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FURTHER STUDIES OF THE HYDRAZINOLYSIS OF 2-ACETAMIDO-1-*N*-ACYL-2-DEOXY-β-D-GLUCOPYRANOSYLAMINES*

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ABSTRACT

1-Deoxy-D-fructose hydrazone is shown to be the major product of the hydrazinolysis of 2-amino-2-deoxy-D-glucose hydrazone and can be detected as a minor product of the hydrazinolysis of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine. The hydrazinolysis conditions (100°, 30 h, no catalyst) of Bayard and Montreuil are inefficient for the cleavage of the amide groups of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine, unchanged starting-material and 1-*N*-acetyl-2-amino-2-deoxy-β-D-glucopyranosylamine accounting for 70% of the product mixture. Under the same conditions, the hydrazinolysis of 2-acetamido-1-*N*-(L-β-asparty1)-2-deoxy-β-D-glucopyranosylamine gave 2-amino-2-deoxy-D-glucose hydrazone as major product together with ~14% of 1-deoxy-D-fructose hydrazone. Participation by the carboxyl group of the asparagine residue is invoked to account for the greater reactivity of the asparagine derivative. The hydrazinium sulphate-catalysed hydrazinolysis gave a higher yield (44%) of 1-deoxy-D-fructose hydrazone when applied to the asparagine derivative. The implications of these results in relation to the hydrazinolysis of glycopeptides and glycoproteins are discussed.

INTRODUCTION

Hydrazinolysis is a convenient reaction for the isolation of the asparagine-bound carbohydrate moieties of glycoproteins[1–7]. It also *N*-deacetylates *N*-acetylhexosamine and *N*-acetylneuraminic acid residues, and the resulting glycanamine can be degraded regiospecifically by the nitrosation procedure[8]. However, excessively vigorous conditions can cause degradation, and two sets of conditions are currently used for such hydrazinolyses: (*a*) the hydrazinium sulphate-catalysed reaction[9] as modified by Kochetkov and his co-workers[10], which involves heating at 105° for 10 h, and (*b*) the uncatalysed reaction used by Bayard and Montreuil[11], which involves heating at 100° for 30 h.

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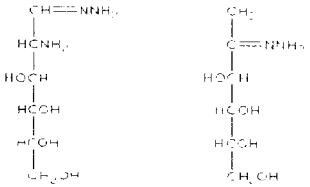
Using 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine as a model for the sugar–asparagine linkage, we have shown[12] the major product of hydrazinolysis to be 2-amino-2-deoxy-D-glucose hydrazone, but a minor product was not identified. We now report the identification of this minor product and compare the above two sets of hydrazinolysis conditions.

RESULTS

When 2-amino-2-deoxy-D-glucose hydrochloride was dissolved in anhydrous hydrazine, the acyclic hydrazone **1** was formed, as shown by ^{13}C-n.m.r. spectroscopy[12]. When the hydrazine solution was heated in a closed tube at 100° for 30 h, ^{1}H- and ^{13}C-n.m.r. measurements indicated that, in addition to the hydrazone of 2-amino-2-deoxy-D-glucose, a compound containing a methyl group was present. The singlet in the ^{1}H-n.m.r. spectrum and the chemical shifts (^{1}H, δ 1.84; ^{13}C, 13.8 p.p.m.) suggested the partial structure CH_3-$\overset{\downarrow}{C}$=NNH_2, and the compound was shown to be identical, by comparison of the ^{1}H- and ^{13}C-n.m.r. spectra, with the compound prepared from 1-deoxy-D-fructose and hydrazine. That this compound was 1-deoxy-D-fructose hydrazone (**2**) was confirmed as follows. The product mixture, after removal of hydrazine, was treated with nitrous acid to convert the hydrazones into the corresponding saccharides. The formation of 1-deoxy-D-fructose was monitored by t.l.c. and comparison with an authentic sample. The most prominent signals in the ^{13}C-n.m.r. spectrum corresponded to the major tautomer, 1-deoxy-β-D-fructopyranose[13]; the nitrosation of aldose hydrazones at pH 4.5 gives both the corresponding aldose and the glycosylhydrazine[14]. The nitrosation product was reduced with sodium borodeuteride and acetylated. G.l.c. analysis then revealed a compound with a retention time identical to that of 1-deoxy-L-mannitol (L-rhamnitol) penta-acetate. G.l.c.–m.s. confirmed the 1-deoxyhexitol structure, and the ion at *m/z* 88 (MeCDOAc^{+}) was consistent with the presence of deuterium at C-2. The ^{1}H-n.m.r. spectrum of the product contained two singlets at δ 1.18 and 1.25, which were assigned to H-1 of the epimeric penta-acetates of 1-deoxy-D-mannitol and 1-deoxy-D-glucitol.



