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Stability studies of hydrazide and hydroxylamine-based glycoconjugates in aqueous solution

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ABSTRACT

Glycoconjugates can be readily formed by the condensation of a free-reducing terminus and a strong α-effect nucleophile, such as a hydrazide or a hydroxylamine. Further characterization of a series of glycoconjugates formed from xylose, glucose and *N*-acetylglucosamine, and either *p*-toluenesulfonyl hydrazide or an *N*-methylhydroxylamine, was carried out to gain insight into the optimal conditions for the formation of these useful conjugates, and their stability. Their apparent association constants (9–74 M^{-1}) at pH 4.5; as well, as rate constants for hydrolysis, at pH 4.0, 5.0 and 6.0 (37 °C), were determined. The half-lives of the conjugates varied between 3 h and 300 days. All the compounds were increasingly stable as the pH approached neutrality. Conjugate hydrolysis rates mirrored those found for O-glycoside hydrolysis where conjugates formed from electron-rich monosaccharides hydrolyzed more rapidly.

1. Introduction

The synthesis of glycoconjugates has facilitated a wide variety of techniques for the detailed study of carbohydrates and their interactions in biological systems.[1,2] The types of glycoconjugates that have been synthesized differ greatly depending on the intended use, but the majority of the conjugates are similar in that the reducing terminus of the oligosaccharide is functionalized for conjugate formation. Until recently, most glycoconjugates have been generated by the total synthesis of the oligosaccharide with the desired reducing terminal conjugating functional group installed early in the synthetic procedure. This approach is robust and can reliably generate the desired glycoconjugates but it is labour intensive, requiring many steps.[3,4]

Alternative approaches to glycoconjugate formation that directly functionalize the reducing terminal hemiacetal have been developed. These methods are especially useful when oligosaccharides can be isolated from natural sources or generated enzymatically. Classically, reductive amination has been used for the synthesis of glycoconjugates.[5] Unfortunately, reductive amination results in an acyclic structure at the reducing terminus of the oligosaccharide, and this can have consequences on the glycoconjugate, potentially causing the loss of its biological activity. It is possible to generate glycosyl amines from the free hemiacetal at the reducing terminus, and then directly acylate these derivatives.[6] However, this reaction can be challenging to control due to the instability and potential mutarotation of the intermediary glycosyl amine.[7]

Recently, condensation approaches that enable direct functionalization of the terminal hemiacetal with preservation of the reducing monosaccharide have been re-investigated. These chemoselective conjugations involve condensation with a nucleophile such as an *N*-alkylhydroxylamine,[8] an acylhydrazide,[9–11] a sulfonylhydrazide,[10] a semicarbazide[12,13] or a semithiocarbazide.[14] A thermodynamic mixture of glycosides is produced from these condensation reactions, which for reducing terminal *gluco*-configured monosaccharides is predominately the β-pyranoside.[15,16] The conditions for the chemoselective conjugations are mild and can often be carried out in aqueous solution, making these methods efficient and amenable to work with small quantities of oligosaccharide. The simplicity and efficiency of the conjugations have made them excellent methods for the formation of glycoconjugates for applications such as biotin labelling,[17,18] labelling for mass spectrometry,[19,20] labelling for chromatography,[21,22] formation of glycoarrays,[23–25] the generation of glycopeptide analogues[26–30] or neoglycoproteins,[31] to 'sweeten' the structures of therapeutics,[9,32–34] and recently in the protecting group-free formation of glycosyl donors.[35]

Despite the wide use of these chemoselective methods, the properties of the linkages have not been thoroughly investigated with regard to their stability and rates of hydrolysis under aqueous conditions.[18,32] A more complete understanding of these properties will allow for the condensation conditions for glycoconjugate formation to be optimized and for the conditions under which the glycoconjugates are useful to be elucidated.

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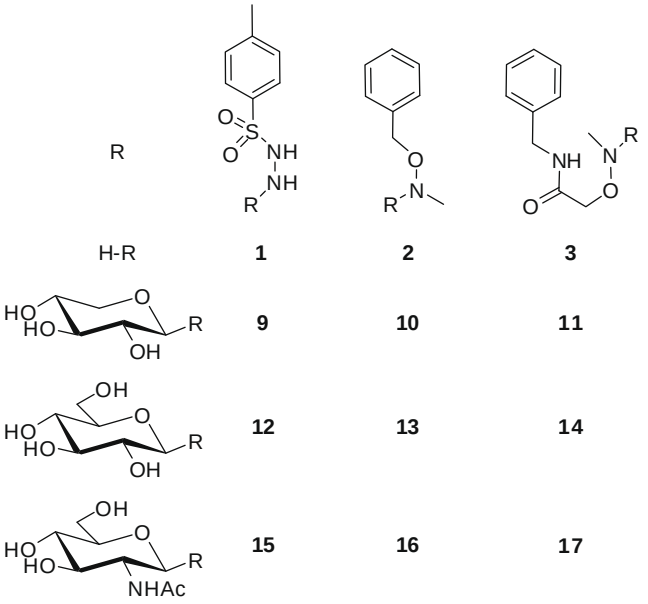


Figure 1. Nine glycoconjugates synthesized and subjected to hydrolysis experiments.

In a previous communication, we reported the hydrolysis rates for derivatized 1-glycosyl-1-benzoylhydrazides. We found them to be unstable in weakly acidic solutions.[36] In this report, we have evaluated nine glycoconjugates (Fig. 1) based on *p*-toluenesulfonylhydrazide and two *N*-methylhydroxylamines. The equilibrium constants for formation of these conjugates in aqueous solution were determined, and the rates of hydrolysis of the conjugates were evaluated at pH 4.0, 5.0 and 6.0. These results have been compared to the established mechanisms for oxime or hydrazone hydrolysis as well as for the hydrolysis of O-glycosides.

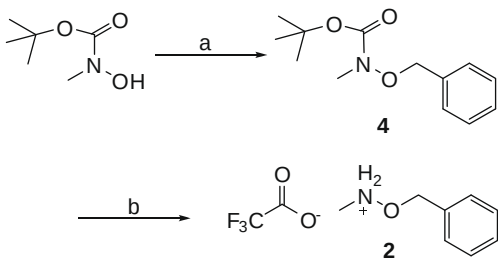
2. Results

2.1. Formation of glycoconjugates

The conjugates are derived from three different monosaccharides: xylose, glucose and *N*-acetylglucosamine. These monosaccharides were chosen for their biological relevance and for the wealth of information that is available about O-glycoside hydrolysis for these monosaccharides. *N*-acetylglucosamine and xylose are the reducing terminal sugars found in the N-linked glycoproteins and the glycosaminoglycans, respectively, thus knowledge of the properties of conjugates derived from these monosaccharides will be directly applicable to conjugates formed from these oligosaccharides. The mechanisms of hydrolysis of the corresponding methyl glycosides of xylose, glucose and *N*-acetylglucosamine have been studied in detail. This knowledge allows parallels to be drawn to the rates of hydrolysis determined for the glycoconjugates studied here, potentially allowing the results obtained to be extrapolated to other glycoconjugates.

