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Note

Hydrolysis rates of 1-glucosyl-2-benzoylhydrazines in aqueous solution

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Abstract—A series of substituted 1-glucosyl-2-benzoylhydrazines were synthesized and their pseudo-first-order rate constants for hydrolysis were determined at pH 4, 5, 6 at 50 °C. All the compounds hydrolyzed quickly ($t_{1/2} < 3$ h) at pH 4.0, but were increasingly stable as the pH approached neutrality.

Keywords: Glycoconjugate; Glycosylhydrazides; Oligosaccharide labelling; Hydrolysis rates

Glycoconjugates are the primary constituents of extracellular matrices and cellular surfaces, where their oligosaccharide moieties have been shown to play important roles in recognition-driven events including cell growth, development, migration and differentiation.[1] The oligosaccharide composition at cell surfaces differs between cell types, and alterations in glycosylation patterns have been associated with specific disease states. Thus, the potential exists to design therapeutic agents that interfere with carbohydrate–protein interactions or exploit the carbohydrates as biomarkers for vaccine development.[2]

Despite the biological relevance of glycoconjugates, their preparation by chemical synthesis is still a challenging task due to the requirements for extensive use of orthogonal protection–deprotection strategies to generate natural *O*- or *N*-glycosidic linkages. This problem becomes acute when only small amounts of isolated oligosaccharides are available and multistep synthetic approaches are not possible. Efforts to circumvent the synthetic challenges of naturally occurring glycosidic linkages have employed alternative conjugation chemistries.

The most commonly used method for glycoconjugate formation is reductive amination, where the reducing end of the polysaccharide is condensed with an amine on the protein or peptide of interest to form a Schiff base, which is then reduced to form a secondary amine.[3] However, reductive amination creates an acyclic structure at the reducing terminus of the oligosaccharide, which may have unintended consequences when the biological activity of the conjugate is evaluated.

Recent developments in chemoselective methods for forming glycoconjugates have provided new routes to multifunctional glycoconjugates of high complexity. These methods use highly nucleophilic functional groups such as *N*-alkylhydroxylamines, hydrazides and semithiocarbazides to specifically condense with the reducing terminus of the oligosaccharide (Fig. 1).[4,5] These methods are very useful for derivatizing the small amounts of oligosaccharides available from biological samples as they can be carried out under mild conditions, in the presence of other functional groups found in biological systems and give the β-pyranosides in a high yield.

Specifically, the chemoselective condensation of hydrazides with reducing saccharides has found wide use for the formation of glycoconjugates for applications including biotin labelling,[6] formation of glycoarrays[7] and the generation of glycopeptide analogues.[8]

Detailed studies of 1-glycosyl-2-acetylhydrazine have determined the saccharide to exist as the β-pyranoside, as a cis/trans equilibrium mixture about the amide bond

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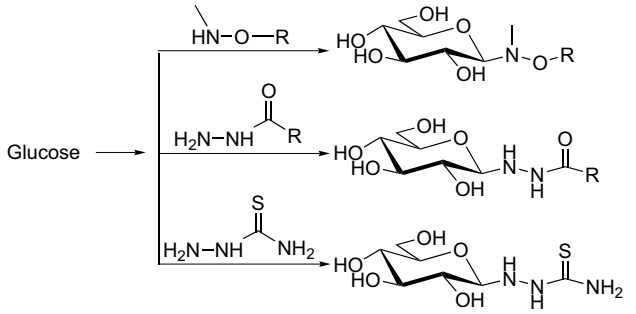

Figure 1. Chemoselective ligation methods based on the reactivity of a free hemiacetal with a potent nucleophile.

and to be rapidly cleaved in the presence of a strong acid (0.1 M H_2SO_4).[9] Contradictory reports regarding the stability of the glycosyl hydrazide linkages under physiologically relevant conditions have appeared in the literature.[10] But, despite the wide utility of this chemoselective method, no quantitative studies have been reported that determine the hydrolytic stability of the glycosylhydrazides. Here we communicate the rates of hydrolysis of a series of 1-glucosyl-2-benzoylhydrazines at the physiologically relevant pH values of 4.0, 5.0 and 6.0 (Fig. 2).

