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UDP-6-deoxy-6-fluoro-α-D-galactose binds to two different galactosyltransferases, but neither can effectively catalyze transfer of the modified galactose to the appropriate acceptor

Cara-Lynne Schengrund [a,*], Pavol Kováč [b]

[a] *Department of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA*

[b] *National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Building 8A, Bethesda, MD 20892-0815, USA*

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Abstract

The effect of substitution of the HO-6 of D-galactose with fluorine on the ability of α-(1→3)-galactosyltransferase (EC 2.4.1.151) and β-(1→4)-galactosyltransferase (EC 2.4.1.22) to catalyze its transfer from UDP to an appropriate acceptor was determined. HPLC analyses indicated that each transferase properly catalyzed formation of the expected product [β-D-Gal-(1→4)-D-GlcNAc] for the β-(1→4)-galactosyltransferase and α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-GlcNAc for the α-(1→3)-D-galactosyltransferase] when UDP-α-D-Gal was the substrate. When UDP-6-deoxy-6-fluoro-α-D-galactose (**6**) was used in conjunction with each transferase, no product indicative of transfer of 6-deoxy-6-fluoro-D-galactose to its respective acceptor sugar was identified. 6-Deoxy-6-fluoro-D-galactose (**3**) was obtained by hydrolysis of methyl 6-deoxy-6-fluoro-α-D-galactopyranoside, synthesized by the selective fluorination of methyl α-D-galactopyranoside with diethylaminosulfur trifluoride (DAST), with aqueous trifluoroacetic acid. Acetylation of **3** gave crystalline 1,2,3,4-tetra-*O*-acetyl-6-deoxy-6-fluoro-β-D-galactopyranose, which was converted to the corresponding 1-α-phosphate and used for the synthesis of **6**.

Keywords: Methyl 6-deoxy-6-fluoro-α-D-galactopyranoside; 6-Deoxy-6-fluoro-D-galactose; 1,2,3,4-Tetra-*O*-acetyl-6-deoxy-6-fluoro-β-D-galactopyranose; UDP-6-deoxy-6-fluoro-D-galactose; Galactosyltransferase

1. Introduction

The effects of replacing a specific hydroxyl group of a sugar with a fluorine atom on the ability of the sugar to function as either a ligand for a lectin [1], a molecule transported by a specific carrier [2], or a substrate for a glycosidase [3], have been reported. The effect of substituting a fluorine for the HO-6 of galactose on the activity of a galactosyl transferase has also been studied using the appropriate nucleotide phosphate derivative [4].

Recently, a number of galactosyltransferases, enzymes that catalyze the transfer of galactose from UDP-α-D-Gal to appropriate acceptors, have been cloned and sequenced. Breton et al. [5] analyzed cDNA sequences for these enzymes and classified them into five

* Corresponding author. Tel.: +1-717-531-8048; fax: +1-717-531-7072.

E-mail address: cschengrund@psu.edu (C.-L. Schengrund)

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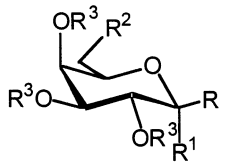
families, four of which have a DxD motif. This motif was proposed to function either in binding of UDP or in the catalytic process. As a first step in determining whether similarities exist in the interaction of different galactosyltransferases with UDP-α-D-Gal, we studied the ability of two galactosyl-transferases to catalyze the transfer of 6-deoxy-6-fluoro-D-galactopyranose from UDP-6-deoxy-6-fluoro-α-D-galactose (**6**) to the appropriate acceptor.

2. Results and discussion

Previous syntheses [4,6] of 6-deoxy-6-fluoro-α-D-galactopyranosyl phosphate (**5**) started with anomeric mixtures of per-*O*-acetyl-6-deoxy-6-fluoro-D-galactopyranose and galactofuranose, formed by acetylation of 6-deoxy-6-fluoro-D-galactose (**3**). In this work, to obtain **6**, we used crystalline 1,2,3,4-tetra-*O*-acetyl-6-deoxy-6-fluoro-β-D-galactopyranose (**4**), obtained as follows. Methyl α-D-galactopyranoside (**1**) was fluorinated with diethylaminosulfur trifluoride (DAST), as previously described for the fluorination of methyl β-D-galactopyranoside [7]. A mixture of products was formed, with methyl 6-deoxy-6-fluoro-α-D-galactopyranoside (**2**) having the slowest mobility upon TLC. The 20–25% yield of pure **2** obtained after silica gel chromatography was comparable to the yield of methyl 6-deoxy-6-fluoro-β-D-galactopyranoside produced, in the same way, from methyl β-D-galactopyranoside [7] as well as the ~20% yield obtained by fluoride ion displacement of a sulfonyloxy group at C-6 of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose [8]. While



