Synthesis and Testing of 2-Deoxy-2,2-Dihalo Glycosides as Mechanism-based Inhibitors of $\alpha$-Glycosidases

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General Methods. $^1$H-NMR and $^{13}$C-NMR were recorded on 200 MHz, 300 MHz, 400 MHz or 600 MHz spectrometers and calibrated to the residual solvent peak. $^{19}$F-NMR was obtained at 188 MHz on a 200 MHz spectrometer or at 282 MHz on a 300 MHz spectrometer. $^{19}$F-NMR Chemical shifts were reported using the $\delta$ scale referenced to CFCl$_3$ ($\delta$= 0.00 ppm). Low resolution mass spectra were recorded using a triple quadrupole mass spectrometer equipped with an electrospray ionization ion source. Reactions were monitored by thin layer chromatography (aluminum backed sheets of silica gel 60F254, 0.2 mm) and were visualized by exposure to 10 % sulfuric acid in methanol followed by charring. Flash column chromatography was performed on 230-400 mesh silica gel.

Fluorination conditions: The general fluorination procedure using acetyl hypofluorite is the following: F$_2$ (5%, 3 × 40 psi) was bubbled into a slurry of NaOAc (1 g) and glacial acetic acid (3 mL) in CFCl$_3$ (30 mL) at -78°C. A solution of 2-fluoro glycal per-O-acetate (1.5 mmol) in CFCl$_3$ (10 mL) was then added to the slurry containing acetyl hypofluorite (in situ). The cooling bath was removed and the reaction was allowed to take place with the reaction progress being followed by TLC analysis. The CFCl$_3$ was removed by evaporation and an aqueous workup followed by column chromatography resulted in purification of the compound.
11.95 g (21.3 mmol) of hexa-O-acetyl-maltal (Hehre, E. J. et al. *J. Biol. Chem.*, 1986, 261, 2147-2153) was dissolved in 350 ml of CH$_3$NO$_2$, 10.0 g (28.2 mmol, 1.3 eq.) of Selectfluor was then added portion wise to the solution over one hour. The mixture was stirred vigorously for two days at room temperature until TLC (petroleum ether: ethyl acetate = 1: 1) showed most of the starting material were consumed. 250 ml of AcOH was then added and the reaction mixture was heated at 100 °C for three days. Evaporated off most of the solvent and re-dissolved it in CH$_2$Cl$_2$. The organic phase was washed with water, saturated NaHCO$_3$ (aq.), water, brine, dried over MgSO$_4$ and concentrated under diminished pressure. Gradient flash column chromatography (petroleum ether: ethyl acetate= 2: 1 to 3:2 to 1: 1) purified the major fraction into a white foam (9.6 g, 15.0 mmol, 70 %) NMR spectra showed that the major fraction was composed of four compounds, with anomic acetate and fluorine having all possible configurations, as shown in the reaction scheme above and they were very difficult to separate. $^1$H-NMR was too difficult to assign. $^{19}$F-NMR data (CDCl$_3$, 282 MHz): -220.3 (ddd, $J = 51.0$ Hz, $J = 26.5$ Hz, $J = 19.0$ Hz), -204.6 (ddd, $J = 48.5$ Hz, $J = 27.5$ Hz, $J = 6.0$ Hz), -203.2 (dd, $J = 48.5$ Hz, $J = 11.5$ Hz), -201.4 (dd, $J = 51.0$ Hz, $J = 14.0$ Hz) MS: Calcd for C$_{26}$H$_{35}$FO$_{17}$+Na$: 661.2. Found: 661.2. This material was directly used for the next step without any further purification.
9.6 g (15.0 mmol) of the mixture from the previous step was dissolved in dry CH₂Cl₂ (300 mL), treated with 32 ml of 33% HBr in acetic acid and allowed to react for 3 days at 0°C. Stopped the reaction by washing the organic phase successively with water, saturated aqueous NaHCO₃ (aq.), water and brine and then dried it over MgSO₄. After removing the solvent in vacuo, two major products identified by NMR was 3,6-di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-α-(1,4)-d-glucosyl)-2-deoxy-2-fluoro-α-glucosyl bromide and 3,6-di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-α-D-glucosyl)-2-deoxy-2-fluoro-α-mannosyl bromide. (Only anomic region of the NMR for the product mixture is shown here). 

