Supporting Information

Heavily fluorinated carbohydrates as enzyme substrates: oxidation of tetrafluorinated galactose by galactose oxidase

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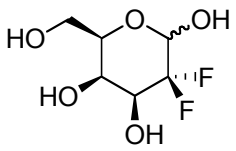
Contents

1) Synthesis and characterisation of 2-deoxy-2,2-difluoro-D-*lyxo*-hexopyranose (3)



$C_6H_{10}F_2O_5$
Exact Mass: 200,05
Mol. Wt.: 200,14
C, 36,01; H, 5,04; F, 18,99; O, 39,97

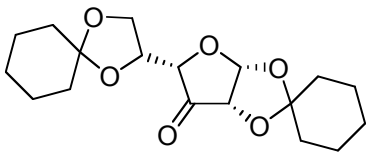
To solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2,2-difluoro-D-*lyxo*-hexopyranose (145 mg, 0.47 mmol, 1 equiv) in MeOH (2 mL), NaOMe (12.8 mg, 0.23 mmol, 0.5 equiv) was added. The mixture was stirred at 0 °C for 105 min then at RT for 45 min. Amberlite IR-120 was added until neutral. The resin was filtred off and the filtrate was concentrated *in vacuo*. The crude mixture was then purified by column cromatography (EtOAc) to give 68 mg of **3** (72%) as white solid.

R_f (DCM/MeOH)(90:10): 0.29; **Mp** 146 °C; **$[\alpha]_D$** +49.4° (c 0.50, MeOH); **IR** (neat) 3301 (br. s), 2923 (br. w), 1837 (w), 1414 (w), 1250 (w), 1179 (w), 1138 (m), 1098 (s), 1041 (ws) cm^{-1}; **^{1}H NMR** (400 MHz, D_2O, α/β ~1.5:1) δ 5.27 (0.6H, d, *J* 8.0 Hz, $H_α$-1), 4.94 (0.4H, d, *J* 16.1 Hz, $H_β$-1), 4.24–4.04 (m, $H_α$-3, $H_β$-3, $H_α$-4, $H_β$-4, $H_α$-5,), 3.81–3.75 (m, $H_β$-5, $2H_α$-6, $2H_β$-6) ppm; **^{13}C NMR** (100 MHz, D_2O) δ 120.2 (dd, *J* 252.4, 246.1 Hz, C-$2_α$), 120.1 (dd, *J* 255.6, 248.8 Hz, C-$2_β$), 95.5 (dd, *J* 29.3, 19.8 Hz, C-$1_β$), 94.5 (dd, *J* 38.1, 28.5 Hz, C-$1_α$), 78.7 (C-$5_β$), 73.9 (C-$5_α$), 42.3 (t, *J* 18.6 Hz, C-$3_β$), 72.0 (d, *J* 7.0 Hz, C-$4_α$), 71.5 (d, *J* 7.8 Hz, C-$4_β$), 69.3 (dd, *J* 19.4, 18.2 Hz, C-$3_α$), 64.0 (C$6_α$), 63.8 (C-$6_β$) ppm; **^{19}F NMR** (282 MHz, D_2O, α/β ~2.4:1 (after 72 h in D_2O) δ –118.9 (0.7F, d, *J* 253.6 Hz, $F_α$), –120.66 (0.7 F, d, *J* 253.6 Hz, $F_α$), –129.7 (0.3 F, d, *J* 245.0 Hz, $F_β$), –137.9 (0.3 F, d, *J* 245.0 Hz, $F_β$) ppm; **ESI$^+$-MS** m/z: 223 $(M+Na)^+$; **HR-ESI$^+$-MS** (ES^+) calcd for $C_6H_{10}O_5F_2Na$: 223.0389, found 223.0390.

2) Synthesis and characterisation of 3-deoxy-3-fluoro-D-galactose (4)

This fluorosugar has been prepared via a slightly different route than reported.