	R	R^1	R^2	R^3
1	H	OMe	OH	H
2	H	OME	F	H
3	H,OH		F	H
4	OAc	H	F	Ac
5	H	OPO_3	F	H
6	H	UDP	F	H

not as good as the overall yield (~42%) obtained by converting methyl α-D-galactopyranoside via four synthetic steps [8], it is a much less laborious, more efficient procedure. The conversion of **2**→**3** was conveniently achieved by hydrolysis with aqueous trifluoroacetic acid (TFA).

The two commonly used O-acetylation procedures [9] (using acetic anhydride with either sodium acetate or pyridine) were used to acetylate D-galactose, and the distribution of isomeric acetates formed was evaluated by TLC and NMR spectroscopy. The products in the crude material obtained by either method produced two bands when examined by TLC. NMR spectroscopy indicated that the sodium acetate–acetic anhydride procedure produced the highest percentage of β-pyranose; therefore, it was used for the synthesis of 1,2,3,4-tetra-*O*-acetyl-6-deoxy-6-fluoro-β-D-galactose (**4**). Material highly enriched in **4** was obtained from the first zone following chromatography of the per-O-acetylated products. Crystallization from ethanol then gave pure **4**. The anomeric configuration in **4** followed from the observed $J_{1,2}$ and $J_{C\text{-}1,H\text{-}1}$ coupling constants.

Initial reaction rates for galactosyltransferase-catalyzed transfer of galactose from UDP-^{3}H-Gal to the appropriate acceptor were determined by monitoring the time over which a linear increase in label associated specifically with the neutral sugar fraction was obtained. For β-(1→4)-galactosyltransferase it was at least 120 min, while for the α-(1→3)-galactosyltransferase it was at least 45 min. Inhibition studies indicated that a five-fold molar excess of **6** relative to UDP-Gal reduced the amount of tritiated galactose transferred from UDP-^{3}H-Gal by more than 50% for each enzyme (Table 1). HPLC analyses indicated that both transferases catalyzed formation of the expected product, β-D-Gal-(1→4)-D-GlcNAc for the β-(1→4)-galactosyltransferase and α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-GlcNAc for the α-(1→3)-galactosyltransferase, when UDP-Gal was the substrate. However, when **6** was used as the substrate, no peaks indicative of product synthesis were seen by HPLC analysis. The results obtained with the β-(1→4)-galactosyltransferase are in agreement with the previously reported observation that when

Table 1
Inhibition by **6** of β-(1→4)- and α-(1→3)-galactosyltransferase-catalyzed transfer of galactose from UDP-Gal to acceptor

	% Inhibition	
[**6**]/[UDP-Gal]	β-(1→4)	α-(1→3)
0	0	0
1	24 ± 4 [a]	39 ± 10
5	55 ± 16	76 ± 4
10	74 ± 2	75 ± 4

[a] Values given are percent inhibition plus and minus the standard deviation of the percent inhibition in replicate measurements.

6 was the substrate, it catalyzed the release of a negligible amount (0.3%) of UDP [4]. The lack of detectable product in this study probably reflects (1) use of a much shorter reaction time (1 h in this study compared with 48 h for the synthesis of 6′-deoxy-6′-fluorolactosamine in [4]), and (2) use of different amounts of β-(1→4)-galactosyltransferase (0.25 mU were used in this study compared to the 7 mU used in initial reaction rate studies and 4 U used to obtain 6′-deoxy-6′-fluorolactosamine in [4]). The inhibition studies indicate for the first time that, while the presence of fluorine at C-6 in D-galactose has little effect on the ability of each enzyme to bind to **6**, it apparently negates their ability to catalyze the transfer of the 6-deoxy-6-fluoro-α-D-galactosyl moiety from UDP to the acceptor sugar.

