5-Fluoro Glycosides: A New Class of Mechanism-Based Inhibitors of Both α- and *β*-Glucosidases

John D. McCarter and Stephen G. Withers*

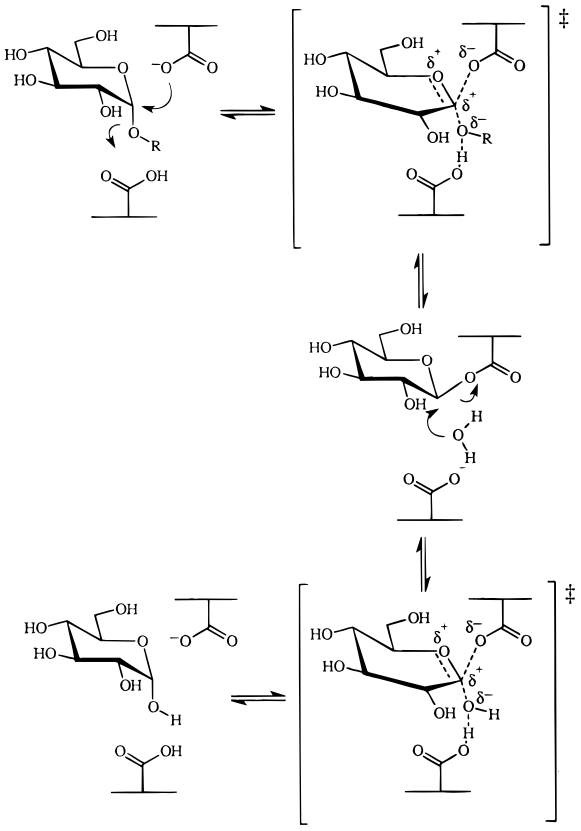
Department of Chemistry, University of British Columbia
2036 Main Mall, Vancouver, British Columbia
Canada V6T 1Z1

Received August 10, 1995

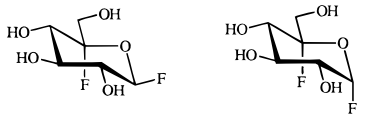
Glycosidase inhibitors have proven to be valuable probes of enzymic mechanism[1] and show considerable promise as therapeutic drugs.[2] Design of inhibitors for these enzymes is best based upon a knowledge of their mechanisms. Retaining glycosidases[3] are generally believed[4−6] to follow a double-displacement mechanism in which a covalent glycosyl−enzyme intermediate is formed and hydrolyzed *via* oxocarbenium ion like transition states, as shown for an α-glucosidase in Scheme 1. A successful strategy for inactivation of retaining *β*-glycosidases involves the use of activated 2-deoxy-2-fluoro glycosides that form a stabilized 2-deoxy-2-fluoroglycosyl−enzyme intermediate that turns over only slowly. Unfortunately, this approach has been notably unimpressive with all α-glycosidases tested.[7,8] Further, the requirement for a fluorine at C2 limits the utility of these inhibitors if the enzyme (e.g., an *N*-acetylhexosaminidase) is intolerant of substitution at this position. This paper describes a novel approach which obviates both these problems and allows inhibition of both α- and *β*-glycosidases through accumulation of a covalent glycosyl−enzyme intermediate, without compromising specificity through substitution of any ring hydroxyl. It also provides substantial evidence against an alternative mechanism for retaining glycosidases involving endocyclic ring opening.[9−11]

5-Fluoro glycosides with good leaving groups, such as **1** and **2**, might be expected to inactivate "retaining" glycosidases by formation of a stabilized 5-fluoroglycosyl−enzyme intermediate through a trapping mechanism analogous to that of the 2-deoxy-2-fluoro glycosides. A sterically conservative fluorine substitution at C5 of a glycosyl oxocarbenium ion exerts electronic effects similar to or greater than those of a C2 fluorine, both atoms being adjacent to centers of developing positive charge.[12] However, crucial transition state binding interactions between the enzyme and the usual C2 substituent[13−15] which are disrupted in the case of the 2-deoxy-2-fluoro sugars are still possible for the 5-fluoro glycosides.