2.1) 1,2:5,6–Di–*O*–cyclohexylidene–α–D–*xylo*-hexofuranos-3-ulose.[1]



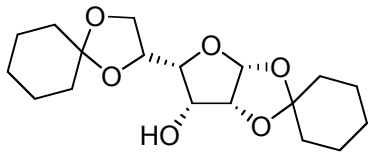
$C_{18}H_{26}O_6$
Exact Mass: 338,17
Mol. Wt.: 338,40
C, 63,89; H, 7,74; O, 28,37

To a solution of 1,2:5,6–di–*O*–cyclohexylidene–α–D–galactofuranose[2] (1.0 g, 2.94 mmol, 1 equiv) in DMSO (12 mL), Ac_2O (8 mL) was added. The mixture was stirred at RT for 12 h. The mixture was concentrated *in vacuo* then purified by column chromatography (pet ether / EtOAc, 90:10) to give a white crystalline solid (520 mg, 52%) which can easily be recrystallised from ether.

R_f (hexane/EtOAc)(60:40): 0.53 ; **Mp** 126–128 °C, lit[1a] 131–132 °C, and[1b] 125–126 °C; **$[\alpha]_D$** +22.6 (c 1.00, $CHCl_3$), lit[1a] +18 (c 0.1, $CHCl_3$), and[1b] +24.0 (c 1.4, $CHCl_3$); **IR** (neat) 2935 (s), 2860 (m), 1770 (s), 1463 (w), 1449 (m), 1366 (m), 1334 (w), 1283 (w), 1250 (w), 1233 (w), 1163 (m), 1144 (m), 1090 (s), 1041 (s), 998 (m) cm^{-1}**; ^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.05 (1H, d, *J* 4.52 Hz, H-1), 4.45 (1H, d, *J* 4.26 Hz, H-2), 4.33 (1H, q, *J* 6.52 Hz, H-5), 4.12–4.01 (3H, m, H-4, 2 × H-6), 1.78–1.30 (20H, m, cyclohexylidene). These data correspond to the literature.[1b] **^{13}C NMR** (100 MHz, $CDCl_3$) δ 207.3 (C-3), 115.7 and 110.5 (2 × C-1'), 102.2 (C-1), 81.7 (C-4), 76.5 (C-2), 75.2 (C-5), 64.8 (C-6), 36.7, 36.3, 36.1, and 34.9 (2 × C-2', 2 × C-6'), 25.0 and 24.7 (2 × C-4'), 23.9, 23.7 (2C), and 23.6 (2 × C-3', 2 × C-5') ppm; **ESI$^+$-MS**: m/z: 393 $(M+MeOH+Na)^+$.

[1] (a) R. C. Tweit, H. W. Sause, *Carbohydr. Res.* 1980, **84**, 175. (b) I. Sakamoto, K. Ichimura, H. Ohrui, *Biosci. Biotechnol. Biochem.* 2000, **64**, 1915. (c) G. S. Forman, A. Scaffidi, R. V. Stick, *Aust. J. Chem.* 2004, **57**, 25.
[2] I. Sakamoto, H. Ohrui, *Biosci. Biotechnol. Biochem.* 2000, **64**, 1974.

2.2) 1,2:5,6–Di–*O*–cyclohexylidene–α–D–gulofuranose.

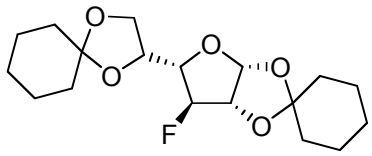


$C_{18}H_{28}O_6$
Exact Mass: 340,19
Mol. Wt.: 340,41
C, 63,51; H, 8,29; O, 28,20

To a solution of 1,2:5,6–di–*O*–cyclohexylidene–α–D–*xylo*-hexofuranos-3-ulose (200 mg, 0.59 mmol, 1 equiv) in MeOH (9 mL), $NaBH_4$ (34 mg, 0.87 mmol, 1.5 equiv) was added. The mixture was stirred at 0 °C for 15 min. The mixture was concentrated *in vacuo* then purified by column cromatography (pet ether / EtOAc, 80:20) to give a white crystalline solid (180 mg, 90%).