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To assess the extent of such degradation in the hydrazinolysis of glycopeptides and glycoproteins, 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine was heated in anhydrous hydrazine at 100° for 30 h (Bayard–Montreuil conditions) and at 105° for 10 h in the presence of hydrazinium sulphate (Kochetkov conditions). The product of hydrazinolysis under Bayard–Montreuil conditions contained approximately equal amounts of starting material, a mono-*N*-acetyl compound, and 2-amino-2-deoxy-D-glucose hydrazone. The mono-*N*-acetyl compound was identified as 1-*N*-acetyl-2-amino-2-deoxy-β-D-glucopyranosylamine from the n.m.r. signals of H-1 (doublet at δ 4.89, $J_{1,2}$ 9.5 Hz) and H-2 (broadened triplet at δ 2.70, $J_{2,3}$ 9 Hz). Of the acetohydrazide released, ~16% was converted into 4-amino-3,5-dimethyl-1,2,4-triazole (Me at δ 2.39 in the ^{1}H-n.m.r. spectrum; see ref. 12).

The hydrazinolysis of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine under Kochetkov conditions has been reported[12]. Repetition of the experiment gave the same results, and 1-deoxy-D-fructose hydrazone was identified as a minor product by ^{1}H- and ^{13}C-n.m.r. spectroscopy. Three singlets (δ 1.84, 1.95, and 2.37) in the ^{1}H-n.m.r. spectrum were assigned to 1-deoxy-D-fructose hydrazone, acetohydrazide, and 4-amino-3,5-dimethyl-1,2,4-triazole, respectively. The yield of 1-deoxy-D-fructose hydrazone, estimated from the ^{1}H-n.m.r. spectrum, was 22%.

The hydrazinolysis of 2-acetamido-1-*N*-(L-β-aspartyl)-2-deoxy-β-D-glucopyranosylamine (GlcNAc–Asn) under Bayard–Montreuil conditions resulted in complete amide cleavage with the formation of 2-amino-2-deoxy-D-glucose hydrazone and a small proportion (~14%) of 1-deoxy-D-fructose hydrazone. Under Kochetkov conditions, the same compound gave a higher yield (~44%) of 1-deoxy-D-fructose hydrazone.

