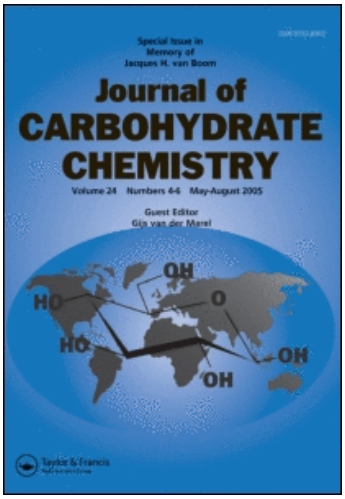

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NMR STUDIES OF SACCHARIDE HYDRAZONES, THIOSEMICARBAZONES AND AZINES: MODEL COMPOUNDS FOR IMMOBILISATION STUDIES

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ABSTRACT

To evaluate possible chemical strategies for the solid-phase immobilization of sugars, studies on the formation and stability of hydrazones, thiosemicarbazones and azines of D-glucose and 2-acetamido-2-deoxy-D-glucose, and the subsequent release of sugars, were carried out. Hydrazone formation was observed to occur under milder conditions than previously reported, thereby minimising the accompanying *N*-deacetylation which occurs with *N*-acetamido sugars. The cyclic β-pyranosyl structures of the saccharide hydrazones, thiosemicarbazones and azines were the preferred isomers in aqueous solution.

INTRODUCTION

Increasing interest in the structure of the oligosaccharide residues from glycoconjugates has occurred as the importance of the conjugated oligosaccharides in a variety of biological functions has become apparent.[1] Structural studies can be facilitated by immobilising the oligosaccharides in techniques such as affinity chromatography,[2] immunoassay,[3] and oligosaccharide modification[4] or synthesis.[5]

Oligosaccharides are generally immobilised through their reducing termini, which are commonly further activated by derivatisation before binding.[6,7] Since the reducing

terminus is the focus of interest, immobilisation studies were carried out with monosaccharides, as models for oligosaccharides. Reducing sugars were bound directly to aminopolystyrene as Amadori compounds[8] and saccharide hydrazones to either isothiocyanatopolystyrene as thiosemicarbazones[9] or to hydroxybenzaldehyde-derivatised polystyrene as azines.[10] Binding occurred efficiently in each case, but the hydrazone bindings had the added advantage of mild reaction conditions and good recoveries of bound saccharides.

To confirm the chemistry involved in the immobilisation of saccharide hydrazones, the formation of model compounds derived from the representative monosaccharides, D-glucose and 2-acetamido-2-deoxy-D-glucose, was investigated by ^{1}H NMR spectroscopy. The studies included optimising reaction conditions to minimise *N*-deacetylation during the formation of the saccharide hydrazones and evaluating their conversion to saccharide thiosemicarbazones and azines. The stabilities of these derivatives and the ease of recovery of the reducing sugars or their saccharide hydrazones, were compared with results of immobilisation studies of saccharide hydrazones.[9,10]

RESULTS AND DISCUSSION

Hydrazinolysis is an important method of cleavage of both *N*- and *O*-linked oligosaccharides from glycoproteins, with the formation of oligosaccharide hydrazones which are subsequently hydrolysed to the parent oligosaccharides. The basic steps of this process have been described,[11] and several model studies have been made of the individual steps.[12-14] During the hydrazinolysis, *N*-deacetylation of acetamido groups occurs, so that a reacetylation step is often necessary.[11] Alternatively, reducing sugars can be converted to their hydrazones at room temperature by treatment with either anhydrous hydrazine,[13] 100% hydrazine hydrate[15] or anhydrous hydrazine or hydrazine hydrate in methanol.[16-18] While *N*-deacetylation can be minimised with methanol as solvent, many saccharides have poor solubility in it, and extended treatment with anhydrous hydrazine or 100% hydrazine hydrate leads to an appreciable amount of *N*-deacetylation.

The formation of hydrazones from reducing sugars and hydrazine hydrate was observed by ^{1}H NMR spectroscopy[12-14,18] in D_2O and in DMSO-d_6 in these studies. The resulting mixture of isomers consisted of open-chain hydrazone and pyranosyl cyclic forms,[13] as well as deacetylated products,[14] the proportions of isomers varying with the

NMR solvent used. Our observations involved the formation of 2-acetamido-2-deoxy-D-glucose hydrazone **1**, since this monosaccharide is found at the reducing terminus of *N*-linked oligosaccharides of glycoproteins and since this also enabled us to to study the effects of different reaction conditions on the *N*-acetyl group. Yields of the various hydrazone isomers, including *N*-deacetylated products, were estimated from their H-1 NMR resonances.