R_f (hexane/EtOAc)(60:40) 0.34; **Mp** 143–145 °C, lit[1a] 151–155 °C; **$[\alpha]_D$** +4.5 (c 1.00, $CHCl_3$); **IR (neat)** 3541 (br. m), 2936 (s), 2857 (m), 1447 (m), 1366 (m), 1333 (w), 1309 (w), 1280 (m), 1250 (w), 1231 (w), 1166 (s), 1144 (w), 1126 (s), 1089 (s), 1073 (s), 1026 (s), 922 (s) cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.78 (1H, d, *J* 4.3 Hz, H-1), 4.66 (1H, dd, *J* 6.5, 4.3 Hz, H2), 4.46 (1H, dt, *J* 8.3, 7.0 Hz, H-5), 4.24–4.16 (2H, m, Ha-6, H-3), 3.86 (1H, dd, *J* 8.3, 5.5 Hz, H-4), 3.71 (1H, dd, *J* 8.5, 7.0 Hz Hb-6), 2.71 (1H, d, *J* 6.0 Hz OH), 1.94–1.28 (20H, m, cyclohexylidene); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 115.9 and 109.9 (2 × C-1'), 105.0 (C-1), 84.6 (C-4), 79.6 (C-2), 75.3 (C-5), 69.6 (C-3), 66.0 (C-6), 36.7, 36.6, 36.3, and 34.7 (2 × C-2', 2 × C-6'), 25.1 and 24.8 (2 × C-4'), 24.0, 23.9, 23.7, and 23.6 (2 × C-3', 2 × C-5') ppm; **MS** (ESI^+): m/z: 363 $(M+Na)^+$. **ESI^+-HRMS** calcd for $C_{18}H_{28}O_6Na$: 363.1778, found 363.1771.

2.3) 1,2:5,6–Di–*O*–cyclohexylidene-3-deoxy-3-fluoro-α-D -galactofuranose.[3]



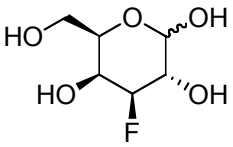
$C_{18}H_{27}FO_5$
Exact Mass: 342,18
Mol. Wt.: 342,40
C, 63,14; H, 7,95; F, 5,55; O, 23,36

A solution of DAST (2.79 mL, 21.1 mmol, 6 equiv), in dry DCM (9 mL) was cooled to 0 °C, treated dropwise with pyridine (1.71 mL, 21.1 mmol, 6 equiv), and then with a solution of 1,2:5,6–di–*O*–cyclohexylidene–α–D–gulofuranose (1.2 g, 3.5 mmol, 1 equiv) in DCM (6 mL). The mixture was stirred at RT for 12h, diluted with DCM, and poured into an aqueous solution of $NaHCO_3$. The aqueous phase was extracted with DCM. The combined organic phases were dried over $MgSO_4$, and evaporated. Column chromatography (pet ether / EtOAc, 90:10) gave a white crystalline solid (820 mg, 68%).

R_f (hexane/EtOAc)(60:40) 0.82; Mp 39–40 °C; **$[\alpha]_D$** +17.9 (c = 0.5, $CHCl_3$); **IR** (neat) 2934 (s), 2861 (m), 1449 (m), 1368 (m), 1096 (s), 1015 (s), 925 (s) cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.90 (1H, d, *J* 3.9 Hz, H-1), 4.84 (1H, dd, *J* 52.0, 3.6 Hz, H-3), 4.72 (1H, dd, *J* 16.1, 3.8 Hz, H-2), 4.33 (1H, q, *J* 6.6 Hz, H-5), 4.12–4.04 (2H, m, H-4 (dd, *J* 6.7, 3.6 Hz is visible), H-6a), 3.82 (1H, dd, *J* 8.4, 6.3 Hz, H-6b), 1.83–1.30 (20H, m, cyclohexylidene); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 114.6 and 110.6 (2 × C-1'), 104.5 (d, *J* 2.9 Hz, C-1), 94.7 (d, *J* = 182.2 Hz, C-3), 84.5 (d, *J* 25.8 Hz, C-2), 84.2 (d, *J* 30.6 Hz, C-4) 74.4 (d, *J* 6.3 Hz, C-5), 65.3 (C-6), 36.7, 36.1, 35.8, and 34.7 (2 × C-2', 2 × C-6'), 25.1 and 24.8 (2 × C-4'), 23.9, 23.7, 23.7, and 23.5 (2 × C-3', 2 × C-5') ppm; **^{19}F NMR** δ –187.6 (s, CHF) ppm; **ESI$^+$-MS** m/z: 365 $(M+Na)^+$; **HR-ESI$^+$-MS** calcd for $C_{18}H_{27}O_5FNa$: 365.1735, found 365.1729.