DISCUSSION

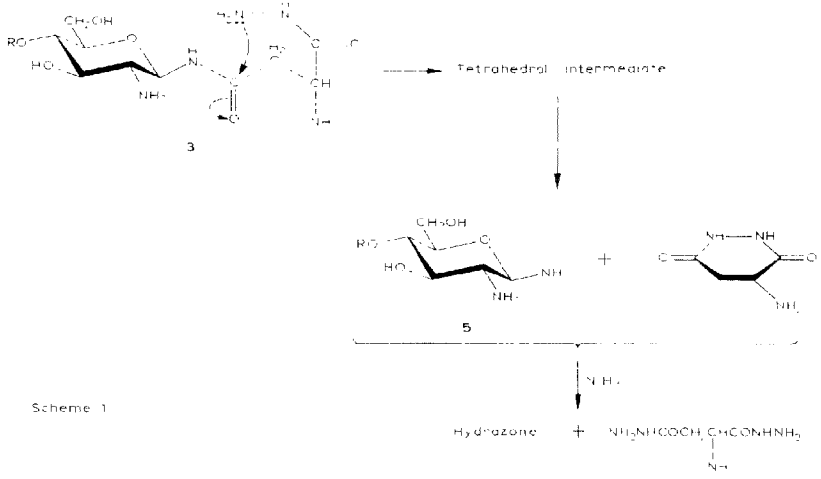
We have shown[12] that 2-amino-2-deoxy-D-glucose hydrochloride is converted into the acyclic hydrazone in hydrazine solution at room temperature, and that the same hydrazone is formed in the hydrazinolysis of 2-acetamido-1-*N*-acyl-2-deoxy-β-D-glucopyranosylamines. The formation of 1-deoxy-D-fructose hydrazone as the major product in the hydrazinolysis of 2-amino-2-deoxy-D-glucose hydrazone is relevant to the hydrazinolysis of glycoproteins and glycopeptides containing asparagine-bound oligosaccharides. Evidence for the partial degradation of the 2-acetamido-2-deoxy-D-glucose residues in glycoproteins appears in several papers[2,15], and the residue linked to asparagine was identified[2] as the one which was partially modified.

The conditions of hydrazinolysis used by Bayard and Montreuil were established using methyl 2-acetamido-2-deoxy-β-D-glucopyranoside as a model[11]. However, this is not an entirely satisfactory model for the cleavage of the *two* amide linkages in the GlcNAc–Asn moiety. The Bayard–Montreuil conditions (100° for

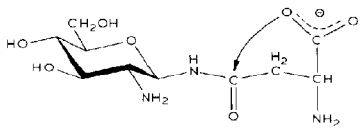
30 h) were very inefficient for the cleavage of the amide groups in 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine, both the starting material (35%) and 1-*N*-acetyl-2-amino-2-deoxy-β-D-glucopyranosylamine (35%) being present in the product. Under Kochetkov conditions, amide cleavage was complete, and degradation to form 1-deoxy-D-fructose hydrazone occurred to the extent of 22%.

The inefficiency of the Bayard–Montreuil conditions is surprising in view of the claim[7] that a reaction time of only 8–12 h at 100° is sufficient to effect the complete release of the asparagine-bound oligosaccharides in glycoproteins and glycopeptides. Reaction for 30 h caused some unspecified degradation of the reducing 2-acetamido-2-deoxy-D-glucose residue. A possible explanation for the difference in efficiency of the hydrazinolysis of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine and of glycoproteins or glycopeptides is that an intramolecular reaction of the intermediate glycopeptide hydrazide (**3**) occurs as shown in Scheme 1. That such neighbouring-group effects are important is shown by the complete amide cleavage achieved when Bayard–Montreuil hydrazinolysis was applied to 2-acetamido-1-*N*-(L-β-aspartyl)-2-deoxy-β-D-glucopyranosylamine. The much greater reactivity of this compound must be due to neighbouring-group participation by the carboxyl group as shown in **4**. The more efficient Kochetkov hydrazinolysis forms 2-amino-2-deoxy-D-glucose hydrazone more rapidly, and thus more degradation to 1-deoxy-D-fructose occurs.