These monosaccharides were condensed with *p*-toluenesulfonylhydrazide (**1**), *O*-benzyl-*N*-methylhydroxylamine (**2**) and *N*-methyl-*O*-(*N*′-benzylacetamide)hydroxylamine (**3**) (Fig. 1). The *p*-toluenesulfonylhydrazide **1** is commercially available while the two *N*-methylhydroxylamines, **2** and **3**, were synthesized.

O-Benzyl-*N*-methylhydroxylamine (**2**) was prepared from *t*-butyl *N*-methyl-*N*-hydroxycarbamate,[37] which was alkylated at the hydroxylamine oxygen using benzyl bromide to give **4**. The Boc-protecting group was removed with TFA in CH_2Cl_2 (1:1) to obtain



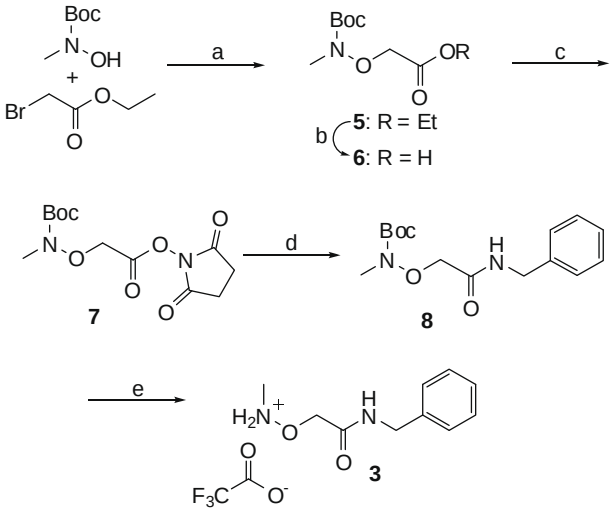
Scheme 1. Reagents and conditions: (a) NaH, BnBr, DMF (85%); (b) TFA, CH_2Cl_2 (1:1) (90%).

the *O*-benzyl-*N*-methylhydroxylamine **2** in an overall yield of 73% (Scheme 1).

N-Methyl-*O*-(*N*′-benzylacetamide)hydroxylamine (**3**) was also prepared from *t*-butyl *N*-methyl-*N*-hydroxycarbamate, shown in Scheme 2, following the method of Niikura et al.[38] Compound **5** was obtained by alkylating the hydroxylamine at oxygen with ethyl bromoacetate. The ester was hydrolyzed with sodium hydroxide in THF to form **6**. The acid **6** was then activated with DCC and coupled with *N*-hydroxysuccinimide to afford the active ester **7**. This compound is a versatile intermediate for forming other *N*-alkylhydroxylamine-based glycoconjugates. Benzylamine was condensed with the succinimide ester to give **8**, which was then deprotected using TFA in CH_2Cl_2 (1:1) to give *N*-methyl-*O*-(*N*′-benzylacetamide)hydroxylamine **3** as the TFA salt.

To determine the optimal conditions for glycoconjugate synthesis, the equilibrium constant for formation of the conjugates was determined using ^{1}H NMR spectroscopy at equimolar concentrations of hydrazide or hydroxylamine at four different concentrations: 50, 75, 100 and 125 mM, in deuterated sodium acetate buffer (pD 4.5). The amounts of conjugate and free monosaccharide were determined by integration of the ^{1}H NMR absorptions. After 4 days of equilibration at 37 °C, no further change was observed in the relative amounts of species present, and the integrated values were used to determine the apparent equilibrium constants under these conditions (Table 1).

As seen from the apparent association constants, the *p*-toluenesulfonohydrazide glycoconjugates (**9**, **12** and **15**) are moderately more stable than the *N*-methylhydroxylamine conjugates. Larger



Scheme 2. Reagents and conditions: (a) NaH, THF (95%); (b) NaOH, THF (86%); (c) DCC, *N*-hydroxysuccinimide, EtOAc (87%); (d) benzylamine, CH_3CN (73%); (e) TFA–CH_2Cl_2 (1:1) (87%).

Table 1
Equilibrium constants for the glycoconjugate formation

Monosaccharide	K_a (M^{-1})[a] (compound)		
Xyl	74[b] (**9**)	21 (**10**)	20 (**11**)
Glc	65[b] (**12**)	18 (**13**)	19 (**14**)
GlcNAc	18[b] (**15**)	16 (**16**)	9 (**17**)

[a] K_a = [Glycoconjugate]/[free nucleophile][free hemiacetal]. Apparent association constants determined in D_2O (pD 4.5, 37 °C); values are the average of four determinations at different compound concentrations. Standard deviation was between 6% and 9%.
[b] 3% DMSO-d_6 was used to solubilize the *p*-toluenesulfonylhydrazide.

association constants were observed with more electron-rich monosaccharides (xylose > glucose > *N*-acetylglucosamine). All of the equilibrium constants are small. Thus, only under concentrated conditions do the conjugation reactions proceed to near completion. It has been reported that the formation of some *N*-methylhydroxylamine conjugates does not proceed to completion, and that the products of the condensation reaction with less reactive monosaccharides (i.e., *N*-acetylglucosamine) are produced in low yields.[8,39] These observations may be the result of the small equilibrium constant for the condensation in aqueous solution.

Given the small equilibrium constants for formation of the glycoconjugates, the condensation reactions were carried out under concentrated conditions (0.75 M, equimolar) at pH 4.5 (2 M NH_4OAc). The samples were incubated at 37 °C for 72 h, and glycoconjugates **9**, **12** and **15** were crystallized from isopropanol, while the remaining compounds were purified using column chromatography. The isolated yields from the conjugation reactions were between 80% and 90%, and only β-pyranosides were isolated in all cases.