[3] Fluorination of the corresponding bis-acetonide: (a) P. Kovac, C. P. J. Glaudemans, *Carbohydr. Res.* 1983, **123**, 326; (b) R. Raju, B. F. Castillo, S. K. Richardson, M. Thakur, R. Severins, M. Kronenberg, A. R. Howell, *Bioorg & Med. Chem. Lett.* 2009, **19**, 4122.

2.4) 3-Deoxy-3-fluoro-D-galactose (4).



$C_6H_{11}FO_5$
Exact Mass: 182,06
Mol. Wt.: 182,15
C, 39,56; H, 6,09; F, 10,43; O, 43,92

To 700 mg (2.05 mmol) of 1,2:5,6–di–*O*–cyclohexylidene-3-deoxy-3-fluoro-α-D-galactofuranose TFA/H_2O (9:1, 10 mL) was added. The solution was stirred overnight at RT and evaporated. The crude mixture was then purified by column chromatography (DCM/MeOH, 80:20) to give 440 mg of s.m. and 120 mg of product (32%). To 440 mg (1.28 mmol) of recovered s.m. 5 mL of TFA/H_2O (9:1) were added. The solution was stirred overnight and evaporated. The crude mixture was then purified by column chromatography (EtOAc/MeOH, 90:10) to give 140 mg (34% calculated on 700 mg of total starting material, 60% calculated on 440 mg of s.m. for this specific reaction). In both of the product, even after crystallisation from acetone, TFA was still present: Column chromatography with silica gel/K_2CO_3 (90:10) as the solid phase and EtOAc as eluent was necessary to completely remove the TFA, and gave 120 mg of pure **4** (32%).

R_f (DCM/MeOH)(80:20) 0.25; **Mp** 114–116 °C, lit[4a] 114–116 °C and[4b] 126–127 °C; **$[\alpha]_D$** +79.7° (c = 1.00, MeOH); **IR** (neat): 3261 (br. s), 3293 (br. w), 1644 (w), 1358 (w), 1124 (w), 1151 (m), 1028 (s), 922 (w) cm^{-1}; **^{1}H NMR** (300 MHz, D_2O, α/β ~1 : 1.4) δ 5.30 (0.4H, t, *J* 4.5 Hz, $H_α$-1), 4.74 (0.4H, ddd, *J* 49.0, 10.1, 3.6 Hz, $H_α$-3), 4.60 (0.6H, d, *J* 7.9 Hz, $H_β$-1), 4.55 (0.6H, ddd, *J* 48.1, 9.7, 3.6 Hz, $H_β$-3), 4.25 (0.4H, ddd, *J* 7.4, 3.5, 0.9 Hz, $H_α$-4), 4.19 (0.6H, ddd, *J* 6.2, 3.7, 0.6 Hz, $H_β$-4), 4.10–4.01 (1.2H, m, $H_α$-2 + $H_α$-5), 3.81–3.66 (3.2H, m, $H_α$-6 + $H_β$-2 + $H_β$-5 + $H_β$-6) ppm. These data are similar to literature data.[5] **^{13}C NMR** (100 MHz, D_2O, α/β ~1 : 1.4) δ 98.6 (d, *J* 12.2 Hz, C-$1_β$), 96.0 (d, *J* 182.0 Hz, C-$3_β$), 95.3 (d, *J* 10.6 Hz, C-$1_α$), 93.8 (d, *J* 180.3 Hz, C-$3_α$), 76.6 (d, *J* 7.2 Hz, C-$5_β$), 73.3 (d, *J* 17.8 Hz, C-$2_β$), 72.6 (d, *J* 6.2 Hz, C-$5_α$), 70.4 (d, *J* 16.5 Hz, C-$4_α$), 69.8 (d, *J* 17.5 Hz, C-$2_α$), 69.7 (d, *J* 16.5 Hz, C-$4_β$), 63.6 (d, *J* 2.8 Hz, C-$6_α$), 63.4 (d, *J* 3.5 Hz, C-$6_β$) ppm. **^{19}F NMR** (282 MHz, D_2O, α/β ~1 : 1.3) δ –199.3 (s, F-$3_β$), –203.4 (s, F-$3_α$), ppm; **ESI$^+$-MS** m/z: 205 $(M+Na)^+$; **HR-ESI$^+$-MS** calcd for $C_6H_{11}O_5FNa$: 205.0843, found 205.0483.

