Design and Synthesis of 2′-Deoxy-2′-Fluorodisaccharides as Mechanism-Based Glycosidase Inhibitors That Exploit Aglycon Specificity

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Abstract: Stable, aglycon-specific inactivators of glycosidases have considerable potential as tools in the study of mechanisms of oligosaccharide processing, and possibly as avenues toward new therapeutics. Glycosidases for which the rate-determining step with the natural substrate is the hydrolysis of the glycosyl-enzyme intermediate are shown to be inactivated by the 2′-deoxy-2′-fluoro derivative of this substrate. Thus *Agrobacterium faecalis* β-glucosidase is inactivated by 2′-deoxy-2′-fluorocellobiose according to inactivation parameters of $k_i = 0.018$ min^{-1} and $K_i = 20$ mM. Inactivation is shown to occur *via* the accumulation of the same 2-deoxy-2-fluoroglycosyl-enzyme intermediate as that formed using activated 2-deoxy-2-fluoroglycosides by identification of the labeled peptide in proteolytic digests. Thus, interactions between the enzyme and the sugar aglycon provide sufficient transition state stabilization to allow formation and trapping of the glycosyl-enzyme. β-Glucocerebrosidase, a β-glucosidase specific for hydrolysis of glucocerebrosides, is not inactivated by 2′-deoxy-2′-fluorocellobiose, thereby demonstrating the aglycon specificity of this class of inactivator.

Introduction

Previously reported covalent glycosidase inactivators have included conduritol epoxides,[1] glycosylmethyltriazenes,[2] *N*-(haloacetyl)glycosylamines,[3] and 2-deoxy-2-fluoroglycosides with good leaving groups such as 2,4-dinitrophenolate or fluoride.[4,5] Each of these inhibitor classes relies upon the presence of either a reactive functional group or activated leaving group in the molecule which results in covalent labeling of the enzyme. Glycon specificity is conferred by structural similarity of the inactivator to the appropriate sugar in the glycon portion of the molecule, but none of these inactivators exploit aglycon specificity, which is sacrificed to permit incorporation of a reactive or activated, but non-natural, moiety. Glycosidases featuring multiple subsites, such as the paradigmatic lysozyme, bind oligosaccharide substrates in two or more adjacent active site regions. The glycon, or catalytic, subsite contains the catalytic residues and binds the nonreducing end sugar proximal to the bond to be cleaved while the aglycon subsite binds the "aglycon" leaving group portion of the substrate, which may, depending on the enzyme, be one or more sugars, a complex oligosaccharide, a fatty acyl group, or various other moieties.

We report the design of a covalent, mechanism-based inactivator of a retaining glycosidase[6] that incorporates full aglycon, and essentially full glycon, specificity. Inhibitors of this class have a single sterically conservative modification, differing from the natural substrates of the enzyme by the replacement of a single hydroxyl group by fluorine on an oligosaccharide which could potentially consist of several sugar residues of different type and linkage specificity. Such compounds thus have the potential for extremely selective inhibition of specific glycosidases and glycosyltransferases, which could be of considerable significance in the design of the therapeutic agents targeting these enzymes. They should also, unlike inactivators which include an activated leaving group, be extremely stable to spontaneous hydrolysis.

While most glycosidase inactivators function by the (essentially) irreversible reaction of reactive functional groups with active site residues, 2-deoxy-2-fluoroglycosides with good leaving groups inactivate retaining glycosidases by the trapping of a stabilized 2-deoxy-2-fluoroglycosyl-enzyme rendered kinetically accessible by the presence of the activated leaving group. This leaving group accelerates only the first, glycosylation step while the C-2 fluorine slows *both* glycosylation and deglycosylation steps so that the resulting 2-deoxy-2-fluoroglycosyl-enzyme accumulates. With some enzymes, it may be possible to incorporate the *natural aglycon* in place of this unnatural leaving group since the additional binding energy derived from interactions between the aglycon and the enzyme's aglycon-binding subsite may substantially increase the rate of the glycosylation step, resulting in a natural leaving group of nucleofugacity effectively comparable to that of unnatural leaving groups of much lower pK_a. The requirement would therefore be that the deglycosylation step for the natural substrate be slower than glycosylation, and thus most likely be rate-limiting. Indeed, with *Agrobacterium* β-glucosidase, the k_{cat} value for cellobiose (4-*O*-(β-D-glucopyranosyl)-D-glucose), the natural substrate of the enzyme, is 9900 min^{-1}, comparable to that of *p*-nitrophenyl β-D-glucoside (10 100 min^{-1}) or 2,4-dinitrophenyl β-D-glucoside (10 700 min^{-1}),[7,8] despite 10^7-10^{10}-

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(6) Retaining glycosidases catalyze the hydrolysis of glycosidic bonds with net retention of anomeric configuration, presumably by a double-displacement mechanism involving attack of an enzymic nucleophile, leading to formation of a glycosyl-enzyme intermediate ("glycosylation") and subsequent hydrolysis of the intermediate ("deglycosylation").[18]

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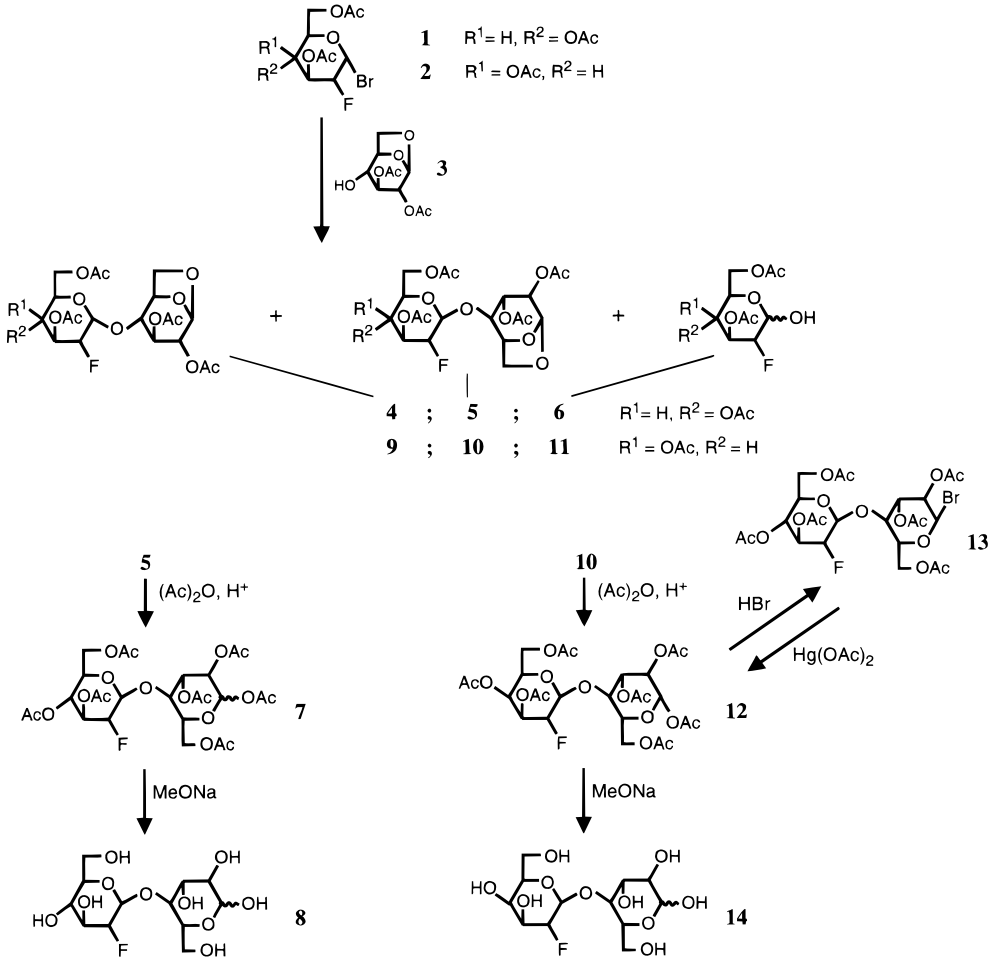
Scheme 1



