing sodium dithionite (10 mg) was kept at room temperature for 16 h. HPLC analysis as described above indicated that the conversion was greater than 95%. The turnover product **26** was purified by an HPLC C_{18} semipreparative column eluted with 50 mM triethylammonium acetate buffer (pH 6.8) containing 1.5% acetonitrile. Lyophilization of the pooled HPLC fractions yielded 14 mg of **26**: ^{1}H NMR (D_2O, 500 MHz) δ 1.30 (18H, t, $J = 7.5$, Et_3N-*Me*), 3.22 (12H, q, $J = 7.5$, Et_3N-CH_2), 3.74 (1H, dd, $J = 12.0$, 4.5, 6-H), 3.78 (1H, dd, $J = 12.0$, 7.5, 6-H), 4.12 (1H, t, $J = 3.0$, 4-H), 4.17−4.34 (5H, m, 3-H, 5-H, 4′-H, 5′-H_2), 4.40 (2H, m, 2′-H, 3′-H), 4.72 (1H, ddt, $J = 49.0$, 9.5, 3.5, 2-H), 5.84 (1H, dd, $J = 7.5$, 3.5, 1-H), 6.01 (1H, d, $J = 8.0$, 5″-H), 6.02 (1H, d, $J = 4.5$, 1′-H), 8.00 (1H, d, $J = 8.0$, 6″-H); ^{13}C NMR (D_2O, 125 MHz) δ 9.8 (Et_3N-*Me*), 48.2 (Et_3N-*C*H_2), 62.3 (C-6), 66.5 (d, $J = 4$, C-5′), 69.2 (d, $J = 17$, C-3), 71.2 (d, $J = 9$, C-4), 71.3 (C-2′), 73.4 (C-5), 75.4 (C-3′), 85.0 (d, $J = 9$, C-4′), 89.7 (dd, $J = 184$, 8, C-2), 89.9

(14) Bradford, M. M. *Anal. Biochem*. **1976**, *72*, 248−254.

(C-1′), 94.4 (dd, J = 22, 6, C-1), 104.3 (C-5″), 143.3 (C-6″), 153.4 (C-2″), 167.8 (C-4″); ^{31}P NMR (D_2O) δ −11.1 (d, J = 20), −12.8 (d, J = 20); ^{19}F NMR (D_2O) δ −206.2 (ddd, J = 49.4, 12.1, 3.7); negative ion high-resolution ESIMS calcd for $C_{15}H_{22}FN_2O_{16}P_2Na$ [M − H]$^-$ 567.0429, found m/z 567.0416.



26



27

Production and Characterization of UDP-[3-F]Gal*p* (27). A mixture of UDP-[3-F]Gal*f* (**10**, 10 mg) and the purified mutase (7.1 mg) in 1.1 mL of 100 mM potassium phosphate buffer containing sodium dithionite (10 mg) was kept at room temperature overnight. The turnover product **27** was isolated by HPLC as described above for the purification of **26**. Lyophilization of the pooled HPLC fractions yielded 8 mg of the product: ^{1}H NMR (D_2O, 500 MHz) δ 1.30 (18H, t, J = 7.5, Et_3N-*Me*), 3.22 (12H, q, J = 7.5, Et_3N-CH_2), 3.77 (1H, dd, J = 12.0, 5.5, 6-H), 3.81 (1H, dd, J = 12.0, 7.0, 6-H), 4.12 (1H, tt, J = 8.5, 3.5, 2-H), 4.19−4.36 (5H, m, 4-H, 5-H, 4′-H, 5′-H_2), 4.40 (2H, m, 2′-H, 3′-H), 4.84 (1H, ddd, J = 49.5, 8.5, 3.5, 3-H), 5.72 (1H, dt, J = 7.0, 3.5, 1-H), 5.99 (1H, d, J = 8.5, 5″-H), 6.01 (1H, d, J = 3.5, 1′-H), 7.98 (1H, d, J = 8.5, 6″-H); ^{13}C NMR (D_2O, 125 MHz) δ 9.8 (Et_3N-*Me*), 48.2 (Et_3N-CH_2), 62.2 (C-6), 66.5 (d, J = 6, C-5′), 68.6 (dd, J = 19, 9, C-2), 68.9 (d, J = 17, C-4), 71.2 (C-2′), 73.9 (d, J = 6, C-5), 75.4 (C-3′), 84.8 (d, J = 9, C-4′), 91.7 (C-1′), 92.5 (d, J = 181, C-3), 97.4 (dd, J = 11, 7, C-1), 104.3 (C-5″), 143.2 (C-6″), 153.7 (C-2″), 168.2 (C-4″); ^{31}P NMR (D_2O) δ −10.5 (d, J = 21), −12.2 (d, J = 21); ^{19}F NMR (D_2O) δ −201.6 (doublet of quintet, J = 49.4, 6.2); negative ion high-resolution ESIMS calcd for $C_{15}H_{22}FN_2O_{16}P_2Na$ [M − H]$^-$ 567.0429, found m/z 567.0456.

Equilibrium Constants. The equilibrium constant, K_{eq}, was calculated from the ratio of product to substrate at equilibrium. The ratios were determined for both the forward and reverse directions by integration of the corresponding peaks from HPLC chromatograms. Specifically, a 200 μL incubation mixture containing 65 μM mutase, 20 mM sodium dithionite, and 1 mM UDP-[2-F]Gal*f* (**9**) or UDP-[3-F]Gal*f* (**10**) in 100 mM potassium phosphate buffer (pH 7.6) was allowed to reach equilibrium, as determined by the constant product/substrate ratio by HPLC. The same procedure was also repeated with a 200 μL incubation of 65 μM enzyme and 1 mM UDP-[2-F]Gal*p* (**26**) or UDP-[3-F]Gal*p* (**27**) in 100 mM potassium phosphate buffer (pH 7.6) containing 20 mM sodium dithionite.

