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HYDRAZINOLYSIS–*N*-REACETYLATION OF GLYCOPEPTIDES AND GLYCOPROTEINS. MODEL STUDIES USING 2-ACETAMIDO-1-*N*-(L-ASPART-4-OYL)-2-DEOXY-β-D-GLUCOPYRANOSYLAMINE*

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ABSTRACT

2-Acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine (**1**) was used as a model glycopeptide to study the hydrazinolysis–*N*-reacetylation procedure. The major, initial product was the β-acetohydrazide derivative of 2-acetamido-2-deoxy-D-glucose (**2**) which gave 2-acetamido-2-deoxy-D-glucose (**5**) after exposure to acidic conditions. Very mild conditions of hydrolysis of **2** gave a 75–80% overall yield of **5** from **1** after the hydrazinolysis–*N*-reacetylation procedure. Several other minor compounds were detected which were not converted into **5** upon mild acid hydrolysis, indicating that 20–25% of product cannot be recovered as **5** at the reducing end of oligosaccharides.

INTRODUCTION

Hydrazinolysis–*N*-reacetylation of glycopeptides and glycoproteins bearing asparagine-linked oligosaccharides is a useful procedure for cleavage of the intact oligosaccharides from the peptide moiety. First used successfully as early[2] as 1966, the procedure has recently been applied widely[3–5]. Since hydrazine attacks amide bonds indiscriminately, the oligosaccharide is also *N*-deacetylated during the procedure and the peptide converted into amino acid hydrazides.

The potential for use of hydrazine-released oligosaccharides in chemical syntheses has been realized by investigators for some time, but hesitancy to use such oligosaccharides has been based on a lack of knowledge of the structures that arise from the asparagine-linked 2-acetamido-2-deoxy-D-glucose residue during the hydrazinolysis–*N*-reacetylation procedure.

Williams and assoc.[6–8] have been the first investigators to study this problem

*A preliminary report has been presented[1].
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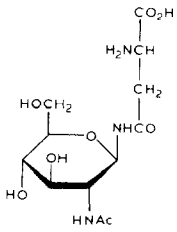

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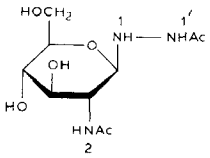

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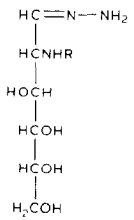

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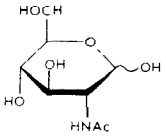
4 R = Ac



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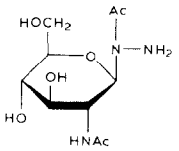

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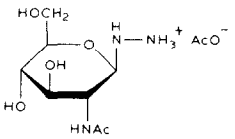

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in a detailed and systematic way. They have found the hydrazone derivative of 2-amino-2-deoxy-D-glucose (**3**) to be the main product after hydrazinolysis; very little free 2-amino-2-deoxy-D-glucose was found at this stage. However, other investigators[2–5] have noted the appearance of a 2-acetamido-2-deoxy-D-glucose reducing residue at the end of oligosaccharides after *N*-reacetylation. The goal of the present investigation was to identify which structure or structures gave free 2-acetamido-2-deoxy-D-glucose (**5**) during the *N*-acetylation step, and to define conditions to maximize the yield of this sugar at the reducing end of hydrazine-released oligosaccharides. By use of 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine (**1**) as a model glycopeptide to study the hydrazinolysis–*N*-reacetylation procedure, we have identified the initial major product, 1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetylhydrazine (**2**). Mild conditions for complete hydrolysis of **2** into 2-acetamido-2-deoxy-D-glucose (**5**) are described, and procedures for the chemical synthesis of **2** are given.