fold differences in leaving group K_a (the pK_a of the 4-position hydroxyl of glucose is ~14, *versus* pK_a values of 7.1 and 3.9, respectively, for *p*-nitrophenolate and 2,4-dinitrophenolate). Deglycosylation has been shown to be rate-limiting with this enzyme for these two aryl glycosides and must therefore also be rate-limiting for cellobiose.[7] Thus binding interactions of the enzyme with the sugar aglycon increase the effective leaving group ability of the sugar 10^7-fold.[9] If this binding energy is sufficient to ensure that the deglycosylation step with a 2′-deoxy-2′-fluoro disaccharide is rate-limiting, then the resulting intermediate (identical to that found upon release of 2,4-dinitrophenolate or fluoride) would be expected to accumulate, inactivating the enzyme. In order to test this hypothesis, the fluorinated disaccharides 4-*O*-(2-deoxy-2-fluoro-β-D-glucopyranosyl)-D-glucose (2′-deoxy-2′-fluorocellobiose, **8**) and 4-*O*-(2-deoxy-2-fluoro-β-D-galactopyranosyl)-D-glucose (2′-deoxy-2′-fluorolactose, **14**) were synthesized.

Results and Discussion

Synthesis of β-1,4-Linked 2′-Deoxy-2′-fluorodisaccharides. 2′-Deoxy-2′-fluorocellobiose (8). Two methods were used to synthesize the title compound **8**. The less stereoselective approach resulted in the desired diastereomeric fluorodisaccharide as the minor diastereomer but required fewer synthetic steps and gave good mass recovery of fluorodisaccharide anomers. The stereoselective approach required four additional synthetic steps (thiolation, benzylation, bromination, and hydrogenolysis after coupling) but was highly stereoselective in the key glycosylation step. However, despite the fact that the yield of the protected β-1,4-linked fluorodisaccharide **19** in the coupling step was doubled, the stereoselective approach resulted in lower overall yield (based on the common intermediate **1** of fluorodisaccharides due to the extra steps required.

In the low stereoselectivity approach (Scheme 1), the fluoroglycosyl bromide **1** was reacted with the anhydro sugar **3** in the presence of silver carbonate, silver trifluoromethanesulfonate, and Drierite in acetonitrile. After being stirred overnight at room temperature, the mixture was filtered and chromatographed on silica to give a mixture of the previously reported[10] α-glycoside **4** and the novel β-1,4-linked fluorodisaccharide **5** along with unreacted **3** and the hydrolysis product **6**. The 2′-deoxy-2′-fluorodisaccharides were isolated in ~50% yield (based on the bromosugar) with an α/β anomeric ratio of ~70/30 as determined by ^{1}H-NMR. The protected fluorocellobiose derivative **5** was isolated from the fluorodisaccharide mixture by fractional crystallization, chromatography, and crystallization. Acetolysis of the 1,6-anhydro ring of **5** gave a mixture of anomeric acetates **7** which could not be induced to crystallize. Upon careful chromatography two samples that were enriched in each anomer were isolated and their ^{1}H-NMR spectra determined. Deacetylation of **7** by the Zemplén method[11] using sodium methoxide in methanol gave a quantitative yield of the syrupy 2′-deoxy-2′-fluorocellobiose (**8**).

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(8) Day, A. G.; Withers, S. G. *Can. J. Biochem.* **1986**, *64*, 914.
(9) Based upon the similar enzyme-catalyzed hydrolysis rates observed for the glycosides of *p*-nitrophenol (pK_a = 7.1) and glucose (pK_a ≈ 14).
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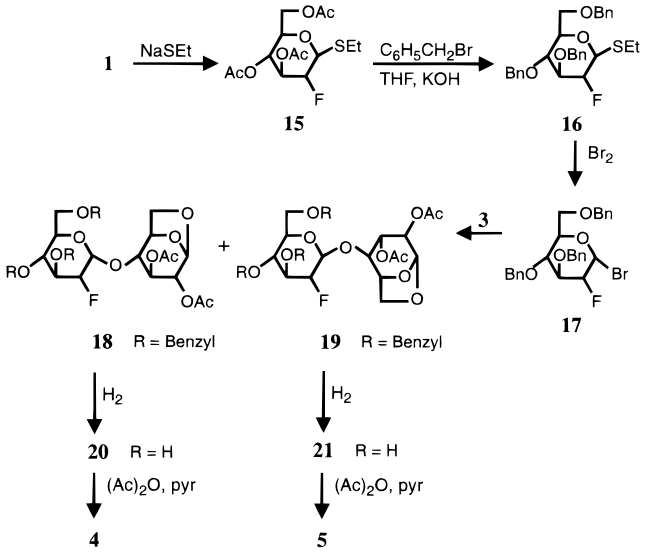
Scheme 2



In the high stereoselectivity approach (Scheme 2) the bromosugar **1** was treated with sodium ethanethiolate to give the ethyl β-thioglycoside **15** exclusively. Replacement of the acetate protecting groups with benzyl ethers was achieved by treating **15** with benzyl bromide in THF in the presence of sodium hydroxide. The newly protected thioglycoside **16** was converted to the bromide **17** by reaction with bromine. Addition of the partially protected anhydro sugar **3** to **17** in dichloromethane in the presence of 1.2 equiv of silver triflate at −78 °C afforded a mixture of the fluorodisaccharides **18** and **19**. Chromatographic separation of the fluorodisaccharides from hydrolyzed and unreacted starting materials gave a 10/90 ratio of the α- and β-1,4-linked fluorodisaccharide products from which the two anomers could be isolated in >95% purity by rechromatography. Hydrogenolysis of the benzyl ethers of **18** and **19** gave the partially protected fluorodisaccharides **20** and **21**. Acetylation of **20** and **21** gave products that were identical to fluorodisaccharides **4** and **5**, respectively.

