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Chemo-Enzymatic Synthesis of Fluorinated Sugar Nucleotide: Useful Mechanistic Probes for Glycosyltransferases

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Abstract—An effective procedure for the synthesis of 2-deoxy-2-fluoro-sugar nucleotides via Selectfluor-mediated electrophilic fluorination of glycals with concurrent nucleophilic addition or chemo-enzymatic transformation has been developed, and the fluorinated sugar nucleotides have been used as probes for glycosyltransferases, including fucosyltransferase III, V, VI, and VII, and sialyl transferases. In general, these fluorinated sugar nucleotides act as competitive inhibitors versus sugar nucleotide substrates and form a tight complex with the glycosyltransferase.

Carbohydrates displayed on the cell surface mediate important molecular recognition processes including bacterial and viral infections, cell adhesion in inflammation and cancer metastasis, immune response, differentiation, fertilization and development.[1] These complex oligosaccharide structures are assembled through the action of a number of enzymes, predominately by a family of at least 100 glycosyltransferases which transfer the sugar moiety from an activated sugar nucleotide donor to an acceptor sugar.[2,3] Selective inhibition of these enzymes has received increasing attention for the promise of new therapeutics; however, the effort has been met with only limited success, and continuing studies are necessary for an understanding of their mechanisms before selective therapeutics can be developed. Among the many mechanistic probes developed for the study of glycosyltransfer enzymes, fluorinated carbohydrates represent the most commonly used.[4]

Fluorinated substrate analogues have shown historical utility in probing enzyme mechanism. In the studies of the prenyl transfer reaction with fluorinated allylic isoprenoid substrates,[5] the linear free energy results between model solvolysis reactions which proceed through established carbocationic intermediates and enzymatic reactions demonstrated a stepwise mechanism with carbocationic intermediate formation with farnesyl diphosphate synthase. Fluorinated substrate analogues have also been utilized in the study of carbohydrate-modifying enzymes.[6,7] The proposed transition-state structure of most carbohydrate-modifying enzymes, including glycosyltransferases and glycosidases, contains a flattened half-chair conformation with substantial oxocarbenium-ion character at the anomeric position of the glycon donor.[4,8,9] With regard to glycosidases, considerable structural, kinetic, and mechanistic data have been used to validate a general transition-state structure that contains discrete oxocarbenium-ion characteristics[4,10,11] and fluoroglycosides have been used effectively to probe the mechanism of these enzymes.[12–17] Replacement of the 2-OH or 5-H group of glycosyl analogues with the strong electron-withdrawing group fluorine transforms the parent glycoside into a slow substrate for retaining glycosidases, supporting the rate-determining exo-cyclic glycosidic cleavage mechanism. These compounds have been used as mechanism-based inactivators of retaining glycosidases that form a covalent adduct in the enzyme active site.

A similar oxocarbenium-ion transition-state structure has been supported for several glycosyltransferanes. It has been shown that β-1,4-galactosyltransferase (β1,4-GalT) displays a secondary isotope effect with UDP-[1-^{2}H]-galactose ($D_V = 1.21$, $D_{V/K} = 1.05$)[7] and that uridine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactose (UDP-2F-Gal) is a competitive inhibitor of this enzyme ($K_i = 2\,\mu M$).[18,19] More recently, GDP-[1-^{2}H]-fucose was shown to display a secondary isotope effect with α-1,3-fucosyltransferase V

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(FucT-V) ($D_V = 1.26$, $D_{V/K} = 1.22$) and that guanosine 5′-diphospho-2-deoxy-2-fluoro-β-L-fucose (GDP-2F-Fuc) serves as a competitive inhibitor against this enzyme ($K_i = 4.2\,\mu M$).[8] The observation that iminocyclitols designed to mimic the charge distribution of the glycosylcation serve as glycosyltransferase inhibitors and a demonstration of synergistic inhibition by the combination of an iminocyclitol, GDP, and the acceptor sugar to mimic the transition-state structure of a fucosyltransferase reaction further support this mechanistic rationale.[9,18,20,21] However, the lack of structural data for glycosyltransferases has made mechanistic conclusions as well as structure-based inhibitor design very difficult. Although several acceptor-modified glycosyltransferase inhibitors have been shown to be excellent inhibitors of glycosyltransferases, such studies enlightened the nucleophilic role of the acceptor but have not helped elucidate the mechanism of nucleotide displacement and inversion of stereochemistry seen at the donor.[22,23]

Studies with fluorinated disaccharides used to probe the glycosidase mechanism have deduced that fluorine at any position along the non-reducing sugar serves to impart mechanism-based inactivation, inhibition, or slow reactivity. This phenomenon grows more pronounced as the fluorine substituent nears the anomeric position, making 2′-deoxy-1′-fluoro- and 5′-fluoro disaccharides the most potent inhibitors.[4,12,15,16,24,25] We reasoned that flourinated sugar nucleotides would demonstrate greater stability and more pronounced effects when substituted at the 2- or 6-position of the glycon, as supported by the competitive inhibition against β1,4-GalT by UDP-2F-Gal and FucT-V by GDP-2F-Fuc.[9,19] All these fluorinated sugar derivatives were synthesized through a complex multi-step procedure.

Here we describe the effective synthesis of fluorinated sugars and their nucleotides using SelectFluor-mediated reaction with glycals coupled with concurrent chemoenzymatic transformations. We also study the effect of these fluorinated derivatives of nucleotide sugars on the fucosyltransferase family (III, V, VI, and VII), sialyltransferases (α-2,6 and α-2,3), and galactosyltransferases (α-1,3 and β-1,4). The synthesis of guanosine 5′-diphospho-6-deoxy-6-fluoro-β-L-fucose (GDP-6F-Fucose) (**1**) and GDP-2-deoxy-2-fluoro-α-L-fucose (**8**), new fluorinated GDP-fucose analogues, and a one-pot chemo-enzymatic synthesis of UDP-2-deoxy-2-fluoro-α-D-galactose (**13**) are described.

Inhibition studies of FucT-III, FucT-V, FucT-VI, FucT-VII, α-1,3-GalT, β-1,4-GalT, and 2,6ST with four fluorinated donor analogues are described.