Four 1-glucosyl-2-phenylhydrazides were produced using acid-catalyzed condensation of glucose with the respective hydrazide in ethanol (Fig. 2).[11] At the limits of detection by ^{1}H NMR, only the β-pyranosides could be observed in all cases.

The hydrolysis of the conjugates was followed by reverse-phase high performance liquid chromatography (HPLC). A representative time course of an elution profile is presented in Figure 3. As has been previously observed, the 1-glycosyl-2-acetylhydrazines elute as two peaks corresponding to the cis–trans isomers about the amide bond.[9] These peaks were shown to reequilibrate after collection, lyophilization and reinjection. Surprisingly, the cis/trans isomers of the compounds could not be observed by ^{1}H or ^{13}C NMR. In calculation of the overall hydrolysis rate, the areas of the cis and trans isomers were combined. No reverse reaction (glucosylhydrazide formation) was observed under the hydrolysis conditions.

The observed pseudo-first-order rate constant for hydrolysis was determined by directly fitting the areas of 1-gluco-2-benzoylhydrazide remaining as a percent-

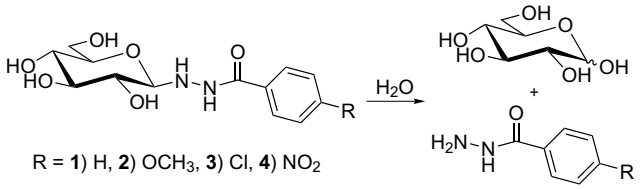

Figure 2. Glucosylhydrazide hydrolysis reactions studied.

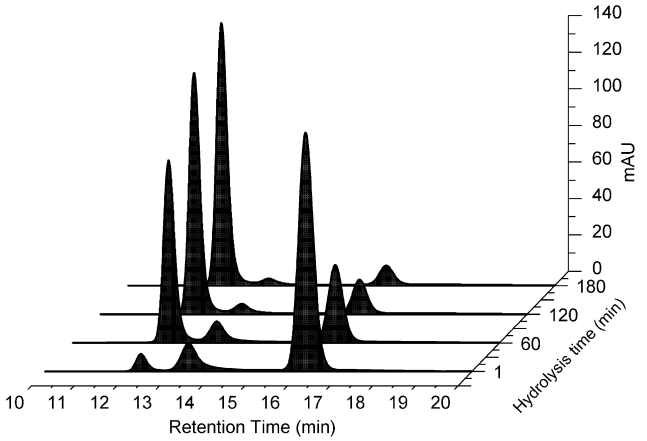

Figure 3. Hydrolysis of 1-glucosyl-2-phenylhydrazide (50 mM NaOAc, 5% MeOH, pH 4.0, 50 °C) was monitored using reverse phase HPLC (C18, MeOH/H_2O gradient 7.2–8.0%, 20 min). Phenylhydrazide (13 min) 1-glucosyl-2-phenylhydrazide appears as two peaks (cis/trans) at retention times 14 min and 16.5 min.

age of total hydrazide observed (Fig. 4). As can be seen in Table 1, the rate of hydrolysis is strongly pH-dependent, slowing significantly as the pH approaches neutrality. This evidently suggests that the rate-limiting step for hydrolysis is acid catalyzed over the pH range investigated. The hydrolysis rates of the conjugates followed the pK_a values for the parent hydrazides with the most stable conjugate being formed with *p*-nitrobenzylhydrazide (pK_a 11.28) followed *p*-chlorobenzylhydrazide (pK_a 12.09), benzylhydrazide (pK_a 12.52) and finally the least stable conjugate was formed with *p*-methoxyhydrazide (pK_a 12.83).[12] This suggests that more electron withdrawn hydrazides may form even more stable conjugates.