2′-Deoxy-2′-fluorolactose (14). In a procedure identical to that for the synthesis of 2′-deoxy-2′-fluorocellobiose (**8**) *via* the low selectivity method, the fluorogalactosyl bromide **2** (Scheme 1) was reacted with the anhydro sugar **3** in acetonitrile in the presence of silver salt promoters. A mixture of the previously reported[10] 2′-deoxy-2′-fluorodisaccharide derivative **9** and the novel 2′-deoxy-2′-fluorolactose derivative **10** was chromatographically isolated along with the unreacted anhydro sugar **3** and the hydrolysis product **11**. The fluorodisaccharides were isolated in a ∼60/40 ratio of α-linked to β-linked anomers and the α-linked anomer was crystallized from the mixture. However, 2′-deoxy-2′-fluorolactose derivative **10** in the mother liquor could not be induced to crystallize despite its predominance (90%), nor could this enriched mixture be further purified by chromatography. In order to obtain a pure sample of **10** the mixture of anomers was treated with acetic anhydride and sulfuric acid (acetolysis of the 1,6-anhydro ring) to give an anomeric mixture of the fully acetylated α- and β-1,4-linked fluorodisaccharides. The α- and β-1,4-linked fluorodisaccharides could be partially separated chromatographically to give an anomeric mixture of the 1-*O*-acetates of compound **12** in high purity. This purified anomeric mixture of acetates could not be crystallized, but transformation of the mixture to the corresponding protected 2′-deoxy-2′-fluorolactosyl bromide **13** and treatment with mercuric acetate give exclusively the β-acetate **12** that crystallized from ether. Deprotection of **12** with methoxide gave the syrupy 2′-deoxy-2′-fluorolactose (**14**).

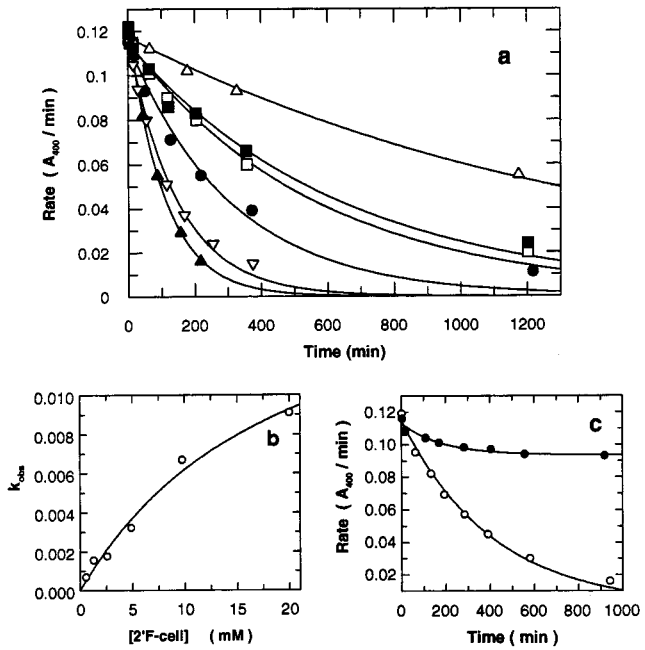


Figure 1. Inactivation of *Agrobacterium* β-glucosidase with **8**. Enzyme was incubated with the following concentrations of **8**: 0.57 mM (△), 1.30 mM (■), 2.60 mM (□), 4.90 mM (●), 9.80 mM (◆), 20.0 mM (▲). (a) Residual activity versus time. (b) Replot of rate constants from (a). (c) Protection against inactivation given by isopropyl β-D-thioglucoside: (○) 3.27 mM **8** alone, (●) 3.27 mM **8** plus isopropyl β-D-thioglucoside (8 mM).

Specific Inactivation of a Glycosidase by a 2′-Deoxy-2′-fluorodisaccharide. 2′-Deoxy-2′-fluorocellobiose (**8**) inactivated *Agrobacterium* β-glucosidase in a time-dependent fashion ($k_i = 0.018 \pm 0.003$ min^{-1}, $K_i = 20 \pm 6$ mM), presumably *via* the accumulation of a 2-deoxy-2-fluoroglucosyl-enzyme intermediate (Figure 1).[12] Evidence for this mechanism was accumulated in a number of ways. Firstly, inactivation was shown not to be due to a contaminating impurity, such as an activated fluorosugar not observed by NMR, by incubating a high concentration of β-glucosidase (0.55 mM) with **8** (1.04 mM) and monitoring loss of activity. Full inactivation was observed on a time scale comparable to that when much lower enzyme concentrations were employed, indicating that the inactivating species must be **8** since were it to be a contaminant present at low levels then there would not have been a sufficient amount to inactivate this concentration of enzyme. Secondly, **8** was shown to be active site-directed since protection against inactivation was afforded by the competitive inhibitor isopropyl β-D-thioglucoside (8 mM, $K_i = 4$ mM),[4] decreasing k_{obs} at 3.27 mM inactivator from 0.0024 to 0.00022 min^{-1}. Thirdly, the formation of a 2-deoxy-2-fluoro-α-glucosyl-enzyme intermediate was indicated by its reaction behavior which was identical to that of the previously characterized species. Thus, freed of excess **8** and incubated in the presence of β-D-glucosylbenzene (100 mM) which acts as a transglucosylation acceptor, reactivation of the isolated 2-deoxy-2-fluoroglycosyl-enzyme was observed, with a k_{reac} of $(2.9 \pm 0.5) \times 10^{-3}$ min^{-1}, comparable to that seen previously (3.3×10^{-3} min^{-1}) with the 2-deoxy-2-fluoroglucosyl-enzyme[13] (produced by reaction with 2-deoxy-2-fluoro-β-D-glucosyl fluoride) at the same concentration of β-D-glucosylbenzene. Further, electrospray mass spectrometry

(12) Kinetic parameters for inactivation were determined essentially as reported previously[4,13] using inactivator concentrations of 0.57, 1.30, 2.60, 4.90, 9.80, and 20.00 mM.