**¹H NMR** (CDCl₃): δ 6.43 (d, J = 4.4 Hz), 6.34 (dd, J = 9.6 Hz, J = 1.5 Hz); 

**¹⁹F NMR** (CDCl₃): δ -182.8 (ddd, J = 49.6 Hz, J = 26.3 Hz, J = 9.7 Hz), -190.3 (dd, J = 49.3 Hz, J = 10.2 Hz). 

**MS**: Calcd for C₂₄H₃₂BrFO₁₅⁺Na⁺: 681.1, 683.1. Found: 681.0, 683.1. This mixture was used without further purification.

The mixture of 2-fluoro-"gluco" and "manno" bromides was dissolved in acetonitrile (250 mL) and triethylamine (50 mL), and allowed to stir for two days at room temperature. The excess base was removed in vacuo, CHCl₃ was added, and the
organic layer was washed with water, saturated aqueous NaHCO₃(aq.), and water, and
dried (MgSO₄). Gradient flash column chromatography (petroleum ether: ethyl acetate= 2:1 to 3:2) gave two major products. By NMR analysis the "gluco" compound had
completely reacted; whereas, the "manno" compound did not undergo the elimination
reaction. 3.1 g (5.4 mmol) of hexa-O-acetyl-2-fluoro maltal 5 was isolated. **¹H NMR**
(CDCl₃, 400 MHz): δ 6.77 (d, 1H, J = 5.2 Hz); 5.45-5.37 (m, 3H), 5.05 (t, 1H,
J = 10.0 Hz), 4.85 (dd, 1H, J = 10.4 Hz, J = 4.0 Hz), 4.47 (m, 1H), 4.40-4.11 (m,
4H), 4.08 (ddd, 1H, J = 10.0 Hz, J = 4.8 Hz, J = 2.4 Hz), 4.00 (m, 1H), 2.111 (s, 3H),
2.108 (s, 3H), 2.106 (s, 3H), 2.099 (s, 3H), 2.044 (s, 3H), 2.027 (s, 3H) **¹³C-NMR**
(CDCl₃, 100 MHz): 170.80, 170.68, 170.53, 170.29, 170.19, 169.81, 142.20 (d, J =
239.0 Hz), 132.16 (d, J = 40.0 Hz), 96.96, 74.61, 74.53, 74.07, 70.62, 69.95, 68.46,
65.87 (d, J = 23.0 Hz), 61.99, 60.70, 20.95 (2C), 20.91, 20.88, 20.82, 20.75 **¹⁹F NMR**
(CDCl₃, 282 MHz): d -164.7 (d, J = 5.2 Hz). **HRMS**: Calcd for C₂₄H₃₁FO₁₅⁺Na⁺: 601.1545. Found: 601.1543
Enzyme Kinetics

All kinetic studies were carried out on a UV/VIS spectrophotometer equipped with a circulating water bath. Quartz or plastic cuvettes with a pathlength of 1 cm path length were used. Measurements were taken at 400 nm, the wavelength of maximal absorbance of 4-nitrophenolate or 2-chloro-4-nitrophenolate. Enzyme concentrations and reaction times were chosen so that less than 10% of the total substrate was hydrolyzed to ensure linear kinetics. Yeast α-glucosidase (EC 3.2.1.20 Type III from yeast) was assayed at 37°C using 4-nitrophenyl α-glucoside substrate in 50 mM phosphate buffer, pH 6.8, containing 0.1% BSA. Human pancreatic α-amylase was purified as previously reported (Rydberg, E. H et al. Protein Sci. 1999, 8, 635-643). It was assayed at 30°C using 2-chloro-4-nitrophenyl α-D-maltotrioside substrate in 50 mM sodium phosphate buffer containing 100 mM NaCl, pH = 6.9.