1 R = $NHCOCH_3$
2 R = NH_2
3 R = OH

$H_2N—NH—COCH_3$
4

Hydrazone formation proceeded rapidly at room temperature, and was complete within six hours in 25% aqueous hydrazine, with less than 10% *N*-deacetylation (Table), but more extensive *N*-deacetylation occurred in 50% and 100% hydrazine hydrate. ^1H NMR spectra in D_2O indicated that the major products were the acyclic *E*-hydrazone **1a** and the cyclic β-pyranosylhydrazine **1b**.[13] The protons of acetohydrazide **4**, resulting from partial *N*-deacetylation of **1** to form 2-amino-2- deoxy-D-glucose hydrazone **2**, occurred at δ1.94 in D_2O, upfield from the saccharide acetyl protons at δ2.02, enabling determination of the extent of *N*-deacetylation. A second doublet at δ7.21 just upfield of the *E* H-1 doublet of **1a** at δ7.23 in D_2O, corresponded to the *E* H-1 proton of **2a**, and a second small doublet at δ4.00, upfield of the β-pyranose H-1 proton of **1b** at δ4.07, to **2b.**

Since hydrazones were prepared as precursors in the immobilisation of saccharides[9,10] it was desirable to determine their stability in aqueous solutions of different pH values. It is known that oligosaccharide hydrazones are stable in anhydrous hydrazine in the absence of alkali.[19] We observed that they are stable also in aqueous alkali, and that their rate of formation is increased in basic solutions (Table), presumably because of base-catalysed ring-opening of the reducing sugar preceding hydrazone formation.[20] If this factor is to be exploited, it is an advantage to use a tertiary amine, such as trimethylamine or triethylamine, which is readily removed by evaporation. The hydrazones were less

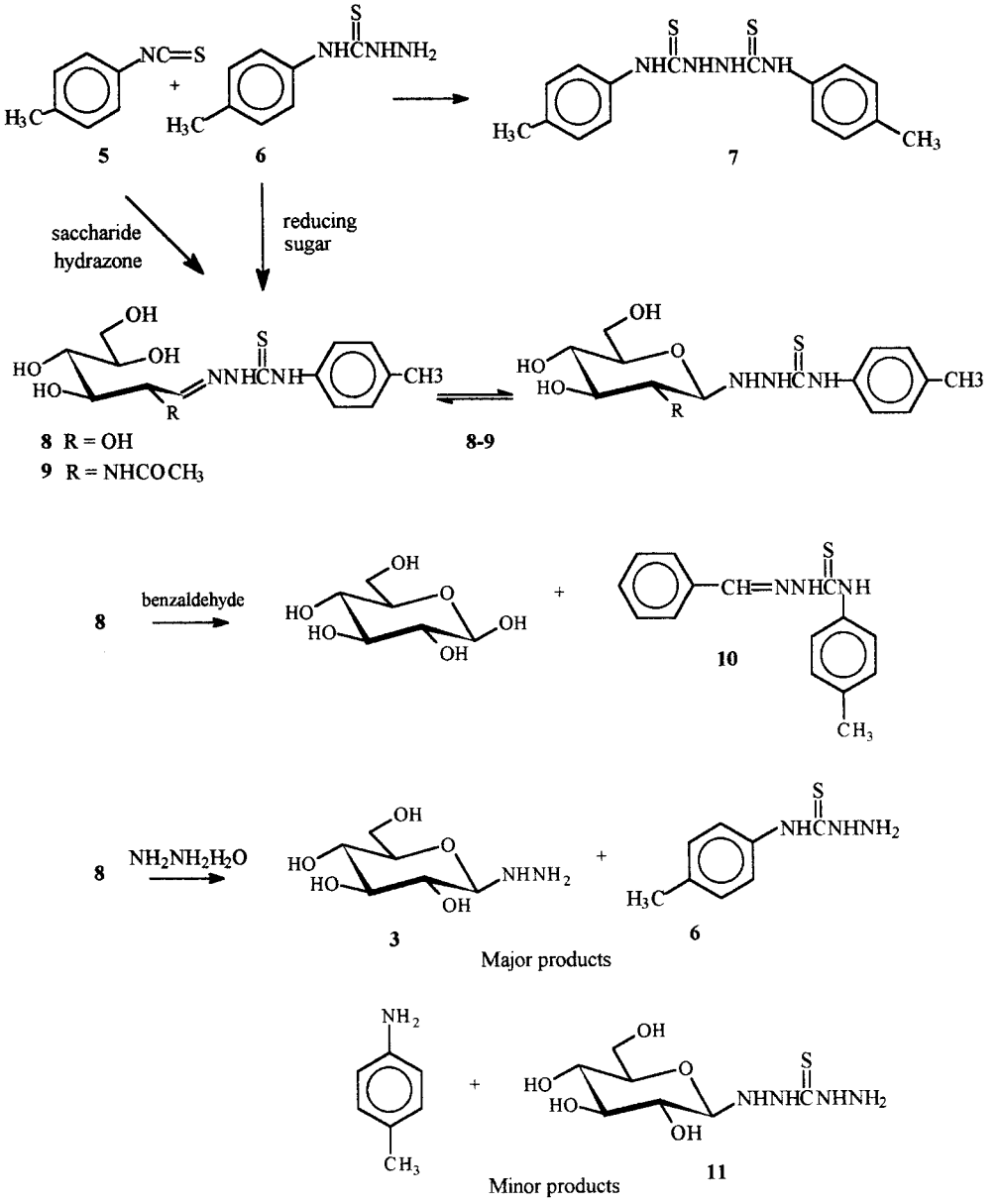
Table. Variation of the yield of hydrazone and extent of deacetylation with concentration of hydrazine, time and added base at room temperature[a]