Positional Isotope Exchange (PIX) Experiments. The labeled UDP-[1-^{13}C,^{18}O]Gal*p* used in these experiments was prepared according to a known procedure.[9,15] The desired product was purified by reversed-phase HPLC as described in the preparation of **9** and **10** and was verified by ^{13}C NMR and mass spectroscopy. The m/z 568.0594 determined by the high-resolution negative ion ESIMS matches well with [M − H]$^-$ 568.0554 calculated for $C_{14}{}^{13}CH_{23}N_2O_{16}{}^{18}OP_2$. For the experiments performed with native enzyme, 10 μL of UDP-Gal*p* mutase (3 μM final concentration) was added to a solution of UDP-[1-^{13}C,^{18}O]Gal*p* (10 mM) in 100 mM potassium phosphate buffer (pH 7.6) prepared with D_2O. The reaction was conducted in an NMR tube, and the total volume of the incubation mixture was 610 μL. The ^{13}C NMR spectra of the resulting sample were recorded before the addition of mutase and at 10, 40, 80, 180, and 300 min after the addition of mutase. For the experiments performed with reduced enzyme, the same conditions were employed except that the substrate concentration was 16 mM and freshly prepared sodium dithionite (20 mM) was included in the buffer. Again, ^{13}C NMR spectra were recorded before the addition of enzyme and at 5, 10, 20, and 40 min after the addition of mutase.

Inhibition of UDP-Galactopyranose Mutase by Fluorinated Analogues. To test whether these fluorinated analogues are inhibitors for the mutase, compounds **9**, **10**, **26**, and **27** (1 mM each) were individually incubated with native UDP-Gal*p* mutase (2 μM) on ice for 1 h in 24 μL of 100 mM potassium phosphate buffer (pH 7.6). The residual enzyme activity was determined as described in the section, Activity Assay. For the protection experiments, compounds **10** and **27** (1 mM each) were individually included in the assay mixture containing UDP-Gal*f* (**2**, 1 mM) as the substrate. Either native UDP-Gal*p* mutase (2.1 μM) or reduced enzyme (53 nM) was added to the mixture, and the activity was determined as described.

Kinetic Analysis of Inactivation of UDP-Galactopyranose Mutase by UDP-[3-F]Gal*f*. In a typical inactivation experiment, an appropriate amount of UDP-[3-F]Gal*f* (**10**) was incubated with mutase (15 μM) in 30 μL of 50 mM potassium phosphate buffer, pH 7.6, at 25 °C. At various time intervals, 3 μL aliquots of the incubation mixture were added to the standard assay cocktail (27 μL) to initiate the reaction. The residual enzyme activity was determined as described in the section, Activity Assay.

Test for Reversibility of the Inactivation by UDP-[3-F]Gal*f*. A sample containing UDP-[3-F]Gal*f* (**10**, 3 mM) and mutase (6 μM) was incubated for 1 h on ice. The inactivated enzyme was dialyzed against 300 mL of 50 mM potassium phosphate buffer containing 15% glycerol (pH 7.6) over 9 h with two changes of buffer. The residual enzyme activity was determined prior to and after the dialysis. A control experiment was also performed under identical conditions without the inactivator. It was noted that the activity of the control sample decreased significantly (>50%) when the dialysis was allowed to run for longer than 9 h.

Results and Discussion

The fluorine-containing sugars, while none are naturally occurring, have attracted much attention due to their high clinical potential as enzyme inhibitors/chemotherapeutic agents.[16] Two fluorinated galactofuranose derivatives, UDP-[2-F]Gal*f* (**9**) and UDP-[3-F]Gal*f* (**10**), were synthesized in this work and used as probes to investigate the catalytic mechanism of UDP-Gal*p* mutase.[17] Since the equilibrium of this enzymatic transformation greatly favors the pyranose over the furanose form (K_{eq} = 0.057),[10a,18] both probes were prepared as furanose sugars. The availability of these furanose derivatives had allowed the enzyme-catalyzed interconversion to be monitored in the reverse reaction (**2** → **1**), thus facilitating the kinetic and mechanistic analyses.

A number of factors were considered in the design rationales of **9** and **10**. First, UDP-Gal*p* mutases isolated from different sources are all flavoproteins. In light of the redox capability of this coenzyme, a mechanism relying on redox recycling of the flavin coenzyme as the driving force for catalysis is certainly feasible. Although failure to recognize the cleavage of the anomeric C−O bond as a requisite step in the interconversion of **1** and **2** has made the mechanism depicted in Scheme 1 invalid, a modified version, incorporating the anomeric C−O bond scission as part of the pathway (see Scheme 5), is still worth considering. Since oxidation of **1** at C-2 or C-3 of the substrate is a key step of this revised mechanism,[19] one of the fluorinated probes is not expected to be processed by the mutase. Instead, it may act as an inhibitor. Second, replacement of a hydroxy group with an isosteric and isoelectronic fluorine at loci adjacent to the anomeric center on the sugar substrate has been shown to slow the catalysis of glycosidases by destabilizing the corresponding carbocation transition states.[20] In our case,

(15) (a) Thiem, J.; Wiemann, T. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1163−1164. (b) Hayashi, T.; Murray, B. T.; Wang, R.; Wong, C.-H. *Bioorg. Med. Chem.* **1997**, *5*, 497−500. (c) Risley, J. M.; Van Etten, R. L. *Biochemistry* **1982**, *21*, 6360−6365.