2.2. Rates of glycoconjugate hydrolysis

To confirm the formation of the hemiacetal and hydrazide or hydroxylamine, hydrolysis products of the conjugates were analyzed by ^1H NMR (3% DMSO-d_6 in 10 mM Na_2DPO_4 buffer at pD 6.0 with *t*-butanol as the internal standard). The hydroxylamine conjugates hydrolyzed to the expected products. However, the *p*-toluenesulfonylhydrazide derivatives **9**, **12** and **15** gave not only the aldose and *p*-toluenesulfonylhydrazide but also *p*-toluenesulfinic and *p*-toluenesulfonic acids. The degradation of *p*-toluenesulfonylhydrazide to *p*-toluenesulfinic and *p*-toluenesulfonic acids had previously been observed.[40,41] Attempts were made to determine the rate of degradation of *p*-toluenesulfonylhydrazide under the hydrolysis conditions, but the data do not fit to a simple first-order process (Fig. 13S, see Supplementary data). Qualitatively, the rate of decomposition of *p*-toluenesulfonylhydrazide is faster than hydrolysis of the conjugate at pH 6.0 but slower than hydrolysis of the conjugate at pH 4.0. Thus, with the analysis carried out here, it is not possible to determine if the *p*-toluenesulfonohydrazide conjugates are hydrolyzing exclusively by an initial C–N bond cleavage, or by competitive pathways involving initial C–N and N–S bond cleavage.

To determine the hydrolysis rates, the glycoconjugates **9**–**17** were incubated at the physiologically relevant pH values of 4.0, 5.0 and 6.0 at 37 °C, and the hydrolysis reaction was followed by HPLC (Fig. 2). The *p*-toluenesulfonohydrazide derivatives **12** and **15** elute as two peaks. This may be due to cis–trans isomerisation about the hydrazide bond. Cis–trans isomerism in glycosylhydrazides has been observed for 1-glycosyl-2-acetylhydrazides[11] and 1-glycosyl-2-benzoylhydrazides.[11,36] When the two isomers were separated, and re-analyzed separately, two peaks were again observed corresponding to re-equilibration of the isomers. In the calculation of the hydrolysis rates, the integrated areas under the peaks derived from both the isomers were combined. Benzyl alcohol was used as an internal standard in all cases. Control experiments showed no glycoconjugate formation upon incubating the nucleophile and hemiacetal at equivalent concentrations (2 mM) under the hydrolysis conditions for time frames consistent with those for the hydrolysis experiments.

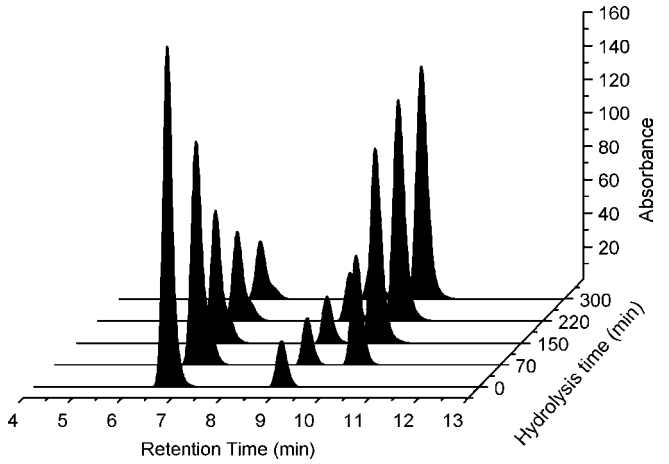


Figure 2. Hydrolysis of *N*-(β-D-xylopyranosyl)-*p*-toluenesulfonohydrazide in 20 mM NaOAc (0.5% DMSO) pH 4.0 at 37 °C. Peaks correspond to glycoconjugate **9** (6.8 min), benzyl alcohol (9.2 min, internal standard) and *p*-toluenesulfonylhydrazide (10.5 min). The corresponding salts of *p*-toluenesulfinic and *p*-toluenesulfonic acids eluted at the solvent front.

The observed rate constants for hydrolysis (pseudo-first-order conditions) were determined by directly fitting the integrated areas indicative of the glycoconjugates remaining compared to an internal standard. The observed half-lives for hydrolysis of conjugates **9**–**17** are presented in Table 2. The pH rate profile is presented in Figure 3. The hydrolysis of all the conjugates fit well to a first-order process despite the possibility of two hydrolysis pathways for the *p*-toluenesulfonohydrazide conjugates (vide supra).

3. Discussion

Methyl α-D-xylopyranoside undergoes specific acid-catalyzed hydrolysis approximately five times faster than methyl α-D-glucopyranoside.[42] The difference in the rate of hydrolysis between these structures correlates well with difference in electron affinity of the substituent at C-5, with glucose being less electron rich than xylose.[43] The rate-limiting step in the hydrolysis reaction is formation of the oxocarbenium ion, and due to the lack of a C-5

Table 2
Half-lives in aqueous solution of glycoconjugates **9**–**17**

Compound	pH 4.0 $t_{1/2}$ (h)	pH 5.0 $t_{1/2}$ (h)	pH 6.0 $t_{1/2}$ (h)
9	2.9	13	29
10	7.8	26	59
11	13	140	540
12	19	48	71
13	11	210	890
14	100	990	5100
15	72	230	1100
16	76	660	2600
17	350	3100	7500

[a] 2 mM sample (20 mM NaOAc pH 4.0, 20 mM NaOAc pH 5.0, 20 mM Na_2HPO_4 pH 6.0) was incubated at 37 °C. 200 μL samples were taken out at different time intervals and quenched with the addition of 400 μL of 4 °C 200 mM Na_2HPO_4 buffer at pH 7.0 and immediately analyzed by HPLC. Standard deviation between replicate runs was between 3% and 5%.

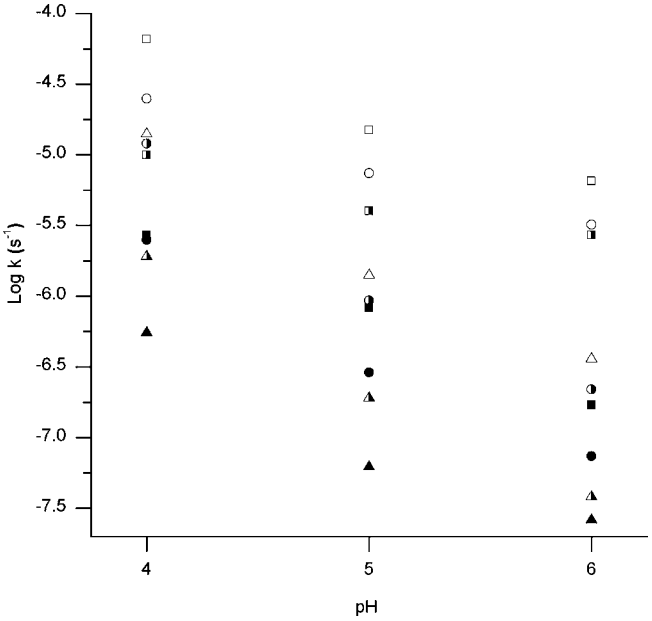


Figure 3. pH rate profile for the glycoconjugates **9–17**. (□) **9**, (○) **10**, (△) **11**, (◨) **12**, (◑) **13**, (◭) **14**, (■) **15**, (●) **16**, (▲) **17**. Each value is an average of two experiments; standard deviation was between 3% and 5%. Buffers: 20 mM NaOAc pH 4.0, 20 mM NaOAc pH 5.0, 20 mM Na_2HPO_4 pH 6.0. 0.5% DMSO was added to maintain solubility for glycoconjugates **9**, **12** and **15**.

