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A Chemoenzymatic Synthesis of UDP-(2-deoxy-2-fluoro)-galactose and Evaluation of its Interaction with Galactosyltransferase

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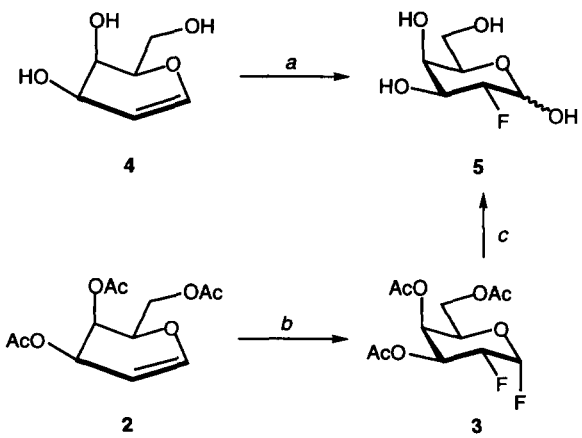
Abstract—Uridine 5′-diphospho-(2-deoxy-2-fluoro)galactose (UDP-2FGal), prepared and characterized for the first time by a chemoenzymatic method, was found to be a competitive inhibitor of β-1,4-galactosyltransferase with a K_i value of 149 μM. This study supports that the glycosyltransferase reaction mechanism proceeds through a glycosidic cleavage transition state with sp^2 character developed at the anomeric center. © 1997 Elsevier Science Ltd. All rights reserved.

Introduction

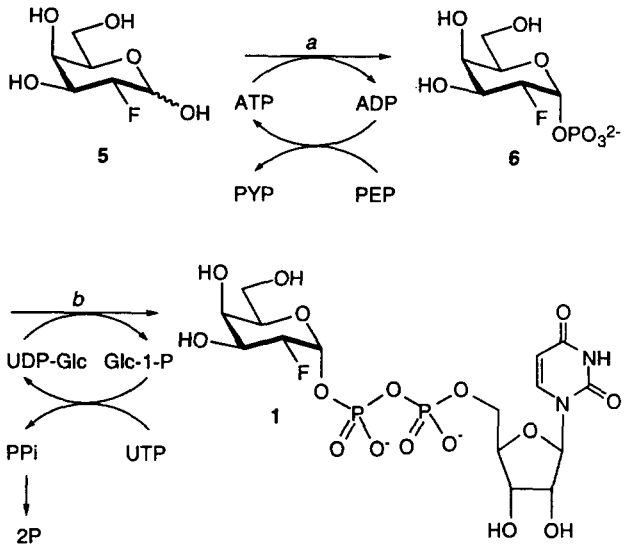
The sugar nucleotide-dependent glycosyltransferases that catalyze the transfer of a sugar from nucleotide to specific oligosaccharides are a group of enzymes with great potential as catalysts for the synthesis of complex oligosaccharides and as targets for inhibition.[1] Although several glycosyltransferases have been evaluated with regard to their specificities and synthetic applications, there are only a limited number of studies on the inhibition and mechanism of these enzymes,[2,3] and in most cases the inhibitors reported are relatively weak with K_i in the mM range. Recently, it has been shown that replacement of the 2- or 5-hydroxyl group of glycosyl analogs by a strongly electron-withdrawing atom such as fluorine has a large decelerating effect on the glycosidase catalyzed hydrolysis. Thus, 2- or 5-fluoroglycosides have been used as mechanism-based inhibitors which are processed by these glycosidases to form a stable covalent adduct.[4] β-1,4-Galactosyltransferase proceeds with a different stereochemistry, inversion of configuration, which stimulated us to prepare nucleotide-linked 2-deoxy-2-fluoroglycosides for investigation of their interaction with glycosyltransferases. Previous studies on the metabolism of 2-deoxy-2-fluorogalactose suggested that UDP-2-deoxy-2-fluorogalactose might be formed in vivo to inhibit glycosylation.[5] UDP-2-deoxy-2-fluorogalactose (or any 2-fluoro-sugar nucleotide), however, has never been prepared for evaluation to support this argument. Reported here is the first synthesis, characterization, and biological evaluation of uridine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactose (UDP-2FGal, **1**) as β-1,4-galactosyltransferase inhibitor.



Scheme 1. (a) XeF_2 (1.8 equivalents), H_2O, rt, 1.5 h, 30%. α:β=1:1.5. (b) XeF_2, $BF_3{\cdot}OEt_2$, Et_2O–benzene, rt, 2.5 h, 78%. (c) 2 N HCl, 90 °C, 2 h, 83%, α:β=1:1.6.