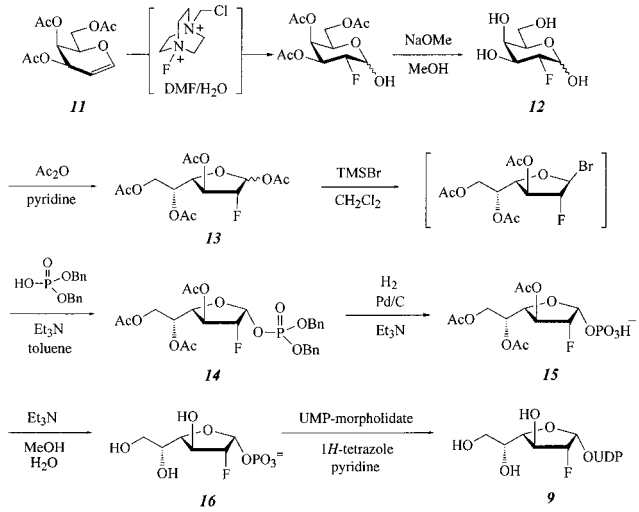
(16) (a) In *Fluorinated Carbohydrates, Chemical and Biochemical Aspects*; Taylor, N. F., Ed.; American Chemical Society: Washington, DC, 1988. (b) Tsuchiya, T. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 91−277.

(17) Burton, A.; Wyatt, P.; Boons, G.-J. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2375−2382.

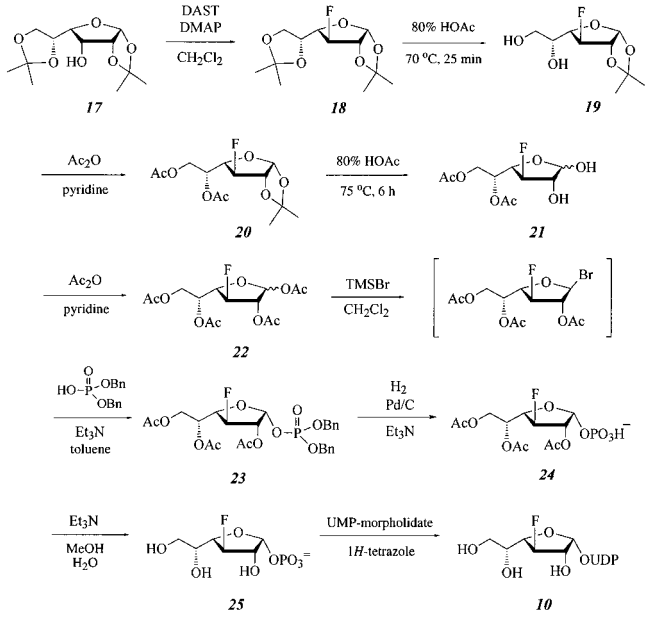
(18) Lee. R.; Monsey, D.; Weston, A.; Duncan, K.; Rithner, C.; McNeil, M. *Anal. Biochem.* **1996**, *242*, 1−7.

(19) It has been suggested that the reaction may proceed via a 2-keto intermediate; however, no detailed scheme has been proposed.[6a]

Scheme 3



Scheme 4



the fluorine group in **9** and **10** is expected to destabilize the putative oxocarbenium ion intermediates/transition states (**5** and **7**), if the mechanism shown in Scheme 2 is operative. The reduction of turnover rate may also allow the reaction intermediates (**5** and/or **7**) to react with enzyme active-site residues, resulting in enzyme inactivation. This strategy has been successfully applied to elucidate the mechanism of the glycosidase-catalyzed reaction which also involves cleavage of the anomeric C−O bond.[20] Thus, these fluorinated probes hold promise to provide insight into the mechanism of this enzyme.

Synthesis of UDP-[2-F]Gal*f* (9). The first key step for the synthesis of **9** was to convert the readily available 2-deoxy-2-fluoro-D-galactose (**12**)[12] to the thermodynamically less favored furanose derivative. Among various methods tested, the acetylation of **12** at elevated temperature gave the best results and led to tetraacetyl galactofuranose **13** (α:β = 1:2) in 28% yield. The prevalence of the trans configuration between the anomeric OH and the fluorine substituent at C-2 is characteristic for 2-deoxy-2-fluorofuranose.[21] As shown in Scheme 3, conversion of **13** to **14** was achieved using bromotrimethylsilane and then with triethylammonium dibenzyl phosphate. A large amount of **13** (65%) was recovered, and the β anomer in the recovered **13** was significantly enriched (α:β = 1:3.6). The lack of spin coupling between H-1 (δ 5.94, $J_{1,2}$ = 4.2 and J_{1H-P} = 6.0 Hz) and 2-F of **14** revealed in both ^{1}H and ^{19}F NMR spectra is consistent with an α-anomeric configuration. The appearance of C-1 at 96.4 ppm in ^{13}C NMR is also in good agreement with the assigned α configuration.[22]

Compound **14** was then debenzylated by catalytic hydrogenation in the presence of triethylamine to afford **15** as a monotriethylammonium salt in good yield. Deacetylation of **15** was readily achieved by treatment with methanol/water and triethylamine, providing 2-deoxy-2-fluoro-α-D-galactofuranosyl phosphate (**16**) as a bistriethylammonium salt in quantitative yield. The final product UDP-[2-F]Gal*f* (**9**) was prepared from **16** and uridine phosphomorpholidate in anhydrous pyridine in the presence of 1*H*-tetrazole. The reaction mixture was purified by size exclusion chromatography and reversed-phase HPLC under conditions similar to those used for the purification of UDP-Gal*f*.[10] The structure of the final product was confirmed by NMR (^{1}H, ^{13}C, ^{31}P, ^{19}F) and high-resolution FABMS. The large electronegativity of fluorine helps spread the ^{1}H NMR signals associated with the galactofuranose moiety over a wide range, thus facilitating the signal assignments by COSY. The purified product was found to be stable in aqueous solution at room temperature and neutral pH, since no decomposition was detected by ^{1}H NMR within 24 h.