Thus, when hydrazinolysis followed by *N*-acetylation is used to isolate the asparagine-bound carbohydrate moieties of glycoproteins or glycopeptides, the



Scheme 1



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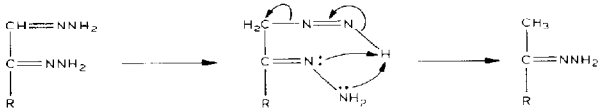
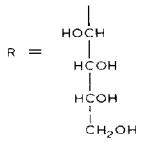
Bayard–Montreuil conditions are preferred to those of Kochetkov in order to minimise the degradation of the 2-acetamido-2-deoxy-D-glucose residue that is linked to asparagine. However, when hydrazinolysis is used also to effect *N*-deacetylation prior to nitrosation, the more efficient Kochetkov-hydrazinolysis is preferred. For example, application of the hydrazinolysis–nitrosation degradation-procedure to glycopeptides which contain the common sequence $(Man)_2Man$-GlcNAc-GlcNAc-Asn would result in partial degradation of only the GlcNAc residue attached to asparagine and hence a diminished yield, after reduction, of 2,5-anhydro-D-mannitol in the degradation product mixture. Such a diminished yield has been found in the product from IgM glycopeptide[16], and we have now identified 1-deoxymannitol by liquid chromatography of the perbenzoate derivative[17]. The sequence $(Man)_2Man$-GlcNAc-Asn would give a mixture of three tetrasaccharides, one with a terminal 2,5-anhydro-D-mannitol residue (normal product), and two with 1-deoxy-D-glucitol and 1-deoxy-D-mannitol residues (degraded products).

Takasaki and co-workers have claimed[7], without citing any evidence, that formation of the glycosylamine **5** and its conversion into glycosylhydrazine are relatively rapid reactions, which are followed by a slow tautomerisation to give the hydrazone that is susceptible to degradation. A more likely reaction-sequence involves nucleophilic attack of hydrazine on the acyclic imine-tautomer of the glycosylamine, to give the hydrazone. We have shown that hydrazones tautomerise





Scheme 2

slowly to the glycosylhydrazines in aqueous medium, although we have not studied the rate of this reaction for hydrazine solutions. In hydrazine solutions, the equilibrium favours the hydrazone tautomers[14].

The 1-deoxy-D-fructose can be envisaged as arising *via* the 1,2-bishydrazone, the formation of which resembles osazone formation. A Wolff–Kishner reaction at C-1, possibly facilitated by intramolecular catalysis involving a six- or seven-membered ring transition-state as depicted in Scheme 2, would then give 1-deoxy-D-fructose hydrazone. A similar reaction at C-2 would give 2-deoxy-D-*arabino*-hexose hydrazone, and it is noteworthy that a 2-deoxy-D-*arabino*-hexitol derivative was recently identified as a minor product of the sequential hydrazinolysis, nitrosation, and reduction of glycopeptides[18].

EXPERIMENTAL

General. — Melting points were determined on a Kofler hot-stage and are corrected. N.m.r. spectra were recorded with Varian HA-100 (^{1}H) and XL-100 (^{13}C) spectrometers. ^{13}C-N.m.r. spectra of hydrazine solutions were measured using D_2O in a coaxial cell for the pulsed lock. Tetramethylsilane and sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate (T.S.P.) were used as references in deuteriochloroform and deuterium oxide solutions, respectively. The apparent coupling constants are the directly observed line-spacings. G.l.c. was performed with a 5% SE30 column (2 m) at 224°. Mass spectra were recorded with an MS9 spectrometer.

Hydrazine solutions, in glass tubes with side-arms and Teflon taps, were degassed, using the freeze–thaw method over nitrogen, and then heated in a Berghof heating-block, the temperature of which was regulated to ±0.5°.

