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BEHAVIOR OF THE 2-ACETAMIDO-2-DEOXY-α-D-GLUCOPYRANOSYL RESIDUE DURING SEQUENTIAL HYDRAZINOLYSIS, *N*-REACETYLATION, REDUCTION, AND METHYLATION OF GLYCOASPARAGINES

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ABSTRACT

The behavior of the 2-acetamido-1-*N*-(L-aspart-4-oyl)-deoxy-β-D-glucopyranosyl residue during sequential hydrazinolysis, *N*-reacetylation, reduction, and methylation analysis was investigated in the model compounds, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn, α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn, and α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-[α-L-Fuc*p*-(1→6)]-β-D-Glc*p*NAc-(1→4)-Asn. The products from the reaction were partly separated, and gas–liquid chromatography–mass spectrometry and fast-atom-bombardment mass spectrometry indicated the presence of 8 by-products in addition to 2-acetamido-2-deoxyglucitol derivatives. It is postulated that these by-products are formed by a series of kinetically competitive processes starting from the key intermediate, 2-acetamido-2-deoxy-D-glucose hydrazone.

INTRODUCTION

Hydrazinolysis is a widely used procedure to isolate the carbohydrate component of glycoproteins[1–5]. After cleavage of the glycosylamine linkage, the freed amino groups are *N*-reacetylated and the resulting oligosaccharides reduced with sodium borohydride. During methylation analysis of the oligosaccharides released by this procedure, 4-*O*-acetyl-2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(methylacetamido)glucitol, which originated from the 2-acetamido-2-deoxy-β-D-glucopyranosyl group linked to asparagine, is obtained in only 60% of the expected yield[2]. A similar result was obtained when the sugar composition of the reduced, *N*-reacetylated oligosacharides was estimated after methanolysis[5]. Recently, Saeed

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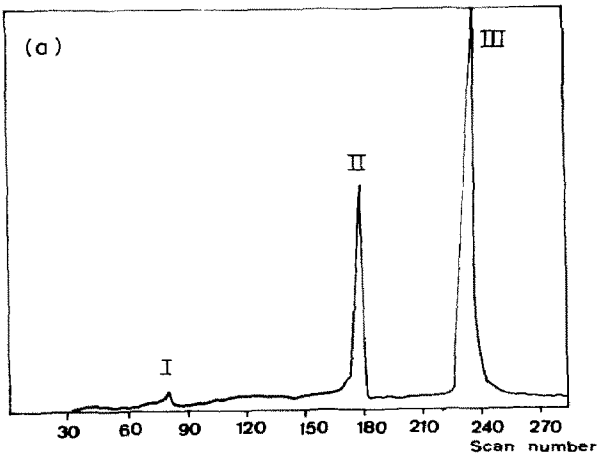
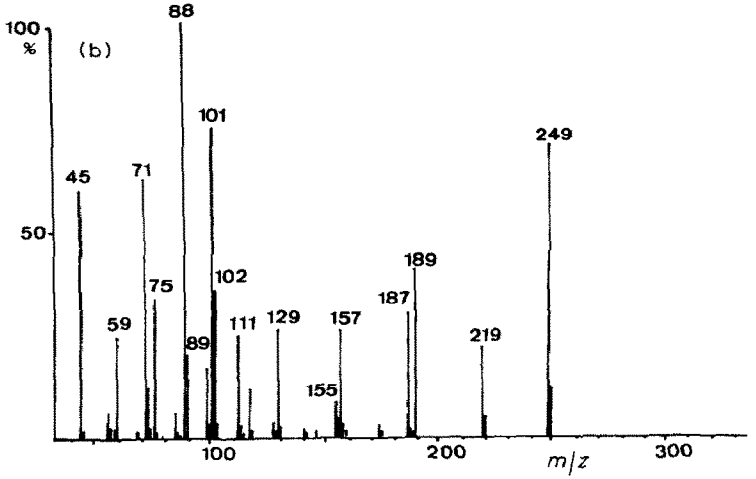