RESULTS AND DISCUSSION

Several compounds were detectable by silver staining when the products of the hydrazinolysis–*N*-reacetylation of **1** were chromatographed on paper. Two major products were present (Fig. 1, C and D), one of which (D) comigrated with authentic **5**. These could be completely separated by running the chromatography for a longer time (45–50 h). The compounds were eluted from preparative-scale chromatograms, lyophilized, and thoroughly dried *in vacuo* in the presence of phosphorus pentaoxide. Recovery of the compounds from the paper was found to

Fig. 1. Paper chromatographic separation of the products of hydrazinolysis–*N*-reacetylation of 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine (**1**) (lane 2). Lanes 1 and 3, authentic **5**. Several products (A–G) were detected by silver staining.

be virtually quantitative; the yields of compound C (55–60%) and D (20–25%) are based on dry weight and deduced structure. Minor products were still mixtures as revealed by ^{1}H-n.m.r. spectrometry, so the estimated yield of the minor products is the difference of the yields of the two major products from 100%. Compound D was identified as **5** by comparison of its 360-MHz ^{1}H-n.m.r. spectrum with that of an authentic sample.

Compound C (Fig. 1) was identified as **2** on the basis of its hydrolysis under mildly acidic conditions to give **5** and acetohydrazide, and by comparison of its ^{1}H-n.m.r. spectrum with that of an authentic sample synthesized by two different routes. The 360-MHz ^{1}H-n.m.r. spectrum of **2** in deuterium oxide (Fig. 2a) showed the presence of two *N*-acetyl groups per sugar molecule. A series of decoupling experiments enabled assignments to be made. The spectrum was not of first order; H-4 and -5 were strongly coupled, which resulted in distortion of these resonance patterns as well as that of H-3, and, to a lesser extent, of those of H-6a and -6b, caused by the effect of "virtual" coupling. A computer-simulated spectrum of the coupling pattern (Fig. 2b) also revealed the effect of "virtual" coupling on the H-3,

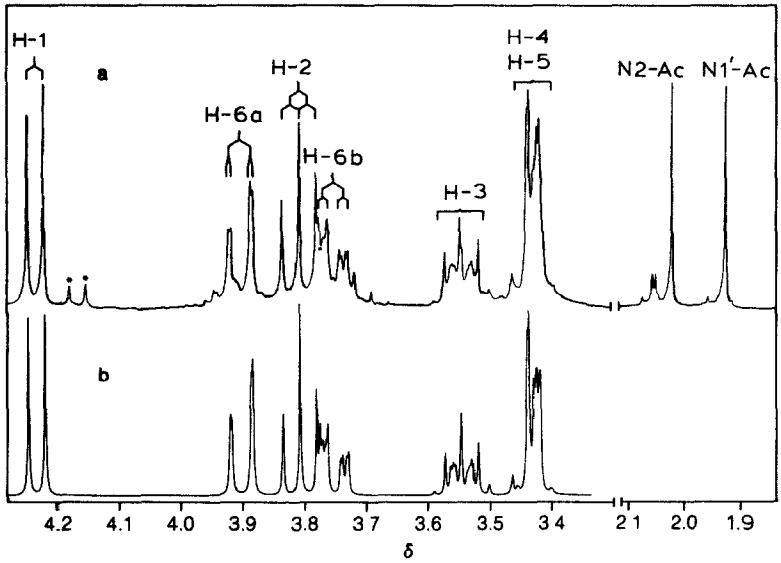


Fig. 2. (a) ^{1}H-N.m.r. spectrum of isolated compound C (Fig. 1) in deuterium oxide. Assignments are indicated. The intensity of the signals in the *N*-acetyl region (δ 1.9–2.1) are shown at ~1/6th of the scale. (b) Computer-simulated spectrum of the major conformer of 1-(2-acetamido-2-deoxy-β-D-gluco-pyranosyl)-2-acetylhydrazine (**2**, compound C, Fig. 1) in deuterium oxide. Only the 7-spin coupling system of the sugar ring protons (downfield region) is shown.

