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# Stability of aminooxy glycosides to glycosidase catalysed hydrolysis

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ABSTRACT

The stability of the amino(methoxy) beta-glycosidic bond to glycosidase catalysed hydrolysis is reported. Beta-*O*-benzyl glucose and beta-*O*-benzyl galactose are substrates hydrolysed by beta-glucosidase and beta-galactosidase from almonds and *Escherichia coli*, respectively. However their beta-*N*-benzyl-(*O*-methoxy)-glucoside and beta-*N*-benzyl-(*O*-methoxy)-galactoside derivatives are competitive inhibitors.


In recent years, the chemoselective ligation of secondary methoxyamine derivatives (sugars, peptides, sterols) with unprotected and non-activated reducing sugars has emerged as an efficient route to access a variety of *N*-(*O*-methoxy)-linked neoglycoconjugates in a manner, which bypasses the need for extensive protecting group manipulation of the donor sugars (Scheme 1).[1] These ligation methods proceed under mildly acidic conditions where the aminoxy group **2** selectively reacts with the aldehyde of the reducing sugar (in its ring opened form) **1a** to give an oxy-imminium intermediate **3** (Scheme 1). Subsequent ring closure results in the predominant formation of the β-linked *N*-(*O*-methoxy)-glycoside **3a** in the pyranose form. There are only a few exceptions to this β-selectivity such as mannose, which favours the α-configured product. Some reactions using galactose and mannose have also been reported where the furanose ligation adducts are observed as minor products.[1,2]

To date, these methods have been employed to prepare a variety of neoglycoconjugates,[1,3–6] glycopeptides[1,7] and glycoproteins.[8] These procedures have also been adapted for the facile neoglycorandomisation of secondary alkoxyamine derivatives of the aglycon portion of glycosylated natural products such as digitoxin,[9,10] colchicine[11] and vancomycin.[12] Fluorous-tagged[2] and alkylamine functionalised methoxyamine ligands[13] have been exploited for the incorporation of reducing sugars onto microarrays. Considering their ease of preparation and the potential uses across a broad spectrum of biological applications we were interested in comparing the relative stability of *O*-linked and *O*-methoxy-*N*-linked glycosides to glycosidase catalysed hydrolysis. The inhibition of bovine liver β-galactosidase by 1 mM concentrations of β-1, 4-N(OMe)-linked disaccharides have previously been reported.[5] However, in these assays, enzyme activity was monitored by the release of the *para*-nitrophenol chromophore from pNP-β-Gal, so any hydrolysis of the N(OMe)-glycosidic bond of the inhibitor(s) would not have been detected under these conditions. Herein, we report the stability studies of two N(OMe)-glycosides, **7** and **8** (Scheme 2), towards enzyme catalysed hydrolysis by retaining β-glycosidases.

Benzyl β-D-glucopyranoside **5**[14] and benzyl β-D-galactopyranoside **6**[15] were prepared as previously described. Aminooxy glycosides **7** and **8** were prepared by stirring a twofold excess of D-glucose or D-galactose with *N*-benzyl-*O*-methyl-hydroxylamine **4**[16] in a 3:1 DMF/AcOH solvent mixture at 40 °C for 6 days and were isolated in 45% and 63% yields, respectively. Proton NMR analysis of the crude product mixture showed exclusive formation of the beta-anomeric products.

Before studying the enzymatic stability of aminooxy glycosides **7** and **8**, it was important to first establish that their O-glycoside analogues **5** and **6** were themselves susceptible to enzymatic hydrolysis using the glycosidases chosen for this study. The $K_m$ value for benzyl-β-D-glucoside **5** was determined using a continuous coupled assay (Scheme 3) where almond β-glycosidase (GlcH) activity was measured by the increase in absorbance at 340 nm due to the $NADP^+$-dependent oxidation of the liberated D-glucose to D-gluconolactone by *Thermoplasma acidophilum* glucose dehydrogenase (GDH). GDH also oxidises D-galactose with comparable efficiency,[17] so the same assay procedures were used to determine substrate kinetics for benzyl-β-D-galactoside **6** with *Escherichia coli* β-galactosidase (GalH). Both **5** and **6** proved to be substrates for their respective β-glycosidases with low millimolar $K_m$ values (Table 1).