(a)
I
II
III
30 60 90 120 150 180 210 240 270
Scan number


100 %
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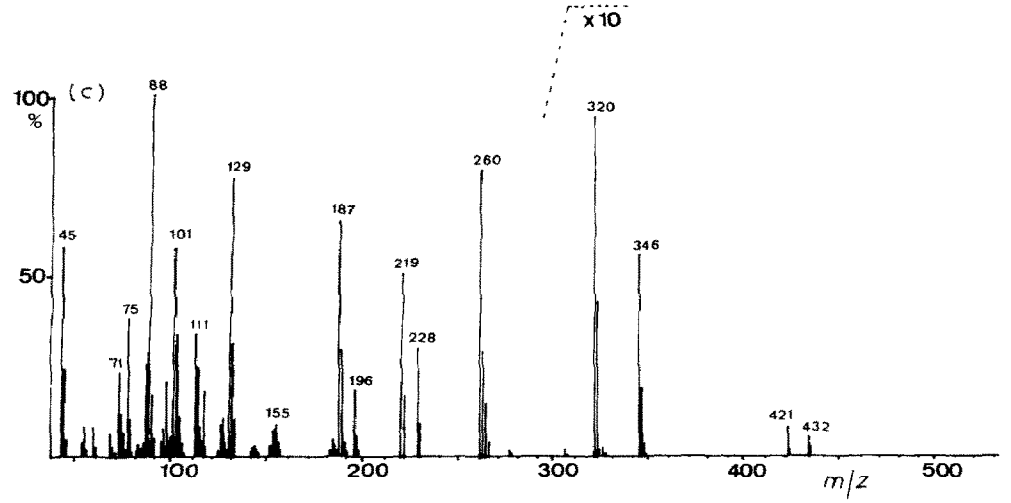
m/z


x10
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(c)
88
320
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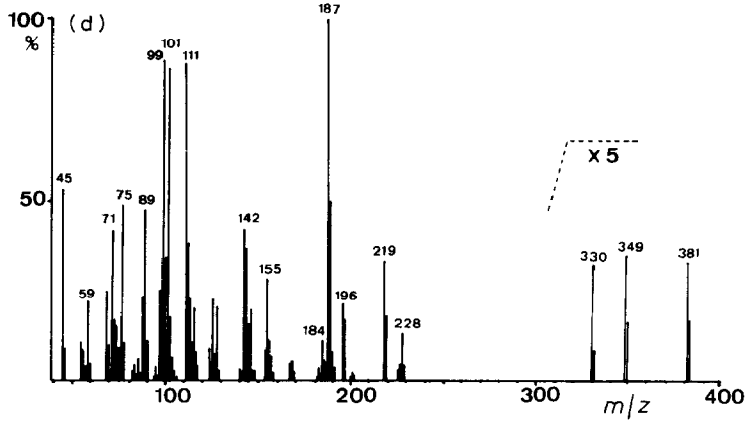


Fig. 1. G.l.c.–m.s. analysis of the products resulting from the sequential hydrazinolysis, *N*-reacetylation, and methylation of **1**: (a) g.l.c. separation; (b) e.i.m.s. of Peak I; (c) e.i.m.s. of Peak II; (d) e.i.m.s. of Peak III.

and Williams[6] have been shown that the major product of the hydrazinolysis of 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine is 2-amino-2-deoxy-D-glucose hydrazone, which is further partially transformed into 1-deoxy-D-fructose hydrazone[7]. We report herein the behavior of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue during sequential hydrazinolysis, *N*-reacetylation, and methylation, with or without prior reduction, of the model compounds, **1, 2,** and **3**. These compounds were subjected to three different reaction sequences: (*a*) hydrazinolysis, *N*-reacetylation, and permethylation; (*b*) hydrazinolysis, *N*-reacetylation, reduction, and permethylation; and (*c*) hydrazinolysis, *N*-reacetylation, and reduction. The resulting products were separated by t.l.c. or l.c., and analyzed either by g.l.c.–m.s. or by fast-atom-bombardment mass-spectrometry (f.a.b.m.s.).