(13) Street, I. P.; Kempton, J. B.; Withers, S. G. *Biochemistry* **1992**, *31*, 9970.

indicated that the mass of the inactivated enzyme was increased from 51 192 ± 6 to 51 354 ± 5, consistent, within error, with the added mass of the expected 2-deoxy-2-fluoroglucosyl moiety (165). Finally, peptic digestion of the inactivated enzyme and anlysis of the fragment peptides by HPLC−tandem mass spectrometry[14] showed that the inhibitor was covalently bound to two peptides with retention times and masses identical to those of the YITENGAC (m/z = 1035) and ITENGAC (m/z = 871) peptides previously labeled by the activated inhibitor 2′,4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucoside, indicating that the same nucleophilic residue, previously identified as Glu-358,[15,21] was labeled by both inhibitors

In keeping with expectations, 2′-deoxy-2′-fluorolactose (**14**) was *not* an inactivator of the β-galactosidase from *Escherichia coli*. The k_{cat} of lactose, the natural substrate of this enzyme, is 2.5-fold *lower* than that of *p*-nitrophenyl β-D-galactoside, a substrate for which galactosylation is rate-limiting.[16] Therefore, accumulation of a 2-deoxy-2-fluorogalactosyl-enzyme derived from 2′-deoxy-2′-fluorolactose was not expected since this compound would similarly be expected to exhibit rate-limiting galactosylation. Indeed, incubation of β-galactosidase with **14** for 1 h at concentrations up to 8.9 mM did not result in inactivation. Instead, **14** was an exceedingly slow substrate of this enzyme, functioning effectively as a competitive inhibitor (K_i = 0.9 mM). This result contrasts sharply with the rapid inactivation ($t_{1/2} \approx$ 3 s at saturation) of *E. coli* β-galactosidase by 2-deoxy-2-fluoro-β-D-galactosyl fluoride (k_i = 13.2 min^{-1}, K_i = 1.3 mM),[5] wherein the fluoride leaving group serves to accelerate the galactosylation step sufficiently such that degalactosylation becomes rate-limiting. However, lactose-hydrolyzing enzymes for which degalactosylation is rate-limiting with the natural substrate would be predicted to be inactivated by **14**.

The aglycon specificity of this class of inactivators was demonstrated by comparing two β-glucosidases with different aglycon specificities. Glucocerebrosidase (GCase) cleaves the β-glucosidic bond in glucosyl ceramide to β-D-glucose and ceramide (*N*-acylsphingosine) and is an essential catabolic enzyme in the lysosomal compartment of mammalian cells. Like *Agrobacterium* β-glucosidase, human GCase is effectively inactivated by 2-deoxy-2-fluoro-β-D-glucosyl fluoride (k_i/K_i = 0.023 min^{-1} mM^{-1}), an inactivator in which the fluoride leaving group serves as an activated, but nonspecific, aglycon with both enzymes.[17] Significantly, no detectable inactivation of GCase by 2′-deoxy-2′-fluorocellobiose (**8**) after 24 h of incubation at concentrations up to 2.2 mM was observed. Evidently, and quite reasonably, binding interactions in the aglycon subsite of GCase which have presumably evolved to specifically bind the *N*-acylsphingosyl moiety, do *not* result in a sufficient increase in the rate of the glycosylation step for the intermediate to accumulate upon reaction of 2′-deoxy-2′-fluorocellobiose with this enzyme. By contrast, reaction of this compound with *Agrobacterium* β-glucosidase, which specifically binds glucose in its aglycon subsite, results in a tremendous acceleration of the glycosylation step, and inactivation of the enzyme. Such specificity may be useful in the design of stable, highly specific inhibitors of glycosidases (such as those involved in glycoprotein processing) whose differences in specificity are based principally upon differences in substrate aglycon structure.

(14) The labeled peptides were identified using tandem mass spectrometry in neutral loss mode, in which the ions are subjected to limited fragmentation by an inert gas in a collision cell.[19] The ester bond between inhibitor and peptide is one of the more labile bonds present, undergoing homolytic cleavage with loss of a neutral sugar. The two quadrupoles are scanned in a linked mode so that *only* those ions differing by the mass of the label could be detected. For a singly-charged peptide, the m/z difference is the mass of the label (165); for a doubly-charged peptide, the m/z difference is half of the mass of the label, and so on. See refs 19 and 20.

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Experimental Section

General Methods. Anhydrous solvents were prepared according to literature[22] procedures except for diethyl ether which was used directly as anhydrous reagent grade. Silica gel used for column chromatography was Merck silica gel 60, and elution was performed at ~8 psi above atmospheric pressure. The acetylated glucal and galactal were obtained from Terochem Laboratories, and the trifluoromethyl hypofluorite was from PCR. 2,3-Di-*O*-acetyl-1,6-anhydro-β-D-glucose was prepared according to literature[23] procedures. Melting points were measured on a Leitz 350 microscope heating stage and are uncorrected. Optical rotations were measured in a 1.0 dm tube with a Perkin-Elmer 141 polarimeter. ^{1}H-NMR spectra were recorded with a Bruker WH-400 spectrometer, using $CDCl_3$ as solvent, unless stated otherwise. Mass spectra were determined on a Varian/MAT CH4B or a Kratos MS50 spectrometer. Elemental analyses were performed at UBC by Mr. P. Borda. Ratios of isomers reported are based on ^{1}H-NMR integration.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-gluco(and galacto)pyranosyl bromide[10] (1 and 2). The bromides **1** and **2** were prepared as described in the literature[10] from 3,4,6-tri-*O*-acetyl-D-glucal (or galactal) and trifluoromethyl hypofluorite in Freon-11. The chromatographically isolated 2-deoxy-2-fluoro-α-D-gluco(or galacto)pyranosyl fluoride was treated with HBr/AcOH, and evaporation of the volatiles followed by coevaporation with toluene (3×) gave crude **1** or **2** which was used in the glycosylation reactions without further purification.