[4] (a) J. S. Brimacombe, A. B. Foster, R. Hems, J. H. Westwood, L. D. Hall, *Can. J. Chem.* 1970, **48**, 3946; (b) J. S. Brimacombe, A. B. Foster, R. Hems, L. D. Hall, *Carbohydr. Res.*, 1968, **8**, 249.
[5] J. N. Barlow, J. S. Blanchard, *Carbohydrate Res.* 2000, **328**, 473.

3) Protein expression and purification

The enzyme was expressed and purified as published.[6] Cultures of 800 mL LB media (containing 100 µg/mL ampicillin) were inoculated with 5 mL pre-culture (OD = 0.5-0.6) containing a single colony of WT GOase. The cells were harvested after overnight incubation at 26 ºC and lysed by sonication. The suspension was centrifuged and the pellet discarded. The supernatant was filtered and loaded onto a Ni-NTA column with a volume of 1mL resin pre-equilibrated in 0.5 mL $NiSO_4$ 0.1M and 5 mL dH_2O. The column was washed with 5mL of wash buffer (NaPi 20 mM, NaCl 500 mM, PMSF 1mM and 30 mM imidazole pH 7.4). The target protein was eluted and the fractions collected using an imidazole linear gradient (30 mM-1M). From SDS-PAGE analysis and subsequent Coomassie staining more than 95% pure protein was acquired. The purified protein preparation was activated with Cu^{2+}. A six times excess of $CuSO_4$ 80 mM was added to the sample and incubated overnight at 4 ºC. The excess copper was removed the following morning using a filter column. The sample was diluted two times to 15 mL with NaPi buffer 20mM and finally concentrated to a volume between 0.25-1 mL. Protein concentrations were determined by measuring the absorbance at 280 nm ($\varepsilon = 1.05 \times 10^5$ M^{-1}). Yields of approximately 7-8 mg pure protein/L culture were obtained.