Hydrazine hydrate (% v/v)	Hydrazine (molar excess)	Time (h)[b]	Base	Yield of products (%)	Deacetyl-ation
3.5	1.1	24		55	0
25.0	11.0	24		100	7
100.0	40.0	24		100	41
anhydrous	10.0	24		100	45
25.0	10.0	3		73	7
50.0	20.0	3		100	20
100.0	40.0	3		100	45
25.0	10.0	2		67	< 5
25.0	10.0	6		100	9
25.0	11.0	24		100	10
2.2	0.5	24	0.1 M NaOH	> 95	0
3.5	1.1	1.5	0.1 M NaOH	91	0
25.0	11.0	1.5	0.1 M NaOH	100	< 5
25.0	11.0	1	0.3 M TEA[c]	100	10

a. ^{1}H NMR spectra recorded in D_2O. b. Reaction times are up to the start of evaporation. c. TEA = triethylamine.

stable in acid solutions[12] and after two days about 40% hydrolysis of **1** was indicated in a D_2O solution at pH 5 by the appearance of the H-1 doublets of α- and β-2-acetamido-2-deoxy-D-glucose.

To underpin studies of the immobilisation of saccharides as thiosemicarbazones,[9] the formation of glucose and 2-acetamido-2-deoxy-D-glucose thiosemicarbazones **8** and **9** (Scheme) in solution, was studied by ^{1}H NMR spectroscopy.[18] It has been suggested[21] that oligosaccharides readily form stable crystalline thiosemicarbazone derivatives due to their insolubility, rather than as a result of their thermodynamic stability. Studies of the formation of the thiosemicarbazone **8** from glucose and 4-tolylthiosemicarbazide **6** (Scheme), using ^{1}H NMR spectroscopy, showed an equilibrium constant of formation[9] of 38 M^{-1}, indicating only modest thermodynamic stability. Consistent with this, an appreciably higher preparative yield (72%) of the water-insoluble 2-acetamido-2-deoxy-D-glucose product **9** was obtained than of the water-soluble glucose product **8** (40%), and



Scheme

both of these reducing sugars bound poorly to immobilised thiosemicarbazide ligands.[9] A more efficient approach to the immobilisation of sugars as thiosemicarbazones was by reaction of their hydrazones with polymer-supported aromatic isothiocyanate ligands[9] (Scheme).

The water-soluble thiosemicarbazone **8** (Scheme) was observed by ^{1}H NMR spectroscopy in both D_2O and DMSO- d_6, to be in the pure cyclic form when prepared by heating glucose with the thiosemicarbazide **6**. When a 50 mM solution of **8** was allowed to stand in D_2O for one week, or when the solution was subsequently heated at 80 °C for four hours, no glucose was observed, indicating that, despite its modest thermodynamic stability, the linkage has considerable kinetic stability, which renders it suitable for immobilisation.