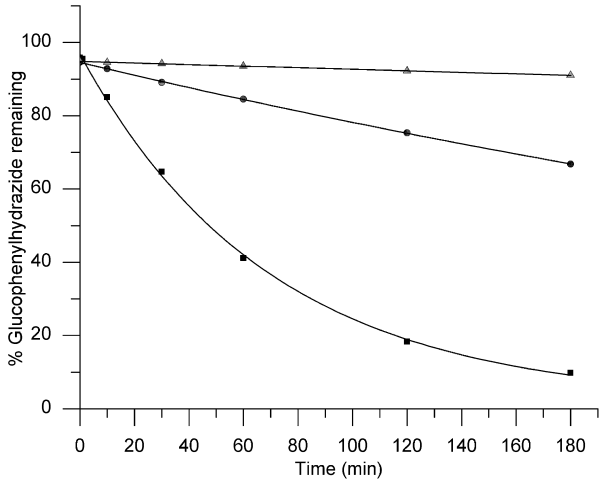

Figure 4. Hydrolysis of 1-glucosyl-2-phenylhydrazide in (■) 50 mM NaOAc (5% methanol) pH 4.0; (●) 50 mM NaOAc (5% methanol) pH 5.0; (▲) 50 mM Na_2HPO_4 (5% methanol) pH 6.0, at 50 °C. Each value represents the average of two experiments.

Table 1. Hydrolysis rates of **1**–**4**

Hydrazide R=	pH 4.0[a]		pH 5.0[a]		pH 6.0[a]	
	k_{obs} (s^{-1}) × 10^{-4}	$t_{1/2}$ (h)	k_{obs} (s^{-1}) × 10^{-5}	$t_{1/2}$ (h)	k_{obs} (s^{-1}) × 10^{-6}	$t_{1/2}$ (h)
OMe (**2**)	2.5	0.78	4.3	4.5	5.0	39
H (**1**)	2.2	0.89	3.2	6.0	3.7	51
Cl (**3**)	1.9	0.99	2.7	7.2	3.7	52
NO_2 (**4**)	1.5	1.3	2.2	8.9	3.4	57

[a] 5 mM sample (50 mM NaOAc, 5% MeOH pH 4.0, 50 mM NaOAc 5% MeOH pH 5.0 or 50 mM NaOAc 5% MeOH pH 6.0) incubated at 50 °C 200 μL samples were taken out at different 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. Standard deviation between replicate runs was between 3% and 8%.

To determine whether the hydrolysis proceeds via general or specific acid catalysis, the rates of hydrolysis were measured at a constant pH but with increasing buffer concentrations from 50 to 500 mM NaOAc (Fig. 5). The minimal acceleration in rate observed at the highest buffer concentration suggests that the hydrolysis is predominantly specific acid catalyzed. Thus, the observed rates can be extrapolated to other buffer systems where glycoconjugates might be used.

Under neutral conditions (pH 7, 50 mM Na_2HPO_4 (5% MeOH)) the samples showed no hydrolysis by ^1H NMR after being kept at room temperature for 37 days, consistent with the determined hydrolysis rates. An extrapolation of the observed rates of hydrolysis to physiological conditions (pH 7.4, 37 °C) yields the half lives of hydrolysis of approximately 50 days.

In conclusion, we have established that the reactions of phenyl hydrazides with glucose occur stereospecifically to give the β-glycopyranosides. The rate of the hydrolysis of these glycosides is strongly influenced by the pH of the solution. Their hydrolysis shows a minor dependence on the electronic properties of the hydrazide, with the conjugate formed with the lowest pK_a hydrazide being the most stable. The derivatives are stable at a neutral pH for extended periods of time and serve as excellent candidates for chemoselective ligation reactions to prepare neo-glycoconjugates. It has been observed that with other carbohydrates, mixtures of glycosides be formed with hydrazides and it is likely that these will hydrolyze at different rates.[9] However, given the hydrolysis rates presented here, caution should be employed when the glycoconjugates are used under mildly acidic conditions, such as those found in the lysosome, as at these pH values the glycoconjugates would rapidly hydrolyze.