Time-Dependent Inhibition

The kinetic parameters for the inactivation of the two α-glycosidases by each inhibitor were determined as follows. Samples of the enzyme were incubated in buffer in the presence of a range of concentrations of the inhibitor. Aliquots (5 or 10 µL) of these inactivation mixtures were removed at time intervals and diluted into assay cells containing a large volume (~ 1 mL) of substrate (at saturating concentrations, 7 × Km, or at least ~ Km). This effectively stops the inactivation both by dilution of the inactivator and by competition with an excess of substrate. The residual enzymatic
activity was determined from the rate of hydrolysis of the substrate, which is directly proportional to the amount of active enzyme. The process was monitored until 80-90% of the enzymatic activity was inactivated. Pseudo-first order rate constants ($k_{obs}$) for each inactivator concentration were calculated from the slopes of the plots of the natural logarithm of the residual enzymatic activity versus time or by fitting plots of the residual activity versus time to a single exponential equation using GraFit (Leatherbarrow, R. J. *GraFit 4.0*; 4.0.19 ed.; Erithacus Software Limited, 1989). These values of $k_{obs}$ were then fit to the equation below describing the inactivation process to obtain values of $k_i$ and $K_i$.

$$k_{obs} = \frac{k_i[I]}{K_i + [I]}$$

In cases where saturation was not observed, $k_i/K_i$ was calculated according to

$$k_{obs} = \frac{k_i[I]}{K_i}$$

where $K_i >> [I]$. In this case, $k_i/K_i$ is given by the slope of the plot of $k_{obs}$ versus $[I]$, which was determined by linear regression using GraFit.

**Protection against Inactivation**

Protection against inactivation was investigated by incubating samples of enzyme in buffer containing the inactivator and in the absence or presence of a competitive inhibitor (at a concentration of ~ $K_i$ or higher). Aliquots were removed at various time intervals, diluted into assay cells containing saturating concentrations of substrate, and the residual enzyme activity monitored by following the release of the 4-nitrophenolate or 2-chloro-4-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**

Reactivations of inactivated α-glucosidase were studied as follows. An appropriate dilution of the inactivated enzyme (~ 400 µL) was concentrated at 4°C using a 10 kDa nominal cut-off centrifugal concentrator to a volume of approximately 50 µL, then diluted with 400 µL of buffer. This was repeated twice, and the retentate was diluted to a final volume of buffer containing 1 mg/mL BSA. The inactivated enzyme was then incubated at 37°C and reactivation was monitored by removal of aliquots (5 or 10 µL) 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 data for non-inhibited control samples.
3,6-Di-O-acetyl-4-O-[2′,3′,4′,6′-tetra-O-acetyl-α-(1,4)-D-glucopyranosyl]-2-chloro-2-deoxy-2-fluoro-α-D-glucopyranosyl chloride (6)

$^1$H-NMR (CDCl$_3$, 300 MHz)
3,6-Di-O-acetyl-4-O-[2′,3′,4′,6′-tetra-O-acetyl-α-(1,4)-D-glucopyranosyl]-2-chloro-2-deoxy-2-fluoro-α-D-glucopyranosyl chloride (6)

$^{13}$C-NMR (CDCl$_3$, 75 MHz)
2-Chloro-2-deoxy-2-fluoro-4-\(O-\alpha-(1,4)-D\)-glucopyranosyl]-\(\alpha\)-D-glucopyranosyl chloride (2)

\(^1\)H-NMR(D\(_2\)O, 300 MHz)
2-Chloro-2-deoxy-2-fluoro-4-O-[α-(1,4)-D-glucopyranosyl]-α-D-glucopyranosyl chloride (2)

$^{13}$C-NMR(D$_2$O, 100 MHz)
3,4,6-Tri-O-acetyl-2-deoxy-2,2-difluoro-α-D-arabinohexopyranose (8)

$^1$H-NMR (CDCl$_3$, 300 MHz)
3,4,6-Tri-O-acetyl-2-deoxy-2,2-difluoro-α-D-arabinohexopyranose (8)