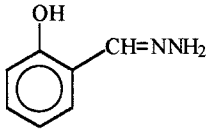
In contrast to **8**, the sparingly soluble thiosemicarbazone **9** of 2-acetamido-2-deoxyglucose precipitated from solution, and was isolated as the acyclic form. Slow ring closure of **9** occurred in DMSO-d_6 containing 10% D_2O, but some acyclic form still remained after three months. As ring closure proceeded, a doublet at δ3.94 ($J_{2,3}$ = 6.4 Hz), assigned to the *E* H-3 proton of the acyclic form of **9**, remained at approximately the same chemical shift, but its coupling constant increased to J = 9.5 Hz and its integral remained equivalent to one proton. This new doublet, with a coupling constant similar to that of the β H-1 proton of the corresponding hydrazone **1** ($J_{1,2}$ = 9.6 Hz), was assigned to the βH-1 proton of the cyclic form of **9**. The H-1 azomethine proton doublet at δ7.57 in the initial spectrum of **9** gradually became smaller, with increasing complexity appearing in the aromatic protons, suggesting the formation of more than one cyclic isomer, also indicated by the appearance of a third methyl peak. A small amount of hydrolysis after three months was indicated by a small doublet at δ4.94, corresponding to the a H-1 proton of 2-acetamido-2-deoxy-D-glucose. When the thiosemicarbazones **8** and **9** were prepared from the hydrazones **1** and **3** respectively and an excess of the isothiocyanate **5** at room temperature, their ^{1}H NMR spectra showed a mixture of cyclic and acyclic isomers in an approximate ratio of 3:2.

The precipitation of the insoluble thiosemicarbazone **9** from aqueous ethanol in its acyclic form, is consistent with reaction of the aldehydo form of 2-acetamido-2-deoxy-D-glucose with the thiosemicarbazide **6**, while the recovery of the soluble thiosemicarbazone **8** from aqueous ethanol in its fully cyclic form after heating indicates the tendency towards

cyclisation also observed in saccharide hydrazones.[12,18] The mixture of acyclic and cyclic isomers, formed in the reaction between saccharide hydrazones and the isothiocyanate **5** at room temperature, may be due to the saccharide hydrazone reactants being partly in the ring form, as well as to further cyclisation after thiosemicarbazone formation.

Studies of the cleavage of saccharide thiosemicarbazones were carried out with the water-soluble derivative **8**, to confirm the chemistry of recovery of sugars[9] immobilised as thiosemicarbazones (Scheme). Cleavage of **8** by hydrazine hydrate was shown by TLC to be complete within thirty minutes at room temperature and, after one hour's reaction followed by evaporation, the ^{1}H NMR spectrum in DMSO-d$_6$ showed no starting materials and the expected major products of the hydrazone **3** (about 84% cyclic isomer) and the thiosemicarbazide **6** (Scheme). A small amount of 4-toluidine was also present. A corresponding small amount of the expected aldose thiocarbazone **11** could not be definitely established, but a small doublet at δ7.33 may have been the H-1 doublet of the acyclic form of **11**. Nucleophilic attack by hydrazine on the thione carbon of **8** would be expected, followed by cleavage to form the saccharide hydrazone and **6.** Cleavage of the other C-N bond would explain the small amount of 4-toluidine.

Conversion of **8** to glucose was effected by treatment with carbonyl compounds. Complete reaction occurred when **8** was heated with 10% benzaldehyde in ethanol at 100 °C for three hours, with the formation of glucose and the benzaldehyde thiosemicarbazone **10**. Similar treatment with 25% aqueous acetone resulted in a recovery of about 70% of glucose. The greater effectiveness of cleavage by benzaldehyde than by acetone was also observed with immobilised saccharide thiosemicarbazones.[9]



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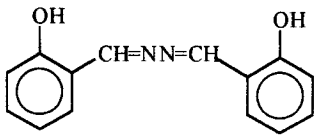

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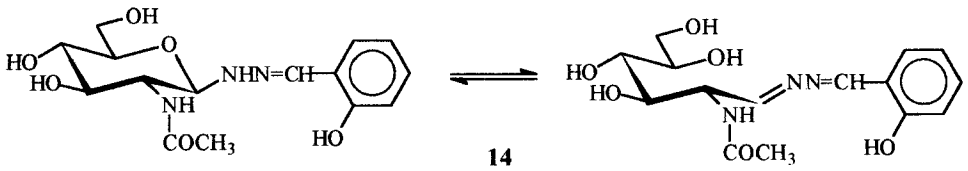