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn

1

α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn

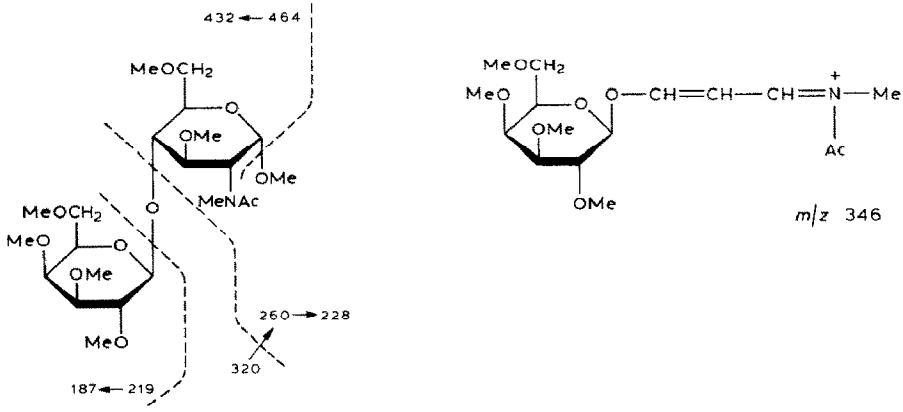
2

α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn
6
↑
1
α-L-Fuc*p*

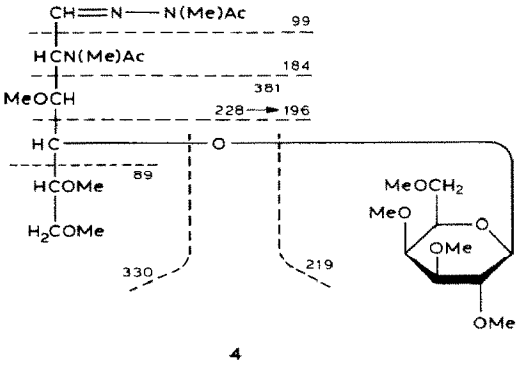
3

RESULTS AND DISCUSSION

The sequential hydrazinolysis (16 h reaction time), *N*-reacetylation, and methylation (method *a*) of **1** without reduction gave a mixture of three products (g.l.c.–m.s.; see Fig. 1): the first (I), which represented less than 2% of the total material, was identified as a permethylated disaccharide β-D-Gal*p*-(1→4)-deoxyhexose, in agreement with the presence of fragments *m/z* 219 (hexose), 189 (deoxyhexose), and 249 (Me–O–CH=O$^+$→deoxyhexose). The second compound (II, ~40% of the total material) was identified as β-D-Gal*p*-(1→4)-D-GlcNAc*p*, as indicated by the presence of fragments *m/z* 219, 260, and 346 (see Scheme 1). The third compound (III, ~60% of the total material) was identified as the acetylhydrazone **4** (see Scheme 2), as clearly indicated by the fragments *m/z* 99, 184, 219, and 330. Since reducing oligosaccharides are partially destroyed during the methylation with sulfinyl carbanion, it was not possible to determine quantitatively the yield of the 2-acetamido-2-deoxy-D-glucose-containing disaccharide. T.l.c. analysis of the mixture obtained after hydrazinolysis and *N*-reacetylation revealed three



Scheme 1. Fragmentation pattern of Peak II, Fig. 1(c).



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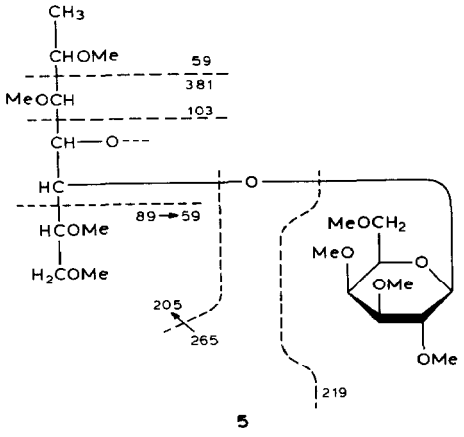
Scheme 2. Fragmentation pattern of Peak III, Fig. 1(d).



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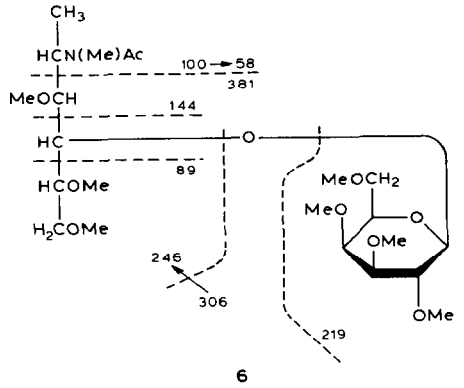
Scheme 3. Fragmentation pattern of Peaks IV and V, Fig. 2(b).



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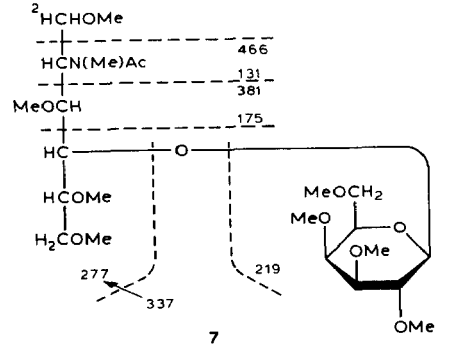
Scheme 4. Fragmentation pattern of Peak VI, Fig. 2(c).