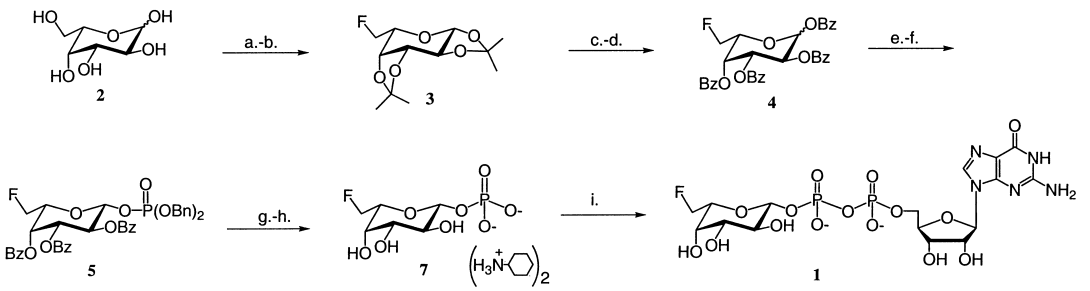
Materials and Methods

General methods

NMR spectra were recorded on Bruker AM-250, AMX-400 or AMX-500 spectrometers. 1H chemical shifts are referenced to residual protic solvent (CDC_{l3}, $\delta_H = 7.26$; -DMSO-d_6, $\delta_H = 2.50$; D_2O $\delta_H = 4.80$) or internal standard TMS ($\delta_H = 0.00$). ^{13}C NMR spectra are proton decoupled and the multiplicity of the signals is 's' unless otherwise noted. ^{13}C chemical shifts are referenced to the solvent signal ($CDCl_3$, $\delta_C = 77.0$; DMSO-d_6, $\delta_C = 39.5$) or to 1,4-dioxane ($\delta_C = 67.6$ in D_2O) as internal standard. ^{19}F NMR spectra were recorded at 376.5 MHz (Bruker AMX-400) and referenced to $CFCl_3$ ($\delta_F = 0.00$) as internal ($CDCl_3$) or external (D_2O) standard. ^{31}P NMR spectra were recorded at 162.0 MHz (Bruker AMX-400) and referenced to 85% H_3PO_4 ($\delta_p = 0.00$) as external standard. Coupling constants were measured in Hertz (Hz). High resolution mass spectra (HRMS) were recorded using fast atom bombardment (FAB) method in a *m*-nitrobenzyl alcohol matrix doped with NaI or CsI. Column chromatography was performed on Mallinckrodt silica gel 60 (230–400 mesh). Analytical thin-layer chromatography was performed using silica gel 60 F_{254} pre-coated glass plates (Merck) and visualized by quenching of fluorescence and (or) by charring after treatment with cerium molybdophoshate. Size exclusion chromatography was performed on Bio-Gel P2 Gel (Bio-Rad Laboratories). Diethyl ether and benzene were distilled from sodium-benzophenone; dichioromethane, acetonitrile, and nitromethane from calcium hydride. Anhydrous pyridine was purchased from Aldrich and used without further purification. Guanosine 5′-diphospho-[U-^{14}C]-β-L-fucose, cytidine 5′-monophospho-*N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid, and uridine 5′-diphospho-[U-^{14}C]-α-D-galactose were purchased from Amersham Life Science. ScintiVerse I scintillation cocktail and $MnCl_2 \cdot 4\,H_2O$ were purchased from Fisher Scientific Company. Protein concentrations were determined with the Coomassie protein staining reagent with albumin standards as purchased from Pierce. LacNAc-β-O-$(CH_2)_5$ CO_2CH_3 was prepared according to published procedures.[26] All other chemicals were purchased from Aldrich. The scintillation counter used was the Beckman LS 3801.

Synthesis of guanosine 5′-diphospho-6-fluoro-β-L-fucopyranoside (GDP-6F-Fuc) (1) (Scheme 1):

(A) 1,2,3,4-Di-*O*-isopropylidene-L-galactopyranoside. Anhydrous $CuSO_4$ (886 mg, 5.55 mmol) (dried at 140 °C under vacuum for 18 h) and anhydrous L-galactose (400 mg, 2.22 mmol) were suspended in dry acetone (9 mL) and treated with concentrated H_2SO_4 (40 μL). The resulting mixture was shaken at room temperature for 24 h. The cupric sulfate was removed by filtration and washed with acetone. The combined organic phases were neutralized by addition of $Ca(OH)_2$, filtered and concentrated. Purification by column chromatography (EtOAc:hexane, 1:1) gave the desired product as a colorless oil (530 mg, 91%). R_f 0.42 (EtOAc:hexane, 2:1). ^1H NMR (400 MHz): δ = 1.32 (3H, s, CH_3), 1.33 (3H, s, CR_3), 1.39 (3H, s, CH_3), 1.51 (3H, s, CH_3), 3.62 (1H, dd, J = 7.3, 11.3 Hz, H_a-6), 3.67 (1H, dd, *J* = 5.4, 11.3 Hz, H_b-6), 3.83–3.88 (1H, m, H-5), 4.28 (1H, dd, *J* = 1.9, 8.1 Hz, H-4), 4.34 (1H, dd, *J* = 2.4, 5.1 Hz, H-2), 4.62 (1H, dd, *J* = 2.4, 7.8 Hz, H-3), 5.49 (1H, d, *J* = 5.1 Hz, H-1). ^{13}C NMR (100 MHz): δ = 21.9, 22.6, 23.7, 23.8 (CH_3), 59.5, 2×67.3, 69.4, 69.6, 95.1 (C-1), 107.1, 107.7 (Cq). HRMS (FAB): calcd for $C_{12}H_{20}O_6$ [$(M + Na)^+$] 283.1158, found 283.1155.



Scheme 1. Synthesis of UDP-6F-L-Gal (1): (a) $CuSO_4$, H_2SO_4 (cat), acetone, 91%; (b) DAST, collidine, DCM, 77%; (c) AcOH, H_2O, 68%; (d) BzCl, pyridine, 70%; 9e) HBr/HOAc, DCM, Ac_2O; (f) Ag_2CO_3, $(BnO)_2P(O)OH$, 3 Å ms, DCM, Et_2O, CH_3CN, 75% over two steps; (g) Pd/C, H_2 toluene, pyridine, NEt_3, 95%; (h) MeOH, cyclohexylamine, 87%; (i) BioRad AG 50 W-X2 (NEt_3-form), quant, then guanosine morpholidate, tetrazole, pyridine, 2 days, 67%.

(B) 1,2,3, 4-Di-*O*-isopropylidene-6-deoxy-6-fluoro-L-galactopyranoside (3). A solution of 1,2,3,4-Di-*O*-isopropylidene-L-galactopyranoside (460 mg, 1.75 mmol) in DCM (8 mL) was treated with collidine (463 μL, 3.51 mmol) and DAST (463 μL, 3.51 mmol) at room temperature and stirred at reflux for 6 h. It was diluted with Et_2O and washed with $NaHCO_3$ (2×), 0.5 N HC1, and brine, dried over $MgSO_4$ and concentrated to give the final product **3** as a colorless oil (360 mg, 77%). R_f 0.72 (EtOAc:hexane, 2:3). ^{1}H NMR (400 MHz): δ = 1.32 (6H, s, CH_3), 1.43 (3H, s, CH_3), 1.53 (3H, s, CH_3), 4.02–4.10 (1H, m, H-5), 4.25 (1H, dd, J = 2.2, 8.1 Hz), 4.33 (1H, dd, J = 2.7, 5.2 Hz), 4.44 (1H, ddd, J = 7.0, 9.7, 47.8 Hz, H_a-6), 4.58 (1H, ddd, J = 5.2, 9.5, 46.2 Hz, H_b-6), 4.60–4.63 (1H, m), 5.53 (1H, d, J = 5.1 Hz, H-1). ^{13}C NMR (100 MHz): δ = 24.4, 24.9, 25.9, 26.0 (CH_3), 66.6 (d, J = 23.3 Hz, C-5), 70.3, 70.4, 70.5 (C-2, C-3, C-4), 82.0 (d, J = 168.7 Hz, C-6), 96.1 (C-1), 108.8, 109.6 (Cq). 19F (367 MHz): δ = −154.9. HRMS (FAB): calcd for $C_{12}H_{19}FO_5$ [(M + H)$^+$] 263.1295, found 263.1292.

(C) 1, 2,3,4-Tetra-*O*-benzoyl-6-deoxy-6-fluoro-L-galactopyranoside (4). A solution of 6-deoxy-6-fluoro-L-galactose (150 mg, 0.823 mmol) in dry pyridine (3 mL) was treated with BzCI (478 μL, 4.12 mmol) and stirred under argon at room temperature for 16 h. It was diluted with EtOAc, washed with 0.5 N HCl, $NaHCO_3$, H_2O, and brine, dried ($MgSO_4$) and concentrated. Purification by column chromatography (EtOAc:hexane, 1:3) gave **4** as a colorless foam (410 mg, 83%). R_f 0.54 (EtOAc:hexane, 1:2). ^{1}H NMR (400 MHz): δ = 4.50–4.55 (1H, m, H-6), 4.62–4.67 (1H, m, H-6), 4.74–4.80 (1H, m, H-5), 6.03 (1H, dd, J = 3.5, 10.8 Hz), 6.10 (1H, dd, J = 3.2, 10.5 Hz), 6.12–6.15 (1H, m), 6.96 (1H, d, J = 3.5 Hz, H-1), 7.25–7.33 (4H, m, H_{arom}), 7.42–7.56 (6H, m, H_{arom}), 7.62–7.68 (2H, m, H_{arom}), 7.81–7.88 (4H, m, H_{arom}), 8.09–8.16 (4H, m, H_{arom}). ^{13}C NMR (100 MHz): δ = 67.6 (C-2, C-3), 63.3 (d, J = 4.4 Hz, C-4), 68.5 (C-2, C-3), 70.1 (d, J = 23.3 Hz, C-5), 80.8 (d, J = 171.6 Hz, C-6), 90.5 (C-1), 128.3, 128.4, 2×128.5, 129.7, 130.0 (signal overlap, C_{arom}), 133.4, 133.5, 133.8, 134.0 (*ipso*-C_{arom}), 164.5, 165.4, 165.7, 165.7 (C=O). HRMS (FAB): calcd for $C_{34}H_{27}FO_9$ [(M + Na)$^+$] 621.1537, found 621.1557.