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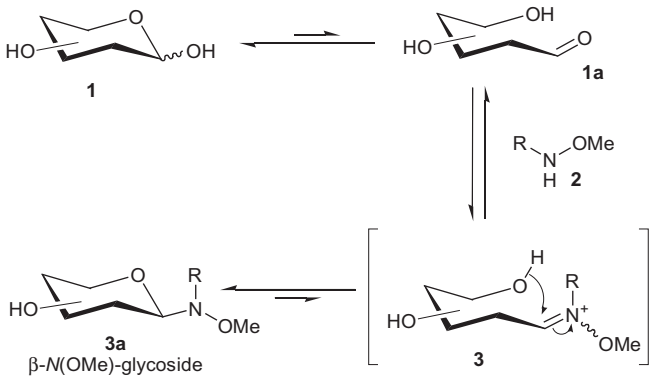
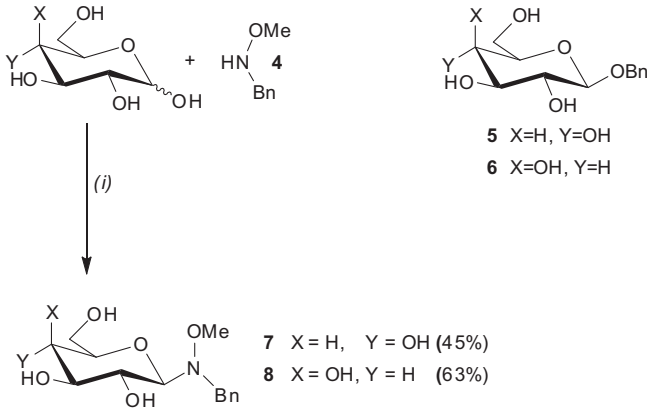
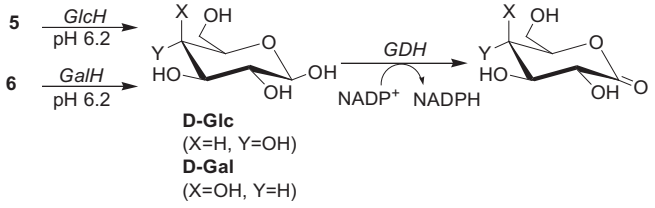



**Scheme 1.** Mechanism for aminooxy glycoside formation via reaction between reducing sugars and O,N-disubstituted hydroxylamines.