Results and Discussion

Schemes 1 and 2 illustrate the synthesis of **1**. The key intermediate 2-deoxy-2-fluorogalactose (**5**) used in the enzymatic synthesis of **1** could be prepared from tri-*O*-acetyl-D-galactal (**2**) by electrophilic fluorinating reagents such as F_2, CF_3OF, AcOF, etc.[6] These gas reagents are unfortunately difficult to handle. In contrast, XeF_2, which is available as a solid, has been used in the fluorination of protected galactal **2** in the presence of a Lewis acid.[7] We have improved the previously reported yield of the difluoro compound **3** to 78% by using an equivalent of XeF_2 and catalytic $BF_3{\cdot}Et_2O$ in ether–benzene. This was then converted to the desired 2-deoxy-2-fluorogalactose (**5**) by heating in 2 N HCl. We also found that **5** can be prepared directly from nonprotected D-galactal (**4**) in water via reaction with 1.8 equivalents of XeF_2 at room temperature,[8] and the reaction is complete in 2 h to give **5** as the only product in 30% yield.[9] This observation greatly simplifies the synthesis of the target molecule.



Scheme 2. (a) Galactokinase, pyruvate kinase, ATP·Na_2, phosphoenolpyruvate·Na_3 (PEP·Na_3) dithiothreitol, cysteine, Mg^{2+}, Mn^{2+}, K^+, HEPES 100 mM, pH 7.4, four days under Ar, rt, 58%. (b) Galactose-1-phosphate uridytransferase, UDP-glucose (UDP-Glc) pyrophosphorylase, pyrophosphatase, UTP·Na_3, UDP-Glc·Na_2, dithiothreitol, pH 8.4, two days under Ar, rt, 67%.

Compound **5** was then enzymatically converted to UDP-2FGal **1** via 2-deoxy-2-fluoro-α-D-galactopyranosyl phosphate (2FGal-1P, **6**) as shown in Scheme 2. In the first step, galactokinase from yeast (Sigma) catalyzes the direct phosphorylation at the anomeric center of **5** with ATP.[10] The reaction was coupled with the phosphoenol pyruvate and pyruvate kinase cofactor regeneration system in order to scale up the reaction and simplify product isolation.[11] The enzymatic reaction produced only the α-form of compound **6** in 58% isolated yield. Compound **1** was then prepared from **6** and UDP-glucose catalyzed by yeast galactose-1-phosphate uridyltransferase (Sigma). The reaction was coupled with the regeneration of UDP-glucose from glucose-1-phosphate using yeast UDP-glucose pyrophosphorylase and pyrophosphatase (both from Sigma).[2,11] The product was further purified by ion-exchange chromatography to obtain **1** in 67% yield (71 mg). Compound **1** was also prepared directly from **5** in 40% yield without isolation of **6**. NMR analysis showed that the axial proton at C2 position of galactose exhibits a characteristic dddd pattern at 4.633 ppm (J=49.5, 10.0, 3.5, 3.0 Hz) due to coupling with fluorine, phosphorus, H1 and H3. An alternative synthesis of **1** via reaction of **6** and uridine 5′-monophosphomorpholidate was explored but the yield of **1** was low and its separation from UMP and unreacted **6** was difficult.[12] However, a procedure recently developed in our laboratory for the morpholidate coupling could prove to be a valuable tool for this type of synthesis.[13]

Compound **1** was then evaluated as inhibitor of β-1,4-galactosyltransferase from bovine milk (Sigma) according to the procedure described previously.[2] Varying UDP-Gal concentrations and inhibitor **1** in the presence of the acceptor *N*-acetyl-α-D-glucosamine, we obtained a series of double reciprocal plots as shown in Figure 1, which demonstrate that compound **1** is a competitive inhibitor versus UDP-Gal. The corresponding 2-deoxy derivative is a good substrate for the transferase that indicates that the 2-hydroxyl group is not critical for catalysis.[11b,14] The apparent K_m of UDP-Gal and K_i of UDP-2FGal were determined to be 127 ± 9 and 149 ± 18 μM, respectively. The K_i of **1** is similar to the K_m of UDP-Gal, which is in agreement with a study of α-1,3-fucosyltransferase V which shows that the affinity for the enzyme is unchanged by the fluorine substitution and only the intrinsic difference in reactivity allows it to be a very good inhibitor.[13] Furthermore, the relative enzyme activity did not decrease after preincubation of the enzyme (5–60 min) in a buffered solution containing **1** and *N*-acetylglucosamine, indicating that the fluoro-sugar nucleotide is not a mechanism-based inactivator. These results support the view that the glycosyltransferase reaction proceeds through a glycosidic cleavage transition state with sp^2 character developed at the anomeric center, consistent with the result based on secondary deuterium isotope effect.[15] Unlike in the case of the glycosidase reaction, the glycosidic bond of the 2-fluoro-sugar nucleotide is inert toward the glycosyltransferase.