(D) Dibenzylphosphoryl 2,3,4-tri-O-benzoyl-6-deoxy-6-fluoro-β-L-galacto-pyranoside (5). To a cooled solution of **4** (150 mg, 0.25 mmol) in DCM (1.5 mL) and Ac_2O (150 μL) was added dropwise 30% HBr-ACOH (600 μL) at 0 °C, and it was stirred for 3 h at room temperature. The mixture was poured onto ice water and extracted with EtOAc. The extracts were successively washed with water, $NaHCO_3$, and brine, dried over anhydrous $MgSO_4$ and concentrated to give the title compound, which was used without further purification. Ag_2CO_3 (138 mg, 0.50 mmol) was added in one portion to a cooled suspension of anomeric bromide, dibenzylphosphate (209 mg, 0.75 mmol) and 3 Å ms (450 mg) in DCM, Et_2O, CH_3CN (1.5 mL each) in a round bottom flask lapped with aluminum foil to shut out light. The mixture was stirred for 15 h at room temperature and filtered through Celite, and the filtrate was concentrated. The residue was chromatographed on silica gel (toluene:EtOAc, 3:1) to give **5** as a colorless oil (145 mg, 75% over two steps). R_f 0.51 (EtOAc:toluene, 1:2). ^{1}H NMR (400 MHz): δ = 4.34–4.41 (1H, m, H-5), 4.51 (1H, d, J = 5.6 Hz), 4.63 (1H, d, J = 5.9 Hz), 4.79 (1H, dd, J = 7.0, 11.6 Hz, CH_aH_bPh), 4.89 (1H, dd, J = 6.7, 11.6 Hz, CH_aH_bPh), 5.10 (1H, dd, J = 7.8, 11.9 Hz, CH_aH_bPh), 5.14 (1H, dd, J = 7.3, 11.6 Hz, CH_aH_bPh), 5.59 (1H, dd, J = 3.2, 10.2 Hz), 5.72 (1H, t, J = 7.6 Hz, H-1), 5.90–5.96 (2H, m), 7.02–7.05 (2H, m, H_{arom}), 7.18–7.37 (12 H, m, H_{arom}), 7.42–7.53 (4H, m, H_{arom}), 7.61–7.66 (1H, m, H_{arom}), 7.78–7.82 (2H, m, H_{arom}), 7.94–7.97 (2H, m, H_{arom}), 8.06–8.09 (2H, m, H_{arom}). ^{13}C NMR (100 MHz): δ = 67.5 (d, J = 5.8 Hz, C-4), 69.5 (d, J = 4.4, CH_2Ph), 69.6 (d, J = 8.7 Hz, C-2), 69.7 (d, J = 5.8 Hz, CH_2Ph), 71.3 (C-3), 73.1 (d, J = 23.3 Hz, C-5), 80.6 (d, J = 173.1 Hz, C-6), 97.0 (d, J = 4.4, Hz, C-1), 127.5, 127.9, 128.3, 128.4, 128.5, 128.7, 129.7, 129.8, 130.0 (signal overlap, C_{arom}), 133.4, 133.6, 133.8 (*ipso*-C_{arom}), 165.2, 165.4 (signal overlap, C=O). ^{19}F (376 MHz): δ = −155.7. ^{31}P (162 MHz): δ = −2.24. HRMS (FAB): calcd for $C_{41}H_{36}FO_{11}P$ [(M + Cs)∼] 887.1034, found 887.1057.

(E) Di(trimethylammonium)phosphoryl-2,3,4-tri-*O*-benzoyl-6-deoxy-6-fluoro-β-L-fucopyranoside (6). A solution of **5** (300 mg, 0.398 mmol) in toluene (2.4 mL) was treated with triethylamine (360 μL) and pyridine (480 μL). The mixture was hydrogenated in an H_2 atmosphere with 10% activated Pd/C (40 mg) as catalyst. After 24 h the mixture was filtered and the filtrate concentrated to give **6** as a colorless foam (290 mg, 95%). R_f 0.44 (^{i}Prop:NH_3 aq:H_2O, 6:3:1). ^{1}H NMR (500 MHz): δ = 1.21 (3H, t, J = 7.4 Hz, $N(CH_2CH_3)_3$), 3.00 (2H, q, J = 7.4 Hz, $N(CH_2CH_3)_3$), 4.43–4.50 (1H, m, H-5), 4.61 (1H, ddd, J = 5.5, 9.5, 46.4 Hz, H_a-6), 4.65 (1H, ddd, J = 5.9, 9.5,

46.8 Hz, H_b-6), 5.60 (1H, t, $J=7.8$ Hz, H-1), 5.72 (1H, dd, $J=3.4$, 10.3 Hz, H-3), 5.76 (1H, dd, $J=7.8$, 10.3 Hz, H-2), 5.93 (1H, bd, $J=3.4$ Hz, H-4), 7.20–7.25 (2H, m, H_{arom}), 7.35–7.55 (6H, m, H_{arom}), 7.63–7.67 (1H, m, H_{arom}), 7.69–7.72 (2H, m, H_{arom}), 7.89–8.05 (4H, m, H_{arom}). ^{13}C NMR (125 MHz): $\delta=14.6$, 36.1 ($N(CH_2CH_3)_3$), 60.8 (d, $J=4.8$ Hz, C-2), 63.2 (d, $J=9.5$ Hz, C-4), 63.7 (d, $J=22.9$ Hz, C-5), 64.5 (C-3), 73.2 (d, $J=169.9$ Hz, C-6), 88.1 (d, $J=4.8$ Hz, C-1), 120.1, 120.2, 120.6, 121.3, 121.5, 121.2 (signal overlap, C_{arom}), 125.2, 125.3, 125.6 (*ipso*-C_{arom}), 157.4, 157.8, 158.1 (C=O). ^{19}F (376 MHz): $\delta=-40.4$. ^{31}P (162 MHz): $\delta=1.20$. HRMS (FAB): calcd for $C_{27}H_{24}FO_{11}P$ [$(M+Na)^+$] 597.0938, found 597.0915.