Synthesis of UDP-[3-F]Gal*f* (10). Diisopropylidene-3-deoxy-3-fluoro-α-D-galactofuranose (**18**)[13] is converted, through a stepwise deprotection and protection, to tetraacetyl furanose **22** as an ~1:1 mixture of α,β anomeric isomers (Scheme 4). Upon treatment with bromotrimethylsilane, the mixture was partially converted to the corresponding tri-*O*-acetyl-3-deoxy-3-fluoro-D-galactofuranosyl bromide, which was sensitive to silica gel. Without purification, this relatively unstable compound was subjected to excess triethylammonium dibenzyl phosphate in toluene,[22] and the resulting mixture was separated on silica gel to give three main fractions. The fastest migrating material was found to be unreacted **22**. The second fraction contained dibenzyl (2,5,6-tri-*O*-acetyl-3-deoxy-3-fluoro-α-D-galactofuranosyl)phosphate (**23α**). Its H-1 signal appears as a doublet of doublets ($J_{1,2}$ = 4.5, $J_{H-1,P}$ = 7.0), with the $J_{1,2}$ value indicative of a cis relationship between 1-H and 2-H.[23] The major product of this reaction was found in the most polar fraction. On the basis of various spectral data, this product was assigned as dibenzyl (2,5,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-galactofuranosyl)phosphate (**23β**). The anomeric proton resonates at δ 5.84 as a doublet ($J_{H-1,P}$ = 5.2). Since no coupling exists between 1-H and 2-H, these protons must be trans to each other.[23] The downfield shift of the C-1 resonance (δ 102.6) as compared to that of a furanose with an α anomeric configuration is also typical for a β isomer. Having the key intermediate **23α** in hand, the synthesis of the target compound was completed via intermediates **24** and **25**, by a sequence similar to that used for the preparation of UDP-[2-F]Gal*f* (**9**). Pure UDP-[3-F]Gal*f* (**10**) was

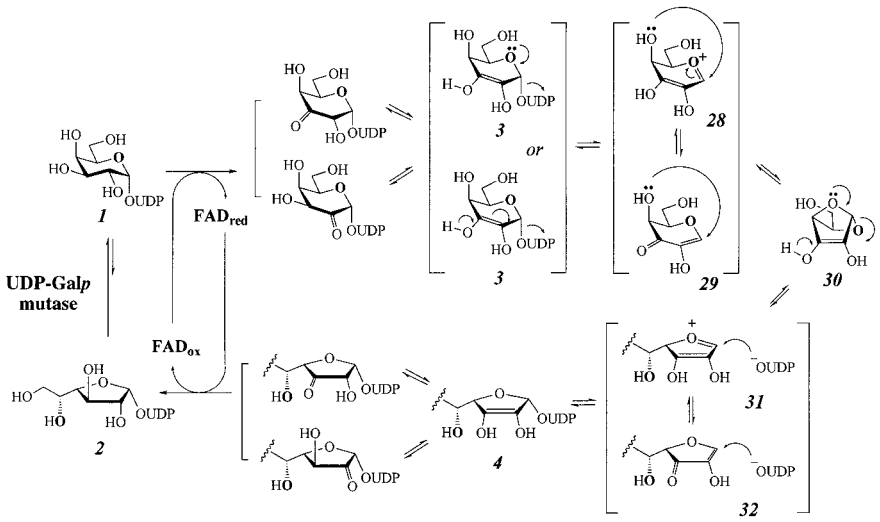
(20) (a) Withers, S. G. *Can. J. Chem.* **1999**, *77*, 1−11. (b) Zechel, D. L.; Withers, S. G. In *Comprehensive Chemistry of Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O.; Eds.; Pergamon: New York, 1999; Vol. 5, pp 279−314 and references therein.

(21) (a) McAtee, J. J.; Schinazi, R. F.; Liotta, D. C. *J. Org. Chem.* **1998**, *63*, 2161−2167. (b) Niihata, S.; Ebata, T.; Kawakami, H.; Matsushida, H. *Bull. Chem. Soc. Jpn.* **1995**, *65*, 1509−1512.

(22) (a) de Lederkremer, R. M.; Nahmad, V. B.; Varela, O. *J. Org. Chem.* **1994**, *59*, 690−692. (b) Tsvetkov, Y. E.; Nikolaev, A. V. *J. Chem. Soc., Perkin Trans. 1* **2000**, 889−891.

(23) Bundle, D. R.; Lemieux, R. U. *Methods Carbohydr. Chem.* **1976**, *7*, 79−86.

Scheme 5



obtained as a bistriethylammonium salt and was found to be stable at room temperature and neutral pH in aqueous solution.

Incubation of UDP-[2-F]Gal*f* and UDP-[3-F]Gal*f* with Reduced UDP-Mutase. When UDP-[2-F]Gal*f* (**9**, 3 mM) was incubated with UDP-Gal*p* mutase (70 μM) in the presence of 20 mM sodium dithionite in a closed vial at 37 °C, over 50% of the substrate was converted to a new product within 5 min, as indicated by HPLC. The retention time for **9** on the C_{18} column was 15.3 min, and that for the new product was 9.3 min. Similar results were also found when UDP-[3-F]Gal*f* (**10**, 3 mM) was incubated with the reduced mutase. The retention times for **10** and its turnover product were 25.2 and 8.5 min, respectively. These products were purified and identified as UDP-[2-F]Gal*p* (**26**) from **9** and UDP-[3-F]Gal*p* (**27**) from **10** by NMR (^{1}H, ^{13}C, ^{31}P, ^{19}F, and COSY) and high-resolution MS. Since the fluorine substituent is chemically inert, the fact that both **9** and **10** can be recognized and processed by the *E. coli* mutase clearly indicates that mechanisms (such as Scheme 5) initiated by the oxidation of 2- or 3-OH during the interconversion of UDP-Gal*p* and UDP-Gal*f* can now be ruled out. This is also consistent with our early conclusion that FAD is not directly involved in the catalysis of UDP-Gal*p* mutase.[10a] It is important to note that a similar approach has been used in a recent report to study the catalysis of mutase from *Klebsiella pneumoniae*.[8] On the basis of the observation that both chemically synthesized **26** and **27** are substrates for the native *Klebsiella* enzyme, a conclusion debunking the involvement of redox chemistry in the mechanism of this enzyme was also reached.