2′-Deoxy-2′-fluorocellobiose (8). Glycosylation in Acetonitrile. 2,3-Di-*O*-acetyl-1,6-anhydro-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl)-α-(and β-)D-glucopyranose (4 and 5). A solution of bromide **1** (0.42 g, 1.35 mmol) in absolute ether (0.8 mL) was added slowly to a stirred solution of the anhydro sugar **3** (3.5 g, 14 mmol) in anhydrous acetonitrile (10 mL) containing silver trifluoromethanesulfonate (CF_3SO_3Ag) (0.307 g, 1.2 mmol), silver carbonate (Ag_2CO_3) (0.817 g, 3 mmol), and powdered Drierite (0.70 g). The resulting mixture was stirred overnight at room temperature and filtered through Celite. The residue was washed with CH_2Cl_2. The combined filtrates were evaporated and the products separated on a silica gel column (1:2 (v/v) hexanes−ethyl acetate) to give the hydrolysis product **6**, a 70:30 mixture of **4** and **5** (300 mg; 41%), and excess anhydro sugar **3**. The fluoromaltose derivative **4** was crystallized in ether from the above mixture of **4** and **5**, and the supernatant was chromatographed on a silica gel column using ether as an eluent. The fractions containing >50% of the more mobile **5** were pooled, and **5** was crystallized from ether and recrystallized from ethanol to a constant melting point. Mp 104−107 °C. $[\alpha]^{22}_D$ −28.2° (c 0.86, $CHCl_3$). ^{1}H-NMR: δ 2.04 (s, 3H × 2, OAc), 2.10, 2.12, 2.14 (each s, 3H × 3, OAc), 3.51 (s, H-4), 3.79 (dd, J_{gem} = 7.5 Hz, $J_{5,6a}$ = 5.5 Hz, H-6a), 3.87 (ddd, $J_{4',5'}$ = 9.5 Hz, $J_{5',6'a}$ = 2.0 Hz, $J_{5',6'b}$ = 5.5 Hz, H-5′), 3.90 (d, H-6b), 4.10 (dd, J_{gem} = 12 Hz, H-6′a), 4.24 (dd, H-6′b), 4.41 (ddd, $J_{1',2'}$ = 8.0 Hz, $J_{2',F}$ = 51 Hz, $J_{2',3'}$ = 9.0 Hz, H-2′), 4.51 (s, H-2), 4.66 (d, H-5), 4.94 (dd, $J_{1',F}$ = 3.0 Hz, H-1′), 4.99 (dd, $J_{3',4'}$ = 9.0 Hz, H-4′), 5.23 (s, H-3), 5.35 (ddd, $J_{F,3'}$ = 14.5 Hz, H-3′), 5.42 (s, H-1). MS (EI): m/z 537 (M + H), 477 (M − CH_3CO_2), 463 (M − $CH_3CO_3CH_2$), 291 (M − aglycon) Anal. Calcd for $C_{22}H_{29}FO_{14}$: C, 49.25; H, 5.45. Found: C, 49.30; H, 5.45.

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1,2,3,6-Tetra-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-*β*-D-glucopyranosyl)-α-(and *β*-)D-glucopyranose (7). Acetolysis of the fluorocellobiose derivative **5** (626 mg, 1.2 mmol) gave an oil which could not be induced to crystallize. Purification of the oil by column chromatography with 19:1 benzene−ethanol gave 609 mg (0.954 mmol, 82%) of an oil consisting of a mixture of the anomeric acetates of **7** (2:1 α/*β*) which again could not be induced to crystallize. Careful rechromatography using the above conditions and isolation of the early and later fractions gave samples of ∼90% anomeric purity from which ^{1}H-NMR spectra and optical rotations were obtained. ∼90% α-acetate. $[\alpha]^{22}_D$ +86.3° (*c* 1.2, $CHCl_3$). ^{1}H-NMR: *δ* 2.00, 2.01, 2.03, 2.05, 2.07, 2.10, 2.15 (each s, 3H × 7, OAc), 3.69 (ddd, $J_{4',5'}$ = 9.9 Hz, $J_{5',6'a}$ = 2.2 Hz, $J_{5',6'b}$ = 4.3 Hz, H-5′), 3.86 (dd, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 10.1 Hz, H-4), 4.04 (dd, J_{gem} = 12.5 Hz, H-6′a), 4.07 (dd, $J_{5,6a}$ = 2.5, H-5), 4.19 (dd, J_{gem} = 12.4 Hz, H-6a), 4.25 (ddd, $J_{1',2'}$ = 7.7 Hz, $J_{2',F}$ = 48.5 Hz, $J_{2',3'}$ = 8.7 Hz, H-2′), 4.34 (dd, H-6′b), 4.48 (d, H-6b), 4.59 (dd, $J_{1',F}$ = 2.7, H-2′), 4.98 (dd, $J_{3',4'}$ = 9.7 Hz, H-4′), 5.01 (dd, $J_{1,2}$ = 3.7 Hz, $J_{2,3}$ = 10.3 Hz, H-2), 5.27 (ddd, $J_{F,3'}$ = 14.7 Hz, H-3′), 5.49 (dd, H-3), 6.26 (d, H-1). Anal. Calcd for $C_{26}H_{35}FO_7$: C, 48.90; H, 5.52. Found: C, 48.63; H, 5.52. ∼90 *β*-acetate. $[\alpha]^{22}_D$ +35.6° (*c* 0.80, $CHCl_3$). ^{1}H-NMR: (the resonances and coupling constants of the fluorinated nonreducing end of the *β*-acetate of **7** were effectively unchanged compared to those of the α-acetate above except for $J_{2',F}$ which increased to 49.9 Hz) *δ* 3.79 (ddd, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = 4.2 Hz, $J_{5,6b}$ = 1.85 Hz, H-5), 3.89 (dd, $J_{3,4}$ = 9.5 Hz, H-4), 4.20 (dd, J_{gem} = 12.25 Hz, H-6a), 4.47 (dd, H-6b), 5.05 (dd, $J_{1,2}$ = 8.3 Hz, $J_{2,3}$ = 9.5 Hz, H-2), 5.27 (dd, H-3), 5.69 (d, H-1).

4-*O*-(2-Deoxy-2-fluoro-*β*-D-glucopyranosyl)-D-glucopyranose (8, 2′-Deoxy-2′-fluorocellobiose). To a syrupy mixture of the α- and *β*-anomeric acetates of **7** (271 mg, 0.425 mmol) under an atmosphere of nitrogen was added 100% methanol (10 mL). The mixture was sonicated until all the syrup had dissolved, after which methanolic $NaOCH_3$ (1.7 M, 0.15 mL) was added and the mixture stirred for 1 h. The resulting yellow solution was neutralized (as indicated on pH paper) with Amberlite IR-120 (H^+) resin and filtered and the residue washed with methanol. The combined filtrates were concentrated *in vacuo*, treated with charcoal, and filtered through Celite to give **8** (138 mg, 94.4%) as a hygroscopic glass after evaporation of methanol and drying under high vacuum. $[\alpha]^{22}_D$ +35.9° (*c* 0.90, H_2O). ^{1}H-NMR (D_2O): (splitting in H-1′ and H-2′ due to anomeric mixture on reducing glucose residue) *δ* 4.17/4.175 (ddd × 2, $J_{1',2'}$ = 7.7 Hz, $J_{2',F}$ = 51.0 Hz, $J_{2',3'}$ = 8.8 Hz, H-2′), 4.65 (d, $J_{1,2}$ = 8.0 Hz, 0.6 H, H-1 *β*-anomer), 4.79/4.795 (d × 2, H-1′), 5.21 (d, $J_{1,2}$ = 3.5 Hz, 0.4 H, H-1 α-anomer). ^{19}F-NMR (D_2O, TFA ref): *δ* −122.45 (dd, $J_{H2',F}$ = 50.6 Hz, $J_{H3',F}$ = 15.3 Hz). Anal. Calcd for $C_{12}H_{21}FO_{10}\cdot(1/2)H_2O$: C, 40.79; H, 6.28. Found: C, 40.66; H, 6.46.

