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O-DEMETHYLATION OF PER-*O*-METHYL DERIVATIVES OF 2-AMINO-2-DEOXYHEXITOLS DURING ACID HYDROLYSIS AND ACETOLYSIS*

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ABSTRACT

Under conditions of acid hydrolysis, acetolysis, and methanolysis, 2-acetamido-2-deoxy-, and 2-deoxy-1,3,4,5,6-penta-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol and -D-galactitol undergo *O*-demethylation, the *N*-methyl group being preserved. Upon acetylation, mainly 2-acetamido-1-*O*-acetyl-2-deoxy-, and 1-*O*-acetyl-2-deoxy-3,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)hexitol derivatives are formed. The previously postulated 2-(*N*-acetylacetamido)-2-deoxy-1,3,4,5,6-penta-*O*-methylhexitol derivatives were not detected.

INTRODUCTION

Alkaline treatment of glycoproteins of the mucin type in the presence of borohydride is a standard procedure used in the structural analysis of these macromolecules[2]. From material of animal origin, complete carbohydrate-chains possessing a terminal 2-acetamido-2-deoxy-D-galactitol residue are usually released upon such treatment. The structure of the carbohydrate chain isolated can then be established after identification by gas–liquid chromatography–mass spectrometry of partially methylated alditol acetates[3], which are formed upon methylation[4], acid hydrolysis, borohydride treatment, and acetylation. 2-Acetamido-2-deoxy-hexosides and -hexitols are *N*-methylated during the process[5]. Hydrolysis of the permethylated polysaccharide chain may present difficulties if it contains amino sugars other than the terminal 2-amino-2-deoxy hexitol residue, because of the fairly drastic conditions usually employed to cleave the glycosidic bonds of 2-amino-2-deoxyhexosides. To overcome this difficulty, an acetolysis–hydrolysis procedure has been elaborated[6] that appeared to give well-nigh quantitative recovery of neutral as well as of amino sugars. However, reports from two laboratories claimed that the acetolysis procedure caused partial *N*-demethylation of the aminosugars, and that *N*-acetylacetamidohexitol derivatives were formed from the totally or partially methylated 2-amino-2-deoxyhexitols during

*A preliminary communication on this work has appeared[1].

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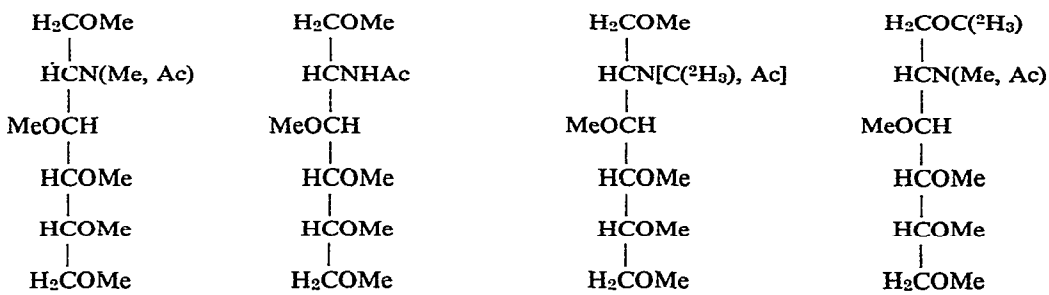
the acetylation step carried out with acetic anhydride[7] for 2 h at 100°, or with acetic anhydride–pyridine[8] for 30 min at 80°.

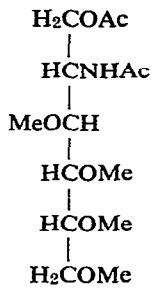
These results appeared somewhat unexpected, as *N*-demethylation of *N*-methylalkylamines does not occur readily, and as the formation of *N*-acetylacetamido derivatives usually requires more-stringent conditions[9] than those just mentioned. In view of the importance of partially methylated alditol acetates in biochemical analysis, the reactions discussed earlier were re-investigated. The results obtained establish that whereas neither acetolysis nor strong acidic hydrolysis with 4M hydrochloric acid for 6 h at 100° cause any appreciable *N*-demethylation of methylated 2-deoxy-2-(*N*-methylacetamido)hexitol derivatives, upon acetolysis, acid hydrolysis, or even methanolysis, the *O*-methyl group at C-1 is partially or entirely lost. *N*-Acetylacetamidohexitol derivatives could not be detected; the compound to which this structure was assigned is in fact a 1-*O*-acetyl-2-deoxy-3,4,5,6-tetra-*O*-methyl 2-(*N*-methylacetamido)-hexitol.