Equilibrium Constants. The reaction catalyzed by UDP-Gal*p* mutase is a reversible process with a K_{eq} value of 0.057 for the conversion of **1** to **2** at 37 °C.[10a] To determine the equilibrium constants for the interconversion of the fluorinated analogues, separate reactions containing each of the furanose and the pyranose analogue were allowed to equilibrate at 37 °C. The reaction was monitored by HPLC until a constant product/substrate ratio was reached. Under these conditions, the equilibrium constants for the forward reaction (**26** to **9**, and **27** to **10**) were determined to be 0.020 and 0.011 for the 2- and 3-fluorinated analogues, respectively. The replacement of a hydroxy group at C-2 or C-3 of the galactose moiety with a fluorine substituent apparently enhances the thermodynamic preference for the pyranose over the furanose form by 0.65 and 1.0 kcal/mol, respectively. The enhanced preference for the pyranose form has also been observed in D-ribose when its 2-hydroxy group is replaced with a flourine.[24]

Table 1. Kinetic Parameters for Reduced UDP-Galactopyranose Mutase

substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m	ratio
UDP-Gal*f* (**2**)	22	27	1.2273	1
UDP-[2-F]Gal*f* (**9**)	65	0.033	0.00051	4.1×10^{-4}
UDP-[3-F]Gal*f* (**10**)	861	5.7	0.0066	5.4×10^{-3}

Kinetic Properties. The kinetic parameters for turnover of **9** and **10** in the reverse direction under reducing conditions were determined by fitting the initial reaction rates to the Michaelis−Menten equation based on nonlinear regression. The K_m values were determined to be 65 and 861 μM for UDP-[2-F]Gal*f* (**9**) and UDP-[3-F]Gal*f* (**10**), respectively. The corresponding k_{cat} values were estimated to be 0.033 and 5.7 s^{-1} (Table 1). In comparison to that of UDP-Gal*f* (**2**), the catalytic efficiencies (k_{cat}/K_m) for UDP-[2-F]Gal*f* and UDP-[3-F]Gal*f* have decreased by approximately 4 and 3 orders of magnitude, respectively. Since the K_m values for UDP-[2-F]Gal*f* (**9**) and UDP-Gal*f* (**2**) are comparable, the substitution of 2-OH with a fluorine appears to have little effect on the interactions in the Michaelis complex. In contrast, the large K_m value for UDP-[3-F]Gal*f* (**10**) implicates the significance of the 3-hydroxy group of the galactose in binding to the reduced enzyme to form the Michaelis complex. Replacing the 3-OH with a fluorine atom may have altered the hydrogen-bonding network essential for the interactions in the Michaelis complex.

As indicated by the k_{cat} values, both **9** and **10** are poorer substrates than UDP-Gal*f* (**2**). The rate reduction is especially profound for **9**, whose fluorine substituent is immediately adjacent to the anomeric center. Such rate retardation is likely due to destabilization of the oxocarbenium ion intermediates or transition states by the electron-withdrawing fluorine group. This inductive effect is expected to be distance-dependent and should result in suppression of the anomeric C−O bond cleavage. Precedents of similar findings are well documented in the glycosidase-catalyzed hydrolysis of pyranosides containing fluorine substituent at C-2 or C-5.[20] Thus, the above results support a mechanism for the catalysis of UDP-Gal*p* mutase involving oxocarbenium ions, such as **5** and/or **7**, as intermediates and/or transition states (Scheme 2). The same argument has also been made for the *Klebsiella* enzyme.[8]

Positional Isotope Exchange (PIX) Catalyzed by Native and Reduced UDP-Gal*p* Mutase. As mentioned earlier, the

(24) Sanderson, P. N.; Sweatman, B. C.; Farrant, R. D.; Lindon, J. C. *Carbohydr. Res.* **1996**, *284*, 51−60.

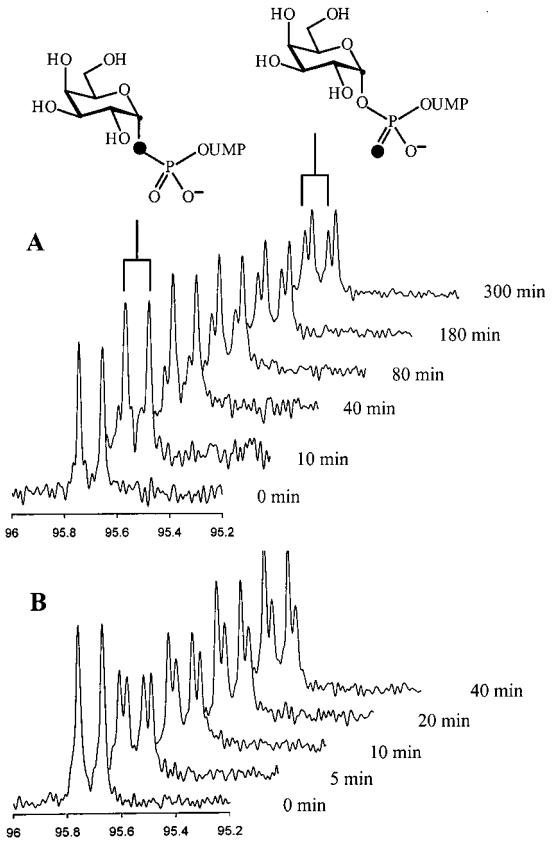


Figure 1. Positional isotope exchange catalyzed by (A) native UDP-galactopyranose mutase and (B) reduced UDP-galactopyranose mutase. Darken atoms indicate ^{18}O labels. See Experimental Section for detailed conditions.