-6a, and -6b resonances, and enabled a determination of the chemical shifts and coupling constants (Table I). The presence of a second H-1 doublet (marked with asterisks) was attributed to a second conformer of **2** in D_2O for the following reasons. The integral ratio of this doublet to that of the H-1 doublet of the major conformer was 1:7 and was always present in the same relative proportion, whether the compound was isolated after hydrazinolysis–*N*-reacetylation of **1** or synthesized

TABLE I

^{1}H-N.M.R. DATA OF THE MAJOR CONFORMER OF 1-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-ACETYLHYDRAZINE (**2**)[a]

Proton	Chemical shift (δ)[b]	*Coupling constant (Hz)*	
H-1	4.227	$J_{1,2}$	9.7
H-2	3.804	$J_{2,3}$	9.9
H-3	3.542	$J_{3,4}$	9.1
H-4	3.429	$J_{4,5}$	9.9
H-5	3.424	$J_{5,6a}$	1.4
H-6a	3.897	$J_{5,6b}$	4.8
H-6b	3.750	$J_{6a,6b}$	−12.2
N2-Ac	2.020		
N1'-Ac	1.923		

[a]For a solution in D_2O at 20°. [b]Values were obtained from computer simulation due to strong H-4–H-5 coupling.

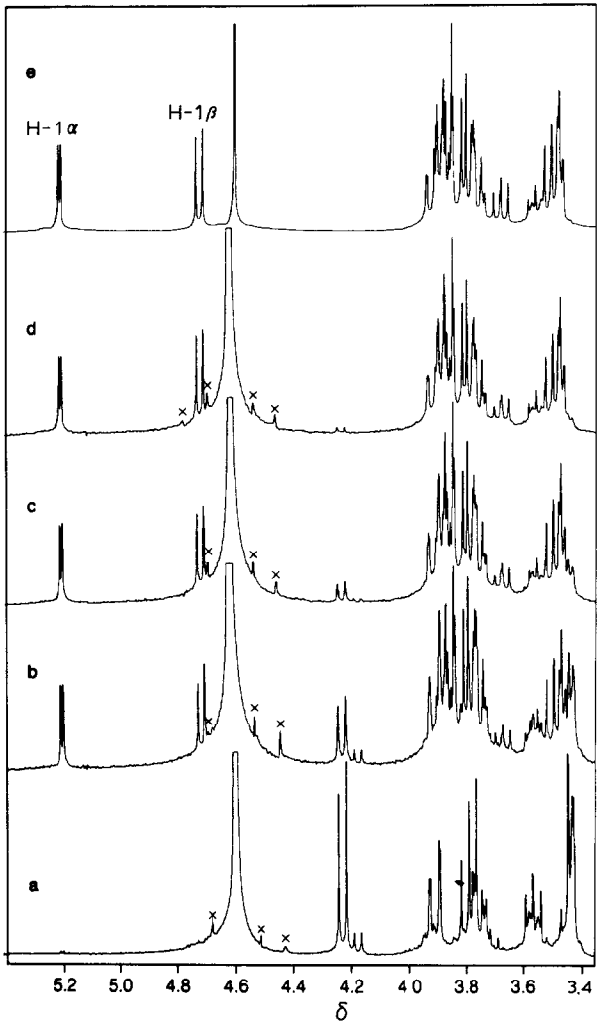


Fig. 3. Hydrolysis of 1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetylhydrazine (**2**) monitored by ^{1}H-n.m.r. Hydrolysis was performed in deuterium oxide containing 50mM H_2SO_4 at 40° in an n.m.r. tube. Shown are the downfield regions of the spectra of: (a) starting compound **2**, and after hydrolysis for (b) 15 min, (c) 45 min, and (d) 85 min. Spectrum (e) is authentic **5** (40°). The *N*-acetyl region of spectrum (d) showed 2 singlets of equal intensity which integrated for 3 protons each, one at δ 2.047 (**5**) and one at δ 2.103 (protonated acetohydrazide). Peaks marked with an x are spinning sidebands.

chemically by two different procedures (see Experimental section). The coupling constant of the H-1 doublet was identical to that of the major conformer (*J* 9.7 Hz), clearly indicating that both conformers were β anomers. Furthermore, when hydrolysis of this compound was followed by 360-MHz ^{1}H-n.m.r. spectrometry in a solution of acidic deuterium oxide, both H-1 doublets decreased in intensity at the same rate, resulting in the same relative intensity of H-1 signals, until the compound was completely hydrolyzed into **5** (Fig. 3).