substituent the xylosyl oxocarbenium ion is of lower energy, leading to faster hydrolysis.[44] Similar effects have been measured for a range of glycosides with deoxy and deoxyfluoro substituents of differing stereochemistry, and in general, the field effect of the substitution on the electron-deficient transition state directly influences the hydrolysis rate.[45] In contrast, the rate of hydrolysis of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside is faster than that of methyl β-D-glucopyranoside, despite the greater electron affinity of the *N*-acetamido group than that of the hydroxyl substituent at C-2. This observation is most likely due to neighbouring group participation of the *N*-acetamido group during hydrolysis.[46] Thus, by analogy, comparison of the rates of hydrolysis of the xylo- and glucopyranoside conjugates **9**–**14** synthesized here provides insight into the importance of the electronic properties of the monosaccharides on the rate of hydrolysis. The comparison of glucose glycoconjugates **12**–**14** with *N*-acetylglucosamine derived conjugates **15**–**17** provides insight into the possible influence of neighbouring group participation on the rate of hydrolysis.

From analysis of the kinetic data, it is apparent that the rates of hydrolysis for all the conjugates investigated are strongly pH dependent (Fig. 3, Table 2). The *p*-toluenesulfonohydrazide conjugates **9**, **12** and **15** hydrolyze more quickly than the *N*-methylhydroxylamine conjugates at pH 5 and 6 by a factor of 2–71.

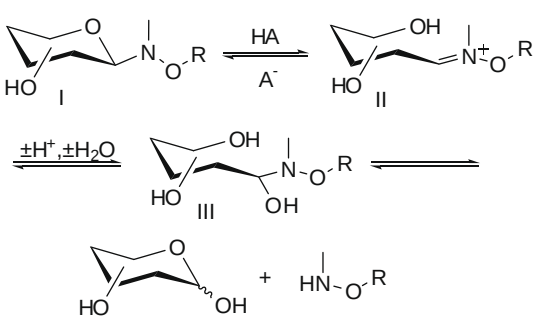
The *N*-(β-D-glucopyranosyl)-*p*-toluenesulfonohydrazide **12** has a half-life of 19 h, and hydrolyzes significantly more slowly than the previously investigated 1-glucosyl-2-benzoylhydrazide.[36] This observation is in agreement with the previous observations that conjugates formed from less basic hydrazides hydrolyze more slowly.[36] Comparison of the *p*-toluenesulfonohydrazide conjugates formed from different monosaccharides **9**, **12** and **15** indicates that the rates of hydrolysis are slower when the monosaccharide has more electron-withdrawing groups on the pyranose ring (xylose (fastest) > glucose > *N*-acetylglucosamine (slowest)). The observation that *N*-acetylglucosamine derivative **15** hydrolyzes more slowly than the corresponding glucose derivative **12** suggests that the field effect of the *N*-acetamido group destabilizes the rate-limiting transition state, and that neighbouring group participation is not a major factor in the hydrolysis mechanism.

From the analysis performed here, it cannot be determined whether the pathway of hydrolysis for the glycosyl-*p*-toluenesulfonohydrazides proceeds exclusively via initial C–N bond cleavage or by two competing pathways involving initial C–N or S–N bond cleavage. The observation that the hydrolysis reaction does fit well to a first-order rate law makes the analysis useful for evaluating experimental conditions under which these conjugates can be applied without completing hydrolysis.

The conjugates formed with *N*-methyl-*O*-benzylhydroxylamine **2** and *N*-methyl-*O*-(*N*′-benzylacetamide)hydroxylamine **3** display a four to ninefold differences in hydrolysis rates across all the individual monosaccharides assessed. Those differences may be explained by the electronic properties of the *N*-methylhydroxylamines, where **3** is more electron-deficient than **2** due to the inductive effect of the amide in comparison to the phenyl ring. As was observed with the glycosyl-*p*-toluenesulfonohydrazide conjugates, the *N*-methylhydroxylamine conjugates formed from the less electron-rich monosaccharides (i.e., *N*-acetylglucosamine) hydrolyze more slowly.

Extensive studies have supported a mechanism of hydrolysis of oximes, and hydrazones over a pH range of ~4.0–6.0 which involves a rate-limiting addition of water at the sp^2 carbon.[47] Over this pH range general base catalysis is observed. It is proposed that the base aids in deprotonation of the nucleophilic water molecule upon attack. We hypothesize that the mechanism of hydrolysis of the *N*-methylhydroxylamine glycoconjugates follows a similar mechanism.

The expected steps in hydrolysis of the hydroxylamine-based glycoconjugates are shown in Scheme 3. The effect of pH on the rate of hydrolysis is reflected in the position of the equilibrium between the conjugate **I** and the ring open oxyiminium species **II** in solution; at lower pH more of species **II** will be present. This is consistent with the observation that more electron-rich conjugates, formed from either electron-rich monosaccharides (e.g., xylose) or more electron-rich hydroxylamines (e.g., **2**), hydrolyze more rapidly because they are more readily protonated. In the electron-poor glycoconjugates it is less favourable to form species **II**. The rate-limiting step is then attack of water on the oxyiminium ion (**II**). Buffer catalysis was observed at pH 4 and pH 6 on the hydrolysis of **13**, which would be expected for a rate-limiting attack of water on the oxyiminium (**II**) (see Supplementary data). After water attack, the carbanolamine (**III**) would rapidly eliminate the *N*-methylhydroxylamine, as the rate of attack of hydroxylamine on aldehydes is rapid and reversible over the pH range investigated.[48,49] Although beyond the scope of this initial investigation, further studies to confirm the buffer ion responsible for the catalysis and an expansion of the pH range investigated are required to support this mechanistic proposal. This mechanism is consistent with that proposed by Dumy and co-workers for the formation of *N*-methyl-*O*-methylhydroxylamine glycoconjugates.[8]



Scheme 3. Putative mechanism for the hydrolysis of *N*-alkylhydroxylamine glycoconjugates.