Hydrazinolysis of 2-amino-2-deoxy-D-glucose hydrazone. — A mixture of 2-amino-2-deoxy-D-glucose hydrochloride (0.2 g) and anhydrous hydrazine (2 mL, Aldrich Chemical Co.) was kept at 100° for 30 h. The ^{13}C-n.m.r. spectrum then showed six strong signals at 13.7, 65.8, 74.6, 76.6, 77.1, and 154.6 p.p.m., together with weaker signals correspondings to 2-amino-2-deoxy-D-glucose hydrazone. The hydrazine was removed *in vacuo* over conc. H_2SO_4, to give a syrup. N.m.r. data (D_2O): ^{1}H, δ 7.73 (s), 7.52 (s), 7.29 (d, J 5.5 Hz), 5.5–4.5 (region obscured by HOD lock), 4.0–3.4 (m), 2.18, 1.96, 1.94, and 1.85 (all s, the strongest absorption being at 1.85); ^{13}C, the six strongest signals corresponded to 1-deoxy-D-fructose hydrazone (see below, C-2 was readily identified in the off-resonance proton-decoupled spectrum as a singlet); weaker signals for 2-amino-2-deoxy-D-glucose hydrazone were present, *e.g.*, 148.5 and 94.1 (C-1 of acyclic and cyclic tautomers); unassigned weak signals included 150.1, 11.7, and 10.7 p.p.m.

1-Deoxy-D-fructose hydrazone. — Crystalline 1-deoxy-D-fructose[13] (86 mg) was dissolved in anhydrous hydrazine (1 mL), and the solution was kept overnight. Hydrazine was removed *in vacuo* over conc. H_2SO_4, to give the syrupy hydrazone. N.m.r. data (D_2O): ^{1}H, δ 1.82 (s, 3 H, H-1) and 3.5–3.8 (m, 5 H); ^{13}C, 13.8 (C-1), 65.4 (C-6), 73.8, 74.9, 76.3, and 157.4 (C-2).

Nitrosation of the product of hydrazinolysis of 2-amino-2-deoxy-D-glucose hydrazone. — The hydrazinolysis product from 2-amino-2-deoxy-D-glucose hydrochloride (2.25 mmol) was dissolved in water (25 mL), and sodium nitrite (0.93 g, 13.5 mmol) added. The solution was cooled in ice, and glacial acetic acid (0.81 mL) was added at intervals to maintain the pH at 6.5. The reaction was monitored by t.l.c. (Kieselgel 60 GF, methanol), using aniline hydrogenphthalate reagent to detect 1-deoxy-D-fructose (orange, R_F 0.6). Ninhydrin was used to detect the hydrazone (yellow–pink, R_F 0.1–0.2). More sodium nitrite (0.69 g) and acetic acid (0.54 mL) were required, the reaction being complete after an overnight period. The solution was deionised by using Amberlite MB-3 resin (300 mL). Evaporation of the filtrate plus washings to dryness gave a syrup (0.294 g) which was analysed by ^{13}C-n.m.r. spectroscopy. Signals corresponding to 1-deoxy-D-fructose were present, the most prominent ones being those of the β-pyranose tautomer: (D_2O) 27.4, 66.0, 72.0, 72.2, 74.8, and 100.9 p.p.m. These values corresponded to those reported by Angyal[13], after allowing for the different reference used*. Many of the signals of the minor tautomers were also detected. Other signals in the anomeric carbon region (90–105 p.p.m.) were probably due to glycosyl azides[14].

To an ice-cooled solution of sodium borodeuteride (48 mg, 1.14 mmol) in water (1 mL) was added dropwise during 10 min a solution of the foregoing product (135 mg) in water (1 mL). After 2 h at room temperature, excess of reducing agent was destroyed with Amberlite IR-120 (H^+) resin (6 mL). The filtrate plus washings were concentrated to dryness, and boric acid was removed from the residue conventionally as methyl borate. The syrupy product (134 mg) was treated with pyridine (2 mL) containing acetic anhydride (1 g) overnight at room temperature. The usual work-up gave a colourless syrup (151 mg). N.m.r. data ($CDCl_3$): 1.18 (s) and 1.25 (s). G.l.c. gave a major peak having a retention time (3.3 min) identical to that of 1-deoxy-L-mannitol penta-acetate, and g.l.c.–m.s. identified a 1-deoxyhexitol penta-acetate containing one deuterium atom. Mass spectrum: *m/z* 304 (0.4), 232 (1.6), 160 (2.5), and 88 (1.1). These ions, less one mass-unit, were present in the mass spectrum of 1-deoxy-L-mannitol penta-acetate, and ions not containing deuterium were present in both spectra, *e.g.*, *m/z* 289 (0.9) and 43 (100).