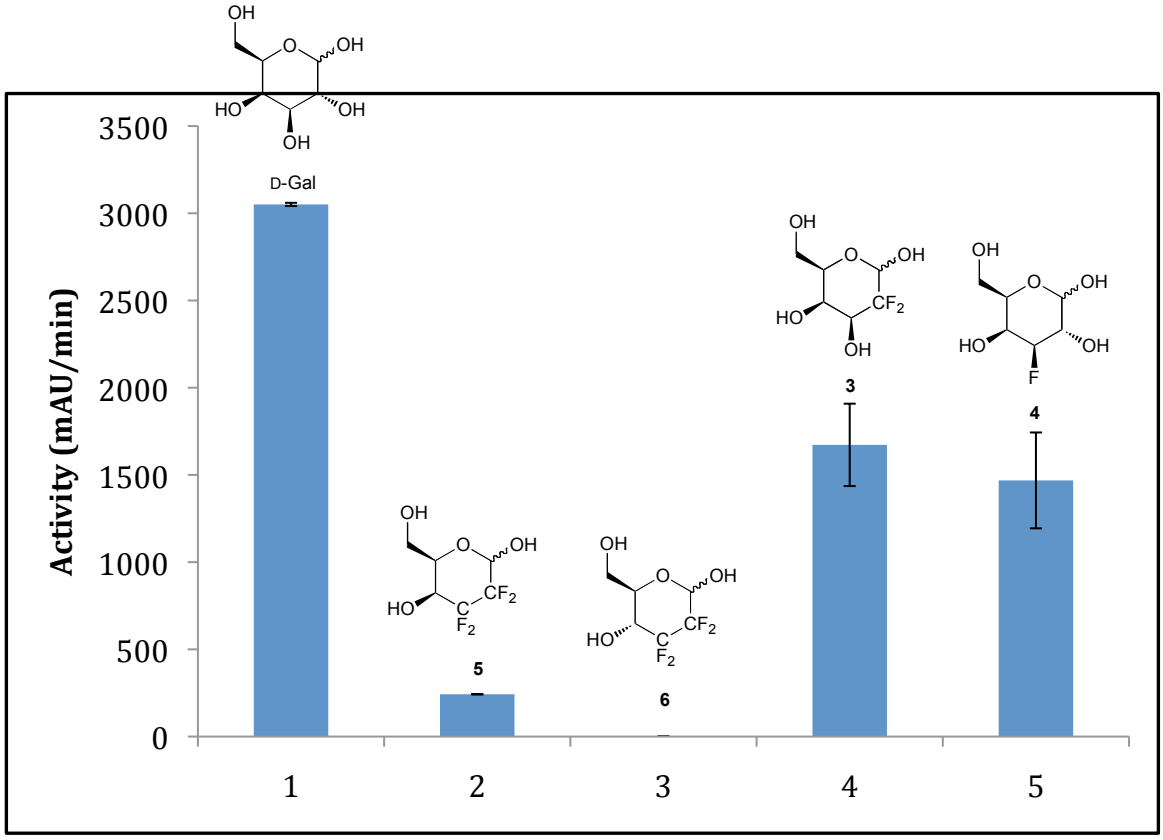
4) Peroxidase assay

The relative activity, kinetics constants and conversion rates of the purified enzyme were measured using an ABTS-HRP coupled assay.[7] For the initial activity measurements, 20 µL protein (12.5 nM final concentration) were mixed with 50 mM of each substrate in NaPi 100 mM pH 7, that contained 0.5 U HRP (Sigma-Aldrich) and 0.1 mg ABTS (Pierce) in a 96-well plate (200 µL total volume). The initial reaction rates were determined by measuring the absorbance change at 420 nm at 30 °C in a SpectraMax M2 (Molecular Devices). Two molecules of ABTS are oxidized for each molecule of H_2O_2 generated by GOase and an extinction coefficient for the ABTS cation radical of $\varepsilon = 36 \times 10^3$ $M^{-1}cm^{-1}$ was used to calculate enzyme activity.

[6] (*a*) J. B. Rannes, A. Ioannou, S. C. Willies, G. Grogan, C. Behrens, S. L. Flitsch, N. J. Turner, *J. Am. Chem. Soc*., 2011, **133**, 8436; (*b*) F. Escalettes, N. J. Turner, *ChemBioChem*, 2008, **9**, 857.
[7] Delagrave, S.*, et al. Protein Eng.*, **2001**, 14, 261-267.

5) Determination of V_{rel}

The peroxidase assay described above (section 4) was used to determine the relative activities of WT GOase against each fluorinated sugar.