$^{13}$C-NMR (CDCl$_3$, 75 MHz)
3,4,6-Tri-O-acetyl-2-deoxy-2,2-difluoro-α-D-arabinohexopyranosyl chloride (9)

1H-NMR (CDCl₃, 300 MHz)

In observe: Ret. = 2.00 h / 25 °C
3,4,6-Tri-O-acetyl-2-deoxy-2,2-difluoro-α-D-arabinohexopyranosyl chloride (9)

$^{13}$C-NMR (CDCl$_3$, 75 MHz)
2-Deoxy-2,2-difluoro-α-D-arabinohexopyranosyl chloride (10)

$^1$H-NMR (D$_2$O, 300 MHz)
2-Deoxy-2,2-difluoro-α-D-arabinohexopyranosyl chloride (10)

$^{13}$C-NMR (D$_2$O, 100 MHz)
1,3,6-Tri-O-acetyl-4-O-[2′,3′,4′,6′-tetra-O-acetyl-α-(1,4)-D-glucopyranosyl]-2-deoxy-2,2-difluoro-α-D-arabinohexopyranose (11)

$^1$H-NMR (CDCl$_3$, 300 MHz)
1,3,6-Tri-O-acetyl-4-O-[2',3',4',6'-tetra-O-acetyl-α-(1,4)-D-glucopyranosyl]-2-deoxy-2,2-difluoro-α-D-arabinohexopyranose (11)

$^{13}$C-NMR (CDCl$_3$, 150 MHz)
3,6-Di-\(O\)-acetyl-4-\(O\)-[2',3',4',6'-tetra-\(O\)-acetyl-\(\alpha\)-(1,4)-D-glucopyranosyl]-2-deoxy-2,
2-difluoro-\(\alpha\)-D-arabinohexopyranose (12)

\(^1\)H-NMR (CDCl\(_3\), 300 MHz)
3,6-Di-\(O\)-acetyl-4-\(O\)-\(\left[2',3',4',6'\right]\)tetra-\(O\)-acetyl-\(\alpha\)-(1,4)-D-glucopyranosyl]-2-deoxy-2,2-difluoro-\(\alpha\)-D-arabinohexopyranose (12)

\(^{13}\)C-NMR (CDCl\(_3\), 75 MHz)

![Graph](image-url)
3,6-Di-O-acetyl-4-O-\([2',3',4',6'-\text{tetra-O-acetyl-}\alpha-(1,4)-\text{D-glucopyranosyl}]\)-2-deoxy-2,2-difluoro-\(\alpha\)-D-arabinohexopyranosyl chloride (13)

\(^1\)H-NMR (CDCl\(_3\), 300 MHz)
3,6-Di-O-acetyl-4-O-[2',3',4',6'-tetra-O-acetyl-α-(1,4)-D-glucopyranosyl]-2-deoxy-2,2-difluoro-α-D-arabinohexopyranosyl chloride (13)

$^{13}$C-NMR (CDCl$_3$, 100 MHz)
2-Deoxy-2,2-difluoro-4-O-[α-(1,4)-D-glucopyranosyl]-α-D-arabinohexopyranosyl chloride (14)

$^1$H-NMR (D$_2$O, 400 MHz)
2-Deoxy-2,2-difluoro-4-O-[α-(1,4)-D-glucopyranosyl]-α-D-arabinohexopyranosyl chloride (14)

$^{13}$C-NMR (D$_2$O, 100 MHz)
2,4,6-Trinitrophenyl 3,6-di-O-acetyl-4-O-[2',3',4',6'-tetra-O-acetyl-\(\alpha\)-\((1,4)\)-D-glucopyranosyl]-2-deoxy-2,2-difluoro-\(\alpha\)-D-arabinohexopyranoside (17)

\(^1\)H-NMR (CDCl\(_3\), 400 MHz)
2,4,6-Trinitrophenyl 3,6-di-O-acetyl-4-O-[2',3',4',6'-tetra-O-acetyl-α-(1,4)-D-glucopyranosyl]-2-deoxy-2,2-difluoro-α-D-arabinohexopyranoside (17)

$^{13}$C-NMR (CDCl$_3$, 100 MHz)