Conclusion

UDP-2FGal **1** was prepared, characterized, and shown to be a competitive inhibitor of β-1,4-galactosyltransferase with respect to UDP-Gal. This result is consistent with the proposal that a significant glycosidic bond cleavage occurs prior to the nucleophile attack on the anomeric position of UDP-Gal.

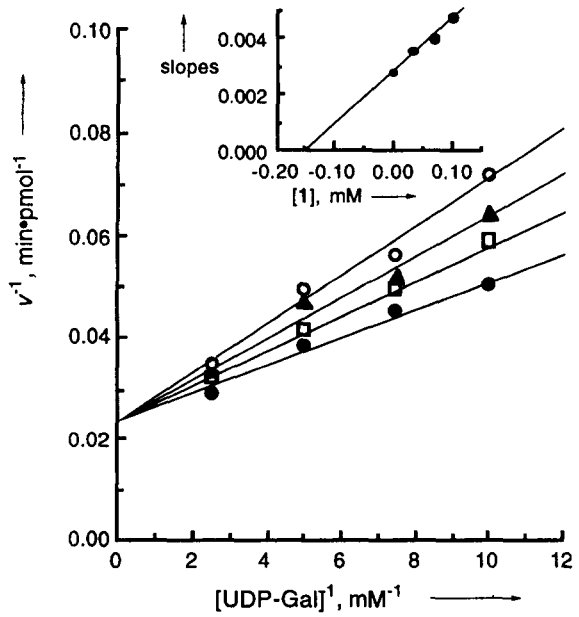


Figure 1. Inhibition kinetics of β-1,4-galactosyltransferase with UDP-2FGal **1** with respect to UDP-Gal in the presence of *N*-acetyl-D-glucosamine (GlcNAc) (1.0 mM) and Mn^{2+} (1.0 mM) in HEPES (100 mM, pH 7.4). The concentrations of inhibitor **1** were 0 mM (●), 0.035 mM (□), 0.070 mM (▲), and 0.105 mM (○). Inset: secondary plot of slopes obtained from the double reciprocal plots versus [**1**].

Experimental

2-Fluoro-D-galactose (5).

From tri-*O*-acetyl-D-galactal (2). To a mixture of tri-*O*-acetyl-D-galactal (870 mg, 3.2 mmol) and XeF_2 (540 mg, 3.2 mmol) in anhydrous Et_2O (17 mL) under Ar was added dropwise a solution of $BF_3{\cdot}OEt_2$ in anhydrous benzene (16 mL). The resulting mixture was then stirred at rt for 2.5 h. The mixture was then washed with a saturated $NaHCO_3$ solution (2×50 mL), water (50 mL), and dried over $MgSO_4$. After filtration, the mixture was evaporated and the residue purified by silica-gel column chromatography with hexane:AcOEt (4:1) to give **3** (770 mg, 78%). Compound **3** (765 mg, 2.5 mmol) was then heated at 90 °C in 2 N HCl for 2 h. The mixture was then allowed to cool to 25 °C, neutralized with K_2CO_3 and evaporated. The residue was taken up with MeOH (10 mL) and the insoluble removed by filtration. After evaporation, the residue was redissolved in H_2O and adsorbed on silica gel (200 mg). Column chromatography with $CHCl_3$:MeOH (50:1–5:4) afforded **5** (629 mg, 83%,α/β = 1:1.6).

From D-galactal (4). A mixture of D-galactal (146 mg, 1.0 mmol), XeF_2 (305 mg, 1.8 mmol), and water (2.5 mL) was stirred at rt for 1.5 h. The workup was similar to the previous procedure. Silica-gel column chromatography with $CHCl_3$:MeOH (50:1–5:4) gave **5** (54.6 mg, 30%, α/β=1:1.5). ^{1}H NMR (D_2O, 500 MHz) δ 5.418 (d, H1, J=4.0 Hz, α-form), 4.789 (dd, H1, J=7.5, 3.0 Hz, β-form), 4.613 (ddd, H2, J=49.5, 10.0, 4.0 Hz, α-form), 4.277 (ddd, H2, J=52.0, 9.5, 8.0 Hz, β-form), 4.08–3.86 (H3, H4, and H5, m), 3.72–3.67 (H6, m). HRMS (FAB) calcd for $C_6H_{11}O_5F$ ($M+Na^+$) 205.0488; found 205.0482. The data are consistent with the literature values.[6b]

2-Deoxy-2-fluoro-α-D-galactopyranosyl phosphate (6). To a solution of HEPES buffer (100 mM, pH 7.4, 9 mL) containing $MgCl_2{\cdot}6H_2O$ (10 mM), $MnCl_2{\cdot}4H_2O$ (5 mM), KCl (20 mM), ATP·Na_2 (18 mg, 33 μmol), phospho(enol)pyruvate·Na_3 (111 mg, 475 μmol), and cysteine (8 mg, 66 μmol) was added **5** (60 mg, 327 μmol), dithiothreitol (13 mg, 82 μmol), galactokinase (5 units) and pyruvate kinase (200 units), and the reaction mixture was then stirred at rt under argon. After four days, $BaCl_2{\cdot}H_2O$ (255 mg, 1.0 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 obtained precipitate was washed with cold H_2O:acetone (1:1, 2×5 mL) and acetone (5 mL). After the resulting powder was dried in vacuo, the barium salt of **6** was obtained (75 mg, 58%). An aliquot was treated with ion-exchange resin (Dowex 50W-X8, H^+ form), neutralized with NaOH and evaporated for characterization. ^{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).