(F) Di(cyclohexylammonium)phosphoryl 6-fluoro-β-L-fucopyranoside (7). The above phosphate **6** (280 mg, 0.367 mmol) was dissolved in dry MeOH (3 mL) and cyclohexylamine (3 mL) was added. It was heated at reflux for 4 h. When quantitative debenzoylation was achieved the mixture was concentrated and fractioned between water and $CHCl_3$. The water phase was washed three times with $CHCl_3$ and then concentrated to dryness by coevaporating MeOH. The product was dissolved in hot EtOH and precipitated by addition of acetone. Filtration gave **7** as a crystalline colorless solid (147 mg, 87%). R_f 0.36 (^{i}Prop:NH_3 aq:H_2O, 6:3:1). ^{1}H NMR (400 MHz): $\delta=1.08$–1.14 (2H, m, $C_6H_{11}NH_2$), 1.23–1.34 (8H, m, $C_6H_{11}NH_2$), 1.56–1.62 (2H, m, $C_6H_{11}NH_2$), 1.71–1.78 (4H, m, $C_6H_{11}NH_2$), 1.91–1.98 (4H, m, $C_6H_{11}NH_2$), 3.05–3.11 (2H, m, $C_6H_{11}NH_2$), 3.51 (1H, dd, $J=7.8$, 10.0 Hz, H-2), 3.65 (1H, dd, $J=3.3$, 9.9 Hz, H-3), 3.91 (1H, d, $J=2.4$ Hz, H-4), 3.93–3.96 (1H, m, H-5), 4.52–4.58 (1H, m), 4.63–4.67 (1H, m), 4.83 (1H, t, $J=7.7$ Hz, H-1). ^{13}C NMR (100 MHz): $\delta=26.3$, 26.8, 32.8, 52.8 (signal overlap, $C_6H_{11}NH_2$), 70.6 (d, $J=7.2$ Hz, C-2, C-4), 74.4 (d, $J=5.8$ Hz, C-2, C-4), 74.8 (C-3), 75.9 (d, $J=20.2$ Hz, C-5), 85.5 (d, $J=163.2$ Hz, C-6), 100.0 (d, $J=2.9$ Hz, C-1). ^{31}P (162 MHz) $\delta=2.60$. ^{19}F (3.76 MHz) $\delta=-162.5$. HRMS (FAB): calcd for $C_6H_{12}FO_8P$ [$(M+2Cs)^+$] 526.8284, found 526.8264.

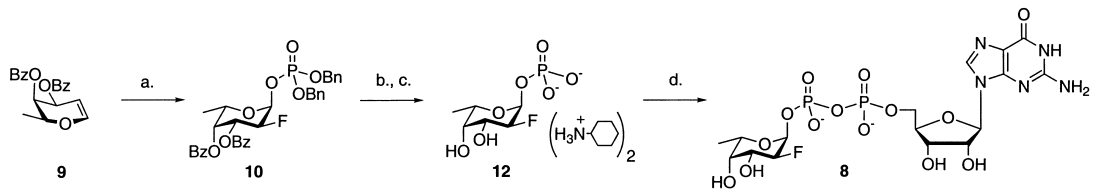
(G) Guanosine 5′-diphospho-6-fluono-β-L-fucopyranoside (1). **7** (60.0 mg, 0.130 mmol) was dissolved in water (2 mL), applied to a Bio-Rad AG 50 W-X2 cation-exchange column (Et_3N^+-form, 2.5×6 cm), and eluted with water (150 mL). The solution was evaporated, coevaporated with MeOH (2×5 mL), and dried for 3 days under vacuum to give tnethyiammonium β-L-fucopyranosyl phosphate. After addition of 4-morpholino-*N*-*N*′-dicyclohexylcarboxamidinium guanosine 5′-monophosphomorpholidate (151 mg, 0.209 mmol) it was coevaporated with dry pyridine (3×5 mL). 1*H*-Tetrazole (29 mg) and dry pyridine (1.25 mL) were added, and the solution was stirred at room temperature. After 2 days, the mixture was diluted with water (3 mL) to become a clear solution, and it was extracted with DCM (3×5 mL). The aqueous phase was concentrated and applied to a Bio-Gel P-2 column (2.5×70 cm), eluted with 50 mM NH_4HCO_3, to give the final product **1** as a white solid after lyophilization (53.2 mg, 67%). R_f 0.25 (^{i}Prop:1 M NH_4OAc, 2:1) ^{1}H NMR (400 MHz): $\delta=3.60$ (1H, dd, $J=7.6$, 10.0 Hz, H-2″), 3.68 (1H, dd, $J=3.2$, 10.0 Hz, H-3″), 3.94 (1H, d, $J=3.2$ Hz, H-4″), 3.95–3.99 (1H, m, H-5″), 4.18–4.21 (2H, m, H-5′), 4.30–4.34 (1H, m, H-4′), 4.48–4.52 (1H, m, H-3′), 4.52–4.57 (1H, m, H-6″), 4.65–4.69 (1H, m, H-6″), 4.73 (1H, t, $J=5.7$ Hz, H-2′), 4.96 (1H, t, $J=7.8$ Hz, H-1″), 5.88 (1H, d, $J=6.0$ Hz, H-1′), 8.07 (1H, s, H-8). ^{13}C NMR (100 MHz): $\delta=67.8$ (d, $J=4.3$ Hz, C-5′), 70.5 (d, $J=5.8$ Hz, C-2″, C-4″), 72.9 (C-3′), 73.6 (d, $J=7.2$ Hz, C-2″, C-4″), 74.5 (C-3″), 76.1 (d, $J=24.7$ Hz, C-5″), 76.2 (C-2″), 85.1 (d, $J=165.8$ Hz, C-6″), 86.2 (d, $J=10.1$ Hz, C-4′), 89.3 (C-1′), 100.9 (d, $J=7.3$ Hz, C-1′), 118.6 (C-5), 140.0 (C-8), 154.2 (C-4), 156.3 (C-2), 161.3 (C-6). ^{19}F (376 MHz): $\delta=-161.0$. ^{31}P (162 MHz): $\delta=-$ 12.3 (d, $J=19.5$ Hz), −10.4 (d, $J=20.7$ Hz). MS (ESI): calcd for $C_{16}H_{24}O_{11}FN_5P_2$ [$(M-H)^-$] 606, found 606; [$(M-2H)^{2-}$] 303, found 303.

Synthesis of guanosine 5′-diphospho-2-deoxy-2-fluoro-α-L-fucopyranoside (GDP-α2F-Fuc) (8) (Scheme 2)

(A) (2,3-dibenzoyl-2-fluoro-α-1-fucopynanosyl) dibenzyl phosphate (10). This was prepared from dibenzoyl fucal (**9**) by our published procedure.[27]

(B) Di(cyclohexylammonium) 2-deoxy-2-fluoro-a-fucopyranosyl phosphate (12). 10 was fully deprotected following the same procedure as its β analogue.[8] ^{1}H NMR (600 MHz, D_2O): 5.54 (dd, $J_{1\text{-}P}=7.4$ Hz, $J_{1\text{-}2}=4.0$ Hz, 1H, 1); 4.55 (dddd, $J_{1\text{-}2}=3.5$ Hz, $J_{3\text{-}2}=10.1$ Hz, $J_{2\text{-}1}=2.2$ Hz, $J_{2\text{-}F}=50.0$ Hz, 1H, 2); 4.20 (q, $J_{5\text{-}6}=6.6$ Hz, 1H, 5); 4.09 (ddd, $J_{3\text{-}2}=10.1$ Hz, $J_{3\text{-}F}=13.1$ Hz, $J_{3\text{-}1}=3.5$ Hz, 1H, 3); 3.81 (t, $J_{F\text{-}4}=J_{3\text{-}1}=3.5$ Hz, 1H, 4); 1.13 (d, $J_{5\text{-}6}=6.6$ Hz, 3H, 6); ^{13}C NMR (150 MHz, D_2O): 92.86 (dd, $J_{C\text{-}P}=5.5$ Hz $J_{C\text{-}F}=22.0$ Hz, 1); 89.16 (dd, $J_{C\text{-}P}=3.7$ Hz, $J_{C\text{-}F}=184.12$ Hz, 2); 73.15 (d, $J=8.2$ Hz, 4); 68.65 (d, $J=17.4$ Hz, 3); 68.23 (5); 16.01 (6). ^{31}P NMR (161 MHz, D_2O): 0.17; ^{19}F NMR (376 MHz, D_2O): −202.31 (dd, $J=12.7$ Hz, $J=50.8$ Hz).