14

The saccharide azine **14** was formed from the saccharide hydrazone **1** and 2-hydroxybenzaldehyde for ^{1}H NMR spectroscopy studies, to enable investigation of the slight instability observed in the immobilisation of saccharide hydrazones as azines to hydroxybenzaldehyde ligands.[10] Under the reaction conditions used, the β-pyranosyl structure of **14**, characterised by its H-1 proton doublet at δ4.43 ($J_{1,2}$ = 9.7) in DMSO-d_6, was the only isomeric form observed. When traces of occluded hydrazine were present in the saccharide hydrazones, the corresponding benzaldehyde hydrazones **12** and azines **13** were detected in the ^{1}H NMR spectra.

The potential for hydrolysis exists at both C-N bonds of a saccharide azine and about 10% of release of monosaccharides immobilised as azines was observed in neutral aqueous solutions.[10] The saccharide azine **14** was unchanged after standing for 20 min in 0.01 M sodium hydroxide, and after three days in a solution of DMSO-d_6 with 10% of water. However, acid hydrolysis was observed when five successive aliquots of 30 μL of deuterated hydrochloric acid (DCl), each containing 30 mmol of acid, were then added to **14** (30 mmol) in DMSO-d_6 over a period of thirty minutes, after which time only about 40% of **14** remained. Signals appeared corresponding to 2-hydroxybenzaldehyde and its symmetrical azine **13**, in a ratio of 2:3, and to the hydrazone **1**, indicating rapid hydrolysis at the C=N double bond attached to the aromatic ring. There was no evidence of reducing sugar formation.

CONCLUSIONS

These NMR studies on model compounds supported the observations made in immobilisation studies. The ease of formation of saccharide hydrazones from reducing sugars and hydrazine under conditions which are sufficiently mild to minimise *N*-deacetylation, and the stability of aqueous solutions of saccharide hydrazones under a range of pH values, was confirmed.[13] The desirability of removing the last traces of occluded hydrazine, particularly for further solution reactions, was noted.

NMR studies on the water soluble thiosemicarbazone **8** confirmed both the high kinetic stability of the saccharide thiosemicarbazone binding to polymers and the chemistry of cleavage of the binding. The action of aqueous acid on the saccharide azine **14** indicated that the limited thermodynamic stability of immobilised saccharide azines in neutral aqueous solutions[10] was due to hydrolysis at the C=N bond attached to the aromatic ring.

EXPERIMENTAL

General methods. All NMR spectra were measured in D_2O or in DMSO-d_6 with D_2O exchange, on a Varian XL 400 NMR spectrometer. DMSO-d_6 spectra were referenced to TMS, and D_2O spectra to the HDO peak, which had been determined at different temperatures relative to trimethylsilylpropanesulfonic acid (TPS). Reporter group resonances are shown. The mass spectrum was recorded on a Finnigan TSQ 9000 spectrometer. Thin-layer chromatography was run on aluminium plates precoated with silica gel 60 (E. Merck, Darmstadt, Germany) in ethanol-ethyl acetate (1:4 v/v); aromatic derivatives were detected under short UV light (254 nm) and reducing sugars by spraying with 10% sulfuric acid and charring. Chemicals used were of analytical grade.