Glycosylation with Benzyl-Protected Glucosyl Bromide at Low Temperature. Ethyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-*β*-D-thioglucopyranoside (15). A solution of the fluoroglucosyl bromide **1** (5.6 g, 15.1 mmol) in anhydrous methanol (10 mL) was treated with a 2.0 mL portion of an ethanethiolate solution (10 mL) (prepared by dissolving potassium (700 mg, 17.9 mmol) portionwise in anhydrous methanol (10 mL) followed by ethanethiol (2.0 mL) over 15 min. After stirring for 0.5 h, the violet-brown solution was filtered and concentrated *in vacuo* to a syrup. The syrup was acetylated by refluxing in acetic anhydride (35 mL) and anhydrous sodium acetate (3.5 g) for 10 min. The cooled mixture was poured onto 150 g of crushed ice and vigorously stirred. The precipitate was filtered, washed thoroughly with water, and crystallized (dichloromethane−hexanes) to give **15** (4.15 g; 65%). Mp 124−126.5 °C. ^{1}H-NMR: *δ* 1.34 (t, CH_2CH_3), 2.04, 2.08, 2.09 (each s, 3H × 3, OAc), 2.76 (m, CH_2CH_3), 3.73 (m, $J_{4,5}$ = 10 Hz, $J_{5,6a}$ = 2.5 Hz, $J_{5,6b}$ = 5.0 Hz, H-5), 4.12 (dd, J_{gem} = 12.4 Hz, H-6a), 4.24 (dd, H-6b), 4.32 (ddd, $J_{1,2}$ = 10 Hz, $J_{2,F}$ = 49.5 Hz, $J_{2,3}$ = 9 Hz, H-2), 4.60 (dd, $J_{1,F}$ = 1.5, H-1), 5.03 (dd, $J_{3,4}$ = 9.5 Hz, H-4), 5.32 (m, $J_{3,F}$ = 14.5 Hz, H-3). MS (EI): *m*/*z* 352 (M), 291 (M − SEt), 272 (291 − F). Anal. Calcd for $C_{14}H_{21}FO_7S$: C, 47.72; H, 6.01. Found: C, 47.72; H, 6.03.

Ethyl 3,4,6-Tri-*O*-benzyl-2-deoxy-2-fluoro-*β*-D-thioglucopyranoside (16). To a stirred solution of the acetylated ethyl thioglycoside **15** (3.0 g) in redistilled tetrahydrofuran (20 mL) was added powdered potassium hydroxide (8.0 g) followed by dropwise addition of benzyl bromide (10 mL). After refluxing for 15 h and cooling, the mixture was filtered through Celite and the residue washed thoroughly with dichloromethane. The filtrate was evaporated *in vacuo* and the residue dried at 250 °C under high vacuum. The syrup was purified on a column of silica gel (300 g, hexanes−ether, 4:1) to give syrupy **16** (3.6 g, 86%). ^{1}H-NMR: *δ* 1.31 (t, *J* = 8 Hz, CH_2CH_3), 2.74 (m, CH_2-CH_3), 3.47 (ddd, $J_{4,5}$ = 10 Hz, $J_{5,6a}$ = 4.5 Hz, $J_{5,6b}$ = 2 Hz, H-5), 3.62 (dd, $J_{3,4}$ = 9 Hz, H-4), 3.66 (dd, J_{gem} = 11.5 Hz, H-6a), 3.73 (dd, H-6b), 3.76 (ddd, $J_{2,3}$ = 8.5 Hz, $J_{F,3}$ = 15 Hz, H-3), 4.35 (ddd, $J_{1,2}$ = 10 Hz, $J_{2,F}$ = 49.5 Hz, H-2), 4.48 (dd, $J_{1,F}$ = 2 Hz, H-1), 4.54 (×2), 4.575, 4.74, 4.83, 4.91 (each d, 1H × 6, J_{gem} − 11 Hz, CH_2Ph), 7.15−7.37 (m, 15 H, Ar*H*). MS (EI): *m*/*z* 476 (M − HF), 405 (M − $PhCH_2$).

3,4,6-Tri-*O*-benzyl-2-deoxy-2-fluoro-α-D-glucopyranosyl Bromide (17). To a stirred solution of the benzylated ethyl thioglycoside **16** (940 mg) in anhydrous ethyl ether (15 mL) was added, dropwise, a solution of bromine (0.15 mL in 3 mL of ether). After 1 h the solution was coevaporated with toluene (2 × 4 mL) and the residue dried *in vacuo* to give the benzylated fluoroglucosyl bromide **17** as a moderately viscous amber syrup which was used in the glycosylation reaction without further purification.

2,3-Di-*O*-acetyl-1,6-anhydro-4-*O*-(3,4,6-tri-*O*-benzyl-2-deoxy-2-fluoro-α-(and *β*-D-glucopyranosyl)-*β*-D-glucopyranose (18 and 19). The procedure of Kronzer and Schuerch[24] was modified for the following glycosylation. Silver trifluoromethanesulfonate (233 mg, 0.91 mmol) was dried in the reaction flask for several hours *in vacuo*, the flask was cooled to −78 °C, and a solution of bromide **17** (300 mg in 2 mL of CH_2Cl_2) was slowly added to the stirred mixture. After 10 min a solution of the anhydro sugar **3** (200 mg in 2 mL of CH_2Cl_2) was slowly added to the above mixture. After 30 min at −78 °C the mixture was allowed to warm under ambient conditions for 10 min. The reaction was terminated by shaking with excess saturated sodium hydrogen carbonate. The mixture was filtered through Celite and the organic layer washed with water, dried over sodium sulfate, filtered, and evaporated to a syrup. The syrup was purified by column chromatography (hexanes−ethyl acetate, 3:2) to give 248 mg (62.5%) of a 9:1 mixture of the *β*-linked to α-linked disaccharides **19** and **18**. Careful rechromatography (benzene−ethyl acetate, 4:1) gave **18** and **19** each in >95% anomeric purity, with the *β*-linked **19** as the more mobile component. ^{1}H-NMR (**18**): *δ* 2.05, 2.09 (each s, 3H × 2, OAc), 3.57 (ddd, *J* = 2, 3.5, 9.5 Hz, H-5′), 3.60 (br s, H-4), 3.70−3.81 (m, 4 H, H-3′, H-4′, H-6′a, H-6′b), 3.82 (dd, J_{gem} = 7.7 Hz, $J_{5,6}$ = 5.5 Hz, $H\text{-}6_a$), 3.98 (d, $H\text{-}6_b$), 4.455 (ddd, $J_{1',2'}$ = 8 Hz, $J_{2',3'}$ = 8 Hz, $J_{2',F}$ = 51 Hz, H-2′), 4.59 (br s, H-2), 4.73 (d, H-5), 4.77 (dd, $J_{1',F}$ = 3.3 Hz, H-1′), 4.465, 4.57, 4.61, 4.76, 4.85, 4.91 (each d, 1H, × 6, J_{gem} − 11 Hz, CH_2Ph), 5.35 (dd, $J_{2,3}$ = 1.5 Hz, $J_{3,4}$ = 1.5 Hz, H-3), 5.49 (s, H-1), 7.13−7.35 (m, 15 H, Ar*H*). ^{1}H-NMR (**19**): *δ* 2.08, 2.09 (each s, 3H × 2, OAc), 3.55 (br s, H-4), 3.61 (dd, $J_{3',4'}$ = 9.0 Hz, $J_{4',5'}$ = 10.5 Hz, H-4′), 3.67 (ddd, $J_{5',6'a}$ = 2.5 Hz, J_{gem} = 10.5 Hz, H-6′a), 3.68 (dd, $J_{5',6'b}$ = 4.0 Hz, H-6′b), 3.71 (dd, $J_{5,6a}$ = 5.5 Hz, J_{gem} = 8.0 Hz, H-6a), 3.96 (d, $J_{5,6b}$ = 0.5 Hz, H-6b), 4.04 (ddd, H-5′), 4.15 (ddd, $J_{2',3'}$ = 9.0 Hz, $J_{F,3'}$ = 12.2 Hz, H-3′), 4.51 (ddd, $J_{1',2'}$ = 4.0 Hz, $J_{2',F}$ = 50 Hz, H-2′), 4.58 (br s, H-2), 4.66 (br d, H-5), 4.835 (dd, $J_{2,3}$ = 1.5 Hz, $J_{3,4}$ = 1.5 Hz, H-3), 4.455, 4.475, 4.555, 4.735, 4.83, 4.87 (each d, 1H, × 6, J_{gem} − 11 Hz, CH_2Ph), 5.30 (d, H-1′), 5.44 (s, H-1), 7.145−7.41 (m, 15 H, Ar*H*).