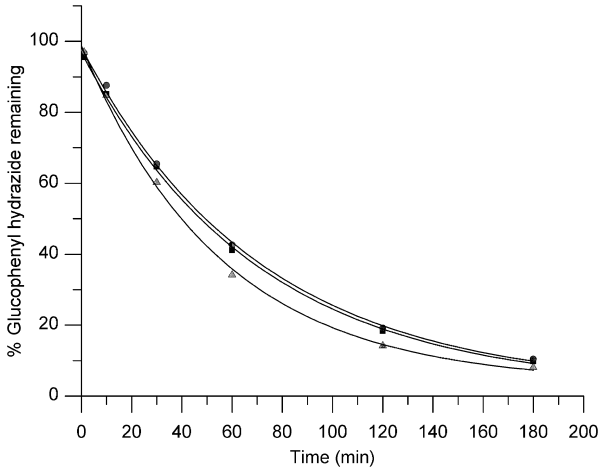

Figure 5. Determination of specific acid hydrolysis of 1-glucosyl-2-phenylhydrazide in (■) 50 mM NaOAc (5% methanol) pH 4.0; (●) 250 mM NaOAc (5% methanol) pH 4.0; (▲) 500 mM NaOAc (5% methanol) pH 4.0, at 50 °C. Each value represents the average of two experiments.

1. Experimental

1.1. General methods

NMR spectra were recorded on Varian NMR-400 and Mercury-400 MHz spectrometers. Chemical shifts were reported in ppm using the solvent residue signals as reference. HPLC analyses were performed on Dionex BioLC (PDA-100 Photodiode Array Detector, GS50 Gradient pump and A550 Autosampler) using a Waters Symmetry® C18 reverse phase analytical column. The buffers used in hydrolysis experiments were (A): 50 mM NaOAc (5% MeOH) at pH 4.0; (B): 50 mM NaOAc (5% MeOH) at pH 5.0 and (C): 50 mM Na_2HPO_4 (5% MeOH) at pH 6.0.

1.1.1. 1-Glucosyl-2-benzoylhydrazine (1). To a suspension of glucose (477 mg, 2.7 mmol) in 2 mL of ethanol and two drops of glacial acetic acid was added phenylhydrazide (540 mg, 4.0 mmol). The reaction mixture was stirred and heated at reflux for 3 h. The product precipitated and was purified by hot ethanol filtration and gave 654 mg (83%) of **1**. ^1H NMR (400 MHz, D_2O): δ 7.75 (m, 2H, Ar), 7.63 (m, 1H, Ar), 7.53 (m, 2H, Ar), 4.22 (d, 1H, $J_{1,2} = 9.0$ Hz, H-1), 3.92 (dd, 1H, $J_{6a,6b} = 12.2$ Hz, $J_{5-6a} = 2.1$ Hz, H-6_a), 3.73 (dd, 1H, $J_{5-6b} = 5.7$ Hz, H-6_b), 3.57 (t, 1H, $J_{3,4} = 9.0$ Hz, H-3), 3.46 (ddd, 1H, $J_{4,5} = 9.8$ Hz, H-5), 3.40 (t, 2H, $J_{2,3} = 9.3$ Hz, H-4, H-2); ^{13}C NMR (100 MHz, D_2O): δ 170.8, 132.5, 132.0, 128.9 (2), 127.4 (2), 90.0, 77.0,

76.4, 70.8, 69.7, 61.0; ESI-MS m/z calcd for $[C_{13}H_{18}N_2O_6+Na]^+$: 321.1064. Found: 321.1057.

1.1.2. 1-Glucosyl-2-*p*-methoxybenzoylhydrazine (2). Using the same procedure as described for compound **1**, glucose (300 mg, 1.7 mmol) and *p*-methoxyphenylhydrazide (415 mg, 2.5 mmol) gave 363 mg (66%) of **2**. ^{1}H NMR (100 MHz, D_2O): δ 7.79 (d, 2H, $J = 8.9$ Hz, *pO*MePh), 7.11 (d, 2H, $J = 8.9$ Hz, *p*O-MePh), 4.25 (d, 1H, $J_{1,2} = 9.0$ Hz, H-1), 3.94 (dd, 1H, $J_{6a,6b} = 12.4$ Hz, $J_{5-6a} = 2.3$ Hz, H-6_a), 3.91 (s, 3H, –OCH_3), 3.75 (dd, 1H, $J_{5-6b} = 5.8$ Hz, H-6_b), 3.58 (t, 1H, $J_{3,4} = 9.0$ Hz, H-3), 3.47 (ddd, 1H, $J_{4,5} = 9.8$ Hz, H-5), 3.41 (t, 1H, H-4), 3.40 (t, 1H, $J_{2,3} = 9.1$ Hz, H-2); ^{13}C NMR (100 MHz, D_2O): δ 170.2, 162.4, 129.5 (2), 124.5, 114.2 (2), 90.2, 77.1, 76.4, 70.9, 69.8, 61.0, 55.6; ESI-MS m/z calcd for $[C_{14}H_{20}N_2O_7+Na]^+$: 351.1166. Found: 351.1162.