4. Conclusions

Efficient methods to synthesize glycoconjugates enable a wide variety of biophysical tools to be used to study the properties of carbohydrates. Here, we have investigated the equilibrium constants for formation, as well as the rates of hydrolysis of *N*-methylhydroxylamine and *p*-toluenesulfonylhydrazide glycoconjugates with xylose, glucose and *N*-acetylglucosamine. The equilibrium constants for formation of all the conjugates are small, between 9 M^{-1} and 74 M^{-1}, revealing the importance of carrying out the conjugation reactions under concentrated conditions. In cases where only small amounts of oligosaccharides are available, this can be addressed by using excess nucleophile, provided that a facile separation method for the glycoconjugate product is available.[25] In all cases, the hydrolysis rates of the conjugates accelerated under increasingly acidic conditions and the half-lives of hydrolysis of these conjugates suggest that caution should be employed when using these conjugates below pH 6.0. The hydrolysis rates are strongly affected by the electronic properties of the monosaccharide involved, as electron-rich monosaccharides hydrolyze significantly more quickly than electron-poor monosaccharides. Given the trends in hydrolysis rates of these conjugates, which parallel those observed with the well studied O-glycosides, it is possible to use the results presented here as a practical guide to extrapolate to the conditions under which a glycoconjugate formed from a novel mono- or oligosaccharide may be useful.

5. Experimental

5.1. General methods

HPLC analyses were performed on a Dionex BioLC (PDA-100 Photodiode Array Detector, GS50 Gradient pump and A550 Autosampler) using a Waters Symmetry® C18 5 μm (4.6 × 150 mm) reverse phase analytical column. All NMR spectra were recorded at 25 °C with either a Varian 400 MHz (AutoX8308-400 probe) or a Mercury 400 MHz (ATB8123–400 probe) spectrometer. Chemical shifts were reported in ppm (δ scale) using the solvent residue signals as reference, and assignments were made by gCOSY spectroscopy. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constant. High resolution mass spectra were obtained from an ABI/Sciex QStar mass spectrometer with an ESI source.

5.1.1. Hydrolysis methods

Each glycoconjugate sample was prepared in duplicate at 2 mM (20 mM NaOAc pH 4.0, 20 mM NaOAc pH 5.0 or 20 mM phosphate pH 6.0) and incubated at 37 °C. Glycoconjugates **9**, **12** and **15** required 0.5% DMSO for solubility. Benzyl alcohol (1 mM) was used as an internal standard for all runs. 200 μL samples were taken out at time intervals and quenched with the addition of 400 μL of 4 °C 200 mM phosphate buffer at pH 7.0 and immediately analyzed by HPLC (15–25% CH_3CN in H_2O, 4 min–15 min gradient). The observed pseudo-first-order rate constants for the hydrolysis were determined by directly fitting the areas of each glycoconjugate remaining as a percentage to an exponential decay using ORIGIN 7.0, defined by the equation: $v = k_{obs} \cdot$ [% glycoconjugate remaining].

5.1.2. Determination of equilibrium constants, K_a

The K_a values were determined using ^{1}H NMR spectroscopy by integrating the absorption of the methyl peak corresponding to the *N*-methylhydroxylamine or the methyl peak of the *p*-toluenesulfonylhydrazide. The reactions were followed using four different solutions, each containing the corresponding sugars: xylose, glucose or *N*-acetylglucosamine, and the corresponding nucleophile: **1**, **2** or **3**, in equimolar amounts at 50, 75, 100 and 125 mM using 500 mM deuterated sodium acetate buffer, pD 4.5. 3% DMSO-d_6 was added for solubility when using *p*-toluenesulfonyl hydrazide. The samples were incubated at 37 °C for 4 days, and their ^{1}H NMR spectra were measured. Product and remaining reagent concentrations were then determined using integration values. Degradation of the *p*-toluenesulfonylhydrazide into *p*-toluenesulfinic and *p*-toluenesulfonic acids was corrected for in the calculations. The represented K_a value is an average of the four samples.

5.2. *O*-Benzyl-*N*-methylhydroxylamine (2)

Compound **4** (5.0 g, 21 mmol) was dissolved in 40 mL CH_2Cl_2–TFA (1:1) and stirred at rt for 22 h. The solvent was then removed under reduced pressure, and the product was purified with column chromatography (pentane) to afford 2.6 g of **2** as oil, yield 90%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.78 (s, 2H, NH_2), 7.37 (s, 5H, Ar), 5.04 (s, 2H, OCH_2), 2.91 (s, 3H, NCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.0, 129.8, 129.5 (2), 129.1 (2), 76.4, 35.9; HRMS *m*/*z* calcd for $C_8H_{12}NO$ $[M+H]^+$: 138.0913, found: 138.0915.

5.3. *N*-Methyl-*O*-(*N*′-benzylacetamide)hydroxylamine (3)

Compound **8** (0.9 g, 3.1 mmol) was dissolved in 10 mL CH_2Cl_2–TFA (1:1) and stirred at rt for 1 h. The solvent was then removed under reduced pressure, and the product was crystallized from Et_2O–pentane to give 0.81 g of the TFA salt **3** as white plates, yield 87%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.18 (s, 2H, NH_2CH$_3$), 7.36–7.26 (m, 5H, Ar), 7.14 (s, 1H, CONH), 4.54 (s, 2H, OCH_2), 4.46 (s, 2H, PhCH_2), 2.88 (s, 3H, NCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.4, 137.3, 129.0 (2), 128.0, 128.0 (2), 71.6, 43.5, 37.4; HRMS *m*/*z* calcd for $C_{10}H_{15}N_2O_2$ $[M+H]^+$: 195.1128, found: 195.1123.

5.4. *t*-Butyl *N*-benzyloxy-*N*-methylcarbamate (4)

A 60% oil dispersion of NaH (1.6 g, 40 mmol) was added to *t*-butyl *N*-methyl-*N*-hydroxycarbamate (5.3 g, 36 mmol) in 20 mL anhydrous DMF and stirred at 0 °C for 30 min under nitrogen. Benzyl bromide (4.7 mL, 40 mmol) was added, and the reaction mixture was stirred for 16 h. The reaction mixture was diluted with pentane (150 mL), and the organic layer was then washed with water (3 × 50 mL) and brine (40 mL), dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (CH_2Cl_2–pentane, 2:3) to afford 7.8 g of **4** as pale yellow oil, yield 85%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42–7.31 (m, 5H, Ar), 4.81 (s, 2H, OCH_2), 3.05 (s, 3H, NCH_3), 1.40 (s, 9H, C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 157.2, 135.8, 129.6 (2), 128.6, 128.5 (2), 81.4, 76.6, 37.0, 28.4 (3); HRMS *m*/*z* calcd for $C_{13}H_{19}NO_3$ $[M+Na]^+$: 260.1257, found: 260.1260.