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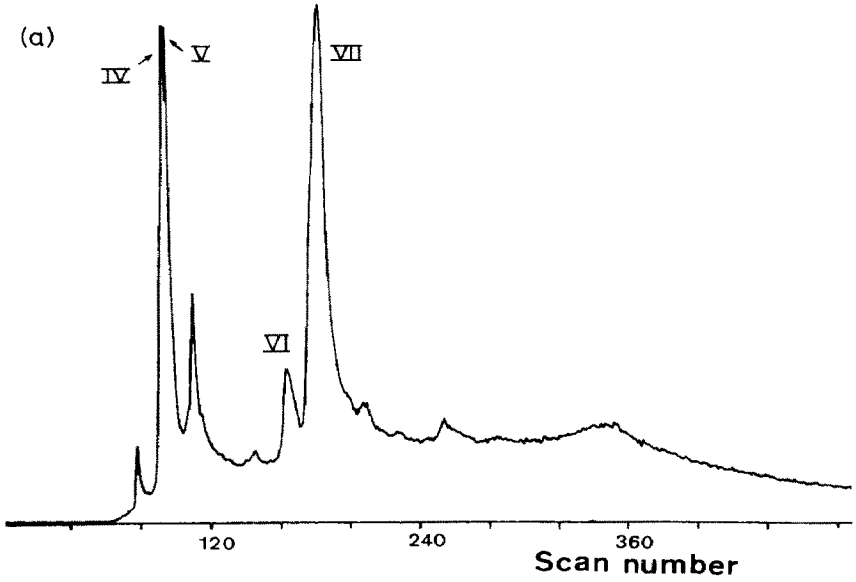
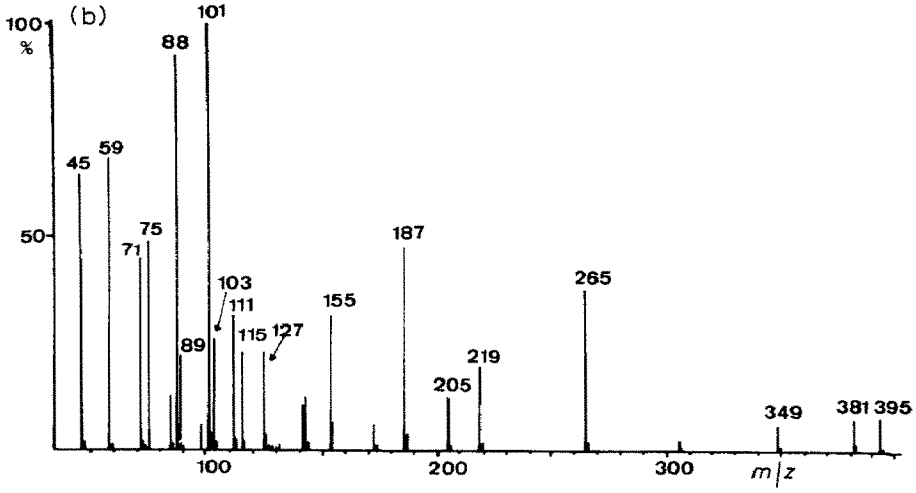
Scheme 5. Fragmentation pattern of Peak VII, Fig. 2(d).

products that could be separated on a preparative scale by l.c. Their relative amounts were in the ratios 1:15:9. After permethylation, followed by g.l.c.–m.s. analysis, the three products were identified separately as compounds I, II, and III. Thus, we confirmed the results of Saeed and Williams[6], who characterized 2-amino-2-deoxy-D-glucose hydrazone in the hydrazinolyzate of 2-acetamino-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine. Under our experimental conditions, the occurrence of a 1-deoxy-D-fructose hydrazone-containing disaccharide was not observed.

In a second experiment, the *N*-reacetylated material obtained after hydrazinolysis of **1** was reduced with $NaBH_4$ or NaB^2H_4 prior to methylation (method *b*). Three major products (IV, V, and VII), and a minor one VI that