1.1.3. 1-Glucosyl-2-*p*-chlorobenzoylhydrazine (3). Using the same procedure as described for compound **1**, glucose (150 mg, 0.8 mmol) and *p*-chlorophenylhydrazide (284 mg, 1.6 mmol) gave 182 mg (66%) of **3**. ^{1}H NMR (400 MHz, D_2O): δ 7.76 (d, 2H, $J = 8.5$ Hz, *p*ClPh), 7.58 (d, 2H, $J = 8.5$ Hz, *p*ClPh), 4.26 (d, 1H, $J_{1,2} = 9.0$ Hz, H-1), 3.94 (dd, 1H, $J_{6a,6b} = 12.2$ Hz, $J_{5-6a} = 1.9$ Hz, H-6_a), 3.75 (dd, 1H, $J_{5-6b} = 5.8$ Hz, H-6_b), 3.58 (t, 1H, $J_{3,4} = 9.0$ Hz, H-3), 3.47 (ddd, 1H, $J_{4,5} = 9.7$ Hz, H-5), 3.41 (t, 1H, H-4), 3.40 (t, 1H, $J_{2,3} = 9.1$ Hz, H-2); ^{13}C NMR (100 MHz, D_2O): δ 169.8, 138.1, 130.7, 129.0 (4), 90.0, 77.1, 76.4, 70.9, 69.7, 61.0; ESI-MS m/z calcd for $[C_{13}H_{17}N_2O_6Cl+Na]^+$: 355.0667. Found: 355.0667.

1.1.4. 1-Glucosyl-2-*p*-nitrobenzoylhydrazine (4). Using the same procedure as described for compound **1**, glucose (820 mg, 4.6 mmol) and *p*-nitrophenylhydrazide (1.5 g, 8.4 mmol) gave 940 mg (60%) of **4**. ^{1}H NMR (400 MHz, D_2O): δ 8.38 (d, 2H, $J = 8.7$ Hz, *p*NPh), 7.98 (d, 2H, $J = 8.7$ Hz, *p*NPh), 4.29 (d, 1H, $J_{1,2} = 8.9$ Hz, H-1), 3.93 (dd, 1H, $J_{6a,6b} = 12.2$ Hz, $J_{5-6a} = 2.1$ Hz, H-6_a), 3.76 (dd, 1H, $J_{5-6b} = 5.8$ Hz, H-6_b), 3.59 (t, 1H, $J_{3,4} = 9.0$ Hz, H-3), 3.49 (ddd, 1H, $J_{4,5} = 9.7$ Hz, H-5), 3.42 (t, 2H, $J_{2,3} = 9.3$ Hz, H-4, H-2); ^{13}C NMR (100 MHz, D_2O): δ 168.8, 149.8, 138.3, 128.8 (2), 124.0 (2), 90.0, 77.1, 76.5, 70.9, 69.7, 61.0; ESI-MS m/z calcd for $[C_{13}H_{17}N_3O_8+Na]^+$: 366.0925. Found: 366.0907.

1.2. General procedure for hydrolysis experiments

A 5 mM solution of the hydrazide was prepared by dissolving compound **1** (3.3 mg, 10 μmol) in 1.9 mL buffer A and 100 μL MeOH. The solution was incubated at 50 °C in a water bath, and 200 μL samples were taken out at defined time intervals and quenched by the addition of 400 μL of 4 °C 200 mM phosphate buffer at pH 7.0. The sample was kept on ice until the analysis was carried out by HPLC. The data was fit to an exponential decay using the software Origin, $v = k$[glucosylhydrazide], where k equals the pseudo-first-order kinetic constant for the appearance of the product.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.12.015.

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