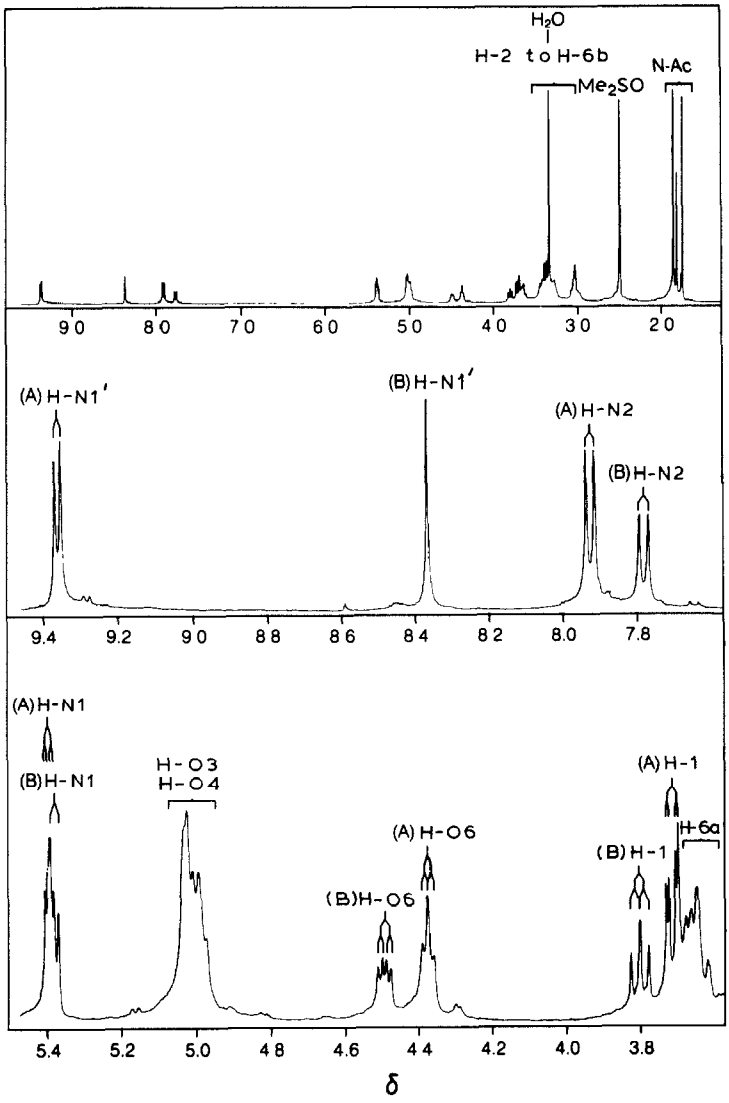


Fig. 4. ^{1}H-N.m.r. spectrum of isolated compound C (Fig. 1) in $(CD_3)_2SO$, with expansions of two regions. All resonances downfield of δ 4.0 were exchangeable, and disappeared rapidly upon adding D_2O. Assignments of the major conformer (A) and the minor conformer (B) are indicated. Labelling of protons on nitrogen atoms is shown in structure **2**.

The ^{1}H-n.m.r. spectrum of a di($^2H_3$)methyl sulfoxide solution of **2** (Fig. 4) showed again two conformers, but in the ratio of 1.8:1.0, and decoupling experiments as well as deuterium oxide exchange enabled assignment of all exchangeable protons (Table II). Most importantly, H-1 of both conformers was coupled to H-2 and to a single proton on a nitrogen atom; this proton was in turn coupled to a single proton on a second, adjacent nitrogen atom. This identified the anomeric derivative as the acetohydrazide **2**, ruling out the hydrazine derivatives **6**

TABLE II

^{1}H-N.M.R DATA OF THE EXCHANGEABLE AND H-1 PROTONS OF THE CONFORMERS OF 1-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-ACETYLHYDRAZINE (**2**)[a]