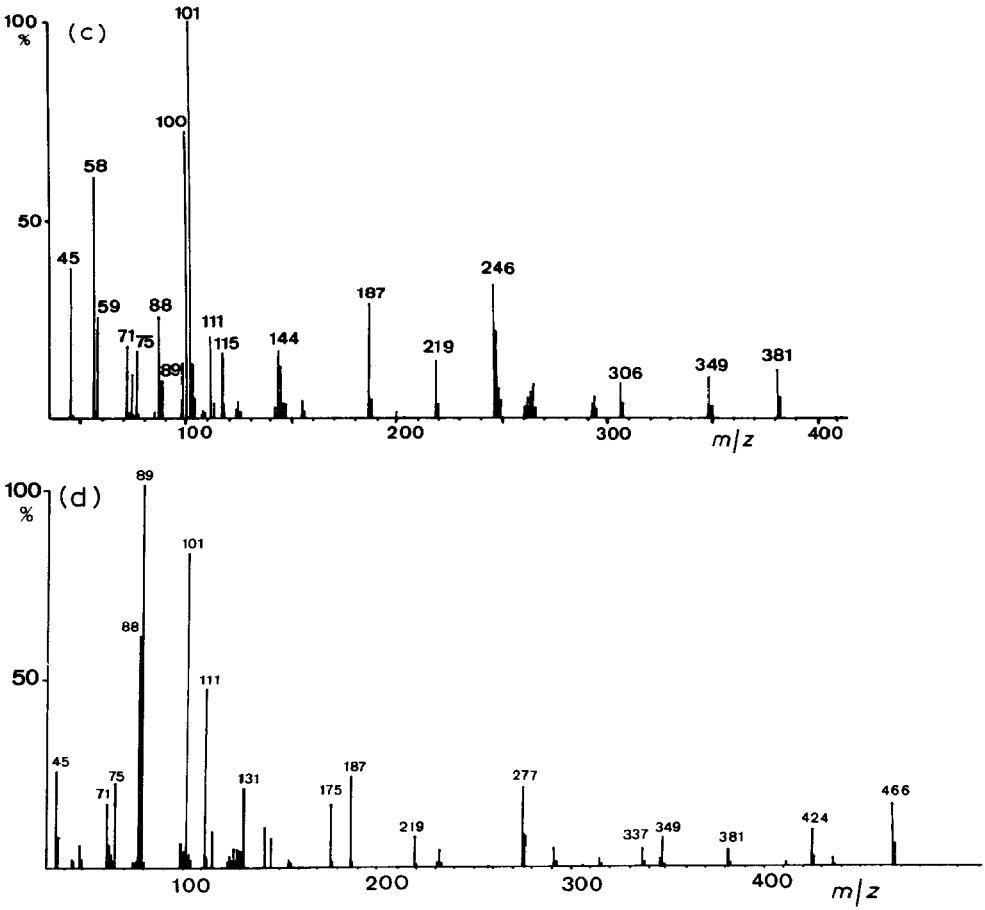


Fig. 2. G.l.c.–m.s. analysis of the products obtained by sequential hydrazinolysis, *N*-reacetylation, reduction, and methylation of **1**: (a) g.l.c. separation; (b) e.i.m.s. of Peaks IV and V; (c) e.i.m.s. of Peak VI; and (d) e.i.m.s. of Peak VII.

represented ~5% of the total material, could be characterized (Fig. 2). Compounds IV and V (~35% of the total material) gave identical mass spectra. When reduced with $NaBH_4$, IV and V showed characteristic fragments at *m/z* 205 (deoxyhexitol), 265 (Me–O–CH=O^+→deoxyhexitol), and 103 (Me–O–CH_2–CH_2–CH=O^+Me for 2-deoxy- or Me–CH(OMe)–CH=O^+Me for 1-deoxy-hexitol) (see Scheme 3). After reduction with NaB^2H_4, the fragments *m/z* 206, 266, 104, and 60 were observed. Since the positive charge could only be carried by an *O*-methyl group in this fragment, the existence of a 2-deoxy structure is excluded. The structures of IV and V are therefore proposed to be permethylated disaccharide 1-deoxyalditol epimers (**5**). Compound VI gave identical spectra after reduction with $NaBH_4$ and with NaB^2H_4. It was identified as the disaccharide **6**, based on the presence of the fragments *m/z* 246, 144, 100, and 58 (see Scheme 4), and compound V (~60% of the

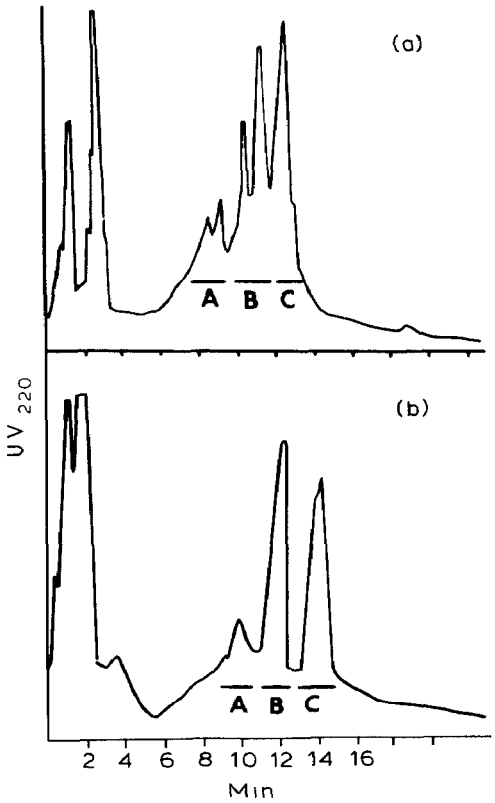


Fig. 3. L.c. fractionation patterns of reduced oligosaccharides resulting from the hydrazinolysis and *N*-reacetylation of: (a) **2** and (b) **3**.

total material) was shown to be **7** (see Scheme 5). Essentially the same results were obtained when the mixture produced by hydrazinolysis and *N*-reacetylation was separated by l.c. prior to reduction and permethylation. The main component (II) was quantitatively converted into VII, and the acetylhydrazone-containing disaccharide **4** yielded IV and V.