Scheme 1. Presumed Mechanism of a Retaining α-Glucosidase



Synthesis of the 5-fluoroglycosyl fluorides hinged upon the known radical photobromination reaction at C5 of per-O-acetylated *β*- and α-glucosyl fluorides.[16−18] Fluorination of these 5-bromoglucosyl fluorides and deacetylation afforded products **1** and **2**, which were purified by chromatography and characterized.[19]



1 **2**

* To whom correspondence may be addressed. Telephone: 604-822-3402. FAX: 604-822-2847. E-mail: withers@chem.ubc.ca.

(1) Lalegerie, P.; Legler, G.; Yon, J. M. *Biochimie* **1982**, *64*, 1977. Legler, G. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 319.
(2) Truscheit, E.; Frommer, W.; Junge, B.; Muller, L.; Schmidt, D. D.; Wingender, W. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 744. Hughes, A. B.; Rudge, A. J. *Nat. Prod. Rep.* **1994**, 135.
(3) "Retaining" glycosidases catalyze the hydrolysis of glycosidic bonds with net retention of anomeric configuration, presumably *via* a double displacement mechanism involving the formation (glycosylation) and breakdown (deglycosylation) of a covalent glycosyl−enzyme intermediate.
(4) Koshland, D. E. *Biol. Rev.* **1953**, *28*, 416.
(5) Sinnott, M. L. *Chem. Rev.* **1990**, *90*, 1171.
(6) Kempton, J. B.; Withers, S. G. *Biochemistry* **1992**, *31*, 9961.
(7) Withers, S. G.; Rupitz, K.; Street, I. P. *J. Biol. Chem.* **1988**, *263*, 7929.
(8) McCarter, J.; Adam, M.; Braun, C.; Namchuk, M.; Tull, D.; Withers, S. G. *Carbohydr. Res.* **1993**, *249*, 77.
(9) Fleet, G. W. J. *Tetrahedron Lett.* **1985**, 5073.
(10) Post, C.; Karplus, M. *J. Am Chem. Soc.* **1986**, *108*, 1317.
(11) Franck, R. W. *Bioorg. Chem.* **1992**, *20*, 77.
(12) Modeling studies (Winkler, D. A.; Holan, G. *J. Med. Chem.* **1989**, *32*, 2084. Kajimoto, T.; Liu, K. K.-C.; Pederson, R. L.; Zhong, Z.; Ichikawa, Y.; Porco, J. A. J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6187) have indicated that the greatest *difference* in partial charge between a ground state sugar and the corresponding glycosyl oxocarbenium ion is at O5 rather than C1.
(13) Wentworth, D. F.; Wolfenden, R. *Biochemistry* **1974**, *13*, 4715.
(14) Roeser, K.-R.; Legler, G. *Biochim. Biophys. Acta* **1981**, *657*, 321.
(15) McCarter, J.; Adam, M.; Withers, S. G. *Biochem. J.* **1992**, *286*, 721.
(16) Ferrier, R. J.; Tyler, P. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1528.
(17) Praly, J. P.; Descotes, G. *Tetrahedron Lett.* **1987**, *28*, 1405.
(18) Somsák, L.; Ferrier, R. J. *Adv. Carbohydr. Chem. Biochem.* **1991**, *49*, 37.

(19) Bromination of per-O-acetylated *β*- and α-glucosyl fluorides with N-bromosuccinimide (*hν*, *N*-bromosuccinimide, CCl_4) yielded the protected 5-bromoglucosyl fluorides. Fluorination (Igarashi, K.; Honma, T.; Irisawa, J. *Carbohydr. Res.* **1969**, *11*, 577) of the *β* anomer ($AgBF_4$, toluene) afforded the protected 5-fluoro-*β*-D-glucosyl fluoride in low yield. 5-Fluoro-α-glucosyl fluoride was synthesized by treatment of the 5-bromo α anomer with fluoride (AgF, CH_3CN), followed by HF/pyridine. Deacetylation (NH_3, CH_3OH) and chromatography (27:2:1 $EtOAc/CH_3OH/H_2O$) on silica gel yielded products **1** and **2**, which were characterized by NMR and elemental analysis. **1**: ^{1}H NMR (D_2O, 400 MHz, TMS reference) *δ* 5.35 (dd, $J_{1,F1}$ = 54 Hz, $J_{1,2}$ = 8.0 Hz, 1 H, H-1), 3.8−3.4 (m, 5 H, H-2−H-6,6′); ^{19}F NMR (D_2O, 188 MHz, CF_3CO_2H reference) −60.5 (ddd, $J_{4,F5}$ = 22 Hz, $J_{F5,6'}$ = $J_{F5,6'}$ = 6 Hz, F-5), −74.2 (dd, $J_{1,F1}$ = 54 Hz, $J_{2,F1}$ = 14 Hz, F-1). Elemental anal. Calcd for $C_6H_{10}O_5F_2 \cdot 0.5H_2O$: C, 34.46; H, 5.30. Found: C, 34.57; H, 5.72. **2**: ^{1}H NMR (D_2O) *δ* 5.75 (dd, $J_{1,F1}$ = 56.5 Hz, $J_{1,2}$ = 3.0 Hz, 1 H, H-1), 4.0−3.4 (m, 5 H, H-2−H-6,6′); ^{19}F NMR (D_2O) −53.5 (m, F-5), −65.8 (ddd, $J_{1,F1}$ = 56.5 Hz, $J_{2,F1}$ = 25.7 Hz,[4] $J_{F5,F1}$ = 21.2 Hz, F-1). Elemental anal. Calcd for $C_6H_{10}O_5F_2$: C, 36.01; H, 5.04. Found: C, 35.56; H, 5.20.