5.5. Ethyl (*t*-butoxycarbonyl-*N*-methylaminooxy)acetate (5)

A 60% oil dispersion of NaH (136 mg, 3.4 mmol) was added to a solution of *t*-butyl *N*-methyl-*N*-hydroxycarbamate (500 mg, 3.4 mmol) in THF (10 mL) and stirred at 0 °C for 30 min under nitrogen. Ethyl bromoacetate (452 μL, 4.1 mmol) was added, and the reaction mixture was stirred for 4 h at rt. The reaction mixture was then diluted with EtOAc (150 mL), and the organic layer was washed with water (3 × 50 mL) and brine (2 × 15 mL), dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (1:9 EtOAc–pentane) to afford 755 mg of **5** as pale yellow oil, yield 95%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.45 (s, 2H, OCH_2CO), 4.24 (q, 2H, J = 7.1 Hz, OCH_2CH$_3$), 3.21 (s, 3H,

NCH_3), 1.49 (s, 9H, C(CH_3)$_3$), 1.30 (t, 3H, J = 7.1 Hz, OCH$_2$CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.5, 157.9, 82.1, 72.2, 61.2, 38.5, 28.4 (3), 14.3; HRMS m/z calcd for $C_{10}H_{19}NO_5Na$ [M+Na]$^+$: 256.1155, found: 256.1154.

5.6. (*t*-Butoxycarbonyl-*N*-methylaminooxy)acetic acid (6)

Compound **5** (689 mg, 3.0 mmol) was dissolved in THF (6 mL) and solution of NaOH (240 mg, 6.0 mmol) in H_2O (2 mL) was added and the solution was stirred for 4 h at rt. The reaction mixture was then diluted with EtOAc (50 mL), and the organic layer was washed with 1 M HCl (2 × 50 mL), water (2 × 50 mL) and brine (1 × 20 mL), dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (CH_2Cl_2) to afford 519 mg of **6** as pale yellow oil, yield 86%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.46 (s, 2H, OCH_2), 3.14 (s, 3H, NCH_3), 1.51 (s, 9H, C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.7, 159.9, 85.2, 73.4, 37.9, 28.2 (3); HRMS m/z calcd for $C_8H_{15}NO_5Na$ [M+Na]$^+$: 228.0842, found: 228.0850.

5.7. (*t*-Butoxycarbonyl-*N*′-methylaminooxyacetyl)-*N*-hydroxysuccinimide ester (7)

To a stirred solution of **6** (750 mg, 3.6 mmol) in EtOAc (25 mL) were added *N*-hydroxysuccinimide (630 mg, 1.5 equiv) and DCC (900 mg, 1.2 equiv). The reaction mixture was then stirred at rt for 2 h. The solid was removed by filtration and washed with EtOAc. The organics were then washed with a solution of sodium bicarbonate (1 M, 2 × 30 mL) and dried over $MgSO_4$. After filtration the solvent was removed under vacuum, and the resulting white solid was recrystallized from EtOAc–hexanes giving 960 mg of **7,** yield 87%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.80 (s, 2H, OCH_2), 3.19 (s, 3H, NCH_3), 2.86 (s, 4H, NCOCH_2), 1.50 (s, 9H, C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.8 (2), 165.2, 158.0, 82.6, 70.0, 38.9, 28.3 (3), 25.7 (2); HRMS m/z calcd for $C_{12}H_{18}N_2O_7Na$ [M+Na]$^+$: 325.1013, found: 325.1006.

5.8. (*t*-Butoxycarbonyl-*N*-methyl-*O*-(*N*′-benzylacetamide))hydroxylamine (8)

To a solution of **7** (186 mg, 0.62 mmol) in CH_3CN (15 mL) was added benzylamine (107 μL, 0.74 mmol). The reaction mixture was stirred under nitrogen for 30 min. The reaction mixture was then diluted with EtOAc (100 mL), and the organic layer was washed with 1 M $NaHCO_3$ (2 × 50 mL), 1 M HCl (2 × 50 mL), H_2O (2 × 50 mL) and brine (50 mL), dried over $MgSO_4$, filtered and concentrated. This afforded **8** as a colourless oil (169 mg), yield 93%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.51 (s, 1H, CON*H*), 7.27–7.18 (m, 5H, Ar), 4.47 (d, 2H, J = 6.0 Hz, PhCH_2), 4.32 (s, 2H, OCH_2), 3.05 (s, 3H, NCH_3), 1.37 (s, 9H, C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.9, 158.0, 138.2, 128.6 (2), 127.8 (2), 127.3, 83.0, 73.5, 42.9, 37.4, 28.1 (3); HRMS m/z calcd for $C_{15}H_{22}N_2O_4Na$ [M+Na]$^+$: 317.1471, found: 317.1465.

5.9. *N*-(β-D-Xylopyranosyl)-*p*-toluenesulfonohydrazide (9)

The title compound (3.5 g) was prepared by making a 0.75 M D-xylose (2.0 g, 13.3 mmol) and *p*-toluenesulfonylhydrazide (2.6 g, 13.7 mmol) solution in 2 M NH_4OAc buffer pH 4.5 and incubating at 37 °C for 72 h. The solution was then lyophilized, and the product was recrystallized from isopropanol, yield 83%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.79 (d, 2H, J = 8.3 Hz, Ar), 7.39 (d, 2H, J = 8.3 Hz, Ar), 3.79 (dd, 1H, $J_{5a,5b}$ = 11.3, $J_{4,5a}$ = 5.4 Hz, H-5a), 3.70 (d, 1H, $J_{1,2}$ = 8.7 Hz, H-1), 3.47–3.40 (m, 2H, H-2, H-4), 3.28 (under CD_3OD peak, 1H, H-3), 3.07 (dd, $J_{5a,5b}$ = 11.3, $J_{4,5b}$ = 10.7 Hz, H-5b), 2.43 (s, 3H, PhCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 145.1, 137.1, 130.6 (2), 129.1 (2), 92.4, 78.2, 71.4, 71.2, 68.3, 21.5; HRMS m/z calcd for $C_{12}H_{18}N_2O_6NaS$ [M+Na]$^+$: 341.0777, found: 341.0788.