In order to corroborate the exact structure of the compounds obtained after the reduction step, asparagine-linked oligosaccharides **2** and **3**, the higher-molecular weights of which facilitated the f.a.b.m.s. analysis of the underivatized material, were investigated. Hydrazinolysis (16 h), *N*-reacetylation, and reduction with $NaBH_4$ (method *c*), followed by preparative l.c. yielded three major fractions A, B, and C for each oligosaccharide (Fig. 3), which were analyzed by negative-ion f.a.b.m.s. (Fig. 4). The major fragments (see Scheme 6) resulting from **2** and **3** are the pseudomolecular ions M − 1 at *m/z* 749 and 805, and 895 and 951, respectively, which were attributed to the corresponding oligosaccharides having a terminal 2-acetamido-2-deoxyglucitol (**14** and **15**) or a 2-acetamido-2-deoxyglucose hydrazone residue (**18** and **19**). The simultaneous presence of M − 1 ions at *m/z* 805 (**20**) and 951 (**21**) suggested that the acetylhydrazono compounds **18** and **19** resulted from an

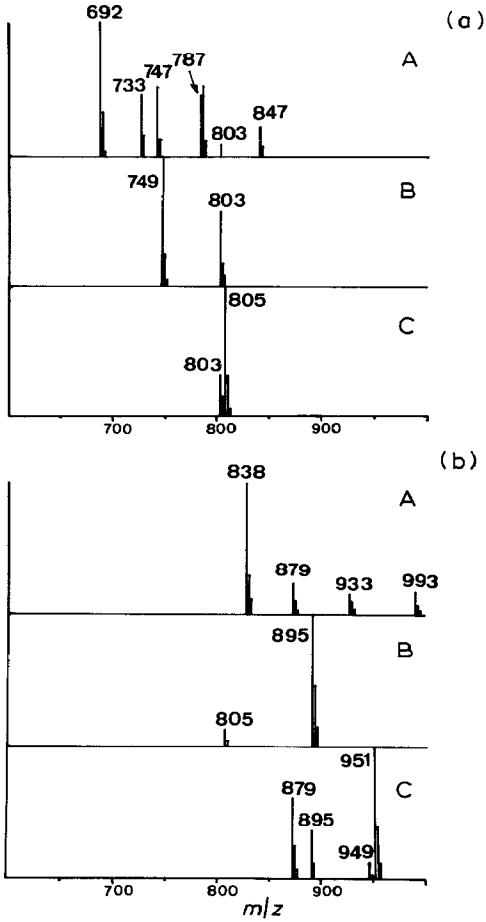
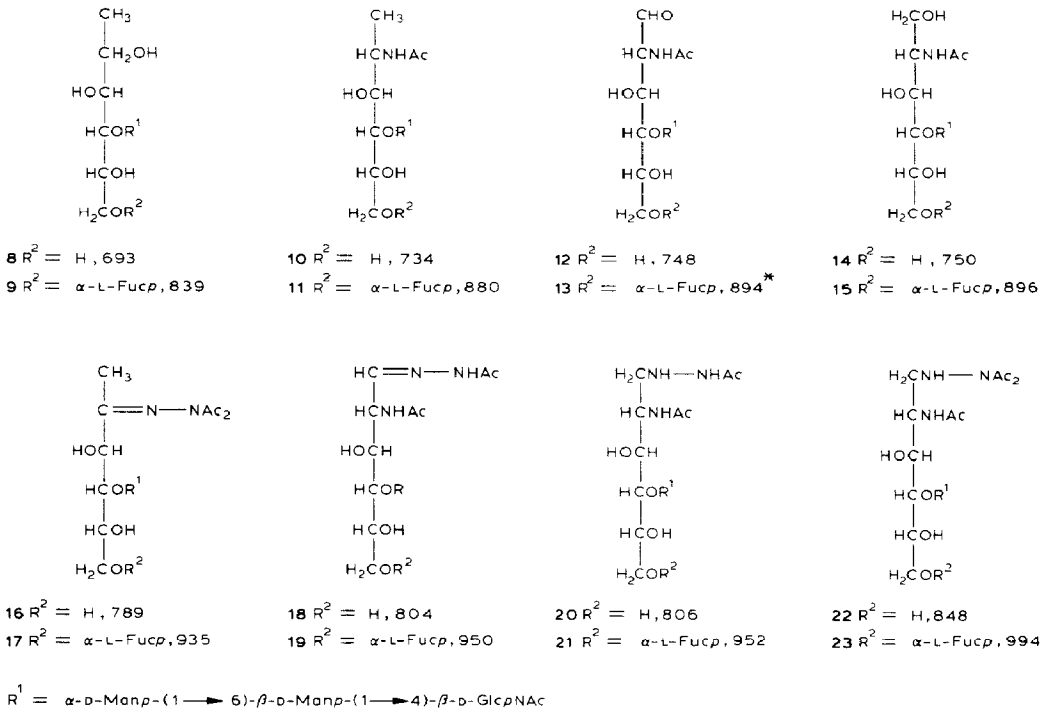


Fig. 4. Molecular-ion region of the f.a.b. mass spectra of fractions A, B, and C obtained from **2** (a) and **3** (b).

incomplete reduction. This may also hold for compound **12**. Compounds **22** and **23** (M − 1, *m/z* 847 and 993) can be regarded as products formed by reduction of the diacetylated hydrazone. The position of the additional acetyl group remains undefined.