Proton	*Chemical shift (δ)*		*Coupling constant (Hz)*		
	Major conf. (A)	*Minor conf. (B)*		*Major conf. (A)*	*Minor conf. (B)*
H-1	3.708	3.797	$J_{H\text{-}1,H\text{-}2}$	9.0	9.0
H-N1	5.388	5.375	$J_{H\text{-}1,H\text{-}N1}$	2.9	8.6
H-N1′	9.356	8.362	$J_{H\text{-}N1,H\text{-}N1'}$	5.8	≤1
H-N2	7.920	7.776	$J_{H\text{-}2,H\text{-}N2}$	8.6	8.6
H-O3	5.01[b]	5.01[b]			
H-O4	5.01[b]	5.01[b]			
H-O6	4.371	4.489			

[a]For a solution in $(CD_3)_2SO$ at 20°. [b]H-O3 and H-O4 signals of both conformers overlapped at a region centered at δ 5.01.

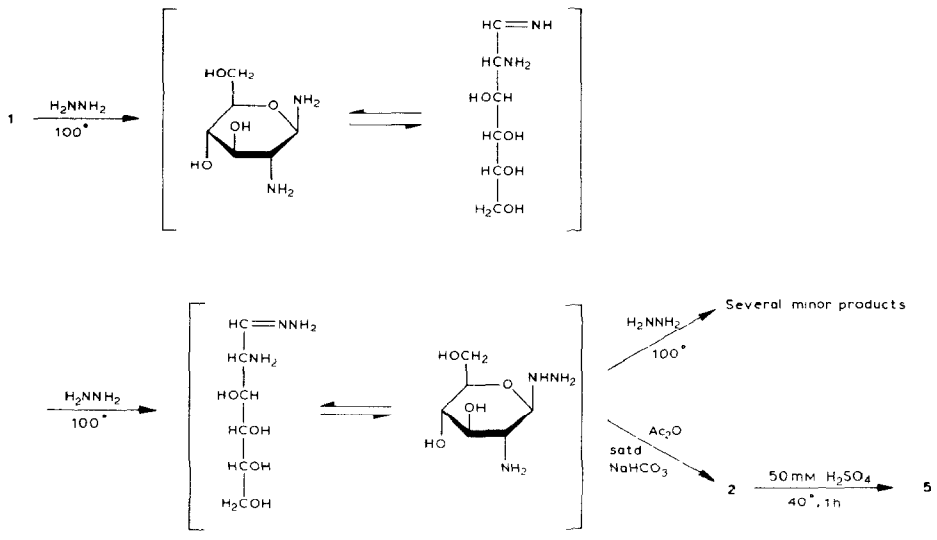
or **7**. The H-2 signal of both conformers was partly masked by the water signal, but irradiation of the H-2 region revealed coupling between H-2 and a proton on the 2-acetamido nitrogen atom, confirming structure **2**. The acetohydrazide group exists in two conformations, which are different with respect to the sugar ring, as evidenced by the differences in coupling constants and chemical shifts of the exchangeable hydrazido protons between the two conformers (Table II). In addition, both H-O6 and H-N2 signals were divided into two regions, indicating a change in the environment of these protons between the conformers. Molecular models of **2** indicated the possibility of an intramolecular hydrogen bond between the hydrazido carbonyl oxygen atom and H-O6. However, whether the observed differences between the conformers are a result of solvent ordering around the molecule or intramolecular hydrogen bonding could not be ascertained.