3. Experimental

General methods.—All reactions were monitored by thin-layer chromatography (TLC), on Silica Gel GHLF plates (Analtech, Inc., Newark, DE). Compounds were visualized using 5% sulfuric acid in 95% ethanol and/or UV light. Column chromatography was carried out using either E. Merck Silica Gel 60 [230–400 mesh ASTM (Bodman Industries, Aston, PA)], Dowex-50W (H^+) from Sigma Chemical Co. (St. Louis, MO), or Bio-Gel P-2, Fine (Bio-Rad Laboratories, Hercules, CA), as appropriate. ^1H and ^{13}C NMR spectra were obtained at 300 and 75 MHz, respectively, using a Varian Mercury spectrometer. When feasible, the assignments were supported by homonuclear decoupling experiments or homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometer. Chemical-ionization mass spectra (CIMS) were measured with a Finnegan 4600 Spectrometer, and optical rotations with a Perkin–Elmer Automatic Polarimeter, model 341. Reactions requiring anhydrous conditions were carried out under nitrogen. Porcine, recombinant α-(1→3)-galactosyltransferase, β-(1→4)-galactosyltransferase from bovine milk, linear B-2 trisaccharide, and *N*-acetyllactosamine were purchased from Calbiochem (San Diego, CA), UDP-Gal [Gal-6-^3H, 60 Ci/mM] from American Radiolabeled Chemicals, Inc. (St. Louis, MO), 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose from Aldrich Chemical Co. (Milwaukee, WI), methyl α-D-galactopyranoside from Sigma Chemical Co., and alkaline phosphatase from calf intestine was from Boehringer Mannheim (Indianapolis, IN). Solutions in organic solvents were concentrated at 40 °C/2 kPa.

Preparation of methyl 6-deoxy-6-fluoro-α-D-galactopyranoside (**2**).—DAST (3.75 mL, 28 mmol) was added with stirring over 30 min, at −40 °C, to a suspension of methyl α-D-galactopyranoside (**1**, 1 g, 5 mmol) in dry CH_2Cl_2 (20 mL). The mixture was stirred at −40 °C for another 30 min and then at room temperature for 3 h. TLC (8:1 CH_2Cl_2–CH_3OH) then showed that essentially all of **1** was consumed and that several products had been formed. After cooling to −20 °C, excess reagent was destroyed by the sequential addition of methanol (10 mL), and $NaHCO_3$ (3 g). The cooling bath was removed, and when the effervescence ceased, the mixture was filtered, and the filtrate was concentrated. The residue was chromatographed (silica gel using a step-gradient of 15:1→8:1 CH_2Cl_2–CH_3OH), to give the product having the slowest mobility, pure **2**: mp 139–141 °C (from acetone); lit. [10], mp 139 °C. Yields in various preparations were between 20 and 25%; ^{13}C NMR (D_2O) δ 101.6 (C-1), 84.2 (d, $J_{6,F}$ 168 Hz, C-6), 71.2 (C-3), 70.6 (d, $J_{4,F}$ 6.8 Hz, C-4), 70.6 (d, $J_{5,F}$ 21.5 Hz, C-5), 70.1 (C-2).

Preparation of 6-deoxy-6-fluoro-D-galactose (**3**).—Compound **2** (1.56 g, 7.6 mmol) was

treated with 50% aq trifluoroacetic acid (86 mL) for 5 h at 100 °C. After concentration and co-evaporation with water to remove TFA, chromatography on silica gel, using a step gradient of 12:1 → 5:1 CH_2Cl_2–CH_3OH, gave first unchanged **2**. Eluted next was **3**. Pure **3** was obtained upon crystallization (H_2O–EtOH, 1.1 g, 76% yield), mp 160–162 °C, lit. [10], 160 °C; ^1H NMR (D_2O) for the anomeric mixture (α:β ~ 1:1): δ 5.28 (d, $J_{1,2}$ 3.5 Hz, H-1a), 4.8–4.48 (m, H-6ab, incl. d at 4.61, $J_{1,2}$ 7.9 Hz, H-1b), 4.32 (ddd, $J_{4,5} < 1$, $J_{5,6a}$ 4.0, $J_{5,6b}$ 7.0, $J_{5,F}$ 16.6 Hz, H-5b), 4.04–3.93 (m, H-4a,b,5a), 4.87 (dd, $J_{2,3}$ 10.1, $J_{3,4}$ 3.1 Hz, H-3a) 3.80 (dd, H-2a), 3.66 (dd, $J_{2,3}$ 10.1, $J_{3,4}$ 3.8 Hz, H-3b), 3.50 (dd, $J_{2,3}$ 9.6 Hz, H-2b); ^{13}C NMR (D_2O) δ 96.52 (C-1b), 92.45 (C-1a), 83.57, 83.22 (2d, $J_{6,F}$ 164.6 Hz, H-6a,b), 73.48 (d, $J_{5,F}$ 19.8 Hz, C-5b), 72.6 (C-3b), 71.8 (C-2b), 69.06 (d, $J_{5,F}$ 18.9 Hz, C-5a), 69.06 (d, $J_{4,F}$ 7.4 Hz, C-4a), 68.94 (C-3a), 68.47 (d, $J_{4,F}$ 7.4 Hz, C-4b), and 68.29 (C-2a).