Saccharide Hydrazones. Typically, glucose (25 mg, 0.139 mmol) or 2-acetamido-2-deoxyglucose (25 mg, 0.113 mmol) were dissolved in hydrazine hydrate (200 μL, 100%, or varying dilutions with water) and stood at room temperature for 1-24 h. In some cases base was also present. When 0.1 M NaOH was used the solution was neutralised with a calculated amount of dilute HCl at the end of the reaction. Evaporation followed under a stream of nitrogen or over 98% H_2SO_4 in a vacuum desiccator. Redissolution in H_2O with reevaporation, or coevaporation with toluene, was carried out to minimise the occluded hydrazine. The products were colourless gums. The hydrazone **1** was obtained as a white powder, after its preparation from 2-acetamido-2-deoxy-D-glucose (500 mg, 2.26 mmol) in 100% hydrazine hydrate solution (1 mL, 20 mmol), by the addition of ethanol (10 mL). The mixture was stored at 4 °C for several days, then filtered and washed with ethanol (3-4 mL), yielding 348 mg (65%). ^{1}H NMR (D_2O) **1a** *E*-isomer δ 2.022 (s, 3H, Ac), 4.01 (dd, 1H, $J_{2,3}$ = 7.5 Hz, $J_{3,4}$ = 2.0 Hz, H-3), 4.58 (dd, 1H, $J_{1,2}$ = 5.4 Hz, $J_{2,3}$ = 7.5 Hz, H-2), 7.23 (d, 1H, $J_{1,2}$ = 5.4 Hz); **1b** β–anomer δ 2.015 (s, 3H, Ac), 4.07 (d, 1H, $J_{1,2}$ = 9.6 Hz, H-1); **2a** *E*-isomer δ 7.21 (d, 1H, $J_{1,2}$ = 5.8 Hz, H-1); **2b** β-anomer δ 4.00 (d, 1H, $J_{1,2}$ = 9.2 Hz, H-1); **3a** *E*-isomer δ 3.91-3.95 (m, 1H, H-3), 4.31 (dd, 1H, $J_{1,2}$ = 6.5 Hz, $J_{2,3}$ = 7.5 Hz, H-2), 7.27 (d, 1H, $J_{1,2}$ = 6.5 Hz, H-1); **3b** β-anomer δ 4.06 (d, 1H, $J_{1,2}$ = 9.0 Hz, H-1); **4** δ 1.942 (s, 3H, Ac).

D-Glucose 4-Tolylthiosemicarbazone (8). (a) From glucose and **6** as described.[9] **(b) From glucose hydrazone** and **5** by a modification of a reported method.[22] **5** (75 mg, 0.5 mmol) in methanol (1.3 mL) was added to glucose hydrazone (20 mg, 0.1 mmol) in water (100 μL) to form a cloudy solution. After mechanical shaking for 3 h, the thick

precipitate of 7 formed from occluded hydrazine in **8**, was removed by centrifuging several times. After evaporation of the supernatant, impurities were removed from the crude product (29 mg) by ethyl acetate extraction, leaving **8** (27 mg, 79%). ^{1}H NMR (D_2O) β-anomer δ 2.33 (s, 3H, Me), 4.16 (d, 1H, $J_{1,2}$ = 9.0 Hz, H-1), 7.24 (d, 4H, J = 2.1 Hz, H_{Ar}); *E*-isomer δ 4.17 (dd, 1H, H-3), 4.24 (dd, 1H, $J_{1,2}$ = 0.9 Hz, $J_{2,3}$ = 9.4 Hz, H-2).

2-Acetamido-2-deoxy-D-glucose 4-Tolylthiosemicarbazone (9). (a) From 2-acetamido-2-deoxy-D-glucose and **6**. 2-acetamido-2-deoxy-D-glucose (100 mg, 0.45 mmol) dissolved in water (1 mL) was added to **6** (85 mg, 0.47 mmol) suspended in ethanol (2 mL), boiled under reflux for 5 h and left at room temperature overnight. The solid product was filtered off and washed well with water and warm ethanol to give a white powder (64 mg, 37%). The residue from evaporation of the supernatant was passed through a silica column. Impurities were eluted with ethyl acetate-ethanol mixtures and further **9** (90 mg, 52%) recovered with ethanol. **(b) From 2-acetamido-2-deoxy-D-glucose hydrazone** and **5**. **5** (200 mg, 1.34 mmol) in methanol (1.7 mL) was added to **1** (25 mg, 0.106 mmol) in water (135 μL). After 3 h mechanical shaking, the insoluble diacylhydrazine by-product **7**, formed from occluded hydrazine in **1**, was removed by centrifuging. The residue from evaporation of the supernatant was passed through a silica column, and **9** (17 mg, 42%) was eluted with ethyl acetate:ethanol (1:1). By omitting the centrifugation step, the yield of **9**, with some contamination by **7**, was increased to 70%. ^{1}H NMR (DMSO- d_6) **9** *E*–isomer δ 1.91 (s, 3H, Ac), 2.29 (s, 3H, Me), 3.94 (d, 1H, $J_{2,3}$ = 6.4 Hz, H-3), 4.64 (dd, 1H, $J_{1,2}$ = 4.8 Hz, $J_{2,3}$ = 6.1 Hz, H-2), 7. 57 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1), 7.17 (d, 2H, J = 8.2 Hz, H_{Ar}), 7.44 (d, 2H, J = 8.2 Hz, H_{Ar}); β-anomer δ 1.84 (s, 3H, Ac), 2.27 (s, 3H, Me), 3.92 (d, 1H, $J_{1,2}$ = 9.5 Hz, H-1); **7** δ 2.27 (s, 3H, Me), 7.13 (d, 2H, J = 8.2 Hz, H_{Ar}), 7.38 (d, 2H, J = 8.2 Hz, H_{Ar}). **7** MS (CI, CH_4): *m/z* 331 (M+1).