Mild acid hydrolysis of **2** gave, in quantitative yield, **5** which was identified by co-chromatography on paper and ^{1}H-n.m.r. spectroscopy. The minimum temperature required for complete hydrolysis with 50mM sulfuric acid for 1 h was 40°, which was determined by treating **2** at temperatures increasing in 5° increments from 25 to 50°, the reaction being monitored by paper chromatography. In mixtures of the products of hydrazinolysis–*N*-reacetylation of **1**, **2** was hydrolyzed in the same temperature-dependent fashion. The hydrolysis of **2** was monitored with time in mildly acidic deuterium oxide solution by ^{1}H-n.m.r. (Fig. 3). The disappearance of the starting compound **2** was paralleled by the appearance of signals corresponding to **5** and protonated acetohydrazide. Acetohydrazide and traces of hydrazine were identified in hydrolyzates by t.l.c., based on their co-migration with the authentic compounds in three different solvent systems. A control experiment in which acetohydrazide alone was exposed to the same hydrolysis conditions revealed a small amount of hydrolysis to hydrazine, so the traces of free hydrazine detected after hydrolysis of **2** arose from acetohydrazide, the initial product of hydrolysis.

At neutral pH, **2** behaved as a neutral compound and could be passed through columns of Dowex 1 (Cl^-) or Dowex 50 (H^+) with no change in its ^1H-n.m.r. spectrum, which adds further evidence against structure **7**.

The β-acetohydrazide derivative **2** likely arose by *N*-acetylation of the hydrazone of 2-amino-2-deoxy-D-glucose (**3**) or its cyclic β-glycosylhydrazine tautomer, which has been reported by Saeed and Williams[6] to be the major product immediately after hydrazine treatment; they detected very little free 2-amino-2-deoxy-D-glucose at this stage. Indeed, the presence of any significant amounts of 2-amino-2-deoxy-D-glucose prior to exposure of products to aqueous conditions is highly unlikely, because sugars are quantitatively converted into their hydrazones in anhydrous hydrazine, even at 25° (see ref. 8 and synthesis of **2** described herein). Therefore, free 2-acetamido-2-deoxy-D-glucose (**5**) arose during and after the *N*-acetylation stage, when the β-acetohydrazide derivative **2** was subject to hydrolysis under the mildly acidic conditions that resulted after exchange of Na^+ for H^+ ions. Depending upon the precise conditions under which acetic acid is removed following the *N*-acetylation stage, variable yields of **5** would be obtained. Other investigators[4] evaporated under reduced pressure, but temperatures and volumes were not reported. The conditions described herein resulted in complete and reproducible hydrolysis of **2**, giving **5** in 75–80% overall yield from the starting compound **1**. The proposed major pathway of the series of reactions occurring during the hydrazinolysis–*N*-reacetylation procedure is outlined in Scheme 1.

Treatment of **2** with mild base for 1 h did not affect its mobility upon paper chromatography, and **2** was still present after mild base treatment of mixtures of the products of hydrazinolysis–*N*-reacetylation of **1**. Compound **2** was clearly stable



Scheme 1

in 100mM $NaOD/D_2O$ for at least 90 min when monitored by 360-MHz ^1H-n.m.r. spectrometry; no signals corresponding to **5** appeared during this time. This is relevant, because if reduction with sodium borotritide is expected to yield 2-acetamido-2-deoxy-D-[^3H]glucitol, it is essential that the acetohydrazide be cleaved from the sugar prior to the reduction, which is usually carried out under basic conditions similar to those used in this investigation.

Minor compounds (fractions E–G, Fig. 1) were found to be mixtures by 360-MHz ^1H-n.m.r. spectrometry, and could not be identified unequivocally. Upfield methyl doublets (δ 1.322, J 6.8 Hz; and δ 1.137, J 6.8 Hz) were attributed to 2-acetamido-1,2-dideoxyhexose, or 1-deoxyhexose, or both molecules. The same upfield doublets were observed when **5** was treated under the same hydrazinolysis–*N*-reacetylation conditions as **1**, and are surmised to result from Wolff–Kishner reduction at C-1 during the hydrazine treatment at 100°. Decoupling experiments clearly showed the methyl groups were terminal on a molecule derived from the sugar moiety. An upfield singlet (δ 1.464) was also routinely observed, and attributed to a 1-methyl-2-hydrazone or 1-methyl-2-keto derivative. Williams and assoc.[6,7] have reported Wolff–Kishner reduction and osazone formation during hydrazine treatment of **1**, which is essentially confirmed herein, although the products observed by ^1H-n.m.r. which showed methyl doublets, could not be the 1-deoxyfructose derivative that they reported. This mixture of minor side-products presents obvious difficulties when on the reducing terminus of an oligosaccharide, especially in separations. Investigations are currently underway to permit separation of oligosaccharides containing a reducing 2-acetamido-2-deoxy-D-glucose residue from the minor products, based on the reactivity of the reducing sugar.