1,2,3,4-tetra-O-*acetyl-6-deoxy-6-fluoro-β*-D-*galactopyranose* (**4**).—Compound **3** (900 mg, 4.7 mmol) was added to a mixture of fused sodium acetate (0.45 g) in acetic anhydride (9 mL) and stirred at 110 °C for 3 h, when TLC (3.5:1 hexane–acetone) showed the reaction to be complete. Excess Ac_2O was destroyed by stirring the mixture with aq $NaHCO_3$. The product was extracted into CH_2Cl_2, and the organic phase was dried and concentrated. TLC showed the presence of two major bands. ^1H NMR spectroscopy indicated the ratio of anomers present to be $\alpha_p:\beta_p:\alpha_f:\beta_f =$ 18.4:52.0:15.4:14.2. Upon chromatography on silica gel (3.5:1 hexane–acetone), the second zone eluted gave **4** in > 90% purity (NMR, 730 mg). The material solidified upon standing and crystallization from EtOH (twice) gave pure **4**, mp 94–96 °C, $[\alpha]_D$ +27.6° (*c* 1.9 $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 5.74 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1), 5.50 (bd, 1 H $J_{3,4}$ ~ 2.9 Hz, H-4), 5.35 (dd, 1 H, $J_{2,3}$ 10.3 Hz, H-2), 5.10 (dd, 1 H, H-3), 4.53 (ddd, 1 H, $J_{5,6a}$ 6.3 Hz, 1 H, $J_{6a,b}$ 9.8 Hz, $J_{6a,F}$ 46.9 Hz, H-6a), 4.44 (ddd, 1 H, $J_{5,6b}$ 5.9 Hz, $J_{6b,F}$ 46.3 Hz, H-6b), 4.11 (m, 1 H, H-5), 2.17, 2.12, 2.05, 2.00 (4 s, 3 H each, 4 $COCH_3$); ^{13}C NMR ($CDCl_3$) δ: 169.97, 169.86, 169.33, 168.88 (4 CO), 92.05 (C-1), 80.19 (d, $J_{6,F}$ 171.9 Hz, C-6), 72.15 (d, $J_{5,F}$ 23.4 Hz, C-5), 70.68 (C-3), 67.70 (C-2), and 66.70 (d, $J_{4,F}$ 5.8 Hz, C-4), 20.69, 20.56, 20.51, 20.46 (4 CH_3). Anal. Calcd for $C_{14}H_{19}FO_9$: C, 48.00; H, 5.47. Found: C, 48.04; H, 5.46.

The mother liquor from crystallization of **4** was combined with material that was unresolved by chromatography, deacetylated with NaOMe–MeOH (Zemplén), and reprocessed as described above.

Preparation of 6-deoxy-6-fluoro-α-D-*galactopyranosyl phosphate* (**5**).—The dipotassium salt of **5** was prepared from **4** (414 mg, 1.18 mmol) as described by MacDonald [11] for α-D-galactose 1-phosphate. The product was purified by chromatography on silica gel using a step-gradient of 10:2:1 → 10:4:0.5 2-propanol–water–CH_3COOH. Yield, 354 mg, 88%: $[\alpha]_D$ +74° (*c* 0.2 H_2O), lit. [6], for the analogous derivative of D-galactose, +81° (*c* 0.2 H_2O); ^1H NMR (D_2O): δ 5.48 (dd, 1 H, $J_{1,2}$ 3.6, $J_{1,P}$ 6.9 Hz, H-1), 4.67, 4.52 (2 m, 1 H each, $J_{6,F}$ 46.6 Hz, H-6a,b), 4.33 (2 t, 1 H, $J_{5,6}$ 5.8, $J_{5,F}$ 17.2 Hz, H-5), 4.02 (dd, 1 H, $J_{4,F}$ 1.1 Hz, $J_{3,4}$ 3.3 Hz, H-4), 3.88 (dd, 1 H, $J_{2,3}$ 10.0 Hz, H-3), 3.74 (ddd, 1 H, $J_{2,P}$ 2.2 Hz, H-2); ^{13}C NMR (D_2O): δ 94.4 (C-1, $J_{C\text{-}1,P}$ 5.7 Hz), 83.65 (C-6, $J_{C\text{-}6,F}$ 163.8 Hz), 69.67 (C-5, $J_{C\text{-}5,F}$ 19.5 Hz), 69.07 (C-3), 69.02 (C-4, $J_{C\text{-}4,F}$ 6.9 Hz), 68.45 (C-2, $J_{C\text{-}2,P}$ 8.0 Hz).