Uridine 5′-diphospho-2-deoxy-2-fluoro-α-D-galactose (1). Compound **6** (70 mg, 176 μmol) was dissolved in H_2O (1 mL) and treated with Dowex 50W-X8 (H^+ form, ca. 0.7 mL) for 20 min. After filtration to remove the resin and washing with H_2O (3×1 mL), the filtrate was neutralized with 1 N KOH. To this solution was added $MgCl_2{\cdot}6H_2O$ (14 mg, 69 μmol), $MnCl_2{\cdot}4H_2O$ (6 mg, 30 μmol), UTP·$Na_3{\cdot}2H_2O$ (37 mg, 63 μmol), UDP-glucose·Na_2 (2 mg, 3.2 μmol) and the solution was adjusted to pH 8.4 with 1 N KOH. After bubbling a stream of Ar into the solution for 30 min, dithiothreitol (9 mg, 60 μmol), galactose-1-phosphate uridyl transferase (5 units), uridine-5′-diphosphoglucose pyrophosphorylase (5 units), and inorganic pyrophosphatase (5 units) was added and the mixture (total volume = 15 mL) was stirred at rt. After 6, 21, and 45 h, 37 mg-portions of UTP·$Na_3{\cdot}2H_2O$ were added and the pH of the mixture was readjusted to 8.4 with 1 N KOH after every addition. After three days, the reaction was stopped by immersing the tube into a bath of boiling water for 90 s. The resulting precipitate was removed by centrifugation and the supernatant was applied to a Dowex 1-X8, (100–200 mesh, Cl^- form, 1.5 × 25 cm) ion-exchange column. After washing the column with H_2O (50 mL), the desired compound was eluted with a gradient of 0.04–0.4 M LiCl in 0.003 N HCl (1.2 L). The fractions containing **1** were combined and adjusted to pH 6.0 with LiOH. After evaporation, the residue was dissolved in MeOH (4 mL) and acetone (40 mL) was further added to the solution. The obtained precipitate was collected and retreated with MeOH and acetone until the supernatant solution was freed from Cl^-. The precipitate was dissolved in H_2O and passed through an ion-exchange resin (Dowex 50W-X8, H^+ form) and neutralized with NaOH to give **1** as the sodium salt (71.8 mg, 67%). ^{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.

Inhibition study

All assays contained 1 mM of $MnCl_2$, 0–0.4 mM of UDP-[6-^{3}H]-galactose, 5 μL of 0.08 mg/mL galactosyltransferase, 1 mM of *N*-acetyl-α-D-glucosamine, 0–0.105 mM of **1**, and 100 mM of HEPES buffer (pH 7.4) in a total assay volume of 0.05 mL. Assays were performed at 25 °C and reactions were halted with the addition of 0.3 mL of distilled, deionized water after 10 min. The reaction mixtures were passed through a pipette column (2 cm) of Sepadex QAE A-25 with

1 mL of distilled, deionized water. The obtained fractions were collected in 10 mL of ScintiVerse I scintillation cocktail. A control reaction without enzyme was used to establish the background, nonenzymatic cleavage rate. Double reciprocal analysis of galactosyltransferase as a function of **1** and UDP-Gal exhibited a competitive pattern. A precise inhibition constant for **1** was determined to be 149 ± 18 μM with a nonlinear, least-square fit of the data to the kinetic equation for competitive inhibition with the Compo FORTRAN program adapted for the Apple Macintosh.[16]

Acknowledgments

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8. The initial reaction mixture was found to be acidic (pH 1.5).

9. This reaction gave only 2-deoxy-2-fluorogalactose **5** as judged by the H1–H2, H2–H3, and H1–F2 coupling constants (J_{H1-H2}=4.0, J_{H2-H3}=10.0, and J_{H1-F2}<0.5 Hz in α-form and J_{H1-H2}=7.5, J_{H2-H3}=9.5, and J_{H1-F2}=3.0 Hz in β-form), which are consistent with the literature values,[6b] whereas the fluorination of glucal under the same condition led to a mixture of 2-deoxy-2-fluoroglucose and 2-deoxy-2-fluoromannose.

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