(C) Uridine 5′-diphospho-2-deoxy-2-fluoro-a-fucopyranose (8). Coupling of **12** with GMP-morpholidate and catalytic 1-*H*-tetrazole carried out via the procedure by Wittmann.[28] ^{1}H NMR (600 MHz, D_2O): 8.07 (s, 1H), 5.84 (d, $J_{1'\text{-}2'}=7.4$ Hz, 1H, 1′), 5.64 (dd, $J_{1\text{-}P}=7.0$ Hz, $J_{1\text{-}2}=3.5$ Hz, 1H, 1); 4.55 (bdddd, $J_{1\text{-}2}=J_{2\text{-}P}=2.2$ Hz, $J_{3\text{-}2}=10.0$ Hz, $J_{2\text{-}F}$



Scheme 2. Synthesis of GDP-2-deoxy-2-fluoro-α-L-fucose (**8**); (a) F-TEDA-OTf then $(BnO)_2POOH$ (54%); (b) H_2 Pd/C; (c) cyclohexylamine (92% over two steps); (d)

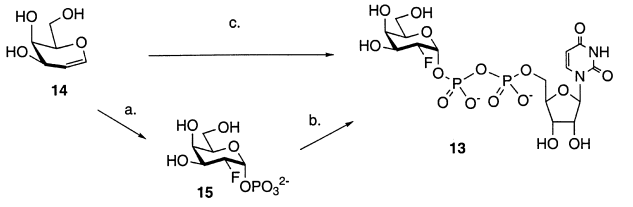
= 49.5 Hz, 1H, 2); 4.44 (dd, J = 3.1 Hz, J = 4.8 Hz, 1H); 4.26 (bs, 1H), 4.18 (q, $J_{5\text{-}6}$ = 6.6 Hz, 1H, 5); 4.12 (bt, J = 4.9 Hz, 1H), 4.07 (ddd, $J_{3\text{-}2}$ = 10.1 Hz, $J_{3\text{-}F}$ = 12.7 Hz, $J_{3\text{-}4}$ = 3.5 Hz, 1H, 3); 3.74 (t, $J_{F\text{-}4} = J_{3\text{-}4}$ = 4.0 Hz, 1H); 1.07 (d, $J_{5\text{-}6}$ = 6.6 Hz, 3H, 6); ^{31}P NMR (161 MHz, D_2O): −10.85 (d, J = 21.4 Hz), −12.51 (d, J = 21.4 Hz); ^{19}F NMR (376 MHz, D_2O): −202.53 (dd, J = 12.7 Hz, J = 53.3 Hz).

Two-step-synthesis of uridine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactose (UDP-2F-Gal) (13) (Scheme 3).

(A) 2-Deoxy-2-fluono-α-D-galactopyranosyl phosphate (15). Galactal (**14**) (100 mg, 684 μmol) and Selectfluor triflate (346 mg, 719 μmol) are dissolved in 3 mL water and stirred 3 h at 25 °C. To the solution is added 1 mL 1M HEPES buffer, pH 7.4, and the solution is adjusted to pH 7.4 with 10 N NaOH and diluted to 10 mL. 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mg/mL bovine albumin, 1 mM ATP, 50 mM phosphoacetate, 1 U galactokinase, and 50 U acetate kinase are added, and the solution is rocked for at 25 °C with additional aliquot of phosphoacetate added after 48 h. The reaction is monitored by TLC (isopropanol:H_2O:30% NH_4OH, 7/2/1). After 4 days, $BaCl_2 \cdot H_2O$ (254.8 mg, 1.04 mmol) was added and the solution was stirred at 4 °C for 6 h. The obtained white precipitate was removed by centrifugation and the precipitate was further washed with H_2O (6 mL). After the supernatant was collected, acetone (1 vol) was added and the cloudy solution was allowed to stand for a day at 4 °C. The solution was centrifuged and the collected precipitate was washed with cold H_2O: acetone (1:1, 5 mL×2) and acetone (5 mL). Treatment of the resulting barium salt of **14** with Dowex 50 Na^+ form followed by drying in vacuo yields the sodium salt of **14** (131 mg, 63%). ^{1}H NMR (D_2O, 500 MHz) δ 5.613 (dd, H1, J = 8.3, 3.8 Hz, 1H), 4.596 (dddd, H2, J = 50.0, 10.0, 3.8, 1.8 Hz, 1H), 4.118 (m, H3 and H5, 2H), 4.007 (m, H4, 1H), 3.697 (m, H6, 2H).

(B) Unidine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactose (13). Compound **15** was enzymatically converted to **13** in 60% yield following published procedures.[19]

One-pot procedure for UDP-2-deoxy-2-fluoro galactose (13). Galactal (**14**) (100 mg, 0.68 mmol) and Selectfluor triflate (346 mg, 0.72 mmol) were dissolved in 3 mL H_2O and stirred at 25 °C for 3 h. To the solution was added 500 μL 1 M Tris–HCl, pH 7.5, and the solution was equilibrated to pH 7.5 with 10 N NaOH. To this solution was added 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM ATP, 64 mM acetylphosphate, and 1 mg/mL bovine serum albumin in a total volume of 5 mL at pH 7.5. 1 Unit of galactokinase and 50 units of AK were added, and solution was incubated at 25 °C. Each 24 h, addition of 100 μL 1 M AcOP, re-equilibration to pH 7.5, and addition of 1 U GK and 50 U AK were repeated for 4 days. The solution was adjusted to pH 8.5 with 10 N NaOH. 4.1 mM UTP, 0.25 mM UDP-glucose, and 2.5 mM β-mercaptoethanol were added to the solution with a total volume of 10 mL with pH 8.5. 10 U inorganic pyrophosphatase, 5 U UDP-glucose pyrophosphory-lase, and 5 U galactose-1-phosphate are then added. The reaction was allowed to incubate at 25 °C for 3 days with addition of 400 μL UTP (followed by equilibration to pH 8.0), 5 U UDP-glucose pyrophosphorylase, and 5 U galactose-1-phosphate uridyltransferase each 24 h. The reaction was halted by addition of 50 U alkaline phosphatase and incubated at room temperature overnight. The product was purified by ion-exchange chromatography (Dowex 1-X8, Cl^- form, 2×10 cm) with a linear gradient of LiCl (0→0.4 M) in 3 mM HCl. Size-exclusion chromatography (BioRad P-2, H_2O, 2×30 cm) was used to desalt the product and gave pure **13** in 60% overall yield. ^{1}H NMR (D_2O, 500 MHz) δ 7.903 (d, H6″, J = 8.5 Hz, 1H), 5.935 (d, H1′, J = 4.0 Hz, 1H), 5.909 (d, H5″, J = 8.5 Hz, 1H), 5.757 (dd, H1, J = 7.5, 4.0 Hz, 1H), 4.633 (dddd, H2, J = 49.5, 10.0, 3.5, 3.0 Hz, 1H), 4.313 (m, H2′ and H3′, 2H), 4.223 (m, H4′, 1H), 4.154 (m, H3, H5 and H5′, 4H), 4.030 (m, H4, 1H), 3.698 (ABq, H6a, J = 12.0, 7.0 Hz, 1H), 3.657 (ABq, H6b, J = 12.0, 5.0 Hz, 1H). HRMS (FAB) calcd for $C_{15}H_{21}N_2O_{16}FP_2Na_2$ ($M + H^+$) 613.0224. found 613.0229.



Scheme 3. One-pot chemo-enzymatic synthesis of UDP-2-deoxy-2-fluoro-galactose (**13**): (a) F-TEDA-CH_2Cl·2Otf, 3 mL H_2O, 3 h; 100 mM Tris–HCl pH 7.4, adjust to pH 7.4 w/10 N NaOH; galactokinase, acetate kinase, ATP, AcOP, βME, BSA, $MgCl_2$, 4 days. 65%: (b) Galactose-1-phosphate uridyltransferase, UDP-glucose pyrophosphorylase, pyrophosphatase, UTP, pH 8.4, 3 days. 67%: (c) F-TEDA-CH_2Cl·2Otf, 3 mL H_2O, 3 h; 100 mM Tris–HCl pH 7.4, adjust to pH 7.4 w/10 N NaOH; galactokinase, acetate kinase, ATP, AcOP, BME, BSA, $MgCl_2$, 4 days. Galactose-1-phosphate uridyltransferase, UDP-glucose, pyrophosphorylase, pyrophosphatase, UTP, pH 8.4, 3 days, 60%.