The occurrence of four compounds in the corresponding Fractions A of **2** and **3** clearly indicates that during the hydrazinolysis several competitive reactions had taken place as outlined in Scheme 7. Starting from 2-acetamido-2-deoxy-D-glucose hydrazone **29**, resulting from the treatment of **2, 3** with hydrazine *via* **28**, a Wolff–Kishner-type reduction may take place at C-1, as indicated by the presence of the 2-acetamido-1,2-dideoxyglucitol derivatives **9** and **11** having mol. wt. 734 and 880, respectively. The hydrazone **29** may also react with an excess of hydrazine in an osazone-type reaction to give **30**, as already proposed by Tang and Williams[7]. Through a six-membered transition state (**27**), the rearrangement of osazone **30** would lead to the 1-deoxy-D-fructose hydrazone derivatives **25** and **26**, which would

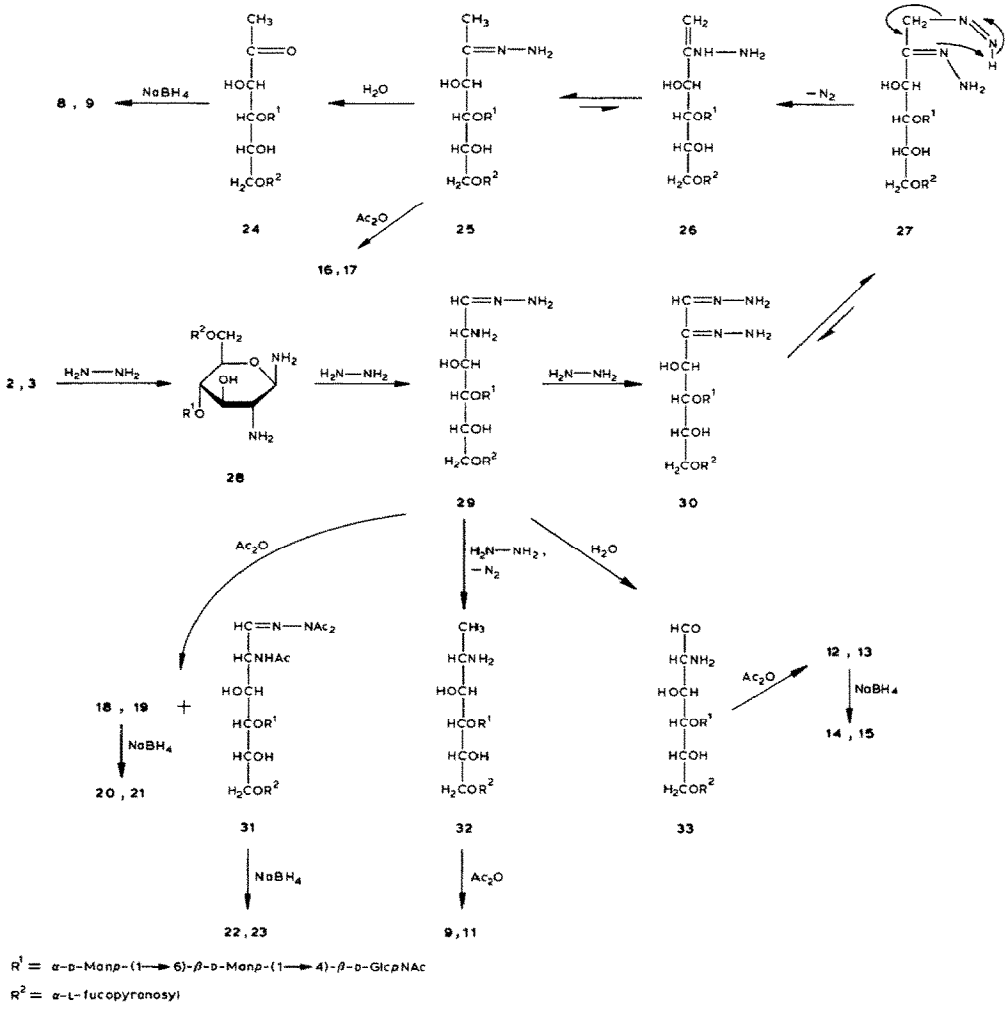


Scheme 6. Mol. wts. and corresponding structures of oligosaccharides produced by sequential hydrazinolysis, *N*-reacetylation, and reduction of **2** and **3**.
*Not detected.

be hydrolyzed to the 1-deoxy-D-fructose derivatives **24**. Finally, reduction would give a mixtures of epimers of 1-deoxy-D-glucitols (**8** and **9**, or **5** from compound **1**). On the other hand, *N*-reacetylation of 1-deoxy-D-fructose hydrazone **25** would furnish compounds **16** and **17**.