0002-7863/96/1518-0241$12.00/0

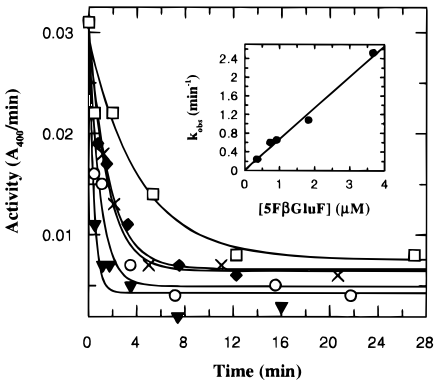


Figure 1. Inactivation of *Agrobacterium* β-glucosidase with **1**. Enzyme was incubated with the following concentrations of **1**: 3.68 μM (▼), 1.84 μM (○), 0.921 μM (×), 0.736 μM (◆), 0.368 μM (□). Inset: replot of rate constants from Figure 1.

5-Fluoro-β-D-glucosyl fluoride (5FβGluF), **1**, inactivated *Agrobacterium faecalis* β-glucosidase according to essentially pseudo-first-order kinetics[20] although inactivation, particularly at lower inactivator concentrations, did not proceed to completion (Figure 1). This is consistent with a kinetic model[21] in which turnover (k_{cat}) of the glycosyl−enzyme is significantly slower than its formation (k_i) at high, but not low, inactivator concentrations. A replot of the rate constants from the initial exponential phase showed no saturation at concentrations up to 3.7 μM (Figure 1, inset), beyond which inactivation became too rapid to accurately measure. Nonetheless, a second-order rate constant of $k_i/K_i = 660$ min^{-1} mM^{-1} was obtained from the slope of this plot. Protection against inactivation was afforded by 4.7 μM castanospermine ($K_i = 3$ μM[22]), the pseudo-first-order rate constant at 1.84 μM 5FβGluF being reduced from 1.07 to 0.18 min^{-1}. When freed of excess inhibitor by ultrafiltration, followed by incubation in buffer at 37 °C, a first-order recovery of enzyme activity was observed ($k_{cat} = 0.082$ min^{-1}, corresponding to $t_{1/2} = 8.5$ min), indicating a catalytically competent intermediate that is capable of normal turnover, but at greatly reduced rates. An electrospray mass spectrum of the 5FGlu-inactivated β-glucosidase showed that the mass of the protein increased from 51 216 ± 6 Da to 51 397 ± 6 Da upon inactivation. This increase of 181 ± 12 Da is that expected for the covalent attachment of *one* 5-fluoroglucosyl moiety (181 Da). A further measure of the effectiveness of this inhibitor is provided by the steady state K_i' value of 0.3 μM determined,[21] one of the best inhibitors yet found for this enzyme.