**Scheme 2.** Reagents and conditions: (i) DMF/acetic acid (3:1), 40 °C, 6 days.




**Scheme 3.** Coupled assay for substrate/inhibitor analyses of **5**–**8**.

Initial incubations of N(OMe)-galactoside **8** (600 μM) with GalH under the coupled assay procedures described above (Scheme 3) exhibited slow $NADP^+$ consumption suggesting enzymatic liberation of galactose from **8**. However, the same rate of $NADP^+$ consumption was also observed when the experiment was repeated in the absence of GalH suggesting either the presence of galactose contamination in the assay mixture or that GDH was able to oxidise **8** in an $NADP^+$-dependent manner. The latter was ruled out because the increase in absorbance at 340 nm reached a plateau long before NADPH levels were depleted. Based on the net increase in absorbance at 340 nm the assay concentration of galactose was determined as 24 μM by enzymatic titration. These initial experiments were performed using a stock solution of **8** (pH 6.2), which had been prepared and stored at room temperature for 20 days. When the assay was repeated using a freshly prepared stock solution of **8** no $NADP^+$ consumption was observed indicating that **8** was subject to slow chemical hydrolysis upon storage in mildly acidic solution. For subsequent enzymatic stability assays, to ensure that any absorbance increase at 340 nm could not be attributed to enzymatic hydrolysis of the N(OMe) linkage, solutions of **7** and **8** were freshly made on the day of use and were first pre-incubated with GDH and $NADP^+$ (30 min) prior to the subsequent addition of the glycosidase enzyme. Under these conditions neither **7** nor **8** showed any evidence of enzymatic hydrolysis Table 1. Further analysis of **7** and **8** showed them to be competitive inhibitors of GalH and GlcH, respectively, with $K_i$ values similar to the $K_m$ values observed for their respective competing substrates, **5** and **6** (Table 1). This demonstrates that the N(OMe) group could serve as a useful bioisostere for the exo-cyclic anomeric oxygen in the design of glycosidase resistant glycoside analogues. It is noteworthy that previous studies of hydrolytic stability of aminooxy glycoconjugates under acidic conditions have demonstrated how they become increasingly stable as the pH approaches neutrality.[18] Collectively, these results suggest that degradation of aminooxy glycoconjugates in vivo is more likely to arise from acid-catalysed, rather than glycosidase-catalysed hydrolysis.

**Table 1**
Substrate and inhibitor properties of **5**–**8** with GlcH and GalH

| Compound | Enzyme | $K_m$ (mM) | $K_i$ (mM) | $V_{max}$ (μmol $min^{-1}$ $mg^{-1}$) |
|---|---|---|---|---|
| **5** | GlcH | 4.77 (±0.53) | — | 0.17 (±0.01) |
| **7**[e] | GlcH | —[a] | 3.00 (±0.57)[c] | —[a] |
| **6** | GalH | 1.37 (±0.08) | — | 0.74 (±0.02) |
| **8**[e] | GalH | —[b] | 2.60 (±0.29)[d] | —[b] |

[a] No hydrolysis of **7** (4 mM) by GlcH (0.23 units $mL^{-1}$).
[b] No hydrolysis of **8** (4 mM) by GalH (11.5 units $mL^{-1}$).
[c] Competitive inhibition w.r.t. **5**.
[d] Competitive inhibition w.r.t. **6**.
[e] GalH is not inhibited by compound **7** and GalH is not inhibited by compound **8**.

## 1. Experimental

### 1.1. General

Chemical reagents and enzymes were purchased from Sigma–Aldrich. Sonication-mediated reactions were carried out using a Branson model 2510 sonicator bath operating at a frequency of 40 kHz. $^1H$ and $^{13}C$ NMR spectra were measured on Varian Unity Plus 400 NMR spectrometer and were referenced to the deuterated NMR solvent. Optical rotations ($[\alpha]_D$) were obtained with a Perkin-Elmer Model 241 Polarimeter, using the specified solvent and concentration, and are quoted in units of $10^{-1}$ deg $cm^2$ $g^{-1}$. High-resolution electrospray mass spectra were conducted at the EPSRC National Mass Spectrometry Service Centre at Swansea University using a Finnigan MAT 900 XLT with polyethyleneimine as the reference compound. *T. acidophilum* glucose dehydrogenase (GDH), *E. coli* β-galactosidase (GalH) and almond β-glucosidase (GlcH) were purchased from Sigma. One unit of GalH activity is defined as the amount of GalH required to hydrolyse 1 μmol of *O*-nitrophenyl-β-D-galactoside per minute under saturating substrate conditions at 37 °C and pH 7.3. One unit of GlcH activity is defined as the amount of GlcH required to liberate 1 μmol of glucose from salicin per minute under saturating substrate conditions at 37 °C and pH 5.0.