Disaccharide 4 from Disaccharide 18 *via* Intermediate 2,3-Di-*O*-acetyl-1,6-anhydro-4-*O*-(2-deoxy-2-fluoro-α-D-glucopyranosyl)-*β*-D-glucopyranose (20). To a prehydrogenated mixture of 10% palladium on charcoal (72 mg) and ethanol (5 mL) was added a solution of the benzylated disaccharide **18** (60 mg, 0.106 mmol) in ethanol (5 mL). The mixture was hydrogenated at atmospheric pressure for 2.5 h, filtered, and concentrated to yield the debenzylated product **20** (34 mg, 0.083 mmol, 78%) as a colorless syrup. The crude syrup was dissolved in acetic anhydride−pyridine (3 mL, 1:1) at 0 °C and stirred at room temperature for 12 h, concentrated, coevaporated with toluene, and purified by column chromatography (benzene−ethyl acetate, 1:1) to give **4** (34 mg; 60% from **18**). This compound produced a ^{1}H-NMR spectrum identical to that of compound **4** synthesized above.

Disaccharide 5 from Disaccharide 19 *via* Intermediate 2,3-Di-*O*-acetyl-1,6-anhydro-4-*O*-(2-deoxy-2-fluoro-α-D-glucopyranosyl)-*β*-D-glucopyranose (21). Treatment of the benzylated cellobiose

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derivative **19** (30 mg, 0.044 mmol) under conditions analogous to those used for the benzylated maltose derivative **18** above gave **5** (16 mg, 66%) after chromatography. This compound produced a ^{1}H-NMR spectrum identical to that of compound **5** synthesized above.

2′-Deoxy-2′-fluorolactose (14). 2,3-Di-*O*-acetyl-1,6-anhydro-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-(and *β*-)D-galactopyranosyl)-*β*-D-glucopyranose (9 and 10). To a solution of the anhydro sugar **3** (3.0 g, 12 mmol) in anhydrous acetonitrile (18 mL) was added silver trifluoromethanesulfonate (900 mg) and silver carbonate (2.76 g). A solution of the bromide **2** (3.0 g, 8.08 mmol) in acetonitrile (5 mL) was slowly added to the vigorously stirred mixture. The mixture was stirred overnight in the absence of light, diluted with dichloromethane (25 mL), and filtered through Celite and the residue washed thoroughly with dichloromethane. The filtrate was concentrated and the residue separated by column chromatography (hexanes−ethyl acetate, 1:1). The disaccharides **9** and **10** were isolated as a mixture (1.7 g, 39%, ∼55:45 α/*β*) along with the hydrolysis product **11** and excess **3**. The previously synthesized α-fluorodisaccharide **9** was crystallized from ether−hexanes. The desired fluorolactose derivative **10** remained as an uncrystallizable syrupy mixture (∼85:15 *β*/α-linked disaccharides). ^{1}H-NMR (**10**): *δ* 2.03, 2.06, 2.10 (s, 3H × 3, OAc), 2.135 (each s, 6H, OAc × 2), 3.60 (br s, H-4), 3.83 (dd, $J_{5,6a}$ = 6.0 Hz, J_{gem} = 8.0 Hz, H-6a), 3.98 (d, H-6b), 3.99 (m, H-6′a), 4.04 (m, H-5′), 4.145 (dd, J_{gem} = 10 Hz, $J_{5',6'b}$ = 5 Hz, H-6′b), 4.595 (d, $J_{2,3}$ = 1.5 Hz, H-2), 4.615 (ddd, $J_{1',2'}$ = 51.5 Hz, $J_{2',3'}$ = 10.0 Hz, H-2′), 4.74 (d, H-5), 4.895 (dd, $J_{1',F}$ = 4.0 Hz, H-1′), 5.125 (ddd, $J_{F,3'}$ = 12.5 Hz, $J_{3',4'}$ = 4.0 Hz, H-3′), 5.275 (dd, $J_{3,4}$ = 1.5 Hz, H-3), 5.40 (dd, $J_{F,4'}$ = 1.5 Hz, H-4′), 5.49 (s, H-1).