Radiolabeled α-1,3-fucosyltransferase activity assay. The activity of FucT V was detected by the GDP-[U-^{14}C]-fucose assay described previously in which GDP-[U-^{14}C]-fucose is separated from [U-^{14}C]-fucosylated product by anion exchange chromatography. Typically, assays contained 10 mM $MnCl_2$, 0.3 m unit of purified FucT V and 25 mM cacodylate buffer (pH 6.2) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were halted with the addition of 0.5 mL of distilled, de-ionized water. GDP-Fuc was separated from the product, Lewis x, with a 1.0 mL Dowexl-X8 pipette column. The reaction mixtures were applied to the column and washed with 0.3 mL distilled, de-ionized water three times. The flow through and the column washes were collected in 10 mL of ScintiVerse I scintillation cocktail. Control reactions, without enzyme, were used to establish the background, non-enzymatic cleavage rate. A typical control reaction of 32672 cpm guanosine-5′-diphospho-L-[U-^{14}C]-fucose would result in 173 cpm of non-enzymatic column flow through. GDP-Fuc concentration was varied (10, 25, 50, 100 μM) at fixed concentrations of GDP-2F-Fuc (0, 20, 40, 80 μM), and the acceptor sugar, LacNAc-β-O-$(CH_2)_5CO_2CH_3$, was kept at twice its K_m level, 0.6 mM. Each assay contained 0.3 m unit of FucT V and 10 mM $MnCl_2$ in a 100 mM MES buffer (pH 6.0). Reactions were 30 min at room temperature. The

precise K_i was determined with a nonlinear least square fit of the data to the equation for competitive inhibition.

Inhibition of α1,3-GalT and β,4-GalT by UDP-2F-Gal. Assays contained 10 mM $MnCl_2$, 0.034 m unit of galactosyltransferase, UDP-[6-^{3}H]-galactose (1 to 100 μM), GlcNAcβOMe (3 mM), inhibitor (1 to 100 μM), bovine serum albumin (1 mg/mL), and 50 mM HEPES buffer (pH 7.4) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were halted by the addition of 0.3 mL of deionized water and immediately applying it to a pipette column of Dowex 1×8 (1 mL). The pipette was washed with an additional 0.9 mL of water (3×0.3 mL). The flow-through and column washes were collected in 11 mL of ScintiVerse I scintillation cocktail. A control reaction without enzyme was used to establish the background count. The precise K_i was determined with a nonlinear, least square fit of the data to the equation for competitive inhibition

Inactivation studies of α1,3-GalT. Assays run as above, with the enzyme samples preincubated as concentrated solutions (50×) in different concentrations of UDP-2F-Gal (0, 1, 0.1, 0.01 mM). Reaction initiated by addition of enzyme after differing times of preincubation (0.5, 4, 10, and 20 mm).

Inhibition of α2,6-sialyl transferase by CMP-3F-Neu5Ac. The assay mixture consisted of 50 μM CMP-Neu5Ac with 250,000 cpm of CMP-[^{14}C]Neu5Ac added as tracer, 10 mM $MnCl_2$, 0.1% Triton CF-54, 20 mM cacodylate, pH 6.0, and 0.5 mU of α′2,6-SialylT, and inhibitor (1 to 100 μM) in a 50-μL reaction mixture. Assays were performed at 37 °C for 30 min. Reactions were halted by the addition of 0.5 mL of 100 mM phosphate buffer, pH 7.0 and immediately applied to a pipette column of Dowex 1×8 (1 mL). The pipette was washed with an additional 1 mL of phosphate buffer (2×0.5 mL). The flow-through and column washes were collected in 15 mL of ScintiVerse I scintillation cocktail. A control reaction without enzyme was used to establish background count. The precise K_i was determined with a nonlinear, least square fit of the data to the equation for competitive inhibition.

Results and Discussion

Synthesis of GDP-6F-fucose

We chose to synthesize **1** as a comparison with the previously described GDP-2-deoxy-2-fluoro-β-L-fucose (**16**) for inhibitory activity against fucosyltransferases. This experiment should determine a correlation between fluorine position and inhibition. The synthesis of this molecule was accomplished by modification of L-galactose (Scheme 1). The previous synthesis of **16**[8] was used as a model for this synthesis following synthesis of the fluorinated monosaccharide.

L-Galactose was first protected at the 1,2- and 3,4- positions with isopropylidene, leaving the 6-OH unprotected. DAST accomplished selective fluorination,[29] displacing the 6-OH to give **3** in a 77% yield. The fluoroglycoside was deprotected, benzoylated, and brominated at the anomeric position. Nucleophilic addition of dibenzyl phosphate with catalytic Ag_2CO_3 yields the protected phosphate **5** in 52% yield over four steps. Complete deprotection and ion exchange is followed by GMP-morpholidate using catalytic 1-*H*-tetrazole,[28] to give **1** with a 55% yield over three steps.

Synthesis of GDP-α-2F-fucose. To understand the stereochemical requirements of the fucosyltransferase enzymes, we chose to synthesize the opposite anomeric stereoisomer of GDP-2F-fucose and investigate its inhibitory properties. An equal or greater inhibition of this molecule to its β analogue will demonstrate a flexible binding domain. The synthesis of this molecule was carried out using a new fluorination–phosphorylation technique from the protected fucal with Selectfluor triflate.[27] Following deprotection to 2-deoxy-2-fluoro fucose-1-phosphate, coupling with GMP morpholidate was conducted according to published coupling procedures.[28]

Dibenzoyl fucal (**9**) was reacted with Selectfluor triflate and dibenzyl phosphate to yield the fluorophosphate **10** in 54% yield. Full deprotection and ion exchange (92%) was followed by GMP-morpholidate coupling with 1-*H*-tetrazole[28] to give **8** in a 56% yield.

Improved synthesis of UDP-2F-galactose. We sought to improve the chemoenzymatic synthesis of **13** to facilitate the large-scale synthesis of this galactosyltransferase inhibitor. Specifically, we wished to develop a one-pot procedure in which fluorination and phosphorylation and nucleotide transfer could be carried out without intermediate purification. Recently, Gastinel et al. solved and refined the 3D structure of the catalytic domain of bovine β-1,4-GalT to 2.4 Å.[30] Co-crystallization and resolution of **13** bound to the enzyme active site will help define the amino acids involved in catalysis. Although high-yielding enzymatic syntheses of the natural substrate UDP-Gal have been published,[31] a one-pot procedure has not been reported.

Previous syntheses of 2-deoxy-2-fluoro galactose have relied upon reductive fluorination of galactal with XeF_2-$BF_3{\cdot}OEt_2$,[19] displacement fluorination of the selectively-protected talopyranoside with DAST,[32] or electrophilic fluorination of the tri-*O*-acetyl galactal with AcOF.[33] With the recent development of a one-pot, fluorination technique using the electrophilic fluorinating reagent F-TEDA-CH_2Cl (Selectfluor), the ability to fluorinate unprotected sugars with high yields in water became available.[34] With the absence of undesirable side products, the result of the reaction with Selectfluor and unprotected galactal is only TEDA-CH_2Cl salt and fluorinated galactose. We reasoned that after the addition of buffer and equilibration of pH, enzymatic reactions should proceed normally.

To verify this hypothesis, we employed a coupled enzymatic assay for galactokinase activity to detect phosphorylation of 2-deoxy-2-fluoro galactose. Galactokinase catalyzes the transfer of phosphorous from ATP to galactose, or 2-deoxy-2-fluorogalactose in this case, to form

galactose phosphate and ADP.[35] The assay monitors the formation of ADP via pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, and NADH. Disappearance of NADH is followed by UV spectroscopy at 340 nM. After reacting 100 mg gaiactal and 1.1 equivalents of Selectfluor (both BF_4 and OTf forms) in 10 mL H_2O for 3 h followed by addition of buffer (100 mM Tris–HCl) and equilibration of the pH to 7.0, galactokinase demonstrated activity with the fluorinated galactose from the Selectfluor triflate reaction. The Selectfluor–BF_4 reaction product showed no activity. We concluded that me TEDA-CH_2CI triflate salt is amenable to buffered enzymatic conditions at physiological pH.