cleavage of the anomeric C−O bond of **1** during catalysis had been demonstrated in a PIX experiment using mutase isolated from *K. pneumoniae*.[9] To confirm that the same pathway is also followed by the *E. coli* enzyme and to investigate if the mechanism remains unaltered for the native and reduced enzymes, the same PIX experiments were carried out with both the native and reduced enzymes. The required doubly labeled UDP-Gal*p* (99% ^{13}C at C-1 and >90% ^{18}O at O_1) was prepared enzymatically as described in the literature.[9,15] This labeled compound and native mutase were incubated at room temperature and analyzed by ^{13}C NMR. The C-1 signal of the labeled UDP-Gal*p* appeared as a doublet (due to its coupling to ^{31}P) at time 0 in the proton-decoupled ^{13}C NMR spectra. As illustrated in Figure 1, a new set of doublets at downfield became visible as incubation continued. The diminishing of the intensity of the original doublet occurred concurrently with the increase of the downfield doublet and was a result of scrambling of the ^{18}O from O_1 to the two nonbridging diphosphate oxygen positions. Therefore, similar to the mechanism of the *K. pneumoniae* enzyme, cleavage of the anomeric C−O bond must also take place during the catalysis of the *E. coli* mutase. Analogous results were also noted when dithionite was included in the reaction mixture, but with greatly enhanced efficiency (Figure 1B). These findings firmly established that the reaction catalyzed by the native as well as the reduced enzymes proceeds through the same mechanism which involves the cleavage of the anomeric C−O bond.

Inhibition of Mutase by Fluorinated Analogues. While compounds **9** and **10** are substrates for the reduced *E. coli* mutase, surprisingly, no turnover products could be detected when **9** and **10** (3 mM each) were incubated with native UDP-Gal*p* mutase (70 μM) at 37 °C for 20 min. Since the equilibrium for this catalysis heavily favors the conversion of furanose to pyranose, the pyranose derivatives, **26** and **27**, are not expected to be substrates under the same conditions. To test whether these fluorinated analogues are inhibitors for the mutase, compounds **9**, **10**, **26**, and **27** (1 mM each) were individually incubated with native UDP-Gal*p* mutase (2 μM) on ice for 1 h, and the resulting mixtures were assayed for their capabilities to catalyze the turnover of UDP-Gal*f* (**2**). As shown in Table 2, all four analogues demonstrated varied inhibitory effects against the native enzyme. It is clear that the 3-F compounds are better inhibitors than the corresponding 2-F analogues, with UDP-[3-F]Gal*f* (**10**) being the most potent inhibitor. Greater than 98% of the enzyme activity was lost when the mutase was pretreated with **10** prior to activity assay. However, the inhibition was less effective when UDP-[3-F]Gal*f* (**10**) or UDP-[3-F]Gal*p* (**27**) (1 mM each) was included in the assay mixture containing UDP-Gal*f* (**2**, 1 mM) as the substrate. Our data showed that the mutase was 81% active in the presence of equimolar amounts of **27** and **2** and retained 13% of its activity upon incubation with equimolar amounts of **10** and **2**. Since the normal substrate **2** can protect the enzyme against **10** and **27**, as demonstrated by the competition assays, the inactivation of UDP-Gal*p* mutase by these flurorinated analogues is most likely active-site-directed.

Table 2. Inhibition of UDP-Galactopyranose Mutase by Fluorinated Analogues[a]

inhibitors	**9**	**10**	**26**	**27**
residual activity of native enzyme preincubated with inhibitor (%)[b]	39	1.4	46	29
residual activity of native enzyme (%)[c]	nd[e]	13	nd	81
residual activity of reduced enzyme (%)[d]	nd	90	nd	94

[a] See Experimental Section for details. [b] Native enzyme (2 μM) was preincubated with inhibitor (1 mM) for 1 h on ice, and the residual activity was determined using **2** as the substrate (1 mM) as described in the section, Activity Assay. [c] Native enzyme (2.1 μM) was added to a mixture of substrate (**2**, 1 mM) and inhibitor (1 mM), and the residual activity was determined as described in the section, Activity Assay. [d] Native enzyme (53 nM) was added to a mixture of substrate (**2**, 1 mM) and inhibitor (1 mM) containing dithionite (20 mM), and the residual activity was determined as described in the section, Activity Assay. [e] Not determined.

Kinetic Study of Inactivation of UDP-Gal*p* Mutase by UDP-[3-F]Gal*f*. Since UDP-[3-F]-Gal*f* (**10**) is the most potent inhibitor in this series, it was selected for further analysis. As illustrated in Figure 2, the activity of this mutase decreased, in a time-dependent fashion, by nearly 80% after 20 min of incubation with **10**. Specifically, the residual enzyme activities were determined to be 26%, 18%, 3.4%, and 1.5% after preincubation at room temperature for 30 min with 0.05, 0.1, 0.5, and 1.0 mM **10**, respectively. The kinetic parameters of the inhibition of **10** on the activity of UDP-Gal*p* mutase were estimated by analyzing the plot of k_{obs} versus inhibitor concentration as depicted in Figure 3. Figure 3A shows a plot of the natural log of the fraction of remaining enzyme activity versus time at various concentrations of **10**. The values of k_{obs} as determined from the slopes of the individual lines were double-reciprocally plotted against the concentrations of **10**, as shown in Figure 3B. The K_I and k_{inact} of the inactivation were determined from Figure 3B and have values of 270 μM and 0.19 min^{-1}. Although the rate of inactivation is slow, the K_I is close to the K_m value (194 μM) found for UDP-Gal*f* (**2**) under the same conditions.[10a] Thus, UDP-[3-F]Gal*f* (**10**) appears to fit well in the active site of native mutase.