1,2,3,6-Tetra-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-*β*-D-galactopyranosyl)-*β*-D-glucopyranose (12). The 1,6-anhydro ring of **10** (∼1.6 g, containing ∼0.27 g of **9** as an inseparable mixture) was acetolyzed as previously described. The resulting syrup was purified by column chromatography (neat diethyl ether) to give an inseparable mixture of the α- and *β*-anomeric acetates of **12** (859 mg, 45%) in a 1.5:1 ratio. A sample of this mixture (750 mg) was dissolved in 45% HBr in AcOH (4.5 mL) and stirred for 0.5 h. The solution was diluted with chloroform (15 mL), washed with water (2 × 15 mL), dried over anhydrous sodium sulfate, filtered, and concentrated. The resulting syrup of crude **13** was dissolved in acetic anhydride (10 mL) containing mercuric acetate (0.75 g) and stirred for 2 h. The solution was diluted with chloroform (30 mL), washed with water (2 × 20 mL), dried over anhydrous sodium sulfate, concentrated, crystallized from ether, and recrystallized from ethanol to give the pure *β*-1,4-linked *β*-*O*-acetate **12** (650 mg, 87%). Mp 96−103 °C (partial melt, resolidifies), 161.5−163 °C. $[\alpha]^{22}_D$ +23.1 (*c* 1.5, $CHCl_3$), $[\alpha]^{22}_D$ −4.4 in $CHCl_3$ for the 2′-OAc analogue. MS (EI): *m/z* 618 (M − HF), 595 (M − CH_3CO), 579 (M − CH_3CO_2), 291 (M − aglycon). ^{1}H-NMR: *δ* 2.06 (s, 6 H, OAc × 2), 2.060, 2.062, 2.098, 2.100, 2.132 (each s, 3H × 5, OAc), 3.81 (ddd, $J_{4,5}$ = 10 Hz, $J_{5,6a}$ = 4 Hz, $J_{5,6b}$ = 2 Hz, H-5), 3.90 (dd, $J_{4',5'}$ = 0.5 Hz, $J_{5',6'a}$ = 7 Hz, $J_{5',6'b}$ = 6.5 Hz, H-5′), 3.94 (dd, $J_{3,4}$ = 9.6 Hz, H-4), 4.06 (dd, J_{gem} = 11.5 Hz, H-6′a), 4.11 (dd, H-6′b), 4.25 (dd, J_{gem} = 12.5 Hz, H-6a), 4.44 (ddd, $J_{1',2'}$ = 7.5 Hz, $J_{2',F}$ = 50.5 Hz, $J_{2',3'}$ = 10 Hz, H-2′), 4.46 (dd, H-6b), 4.59 (dd, $J_{1',F}$ = 4.5 Hz, H-1′), 5.05 (ddd, $J_{3',4'}$ = 3.7 Hz, $J_{F,3'}$ = 12.7 Hz, H-3′), 5.055 (dd, $J_{1,2}$ = 8.4 Hz, $J_{2,3}$ = 9.6 Hz, H-2), 5.295 (dd, H-3), 5.38 (br dd, $J_{F,4'}$ = 3.7 Hz, H-4′), 5.705 (d, H-1). Anal. Calcd for $C_{26}H_{35}FO_{17}$·(1/2)H_2O: C, 48.22; H, 5.60. Found: C, 48.54; H, 5.73.

4-*O*-(2-Deoxy-2-fluoro-*β*-D-galactopyranosyl)-D-glucose (2′-Deoxy-2′-fluorolactose, 14). The hepta-*O*-acetate **12** (54 mg, 0.085 mmol) was treated in a manner identical to that for the production of **8** from **7** to give **14** (27 mg, 88%) as a hygroscopic transparent glass after pumping at high vacuum. $[\alpha]^{22}_D$ +53.7° (*c* 1.2, H_2O), $[\alpha]^{22}_D$ +55.3° (H_2O) for the 2′-hydroxy analogue. ^{1}H-NMR (D_2O): *δ* 5.22 (d, $J_{1,2}$ = 4.0 Hz, H-1 (α-anomer)), 4.38 (m, H-2′ (α-anomer), H-2′ (*β*-anomer)). ^{19}F-NMR (D_2O, TFA ref): *δ* −130.73 (ddd, $J_{H2',F}$ = 51.4 Hz, $J_{H3',F}$ = 14.1 Hz, $J_{H1',F} \approx$ 3 Hz). Anal. Calcd for $C_{12}H_{21}FO_{10}$·(3/4)H_2O: C, 40.28; H, 6.34. Found: C, 40.21; H, 6.58.

Time-Dependent Inactivation. The kinetic parameters for the inactivation of *Agrobacterium* *β*-glucosidase by 2′-deoxy-2′-fluorocellobiose were determined as follows. The enzyme was incubated at 37 °C in 50 mM sodium phosphate buffer, 0.1% BSA, pH 6.8, with the following inhibitor concentrations: 0.57, 1.30, 2.60, 4.90, 9.80, and 20.0 mM. Aliquots (10 *μ*L) of these inactivation mixtures were removed at various time intervals and diluted into assay cells (1 mL) containing *p*-nitrophenyl *β*-D-glucoside at saturating concentration (1 mM). Inactivation was monitored until 80−90% of the enzyme was inactivated. Pseudo-first-order rate constants (k_{obs}) for each inactivator concentration were calculated by fitting the residual activity *versus* time data to a single exponential equation using GraFit.[25] Values of k_i and K_i were determined from these k_{obs} values by fitting to the equation

$$k_{obs} = k_i[I]/(K_i + [I])$$

Protection against Inactivation. Protection against inactivation was investigated as follows. Samples of *Agrobacterium* *β*-glucosidase were incubated at 37 °C in 50 mM sodium phosphate buffer, 0.1% BSA, pH 6.8, containing 2′-deoxy-2′-fluorocellobiose (3.27 mM), in the presence or absence of a competitive inhibitor, isopropyl *β*-D-thioglucoside (8 mM). Aliquots were removed at various time intervals and diluted into assay cells containing saturating concentrations of substrate, and residual activity was monitored by following the release of *p*-nitrophenolate at 400 nm as described above. Pseudo-first-order rate constants for inactivation at the same inactivator concentration, but in the absence or presence of the competitive inhibitor, were determined.

Reactivation of Inactivated Enzyme. An appropriate dilution of the inactivated enzyme (200 *μ*L) was concentrated at 4 °C using 10 kDa nominal cutoff centrifugal concentrators (Amicon Corp., Danvers, MD) to a volume of approximately 15 *μ*L and then diluted with 180 *μ*L of buffer. This was repeated twice, and the retentate was diluted to a final volume of buffer (165 *μ*L) containing 1 mg/mL BSA and the transglycosylation ligand *β*-D-glucosylbenzene (100 mM). The inactivated enzyme was then incubated at 37 °C, and reactivation was monitored by removal of aliquots (10 *μ*L) at appropriate time intervals and assaying as described above. Measured activities were corrected for decreases in activity due to denaturation over this time course using a *noninhibited* control sample. The spontaneous reactivation rate constant, k_3, was determined by fitting the data so obtained to a first-order rate equation, as described above.

Test for Inhibitory Contaminant in 2′-Deoxy-2′-fluorocellobiose. *Agrobacterium* *β*-glucosidase (37 mg/mL, 29 *μ*L, 2.2 × 10^{-8} mol) was incubated with 2′-deoxy-2′-fluorocellobiose (3.78 mM, 11 *μ*L, 4.2 × 10^{-8} mol) in a total volume of 40 *μ*L for 44 h at 37 °C. The molar ratio of inhibitor/enzyme is thus ∼1.9:1. Aliquots were removed at time intervals, diluted appropriately, and assayed with *p*-nitrophenyl *β*-D-glucoside as described previously. The amount of inactivation after ∼25−45 h of incubation was compared to that previously determined after similar incubation times, but with a much greater inhibitor excess.

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