Given this positive result, we attempted the one-pot chemo-enzymatic synthesis of 2-deoxy-2-fluorogalactose phosphate (**15**) (Scheme 1). Galactal (**14**) and Selectfluor triflate were dissolved in 3 mL H_2O and stirred at 25 °C for 3 h. To the solution was added 1 M Tri–HCl, pH 7.5, and the solution was equilibrated to pH = 7.5 with 10 N NaOH. To this solution was added $MgCl_2$, β-mercaptoethanol, ATP, phosphoacetate, bovine serum albumin, galactokinase, and alkaline phosphatase to a total volume of 5 mL. The solution was incubated at 25 °C for 4 days with addition of 1 equiv phosphoacetate each 24 h. $BaCl_2{\cdot}2H_2O$ was added to precipitate barium phosphate. The supernatant was diluted with cold acetone and incubated at 4 °C to precipitate the product. The precipitate was collected by centrifugation, washed with ice cold H_2O/acetone and then cold acetone. The solid was dried in vacuo, and the product was incubated with H_2O and Dowex 50 ion-exchange resin (Na^+ form) to give the sodium salt. The filtrate was dried in vacuo to yield **15** in 65% yield.

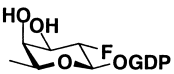
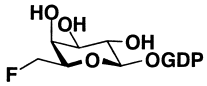
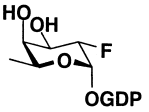
Finally, a one-pot method was applied to the complete synthesis of **13**. The above procedure to make **15** was followed without barium precipitation. Instead, the pH was adjusted to 8.5 with 10 N NaOH. UTP, inorganic pyrophosphorylase, UDP-glucose pyrophosphorylase, galactose-1-phosphate uridyltransferase, and a catalytic amount of UDP-glucose were added to a total volume of 10 mL. The reaction was allowed to incubate at 25 °C for 3 days with addition of extra UTP, UDP-glucose pyrophosphorylase, and galactose-1-phosphate undyltransferase after 24 h. The reaction was halted by addition of alkaline phosphatase. The product was purified by ion-exchange chromatography (Dowex 1×8, Cl^- form) with a linear gradient of LiCl. Size-exclusion chromatography (BioRad P-2) was used to desalt the product and gave pure **13** in 60% overall yield.

Fucosyltransferase inhibition. Fucosyltransferases (FucTs) biosynthesize a variety of oligosaccharides including the sialyl Lewis x(SLe^X), Lewis x (Le^x), Lewis a (Le^a), and sialyl Lewis a (SLe^a) that are central to numerous cell–cell interactions.[36–39] Five human fucosyltransferases (FucTIII-FucTVII) have been cloned, two of which are currently commercially available (FucTV and FucTVI).[3] Mammalian FucTs transfer a fucose moiety from the donor guanosine 5′-diphospho-β-D-fucose (GDP-Fuc) to a carbohydrate acceptor in enzyme-dependent α-1,2-, α-1,3-, α-1,4-, and α1,6-linkages with inversion of anomeric configuration. Several of these enzymes, both bacterial and mammalian, have been studied in great detail mechanistically,[8,21,26,40,41] and attention has recently been focused upon the identification of peptide sequence homology, acceptor binding domains, and catalytic residues.[42–45]

For our experiments, we focused on mammalian fucosyltransferases responsible for the biosynthesis of sialyl trimeric $Lewis^x$, which is a portion of the sugar chains attached to the P-selectin glycoprotein ligand-1 (PSGL-1) glycan.[46] These include FucTIII, V, VI, and VII. FucTV and FucTVI are commercially available (Calbiochem, San Diego, CA), and FucTIII and FucTVII were expressed as baculovirus constructs in cultured insect cells.[47] Inhibition assays were performed and the results are summarized in Table 1. Both **1** and **16** display competitive inhibtion against all four mammalian fucosyltransferases with K_Is at or below K_m values.[44,48,49] Given earlier studies of FucTV, correlating inhibition of **16** with a cationic transition state species,[8] and 90% homology between the studied fucosyltransferases, we deduced that FucTIII, V, VI, and VI perhaps share considerable mechanistic features, including formation of an oxocarbenium-ion species in the transition state (Fig. 1), but the proposed mechanism remains to be proven.

An interesting issue is whether these competitive inhibitors are non-reacting analogues or slow substrates to the FucTs. Whereas it is known that GDP-2F-Fuc acts as a slow substrate for FucTIII, the same molecule is not accepted as a substrate for FucTV or FucTVI.[8,50,51] Within this inhibition study the effect of very slow reactivity is negligible within the assay timeframe, the differential reactivity between the enzymes is indicative of

Table 1. Inhibition of four human fucosyltransferases by GDP-2F-Fuc, GDP-6F-Fuc, and GDP-α-2F-Fuc

	GDP-Fuc K_m (μM)	 (**16**) K_i (μM)	 (**1**) K_i (μM)	 (**8**) K_i (μM)
Fuct-III	33.6	38±4	22±10	—
Fuct-V	18.8	4.0±0.6	3.4±1.0	36±4
Fuct-VI	9.0	10±2.4	1.0±0.5	2±1
Fuct-VII	8.0	21±2	11±2	—

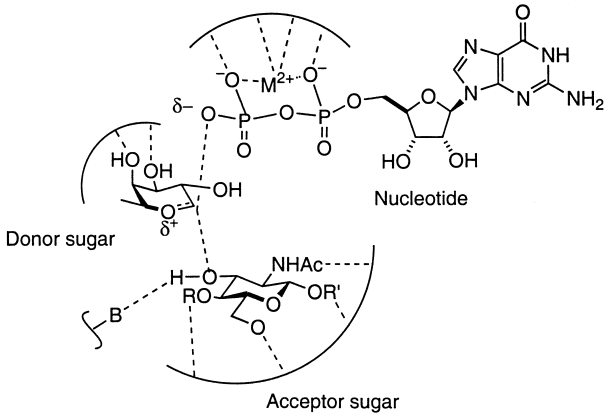


Figure 1. Putative transition-state structure of fucosyltransferases.

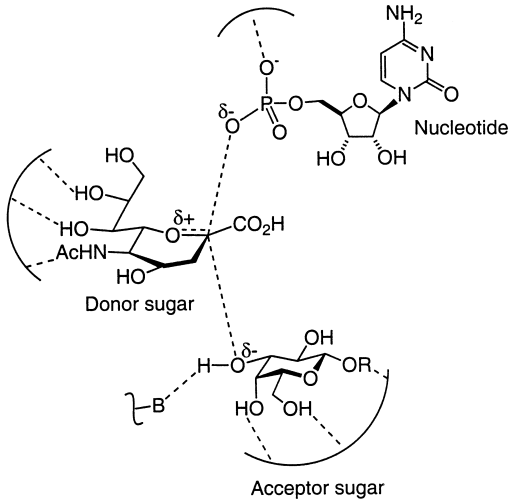


Figure 2. Putative transition-state structure of sialyltransferase.

possible mechanistic differences within the FucT family and merits further study.

Also of interest is the similarity between the K_I values of the two inhibitors, demonstrating that decoration of the glycon at the 2- and 6-positions with fluorine, equidistant from the ring oxygen, provide similar electronic effect. It is therefore valuable to note the similar activity of 2- and 6-fluoro glycosides for future inhibitor design.

Inhibition of **8** with FucTV and FucTVI showed that the unnatural anomeric configuration in the fluorinated analogue provides significant inhibition. This result indicates that the binding domain of fucosyltransferase enzymes for the sugar moiety allows stereochemical flexibility.