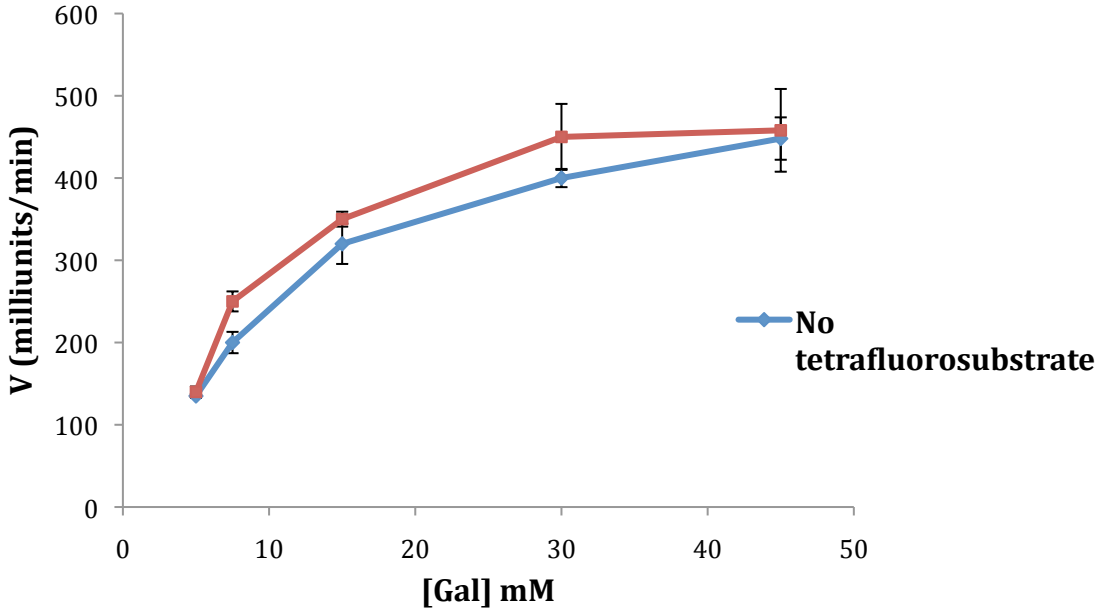
Substrate profile of WT GOase towards galactose and fluorinated analogues of Gal and Glc. The liquid phase assay was performed at substrate concentration of 50 mM and an enzyme concentration of 12.5 nM. Activity was determined by measuring the increase in absorbance at λ = 420 nm. Data points represent means and error is expressed as standard deviation (SD) from duplicate samples.

6) Determination of k_{cat} and K_M

For the kinetic characterisation of the protein, the peroxidase assay (section 4) was used and measurements were performed using a range of substrate concentrations (0.1 – 1.0 M). The kinetic constants k_{cat} and K_M were determined by fitting the data to the Michaelis-Menten equation using the Sigma Plot kinetics module (Systat Software Inc.). All values represent an average of the data closest to the mean ± the standard deviation of triplicate results.

7) Competition experiment

The peroxidase assay was used to determine whether Substrate **5** could act as a competitive inhibitor by assaying D-Gal turnover in the presence of **5**. The reaction rates were first determined as a function of D-Gal concentration in the absence of **5**. The experiment was repeated and a constant concentration of **5** (7.5 mM) was added at each D-Gal concentration. The range of D-Gal concentrations used was 7.5 - 45 mM and each assay was carried out in triplicate. Figure 6.2 illustrates that the enzymatic reaction rate for D-Gal is not decreased in the presence of **5**. The results suggest that **5** is not a competitive inhibitor of WT GOase, but merely a poor substrate.



Michaelis-Menten curves representing the reaction rates at various substrate concentrations in the absence and presence of 2,3-dideoxy-2,2,3,3-tetrafluoro-D-galactose (**5**). The liquid phase assay was performed to determine whether **5** acts a competitive inhibitor. The concentration of **5** was kept constant at 7.5 mM whereas the concentration of Gal was increased from 7.5 mM to 45 mM. The concentration of WT enzyme used 2.5 nM. Activity was determined by measuring the increase in absorbance at λ = 420 nm. Data points represent means and error is expressed as standard deviation (SD) from triplicate samples