EXPERIMENTAL

General methods. — ^1H-N.m.r. spectra were recorded with a Nicolet NT-360 spectrometer located at the Toronto Biomedical n.m.r. Centre. Typical experimental conditions, except where otherwise noted, were as follows. The instrument was operated in the f.t. mode with a full quadrature cycling, a +/− 2000 Hz sweep width, 16k data set, and a 90° (8 μsec) observe pulse. A relaxation delay of 5 s was typically utilized. Data sets were processed with simple exponential apodization of 0.1 Hz. Experiments were usually performed at ambient probe temperature (20°). Deuterated solvents were obtained from Merck Sharp and Dohme, Canada. When D_2O was used as a solvent, exchange with D_2O (99.8 atom %) was obtained by repeated lyophilization, the sample being dissolved in ~0.4 mL of D_2O (99.96 atom %) and placed in a 5-mm tube. Chemical shifts were measured relative to the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) (indirectly through acetone with signal at δ 2.225). For samples in Me_2SO, the lyophilized powder was dissolved in 0.4 mL of di($^2H_3$)methyl sulfoxide (99.5 atom %). Chemical shifts were measured relative to the solvent peak at δ 2.490. Computer simulations of ^1H-n.m.r. spectra were performed on a VAX 11/780 in-

strument (Ontario Cancer Institute) using an interactive version[9] of the LAOCOON 3 program[10]. This program was modified to allow analysis of up to seven spin systems, as it was not suitable, as described[9], for seven spin systems.

Paper chromatography was performed, in descending fashion, on Whatman 3MM paper which was washed with distilled water and air-dried before use. The solvent system was 10:1:2 1-butanol–ethanol–water and all solvents were redistilled prior to use. Chromatography was for 24–48 h, depending on which compounds were to be separated. For preparative chromatography, compounds were streaked at the origin at ~3–5 μmol/cm of width. Chromatograms were air-dried and guide strips were Ag stained. Staining with $AgNO_3$ was essentially as described by Trevelyan *et al.*[11], except that papers were fixed in 1% $Na_2S_2O_3$ instead of an NH_3 solution. T.l.c. of hydrazine and acetohydrazide was performed on Eastman 6064 cellulose sheets in 3 solvent systems: (*A*) 5:1 ethanol–M aqueous HCl, (*B*) 5:1 methanol–M aqueous HCl, and (*C*) 2:1 1,4-dioxane–M aqueous HCl. Compounds were spotted as their hydrochloride salts and detected as u.v. fluorescent derivatives after being sprayed with *p*-dimethylaminobenzaldehyde reagent[12]. R_F values of acetohydrazide · HCl in solvent systems *A*, *B*, and *C*: 0.53, 0.72, and 0.78, respectively; and of hydrazinium chloride 0.19, 0.40, and 0.58, respectively.

Hydrazinolysis–N-reacetylation. — Compound **1** (Sigma) was dried for 4 days *in vacuo* in the presence of P_2O_5 and dissolved (16 μmol/mL) in anhydrous hydrazine (Pierce) which was always transferred in a glove box under Ar, kept dry by continuous circulation through Drierite. The solution was heated for 24 h to 100° in Teflon-sealed vacuum hydrolysis tubes, and then frozen and lyophilized to remove hydrazine. The remaining material was dissolved in saturated $NaHCO_3$ (6.5 μmol of starting material/mL) and *N*-acetylated at room temperature by adding acetic anhydride (0.05 vol.), and repeating the addition after a 10-min interval. After 1 h, Na^+ ions were removed by passing the solution, at 4°, through a column of Dowex 50-X8 (100–200 mesh, H^+, >10 equiv.), washed immediately before use with distilled water. The solution was rapidly frozen and lyophilized. The residue was taken up in a small volume of water and deposited on paper for chromatography. Preparative-scale chromatograms were run and the isolated compounds were eluted with water.