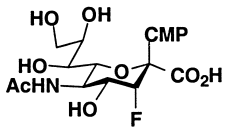
Sialyltransferase inhibition. Sialyltransferases are the enzymes responsible for transfer of sialic acid from cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) to a growing oligosaccharide, often terminating the series at the reducing end.[52] Recent synthesis of potent inhibitors of α-2,6-sialyltransferase (2,6ST) have elucidated important conformational structure of the transition state to include sp^2 character at the anomeric carbon.[53–57] However, a cationic transition-state structure has not yet been demonstrated in mechanistic studies of these enzymes, although one would be predicted given the quaternary structure of the anomeric carbon. Only solvolysis of CMP-Neu5Ac has been shown to display a finite sialyl cation species.[58] Additionally, *N*-Acetyl-3-fluoro-neuraminic acid alone serves as a competitive inhibitor of bacterial and viral sialidases.[59–61] Therefore, inhibition of sialyltransferases by a fluorinated CMP-Neu5Ac derivative will provide additional support for the proposed cationic transition state (Fig. 2).

We chose to study 2,6ST initially due to its commercial availability (Calbiochem). Inhibition assays were performed and are summarized in Table 2. Cytidine 5′-monophospho-*N*-acetyl-3-fluoro-neuraminic acid (**17**)[62] displayed competitive inhibition against 2,6ST with a K_I of 5.7 μM. Compared to the K_m for CMP-Neu5Ac of 15 μM, this result is consistent with a transition-state-structure containing considerable oxo carbenium character.

Galactosyltransferase inhibition. β-1,4-Galactosyltransferase (β-1,4-GalT) has been extensively studied due to its role in the biosynthesis of blood group antigens and sialyl Lewis x.[19,20,63–65] Changes in the in vivo activity of β-1,4-GalT have been shown to be indicative of arthritis and cancer progression.[66,67] A role of β-1,4-GalT in mammalian fertilization as a cell-surface receptor has also been discovered.[68,69] α-1,3-GalT, an enzyme found in pigs and other mammals, is involved in the biosynthesis of xenoantigens responsible for the rejection of xenotransplanted organs in humans.[70] An important distinction of α-1,3-GalT is that a 'retaining' glycosyltransferase, where the anomeric configuration of the transferred galactose moiety remains in alpha configuration, like that of the sugar nucleotide donor uridine 5′-diphosphogalactose (UDP-Gal). β-1,4-GalT is an inverting glycosy1transferase, where the anomeric configuration is reversed in the reaction from α in UDP-Gal to β in the glycoside product. The 'retaining' nature of α-1,3-GalT implies a two-step process for the transfer of UDP-Gal to the acceptor as to that of retaining glycosidases.[9]

β-1,4-GalT catalyzes an inversion of configuration at the anomeric position of the transferred sugar, from α configuration in the sugar nucleotide donor uridine 5′-diphosphogalactose (UDP-Gal) to the transferred β saccharide. Srivastava et al. showed that the 2-deoxygenated derivative of UDP-Gal had kinetic parameters almost identical to the natural substrate with β(1,4)GalT ($K_m = 51$ μM).[71] Given that this hydroxy group may be necessary for substrate binding, an equal or better substrate would affect the oxocarbenium formation, if existing, imparted by removal of the electronegative oxygen at C-2.[72] Takayama et al. demonstrated the inhibition properties of **13** for α-1,3-GalT ($K_i = 2$ μM) and β-1,4-GalT ($IC_{50} = 245$ μM.[8,18] A further piece of mechanistic study could be found in the structural determination of galactosyltransferase by single crystal X-ray diffractometry with a bound inhibitor. Co-crystallization and resolution of UDP-2-deoxy-2-fluorogalactose with this enzyme will help define the amino

Table 2. Inhibition of α2,6ST by CMP-3F-Neu5Ac

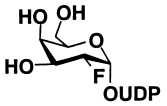
	CMP-Neu5Ac	
	K_m (μM)	(2) K_i (μM)
α2,6ST	15	5.7±2

acids involved in catalysis in order to confirm the mechanistic hypothesis.

In assaying both α and β galactosyltransferase, we hoped to understand inherent mechanistic differences between the two classes of enzyme. Table 3 summarizes the inhibition resufts. The large disparity in IC_{50} values between α and β galactosyltransferase (35-fold difference) may be explained by the mechanistic assumptions made about these enzymes. We can rationalize inhibition of β(1,4)GalT in a manner similar to fucosyltransferases and sialyltransferase above. Formation of the oxocarbenium in displacement of the nucleotide pyrophosphate and prior to addition of the acceptor sufficiently may explain this result (Fig. 3).

α(1,3)GalT, on the other hand, has been predicted to undergo a two-step, double-displacement mechanism in which a nucleophile is transiently involved in forming a glycosyl–enzyme complex.[73] This mechanism is analogous to that of retaining glycosidases, which have been conclusively demonstrated to form a covalent enzyme–glycon intermediate.[14] Here the leaving group (UDP) and the acceptor reside on the same side of the glycon, indicating a synclinal, two-step mechanism. Ichikawa et al. demonstrated inhibition by a charged iminocyclitol, implicating interaction with a carboxylate residue to impart strong binding.[74] Because this proposed mechanism includes the existence of a covalent intermediate between enzyme and the glycon, we investigated enzyme inactivation by **13** of α(1,3)-GalT.[75] 2-Deoxy-2-fluoro glycosides have been shown to inactivate glycosidases following formation of an enzyme–glycon covalent intermediate.[14] Our studies, which analyzed inactivation concentrations of 0, 1, 0.1, and 0.01 mM, showed no enzyme inactivation by the fluorinated nucleotide sugar. Hence, the mechanism may differ significantly from the formation of a covalent intermediate in the two-step reaction of glycosidases.

Table 3. Inhibition of α(1,3)GalT and β(1,4)GalT by UDP-2F-Gal

	GDP-Gal	
	K_m (μM)	K_i (μM)
α(1,3)GalT	17±3	245
β(1,4)GalT	3±6	2.0±0.3

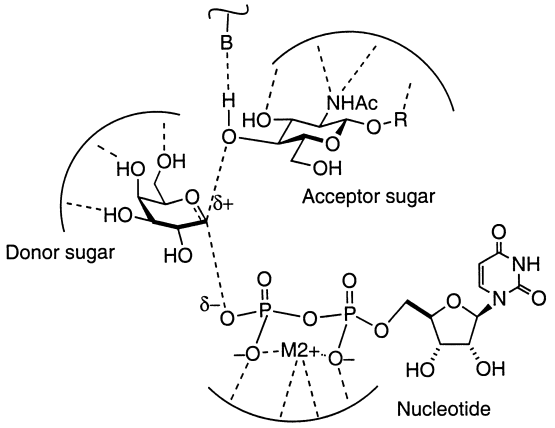


Figure 3. Putative transition-state structure of β-galactosyltransferase.

Conclusion

Here we have provided useful synthetic methods for the preparation of fluorinated sugar nucleotides which may find use as probes of glycosyltransferases. This study demonstrates that the general non-retaining glycosyltransferase reactions may proceed through a charged species associated with the donor glycon within the transition-state structure, most the oxocarbenium-ion species. Alternatively, these fluoro nucleotides may simply bind to the active site as non-productive substrates, and therefore are observed as competitive inhibitors. This trend is seen with human fucosyltransferase III, V, VI, and VII, α(2,6)sialyltransferase, and β(1,4)galactosyltransferase. Also demonstrated is the non-inactivation of α(1,3)galactosyltransferase, indicating that retaining glycosyltransferases may not undergo covalent enzyme-glycon intermediates similar to retaining glycosidases. These fluoro sugar nucleotides should be useful for the structural study of glycosyltransferases as they form a stable tight complex with the enzyme.

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