Preparation of UDP-6-deoxy-6-fluoro-α-D-*galactose* (**6**).—The dipotassium salt of **5** was converted to UDP-6-deoxy-6-fluoro-α-D-galactose using the procedure described by Wittmann and Wong [12] for the synthesis of UDP-Gal. After purification on a Bio-Gel P-2 column with 0.25 M NH_4HCO_3 as the eluant, the sample was lyophilized. Recovered UDP-6-deoxy-6-fluoro-α-D-galactose was dissolved in water and converted to the disodium salt by first adding Dowex 50W (H^+) to adjust the pH to ~ 6, filtering to remove the Dowex, and then adding NaOH to bring the pH to ~ 7. Excess salt was removed by filtration through a YC05 membrane that had a molecular-weight cutoff of 500. ^{31}P and ^1H NMR (D_2O) spectral data agreed with those reported previously [4]. Mass spectral analysis of the disodium salt gave the anticipated *m/z* peak at 613 $[M + 1]^+$.

Enzyme assays.—To determine α-(1→3)-galactosyltransferase activity, the enzyme (0.25 mU) was preincubated for about 15 min at 37 °C with 20 mM *N*-acetyllactosamine in 0.1 M sodium cacodylate buffer, pH 6.5, containing 20 mM $MnCl_2$, 36 U of alkaline phosphatase, and 10 μg of bovine serum albumin (BSA) in a total volume of 40 μL. At the end of the preincubation, the solution was made 2 mM in UDP-Gal or **6** by adding 10 μL of a 10 mM solution of the UDP-Gal or **6** in 0.1 M sodium cacodylate buffer, pH 6.5, containing 20 mM $MnCl_2$, and incubated at 37 °C for 30–35 min. In experiments requiring UDP-^{3}H-Gal, an aliquot that gave ~200,000 cpm was added with the UDP-Gal or UDP-Gal plus **6**. The product was separated from unreacted UDP-sugar and UMP by ion-exchange chromatography on Bio-Rad Ag 1-X8 [13]. When labeled substrate was used, the amount of labeled galactose present in the product was determined by counting in a scintillation counter. The presence of nonspecific label in the eluant was accounted for by subtracting label recovered in reactions carried out in the absence of acceptor sugar from that obtained in its presence. When cold substrate was used, the product was identified by chromatography using a Dionex HPLC (Sunnyvale, CA) with a Dionex CarboPac™ PA1 (4 × 250 mm) column and NaOH (0.1 M) as the solvent. NaOH (0.4 M) was added to the eluant prior to passage through a pulsed amperometric detector.

A similar procedure was used to determine the activity of β-(1→4)-galactosyltransferase. Enzyme (0.25 mU) was preincubated for 15 min at 37 °C with 20 mM 2-acetamido-2-deoxy-D-glucose in 50 mM HEPES, pH 7.2, containing 20 mM $MnCl_2$, alkaline phosphatase (36 U) and BSA. Except for the use of a 1 h incubation time, the rest of the procedure was unchanged from that for α-(1→3)-galactosyltransferase. The ability of **6** to inhibit the transfer of galactose from UDP-^{3}H-Gal to acceptor was monitored by carrying out the reaction in the presence of inhibitor and then determining the amount of label associated with material eluted from the Bio-Rad AG 1-X8 column with water. As described above, the presence of nonspecific label in the eluant was accounted for by subtracting label recovered in reactions carried out in the absence of acceptor sugar.

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