Irreversibility of the Inactivation of Mutase by UDP-[3-F]Gal*f*. It is important to note that the inactivation of the

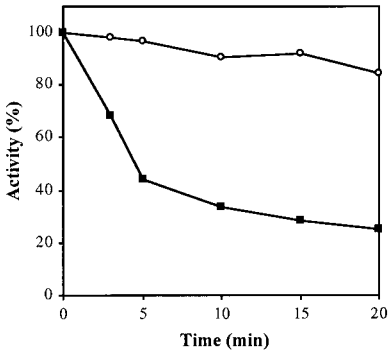


Figure 2. Time-dependent inactivation of UDP-galactopyranose mutase by UDP-[3-F]Gal*f* (**10**) (○, control; ■, with UDP-[3-F]Gal*f*). UDP-galactopyranose mutase (15 μM) was incubated with UDP-[3-F]Gal*f* (**10**, 200 μM) in a reaction volume of 30 μL at room temperature. At the indicated times, aliquots of the reaction were taken out, diluted 10-fold, and assayed as described in the Experimental Section. The control reaction had an equivalent volume of buffer instead of UDP-[3-F]Gal*f*.

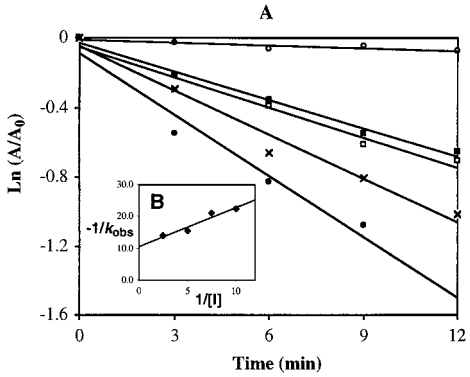
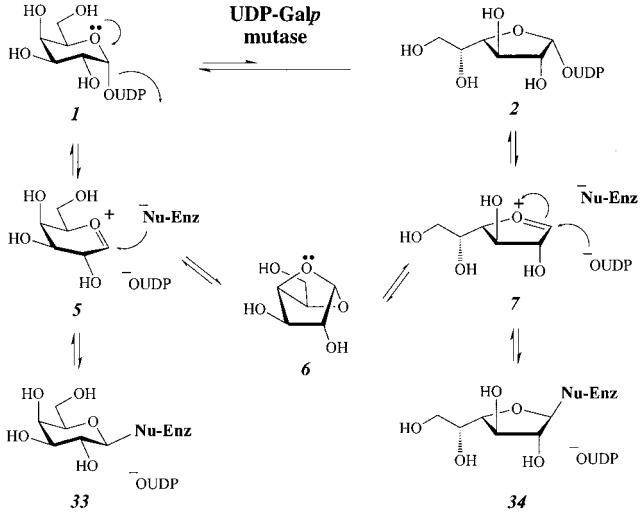


Figure 3. (A) Time- and concentration-dependent inactivation of UDP-galactopyranose mutase by UDP-[3-F]Gal*f* (**10**) (○, 0 μM UDP-[3-F]-Gal*f*; ■, 100 μM; □, 133 μM; ×, 200 μM; ●, 400 μM). UDP-galactopyranose mutase (15 μM) was incubated with the indicated amounts of UDP-[3-F]Gal*f* in a reaction volume of 30 μL at room temperature. At the indicated times, aliquots of the reaction were directly used to assay the activity of the enzyme. The values of k_{obs} were determined from the slopes of the linear fit for the inactivation data obtained with the respective concentration of UDP-[3-F]Gal*f*. (B) Plot of k_{obs} as a function of UDP-[3-F]Gal*f* concentration ([I]). The inset shows the double-reciprocal plot of k_{obs} versus UDP-[3-F]Gal*f* concentration. The data from plot B were used to calculate k_{inact} and K_I, which are reported in the text.

mutase by **10** is practically irreversible. An inactivated enzyme sample with 2% residual activity was found to remain inactive with little recovery of activity (5% residual activity) after extensive dialysis. Since the inactivation is time-dependent, irreversible, and active-site-directed, it likely results from the formation of a covalent adduct between the enzyme and the inactivator. While the above observations may also be ascribed to **10** being a tight binding inhibitor, such a scenario, however, is incompatible with the fact that K_I of **10** is greater than the K_m of the substrate **2**. Unfortunately, attempts to further characterize the inactivated enzyme by MS analysis led to ambiguous results. The inhibitor−enzyme adduct, if it indeed exists, may be labile under the sample preparation conditions. A strategy requiring less destructive treatment will have to be developed to fully analyze the inactivated enzyme in the future.

Reductive Reactivation of Inactivated Mutase. To our great surprise, upon treatment with dithionite, this inactivated enzyme could be fully reactivated, having activity restored to the same level as the reduced enzyme. As expected, when the mutase was incubated with UDP-[3-F]Gal*f* (**10**) or UDP-[3-F]Gal*p* (**27**) and an equivalent of **2** under reducing conditions (20 mM dithionite) for 2 min at 37 °C, little inhibition was observed. As shown in Table 2, the mutase retained greater than 90% of its activity in both cases. It is worth mentioning that compounds **10** and **27** are stable toward dithionite, and no turnover of **10** to **27** was discernible within the detection limit under the above experimental conditions. Thus, the very little inhibitory effect of **10** and **27** under the reducing state is likely a result of depleting the effective concentration of enzyme for the conversion of the normal substrate **2**, and consequently lowering the observed catalytic activity. Evidently, the reduced enzyme is immune to inactivation by **10**, and more significantly, the enzyme being inactivated by **10** can be reductively reactivated.