Mild acid hydrolysis of **2**. — The isolated compound **2** or mixtures of the products of hydrazinolysis–*N*-reacetylation of **1** were treated with 50mM H_2SO_4 for 1 h at various temperatures. The solution was diluted 5-fold with water and immediately passed in succession through coupled columns of Dowex 50-X8 (H^+), to remove acetohydrazide, and Dowex 1-X8 (AcO^-), to remove SO_4 ions, and washed with water (4 column vol.). The eluates were lyophilized and the residues either taken up in water for paper chromatography or in D_2O for ^{1}H-n.m.r. analysis. Hydrolysis, which was monitored with time by ^{1}H-n.m.r., was carried out at 40° in D_2O containing 50mM H_2SO_4. When analysis of acetohydrazide in hydrolyzates was carried out, isolated compound **2** was hydrolyzed at 40° in 0.1M HCl for 1 h. The solution was rotary evaporated to near-dryness, and taken up in

a small volume of water. The resultant solution was deposited directly on t.l.c. sheets, and chromatographed in solvent system *A*, *B*, or *C*, alongside authentic acetohydrazide and hydrazinium chloride as described in the General Experimental section.

Mild alkaline treatment of **2**. — The isolated compound **2** or mixtures of the products of hydrazinolysis–*N*-reacetylation of **1** were treated with 0.1M NaOH at 25° for 1 h. To remove Na^+ ions, the solution was passed through a column of Dowex 50-X8 (H^+; >10 equiv.) and washed with additional water (4 column vol.). The eluate was lyophilized and the residue taken up in water for chromatographic analysis. When base treatment was monitored with time by ^{1}H-n.m.r., compound **2** was treated with 0.1M NaOD in D_2O at room temperature (~20°) for 90 min.

*Synthesis of 1-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-2-acetylhydrazine* (**2**). — (*a*). A solution of 2-acetamido-2-deoxy-D-glucose (**5**, 0.25 mmol) in anhydrous hydrazine (1 mL) was kept for 8 h at 25°, frozen, and lyophilized. A sample from the residue was taken up in D_2O for ^{1}H-n.m.r. analysis. No starting compound **5** was observed as it had been quantitatively converted into the hydrazone **4** (doublet, δ 7.25, H-1). The remainder was taken up in saturated $NaHCO_3$ (10 mL), and two 0.5-mL aliquots of acetic anhydride were added at 10-min intervals. After an additional 10 min, the solution was cooled to 0° for a further 30 min, and loaded onto a column (2.6 × 16 cm) of Dowex 50-X8 (100–200 mesh, H^+) which was washed immediately prior to use with distilled water and maintained at 4°. After washing with additional water (140 mL at 4°), the solution was frozen immediately in a dry ice–ethanol bath and lyophilized. Rotary evaporation at this stage is to be avoided as yields are decreased due to hydrolysis. The dry material was dissolved in a small volume of water and the solution streaked on paper for preparative separation of the products (yield: 55%).

(*b*). 2-Acetamido-2-deoxy-D-glucose (**5**, 0.5 mmol) and acetohydrazide (1.5 mmol, Aldrich) were dissolved in a 15% (v/v) solution of acetic acid in water (0.5 mL). The solution was heated to 50° for 15 h, cooled, and rotary evaporated (37°) to a syrup. This was dissolved in water (0.3 mL) and the solution streaked on paper for preparative separation of the products (yield: 65%). Attempts to crystallize **2** synthesized by routes (*a*) or (*b*) were unsuccessful. The compound migrated, on paper, with R_5 0.85 (the same as compound C in Fig. 1), and the ^{1}H-n.m.r. spectrum of **2** synthesized by either route was identical to that seen in Fig. 2a.

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