Incubation of yeast α-glucosidase with 5-fluoro-α-D-glucosyl fluoride (5FαGluF), **2**, resulted in very rapid inhibition, even when assaying at the shortest possible time intervals.[23] However, complete inhibition was not observed since turnover of the intermediate (k_{cat}) was also rapid, allowing the establishment of a significant steady state rate. Furthermore, incubation of 5FαGluF with α-glucosidase resulted in a slow release of 2 (2.0 ± 0.3) equiv of fluoride. The time course of fluoride release was linear over virtually the entire course of the hydrolysis reaction, even at micromolar concentrations of 5FαGluF, indicating an extremely low K_m ($=K_i'$) value and providing a k_{cat} value of 6.6 min^{-1}. This very tight binding, due to the significant accumulation of an intermediate, precluded direct determination of the K_m value due to the insensitivity of the fluoride electrode at the low concentrations required. However, a steady state K_i' of 1.4 μM was determined, one of the lowest such values yet seen with yeast α-glucosidase.

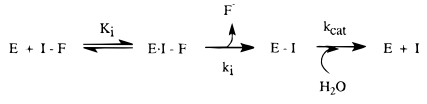
In conclusion, these 5-fluoro α- and β-glycosides are potent mechanism-based inhibitors of α- and β-glycosidases, respectively, forming catalytically competent intermediates that are capable of turnover, at rates reduced 10^5- and 10^3-fold, respectively, from those of the β-[24] and α-D-glucosyl fluoride[25] parent substrates. In addition, the fact that successful inactivation is seen provides further substantial evidence[26] against an enzymic mechanism involving initial endocyclic bond cleavage.[9−11] According to such a mechanism, a reactive fluorohydrin would be generated at C5 which should eliminate fluoride rapidly, releasing a 5-ketoglucose derivative. Thus hydrolysis, not inactivation, would be observed.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada for financial support of this work.

JA952732A

(20) Inactivation of *Agrobacterium* β-glucosidase by 5FβGluF, **1**, was performed by incubation of enzyme (~0.04 mg/mL) in the presence of various concentrations (0.37−3.7 μM) of **1** and removal of aliquots at time intervals for assay of residual activity with *p*-nitrophenyl β-D-fucopyranoside (0.84 mM). Reactivation of the 5FGluF-inactivated enzyme (~0.04 mg/mL) was studied after removal of excess inactivator using 10 kDa nominal cut-off centrifugal concentrators (Amicon Corp., Danvers, MD). The reactivation rate constant, corresponding to k_{cat}, was determined by fitting the data to a first-order rate equation (Leatherbarrow, R. J. *GraFit Version 3.0*; Erithacus Software Ltd.: Staines, U.K., 1990). For experimental details, see: Street, I. P.; Kempton, J. B.; Withers, S. G. *Biochemistry* **1992**, *31*, 9970.

(21) In the equation below, E represents free enzyme, I−F the 5-fluoroglycosyl fluoride, E−I the glycosyl−enzyme, and I the sugar product. If $k_i > k_{cat}$, E−I accumulates and inactivation is observed. If, however, turnover is more rapid, tight apparent binding is observed in steady state determinations, allowing measurement of an apparent inhibition constant $K_i' = K_i/(1 + k_i/k_{cat})$. This equation takes the form of the Michaelis−Menten expression for K_m. See refs 13 and 14.



(22) Namchuk, M. Ph.D. Thesis, University of British Columbia, 1993.

(23) The k_{cat} for 5FαGluF with yeast type III α-glucosidase (Sigma) was determined by monitoring the release of fluoride using an Orion 96-09 combination fluoride ion electrode; 10 μL of stock enzyme (~3.75 mg/mL) was added to cells containing various concentrations of 5FαGluF (99−990 μM) in 50 mM sodium phosphate buffer, pH 6.8 in a final volume of 250 μL incubated at 37 °C. The calculated k_{cat} is corrected for the fact that 2 equiv of fluoride is released. An apparent K_i' value for 5FαGluF under steady state conditions was determined by assaying the activity of α-glucosidase with αPNPGlu in the presence of various concentrations of inhibitor. An appropriate dilution of enzyme (~1.9×10^{-5} mg/mL) was added to cells each containing αPNPGlu (0.1 mM) and various concentrations (1.98−19.8 μM) of 5FαGluF, and the enzymic rates were determined by monitoring the rate of release of *p*-nitrophenolate.

(24) Day, A. G.; Withers, S. G. *Can. J. Biochem.* **1986**, *64*, 914.
(25) Konstantinidis, A.; Sinnott, M. L. *Biochem. J.* **1991**, *279*, 587.
(26) Lehmann, J.; Reinshagen, H. *Justus Liebigs Ann. Chem.* **1970**, *732*, 112.