Scheme 6



Mechanistic Insights Derived from the Inactivation of Mutase by UDP-[3-F]Gal*f*. As mentioned earlier, the catalytic mechanism of UDP-Gal*p* mutase has been proposed to be initiated either by ring distortion to allow attack of O_4 on C-1 to release UDP, or by elimination of UDP first followed by O_4 attack on C-1 (Scheme 2).[9] In both cases, formation of oxocarbenium ions, such as **5** and/or **7**, as intermediates and/or transition states is postulated. The fact that the electron-withdrawing fluorine substituent reduces the k_{cat} of UDP-[2-F]-Gal*f* (**9**) more significantly than that of UDP-[3-F]Gal*f* (**10**) provides strong support for the proposed mechanism. While **9** and **10** are substrates for the reduced mutase, both are inhibitors for the native mutase. Since the inhibition likely involves covalent adduct formation, trapping an active-site nucleophile by the oxocarbenium type intermediates/transition states (**5**/**7**) to form stable adducts (Scheme 6, **33**/**34**) is a conceivable inactivation mechanism. Although the corresponding adduct (**33**/**34**) derived from **9** will be preferentially stabilized by the adjacent electron-withdrawing 2-F group, its formation via **5**/**7** should be considerably suppressed by the inductive effect of 2-F. Thus, it is not too surprising that the mutase was only partially inactivated after treatment with **9** for 60 min. In contrast, the suppression of the formation of oxocarbenium-type intermediates/transition states should be less significant in the case of **10**. Hence, complete inactivation was observed upon incubation with **10** under identical conditions.

The disparity on the response of the native and reduced enzyme toward the inactivation by **10** is intriguing. Early studies had shown that when the enzyme was reduced by dithionite, its catalytic efficiency was increased by more than 2 orders of magnitude.[10a] While both the FAD coenzyme and the cysteine

residues of this mutase were fully reduced under these conditions, the activity enhancement was demonstrated to be solely associated with the reduction of the flavin coenzyme. Reduction of FAD, which involves transformation of the coenzyme from a highly conjugated planar frame to a bent butterfly structure, may induce a conformational change within the enzyme that may become more conducive to catalysis.[25] Since the reduced flavin bears a higher electron density at N-1, this anionic species may be used to stabilize the transiently formed oxocarbenium intermediates/transition states to facilitate catalysis.[25] It is conceivable that the proposed conformational change resulting from reduction of the enzyme-bound FAD may also facilitate the collapse of the putative covalent enzyme−inhibitor adducts (**33**/**34**) to reactivate the enzyme. Likewise, the reduced FAD may play a role to prevent the formation of the covalent adduct or facilitate its breakdown by charge stabilization of the oxocarbenium intermediates/transition states (**5**/**7**). Clearly, more experiments are needed to address whether conformational effects, electronic effects, or a combination of both dictate the ability of reduced FAD to enhance the rate of the mutase reaction and to protect as well as to salvage it from the inactivation by **9** and **10**.

In summary, several enzymes that require flavin and yet catalyze reactions involving no net redox chemistry are known. Members of this group include chorismate synthase,[25] acetolactate synthase,[26] mandelonitrile lyase,[27] tartronate-semialdehyde synthase,[28] and YerE.[29] While the roles of the flavin in these enzymes are not well defined, reduction of the flavin coenzyme in several cases have been shown to have no effect[26,29] or to be detrimental.[28] In contrast, the unexpected increment of activity of UDP-Gal*p* mutase by the reduction of its FAD coenzyme makes this catalyst unique among members of this family.[30] It should be mentioned that the catalysis of chorismate synthase is an exception, since its flavin coenzyme in the reduced form has been demonstrated to be involved in the catalytic cycle, mediating a radical mechanism.[25] However, a similar pathway for UDP-Gal*p* mutase invoking radicals is unlikely as both the oxidized and reduced enzymes are active. Using two fluorinated UDP-galactofuranose analogues, UDP-[2-F]Gal*f* (**9**) and UDP-[3-F]Gal*f* (**10**), as mechanistic probes, we have now confirmed an active role played by the enzyme-bound flavin in the catalysis of UDP-Gal*p* mutase. Reduction of the FAD coenzyme in this mutase can not only enhance the catalytic efficiency of the mutase, but also protect the enzyme from being inactivated by **9** and **10**. Compound **10** is an effective inhibitor for the mutase, and the inactivation is reversible only under reduction conditions. Study of this inactivation has led to the discovery of possible involvement of a covalent intermediate in the mechanism of this mutase. It remains to be established whether the covalent adduct formation is catalytically relevant or is simply an incident of derail from the normal turnover.

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(25) (a) Kerwin, J. F., Jr.; Lancaster, J. R., Jr.; Feldman, P. L. *J. Med. Chem.* **1995**, *38*, 4343−4362. (b) Macheroux, P.; Schmid, J.; Amrhein, N.; Schaller, A. *Planta* **1999**, *207*, 325−334 and references therein.

(26) Chipman, D.; Barak, Z.; Schloss, J. V. *Biochim. Biophys. Acta* **1998**, *1385*, 401−419.

(27) Xu, L.-L.; Singh, B. K.; Conn, E. E. *Arch. Biochim. Biophys.* **1986**, *250*, 322−328.

(28) Cromartie, T. H.; Walsh, C. T. *J. Biol. Chem.* **1976**, *251*, 329−333.

(29) Chen. H.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **1998**, *120*, 11796−11797.

(30) A relevant example is NifL of *Azotobacter vinlandii*, a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via the conformational change of flavin oxidation reduction (Hill, S.; Austin, S.; Eydmann, T.; Jones, T.; Dixon, R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2143−2148).