### 1.2. Benzyl methoxyamine β-D-glucopyranoside (7)

A solution of D-glucose (360 mg, 2 mmol) and *N*-benzyl-*O*-methyl hydroxylamine **4** (137 mg, 1 mmol) dissolved in a 3:1 solvent mixture of DMF/AcOH (16 mL) was stirred at 40 °C for 6 days. The reaction mixture was then concentrated on a rotary evaporator and purified by chromatography eluting with a step-wise solvent gradient of $CH_2Cl_2$/MeOH (9:1 to 8:2) to give **7**

(135 mg, 45%) as a white solid. $[\alpha]_D^{22}$ −6.1 (*c* 1.0, MeOH); $\delta_H$ (400 MHz; $CD_3OD$) 3.02–3.10 (1H, m, H-5), 3.18–3.26 (2H, m, H-4, H-3), 3.43 (1H, t, *J* 9.0, H-2), 3.61 (1H, dd, *J* 12.1, 5.6, H-6a), 3.80 (3H, dd, *J* 12.1, 2.2, H-6b), 3.83 (1H, d, *J* 9.0, H-1), 3.97 (1H, d, *J* 12.6, Ph-C*Ha*Hb), 4.10 (1H, d, *J* 12.6, Ph-CHa*Hb*), 7.13–7.24 (3H, m, 3 × Ar-H), 7.30–7.38 (2H, m, 2 × Ar-H); $\delta_C$ (100 MHz; $CD_3OD$) 57.77 ($CH_2$-Ph), 62.69 (OMe), 63.02 ($CH_2OH$), 71.40 (C-3), 71.69 (C-2), 79.68 (C-4), 79.88 (C-5), 93.38 (C-1), 128.61, 129.40, 131.36, 138.64 (Ph); *m*/*z* ($ES^+$) 317.1707 ($M+NH_4^+$, $C_{14}H_{25}N_2O_6$) requires 317.1703.

### 1.3. Benzyl methoxyamine β-galactopyranoside (8)

Compound **8** was synthesised from D-galactose as described for compound **7** and was obtained in 63% isolated yields as a white solid. $[\alpha]_D^{22}$ −10.3 (*c* 1.0, MeOH); $\delta_H$ (400 MHz; $CD_3OD$) 3.17–3.26 (2H, m, H-3, H-5), 3.64 (1H, dd, *J* 11.5. 5.2, H-6a), 3.70–3.76 (3H, m, H-6b, H-2, H-4), 3.84 (1H, d, *J* 9.0, H-1), 3.96 (1H, d, *J* 12.8, Ph-C*Ha*Hb), 4.09 (1H, d, *J* 12.8, Ph-CHa*Hb*), 7.13–7.24 (3H, m, 3 × Ar-H), 7.30–7.38 (2H, m, 2 × Ar-H); $\delta_C$ (100 MHz; $CD_3OD$) 57.35 ($CH_2$-Ph), 62.47 (OMe), 62.85 ($CH_2OH$), 78.68 (C-5), 76.58 (C-3), 70.75, 69.19 (C-2, C-4), 94.12 (C-1), 138.94, 131.21, 129.34, 128.49 (Ph); *m*/*z* ($ES^+$) 317.1707 ($M+NH_4^+$, $C_{14}H_{25}N_2O_6$) requires 317.1711.

### 1.4. Enzyme assays

Enzyme substrate assays were carried out in a final volume of 200 μL in a 96-well microplate at 37 °C containing GlcH (50 mU) or GalH (2.3 U), PIPES (10 mM), NaOAc (20 mM), EDTA (0.1 mM), $NADP^+$ (0.6 mM), GlcDH (0.9 U) at pH 6.2 and varying concentrations of substrates. Substrate stock solutions were made up using co-solvent mixtures of assay buffer and DMSO; final assay mixtures contained 4% DMSO. Enzyme activity was monitored by the increase in absorbance at 340 nm due to NADPH formation using a Polarstar Optima microplate reader. Initial rates were measured from the linear region of product formation For $K_i$ determinations, inhibition assays were carried out at three or more different inhibitor concentrations and six substrate concentrations. Initial rates ($v$) were measured from the linear region of product formation. Kinetic data were analysed and $K_m$, $V_{max}$ and $K_i$ values determined (by non-linear regression with the appropriate rate equations) using Grafit Version 5 (Erithacus Software Ltd).

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