5.10. *N*-Methyl-*O*-benzyl-*N*-(β-D-xylopyranosyl)hydroxylamine (10)

The title compound (152 mg) was prepared by making a 0.75 M D-xylose (100 mg, 0.67 mmol) and compound **2** (110 mg, 0.8 mmol) solution in 2 M NH_4OAc buffer pH 4.5 and incubating at 37 °C for 72 h. The product was purified by column chromatography (5% MeOH in CH_2Cl_2), yield 84%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.39–7.27 (m, 5H, Ar), 4.75 (s, 2H, PhCH_2), 3.95 (d, 1H, $J_{1,2}$ = 9.0 Hz, H-1), 3.88 (dd, 1H, $J_{5a,5b}$ = 11.2, $J_{4,5a}$ = 5.4 Hz, H-5a), 3.49–3.42 (m, 2H, H-2, H-4), 3.31 (under CD_3OD peak, 1H, H-3), 3.14 (dd, $J_{5a,5b}$ = 11.2, $J_{4,5b}$ = 10.9 Hz, H-5b), 2.69 (s, 3H, PhCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 138.5, 130.2 (2), 129.3 (2), 129.0, 96.4, 79.4, 76.4, 71.7, 71.1, 68.9, 39.3; HRMS m/z calcd for $C_{13}H_{20}NO_5$ [M+H]$^+$: 270.1335, found: 270.1328.

5.11. *N*-Methyl-*O*-(*N*′-benzylacetamide)-*N*-(β-D-xylopyranosyl)hydroxylamine (11)

Using the same procedure as described for compound **10**, D-xylose (41 mg, 0.27 mmol) and **3** (100 mg, 0.32 mmol) gave 76 mg of **11** as white amorphous solid (86%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.33–7.21 (m, 5H, Ar), 4.45 (d, 1H, OCH_{2a}, J = 15.0 Hz), 4.41 (d, 1H, OCH_{2b}, J = 15.0 Hz), 4.34 (d, 1H, ArCH_{2a}, J = 15.1 Hz), 4.28 (d, 1H, ArCH_{2b}, J = 15.1 Hz), 3.98 (d, 1H, $J_{1,2}$ = 9.0 Hz, H-1), 3.88 (dd, 1H, $J_{5a,5b}$ = 11.2, $J_{4,5a}$ = 5.4 Hz, H-5a), 3.48–3.41 (m, 2H, H-2, H-4), 3.31 (under CD_3OD peak, 1H, H-3), 3.16 (dd, $J_{5a,5b}$ = 11.2, $J_{4.5b}$ = 10.6 Hz, H-5b), 2.73 (s, 3H, Ac); ^{13}C NMR (100 MHz, CD_3OD): δ 172.1, 139.6, 129.6 (2), 128.7 (2), 128.3, 96.3, 79.1, 72.4, 71.6, 71.1, 68.9, 43.7, 38.8; HRMS m/z calcd for $C_{15}H_{23}N_2O_6$ [M+H]$^+$: 327.1550, found: 327.1566.

5.12. *N*-(β-D-Glucopyranosyl)-*p*-toluenesulfonohydrazide (12)

Using the same procedure as described for compound **9**, D-glucose (2.0 g, 11.1 mmol) and *p*-toluenesulfonylhydrazide (2.13 g, 11.43 mmol) gave 3.43 g of compound **12** as white solid (89%). ^{1}H NMR (400 MHz, D_2O): δ 7.81 (d, 2H, J = 8.3 Hz, Ar), 7.40 (d, 2H, J = 8.3 Hz, Ar), 3.86 (dd, 1H, $J_{6a,6b}$ = 11.7, $J_{5,6a}$ = 1.9 Hz, H-6a), 3.67 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 3.58 (dd, 1H, $J_{6a,6b}$ = 11.7, $J_{5,6b}$ = 6.2 Hz, H-6b), 3.34 (under CD_3OD peak, 1H, H-3), 3.29 (under CD_3OD peak, 1H, H-4), 3.20–3.11 (m, 2H, H-2, H-5), 2.44 (s, 3H, PhCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 145.1, 137.4, 130.6 (2), 129.1 (2), 91.5, 79.2, 78.2, 71.8 (2), 63.2, 21.5; HRMS m/z calcd for $C_{13}H_{20}N_2O_7NaS$ [M+Na]$^+$: 371.0883, found: 371.0901.

5.13. *N*-Methyl-*O*-benzyl-*N*-(β-D-glucopyranosyl)hydroxylamine (13)

Using the same procedure as described for compound **10**, D-glucose (103 mg, 0.57 mmol) and **2** (94 mg, 0.68 mmol) gave 150 mg of **13** as white solid (88%). ^{1}H NMR (400 MHz, D_2O): δ 7.41–7.30 (m, 5H, Ar), 4.81 (d, 1H, J = 10.4 Hz, OCH_{2a}), 4.78 (d, 1H, J = 10.4 Hz, OCH_{2b}), 4.04 (d, 1H, $J_{1,2}$ = 8.9 Hz, H-1), 3.84 (dd, 1H, $J_{6a,6b}$ = 12.1, $J_{5,6a}$ = 2.2 Hz, H-6a), 3.68 (dd, 1H, $J_{6a,6b}$ = 12.1, $J_{5,6b}$ = 5.1 Hz, H-6b), 3.49 (t, 1H, $J_{3,4}$ = 8.9 Hz, H-3), 3.39 (t, 1H, $J_{3,4}$ = 8.9 Hz, H-4), 3.28 (under CD_3OD peak, 1H, H-2), 3.22 (ddd, 1H, $J_{4,5}$ = 9.5, $J_{5,6b}$ = 5.1, $J_{5,6a}$ = 2.2 Hz, H-5), 2.76 (s, 3H, NCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 138.1, 130.5 (2), 129.4 (2), 129.3, 95.4, 79.6, 79.3, 76.7, 71.8, 71.1, 62.7, 39.4; HRMS m/z calcd for $C_{14}H_{22}NO_6$ [M+H]$^+$: 300.1441, found: 300.1454.