*Hydrazinolysis of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine.* — A solution of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine[12] (0.2 g) in anhydrous hydrazine (2 mL) was kept at 100° for 30 h, and then concentrated *in vacuo* over conc. H_2SO_4, to give a syrupy product. N.m.r. data (D_2O): ^{13}C, strong signals corresponding to the starting material[12] were present plus signals at 178.1, 175.7 (CO of $AcNHNH_2$), 151.2 and 94.7 (C-1 of 2-amino-2-deoxy-D-glucose hydrazone tautomers), 22.6 (Me of $AcNHNH_2$), and 11.8 p.p.m. (Me of triazole); ^1H, δ 7.26 (very weak d, *J* 5.5 Hz), 5.06 (d, *J* 8.5 Hz), 4.89 (d, *J* 9.5 Hz; a probe temperature of 50° was used to move the HOD signal upfield and reveal this

*The chemical shift of 1,4-dioxane with respect to T.S.P. is 69.20 p.p.m.

signal), 4.0–3.3 (m), 2.70 (bt, J 9 Hz), 2.39 (s, Me of triazole), 2.08 (s, Me of mono-*N*-acetyl compound), 2.02 (s, Me of starting material), and 1.95 (s, Me of $AcNHNH_2$); ratios of last 4 s, 10:25:48:58.

*Hydrazinolysis of 2-acetamido-1-*N*-(L-β-aspartyl)-2-deoxy-β-D-glucopyranosylamine.* — (*a*) *Kochetkov conditions.* A mixture of the aspartylglycosylamine (45 mg), hydrazinium sulphate (30 mg), and anhydrous hydrazine (0.5 mL) was heated at 105° for 10 h, and then concentrated to give a syrupy residue. N.m.r. data (D_2O): ^{13}C, 11.8 and 156.2 (triazole), 14.0, 65.5, 73.9, 74.8, 76.4, 157.5 (1-deoxy-D-fructose hydrazone), 22.6 and 175.6 ($AcNHNH_2$), 41.2, 53.4, 174.1, and 176.9 (aspartic hydrazide), 93.7 and 147.1 p.p.m. (weak, C-1 of 2-amino-2-deoxy-D-glucose hydrazone tautomers); ^1H, δ 1.87 (s, Me of 1-deoxy-D-fructose hydrazone), 1.94 (s, weak), 1.98 (s, $MeCONHNH_2$), 2.40 (s, Me of triazole), 2.5 (m, CH_2 of aspartic hydrazide), and 7.28 (d, J 5.5 Hz, H-1 of 2-amino-2-deoxy-D-glucose hydrazone); ratio of Me singlets (1-deoxy-D-fructose hydrazone to rest), 26:59.5.

(*b*) *Bayard–Montreuil conditions.* The aspartylglycosylamine (44 mg) in anhydrous hydrazine (0.5 mL) was heated at 100° for 30 h. N.m.r. analysis of the product, as above, revealed the same products as in (*a*), but the relative amounts of 4-amino-3,5-dimethyl-1,2,4-triazole and 1-deoxy-D-fructose hydrazone were less. In the ^1H-n.m.r. spectrum, the ratio of Me singlets (1-deoxy-D-fructose hydrazone to the rest) was 16:114.

Detection of 1-deoxymannitol in the degradation product from IgM glycopeptide. — The product (1 mg) from the sequential hydrazinolysis, nitrosation, and reduction of IgM glycopeptide[16] was benzoylated, and analysed by liquid chromatography[17]. In the monosaccharide region, peaks corresponding to the benzoates of 1-deoxymannitol and 2,5-anhydro-D-mannitol were detected.

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