Recovery of Sugars. (a) As saccharide hydrazones. (i) When **8** (5 mg) was allowed to stand 30 min in hydrazine hydrate (50 μL), TLC showed the complete disappearance of **8** at R_f 0.4 and the appearance of **6** at R_f 0.8. (ii) **8** (10 mg, 0.03 mmol) was dissolved in hydrazine hydrate (100 μL, 2 mmol), left at room temperature for 1 h and the excess hydrazine evaporated under vacuum over concentrated sulfuric acid. ^{1}H NMR (DMSO-d_6) **6** δ 2.26 (s, 3H, Me), 7.10 (d, 2H, J = 8.3 Hz, H_{Ar}), 7.45 (d, 2H, J = 8.3 Hz, H_{Ar}); 4-toluidine δ 2.12 (s, 3H, Me), 6.46-6.48 (m, 2H, H_{Ar}), 6.81 (d, 2H, J = 8.46 Hz, H_{Ar},). **(b) As reducing aldoses.** (i) When benzaldehyde (30 μL) in ethanol (190 μL) was

added to **8** (2 mg) dissolved in water (80 μL), and the solution heated at 100 °C for 3 h, TLC showed the appearance of benzaldehyde 4-tolylthiosemicarbazone **10** at R_f 0.92 and the disappearance of **6** at R_f 0.70, with glucose at the origin. (ii) Benzaldehyde (50 μL, 0.49 mmol) in ethanol (350 μL) was added to **8** (10 mg, 0.03 mmol) in water (100 μL) and heated at 100 °C for 3 h, after which excess benzaldehyde was evaporated with the solvent under a stream of nitrogen. The NMR spectrum (DMSO-d_6) showed complete conversion of **8** to a mixture of glucose and **10**. (iii) Acetone (60 μl, 0.81 mmol) was added to **8** (7 mg, 0.02 mmol) in water (180 μL), the solution was heated at 100 °C for 3 h and the solvent evaporated under a stream of nitrogen. The ^{1}H NMR spectrum (DMSO-d6) showed a 70% recovery of glucose. ^{1}H NMR (DMSO-d6) **10** δ 2.32 (s, 3H, Me), 7.18 (d, 2H, J = 8.6 Hz, H_{Ar}), 7.43-7.45 (m, 4H, H_{Ar}), 7.89-7.91 (m, 2H, H_{Ar}), 8.17 (s, 1H, H-1).

2'-Hydroxybenzylidenehydrazono-2-acetamido-2-deoxy-D-glucose (14). 2-hydroxybenzaldehyde (130 μL, 0.90 mmol) in methanol (5 mL) was added to **1** (100 mg, 0.45 mmol) in water (400 μL), the solution stirred for 3 h and the solvent evaporated under a stream of nitrogen. The residue was shaken with methanol (2 mL), traces of **12** and **13** removed by centrifuging, and **14** was obtained from the evaporated supernatant. ^{1}H NMR (DMSO-d6) **12** δ 6.80-6.85 (m, 2H, H_{Ar}), 7.10-7.15 (m, 1H, H_{Ar}), 7.21 (dd, 1H, J = 1.7 Hz, J = 7.5 Hz, H_{Ar}), 7.94 (s, 1H, HC=N); **13** δ 6.97–7.00 (m, 2H, H_{Ar}), 7.39-7.44 (m, 1H, H_{Ar}), 7.70 (dd, 1H, J =1.7 Hz, J=8.2 Hz, H_{Ar}), 8.91, (s, 1H, HC=N); **14** β-anomer, δ1.82 (s, 3H, Ac), 4.43 (d, 1H, $J_{1,2}$ = 9.7 Hz, H-1), 6.81-7.00 (m, 2H, H_{Ar}), 7.12-7.17 (m, 1H, H_{Ar}), 7.25 (dd, 1H, J = 1.8 Hz, J = 6.8 Hz, H_{Ar}), 7.90 (s, 1H, HC=N).

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