These pathways are consistent with the presence of the Wolff–Kishner-reduction compound **6**, and with the results of Tang and Williams[7] who found that, under the conditions of hydrazinolysis, 2-acetamido-2-deoxy-D-glucose hydrazone is converted into 1-deoxy-D-fructose hydrazone. The products having mol. wts. 788 and 934 (788 + L-fucosyl residue) remain unidentified.

The quantitative formation of 2-acetamido-2-deoxy-D-glucitol-, 2-acetamido-1,2-dideoxy-D-glucitol-, 1-deoxyhexitol-, and 2-acetamido-1-acetylhydrazo-2-deoxy-D-glucitol-containing oligosaccharides in the ratios ~30:2:3:15 is in good agreement with that obtained after methylation of reduced oligosaccharides derived from **1** (Fig. 2). Recently, it has been shown that hydrazinolysis catalyzed by hydrazinium sulfate is superior to the longer time, uncatalyzed reaction, as judged by the proportion of degradation products[10]. In order to compare the yields of reducing 2-acetamido-2-deoxy-D-glucitol residue recovered after the catalyzed and uncatalyzed reactions, the hydrazinolysis of **3** was performed for 8, 16, and 30 h, in the presence or absence of hydrazinium sulfate. T.l.c. analysis of the *N*-



Scheme 7. Proposed reaction-pathways during hydrazinolysis, *N*-reacetylation, and reduction of **2** and **3**.

reacetylated and reduced oligosaccharides (Fig. 5) showed that some glycoasparagine derivative remained unhydrolyzed after an uncatalyzed reaction of 8 h. The ratio of 2-acetamido-2-deoxy-D-glucitol to 2-acetamido-1-acetylhydrazo-2-deoxy-D-glucitol appears to be the highest after a catalyzed reaction of 30 h.

EXPERIMENTAL

Compounds **1**, **2**, and **3** were isolated from the urine of patients with aspartylglucosaminuria or fucosidosis[8].

Hydrazinolysis was performed in sealed glass tubes for 8 to 30 h at 100°. In

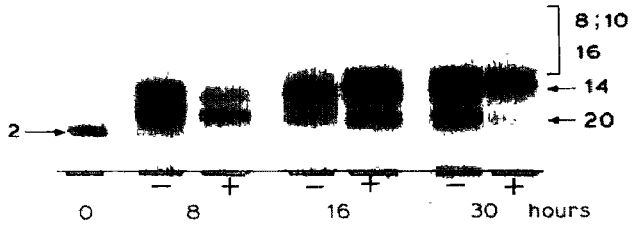


Fig. 5. T.l.c. separation of reduced oligosaccharide resulting from the hydrazinolysis and *N*-reacetylation of **2** at various times, without (−) or with (+) hydrazinium sulfate

some experiments, the reaction was catalyzed with hydrazinium sulfate[10]. Hydrazine was removed under a flow of nitrogen and then *in vacuo* in the presence of H_2SO_4. The residue was dissolved in saturated $NaHCO_3$ (1 mL for 2 mg of material), and acetic anhydride was added every 5 min, in 10-μL portions, during 20 min. After a reaction time of 1 h, the products were de-ionized by the addition of Dowex 50-X2 (H^+, 200–400 mesh) cation-exchange resin. Reduction was performed with $NaBH_4$ or NaB^2H_4, and the mixture was kept overnight at room temperature.

Methylation analysis was performed according to Finne *et al.*[9]. The permethylated oligosaccharides were analyzed by g.l.c. in a capillary column (60 m × 0.35 mm) coated with OV-101 (temperature program: 150–300°, 5°/min). T.l.c. analysis was performed on silica plates (SI 60, Merck) in 2:1:1 1-butanol–acetic acid–water and the sugars were detected with 0.2% orcinol in 20% aqueous H_2SO_4. The *N*-reacetylated oligosaccharides resulting from the hydrazinolysis of **1** were fractionated by liquid chromatography (l.c.) on a Radial Pak column [octadecyl C_{18}(14), 5 μm, 0.4 × 15 cm] eluted with distilled water. The *N*-reacetylated oligosaccharides obtained from **1** and **3** were fractionated on a 5 μm Amino AS-5A column (0.4 × 25 cm, Chromatem 33) eluted with 3:1 acetonitrile–water.

F.a.b.m.s. of the *N*-reacetylated and reduced oligosaccharides obtained from **2** and **3** was performed with a VG Analytical ZAB–HF reversed-geometry mass-spectrometer. The samples (5 μg) were applied to the target in aqueous solution; 1:1 glycerol–1-mercapto-2,3-propanediol (EGA Chemie, Strasbourg, F. 67,000, France) was used as matrix. The target was bombarded with xenon atoms having a kinetic energy equivalent to 9 keV. The spectra were recorded, in negative-ion mode at 7-kV acceleration voltage, in a mass-controlled linear scan at a resolution of 350 p.p.m.

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