5.14. *N*-Methyl-*O*-(*N*′-benzylacetamide)-*N*-(β-D-glucopyranosyl)hydroxylamine (14)

Using the same procedure as described for compound **10**, D-glucose (23 mg, 0.13 mmol) and **3** (50 mg, 0.16 mmol) gave 38 mg of **14** as white amorphous solid (82%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.34–7.22 (m, 5H, Ar), 4.47 (d, 1H, J = 14.9 Hz, OCH_{2a}), 4.43 (d, 1H, J = 14.9 Hz, OCH_{2b}), 4.37 (d, 1H, J = 15.1 Hz, ArCH_{2a}), 4.31 (d, 1H, J = 15.1 Hz, ArCH_{2b}), 4.05 (d, 1H, $J_{1,2}$ = 8.8 Hz, H-1), 3.87 (dd, 1H, $J_{6a,6b}$ = 11.9, $J_{5,6a}$ = 2.1 Hz, H-6a), 3.68 (dd, 1H, $J_{6a,6b}$ = 11.9, $J_{5,6b}$ = 5.2 Hz, H-6b), 3.45 (t, 1H, J = 8.8 Hz, H-3), 3.37 (t, 1H, J = 8.7 Hz, H-4), 3.30–3.21 (m, 2H, H-2, H-5), 2.78 (s, 3H, NCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 172.1, 139.7, 129.6 (2), 128.6 (2), 128.3, 95.4, 79.7, 79.2, 72.5, 71.7, 71.4, 62.7, 43.7, 38.8; HRMS m/z calcd for $C_{16}H_{24}N_2O_7Na$ [M+Na]$^+$: 379.1475, found: 379.1462.

5.15. *N*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*p*-toluenesulfonohydrazide (15)

Using the same procedure as described for compound **9**, *N*-acetyl-D-glucosamine (1.0 g, 9.0 mmol) and *p*-toluenesulfonylhydrazide (0.85 g, 9.1 mmol) gave 2.9 g of compound **15** as white solid (83%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.74 (d, 2H, J = 8.3 Hz, Ar), 7.38 (d, 2H, J = 8.3 Hz, Ar), 3.94 (d, 1H, $J_{1,2}$ = 9.2 Hz, H-1), 3.89 (dd, 1H, $J_{6a,6b}$ = 11.7, $J_{5,6a}$ = 1.7 Hz, H-6a), 3.60 (m, 1H, H-6b), 3.46 (t, 1H, $J_{2,3}$ = 10.1, $J_{1,2}$ = 9.2 Hz, H-2), 3.42–3.37 (m, 1H, H-3), 3.20–3.18 (m, 2H, H-4, H-5), 2.43 (s, 3H, PhCH_3), 2.01 (s, 3H, Ac); ^{13}C NMR (100 MHz, CD_3OD): δ 173.9, 145.1, 137.2, 130.5 (2), 129.1 (2), 91.9, 78.9, 76.2, 72.4, 63.2, 55.0, 23.0, 21.5; HRMS m/z calcd for $C_{15}H_{23}N_3O_7NaS$ [M+Na]$^+$: 412.1148, found: 412.1156.

5.16. *N*-Methyl-*O*-benzyl-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)hydroxylamine (16)

Using the same procedure as described for compound **10**, *N*-acetyl-D-glucosamine (147 mg, 0.67 mmol) and **2** (110 mg, 0.8 mmol) gave 196 mg of **16** as white amorphous solid (86%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.42–7.40 (m, 2H, Ar), 7.36–7.30 (m, 3H, Ar), 4.68 (d, 1H, J = 10.0 Hz, ArCH_{2a}), 4.62 (d, 1H, J = 10.0 Hz, ArCH_{2b}), 4.19 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-1), 3.95 (t, 1H, $J_{1,2}$ = 9.8 Hz, H-2), 3.85 (dd, 1H, $J_{6a,6b}$ = 12.1, $J_{5,6a}$ = 2.2 Hz, H-6a), 3.69 (dd, 1H, $J_{6a,6b}$ = 12.1, $J_{5,6b}$ = 5.3 Hz, H-6b), 3.42 (t, 1H, $J_{3,4}$ = 8.9, $J_{2,3}$ = 9.8 Hz, H-3), 3.33 (t, 1H, $J_{4,5}$ = 9.5, $J_{3,4}$ = 8.9 Hz, H-4), 3.23 (ddd, 1H, $J_{4,5}$ = 9.5, $J_{5,6b}$ = 5.3, $J_{5,6a}$ = 2.2 Hz, H-5), 2.72 (s, 3H, NCH_3), 2.01 (s, 3H, Ac); ^{13}C NMR (100 MHz, CD_3OD): δ 173.4, 138.1, 130.7 (2), 129.3 (2), 129.2, 93.7, 79.6, 77.6, 76.1, 71.6, 62.8, 54.1, 39.1, 23.1; HRMS m/z calcd for $C_{16}H_{24}N_2O_6Na$ [M+Na]$^+$: 363.1526, found 363.1530.

5.17. *N*-Methyl-*O*-(*N*′-benzylacetamide)-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl) hydroxylamine (17)

Using the same procedure as described for compound **10**, *N*-acetyl-D-glucosamine (29 mg, 0.13 mmol) and **3** (31 mg, 0.16 mmol) gave 44 mg of **17** as white amorphous solid (85%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.34–7.28 (m, 4H, Ar), 7.26–7.22 (m, 1H, Ar), 4.47 (d, 1H, J = 14.8 Hz, OCH_{2a}), 4.42 (d, 1H, J = 14.8 Hz, OCH_{2b}), 4.27 (d, 1H, J = 14.7 Hz, ArCH_{2a}), 4.25 (d, 1H, $J_{1,2}$ = 9.7 Hz, H-1), 4.18 (d, 1H, J = 14.7 Hz, ArCH_{2b}), 3.89 (dd, 1H, $J_{6a,6b}$ = 11.9, $J_{5,6a}$ = 2.1 Hz, H-6a), 3.82 (t, 1H, J = 9.7 Hz, H-2), 3.71 (dd, 1H, $J_{6a,6b}$ = 11.9, $J_{5,6b}$ = 5.4 Hz, H-6b), 3.42 (dd, 1H, $J_{4,5}$ = 9.9, $J_{3,4}$ = 8.5 Hz, H-3), 3.30 (under CD_3OD, H-4), 3.25 (ddd, 1H, $J_{4,5}$ = 9.6, $J_{5,6b}$ = 5.4, $J_{5,6a}$ = 2.1 Hz, H-5), 2.75 (s, 3H, NCH_3), 1.95 (s, 3H, Ac); ^{13}C NMR (100 MHz, CD_3OD): δ 173.5, 171.9, 139.8, 129.6 (2), 128.6 (2), 128.3, 93.6, 79.7, 77.3, 72.9, 71.9, 62.7, 54.1, 43.6, 38.6, 23.0; HRMS m/z calcd for $C_{18}H_{27}N_3O_7Na$ [M+Na]$^+$: 420.1741, found: 420.1726.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.11.007.

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