RESULTS AND DISCUSSION

In accord with previous observations[7,8], when 1,3,4,5,6-penta-*O*-methyl-*N*-methylacetamido-glucitol (**1**) was submitted to the acetolysis–hydrolysis procedure of Stellner *et al.*[6], and then acetylated, its quantitative transformation into an apparently homogeneous 2-amino-2-deoxyhexitol derivative was observed by gas–liquid chromatography. The lower values (m/e <202) in the mass spectrum of this derivative were qualitatively (but not quantitatively) very similar to those published[7,8] for 2-(*N*-acetylacetamido)-2-deoxy-1,3,4,5,6-penta-*O*-methyl-D-glucitol. Although for the higher values, that of the ion M + 1 at m/e 336 (1.8%) was in agreement with the calculated molar mass (335), a fragment at m/e 275 (7.8%), indicating formal loss of acetic acid (M — 60), was not compatible with the suggested *N*-acetylacetamido grouping but suggested instead the presence of an *O*-acetyl group. The presence of the latter was also suggested by the ions at m/e 158 (26.6%; $AcOCH_2CHNMeAc^+$) and m/e 98 (the base peak; 158 — 60, $CH_2{=}CNMeAc^+$), which may derive from a 1-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)hexitol derivative. It is noteworthy that previous authors have not observed the M + 1 and M — 60 peaks.



1 **2** **3** **4**



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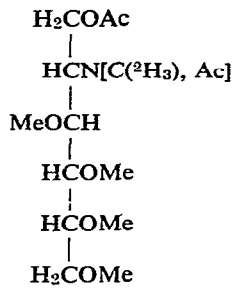

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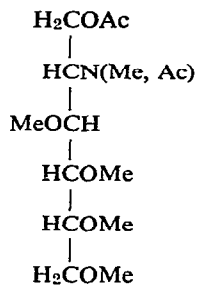

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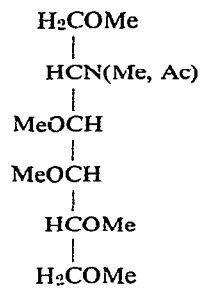

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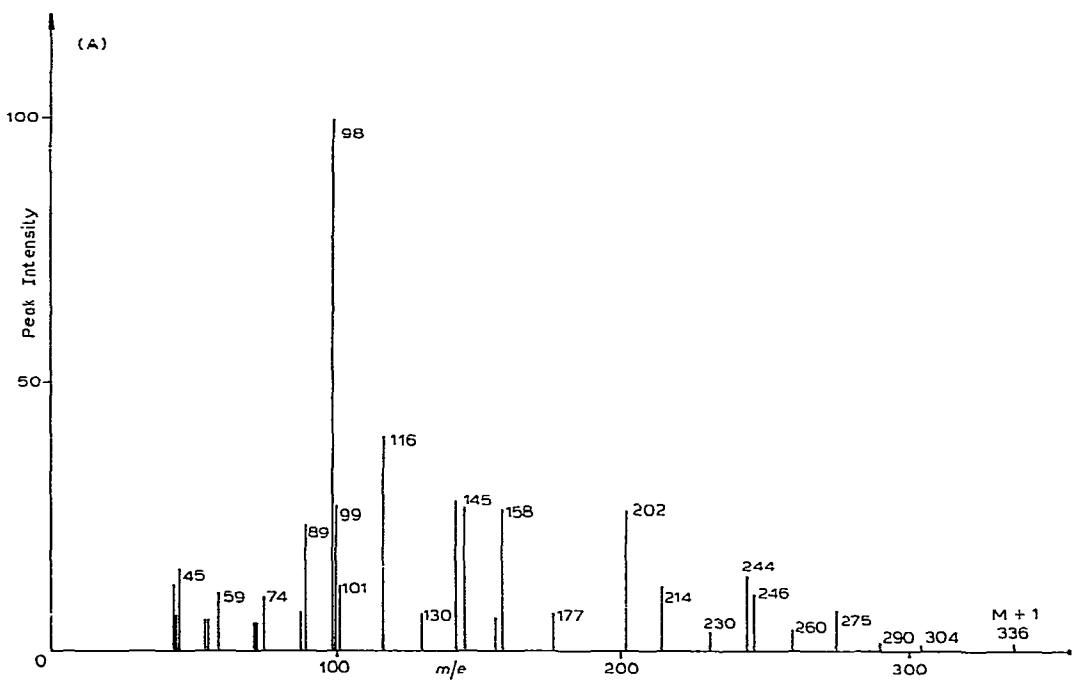
The presence of an *O*-acetyl group in the molecule was confirmed by first treating the material with methanolic ammonia solution in order to remove any *O*-acetyl group (this treatment will not remove *O*-methyl groups, and *N*-acetyl groups are likely to be removed only if a neighboring, free hydroxyl group were present in the molecule), and then methylating with methyl-d_3 iodide–silver oxide in order to mark the position set free during *O*-deacetylation. The single compound detected upon analysis by gas–liquid chromatography–mass spectrometry of the re-acetylated material had the mass spectrum (see **4**, Table I) of a permethylated *N*-(methyl-acetamido)hexitol bearing a trideuteriomethyl group. The introduction of the trideuteriomethyl group on one of the primary alcohol groups of the hexitol was deduced from the presence of a relatively strong (15.6%) peak at *m/e* $(CH_2OCD)_3^+$;



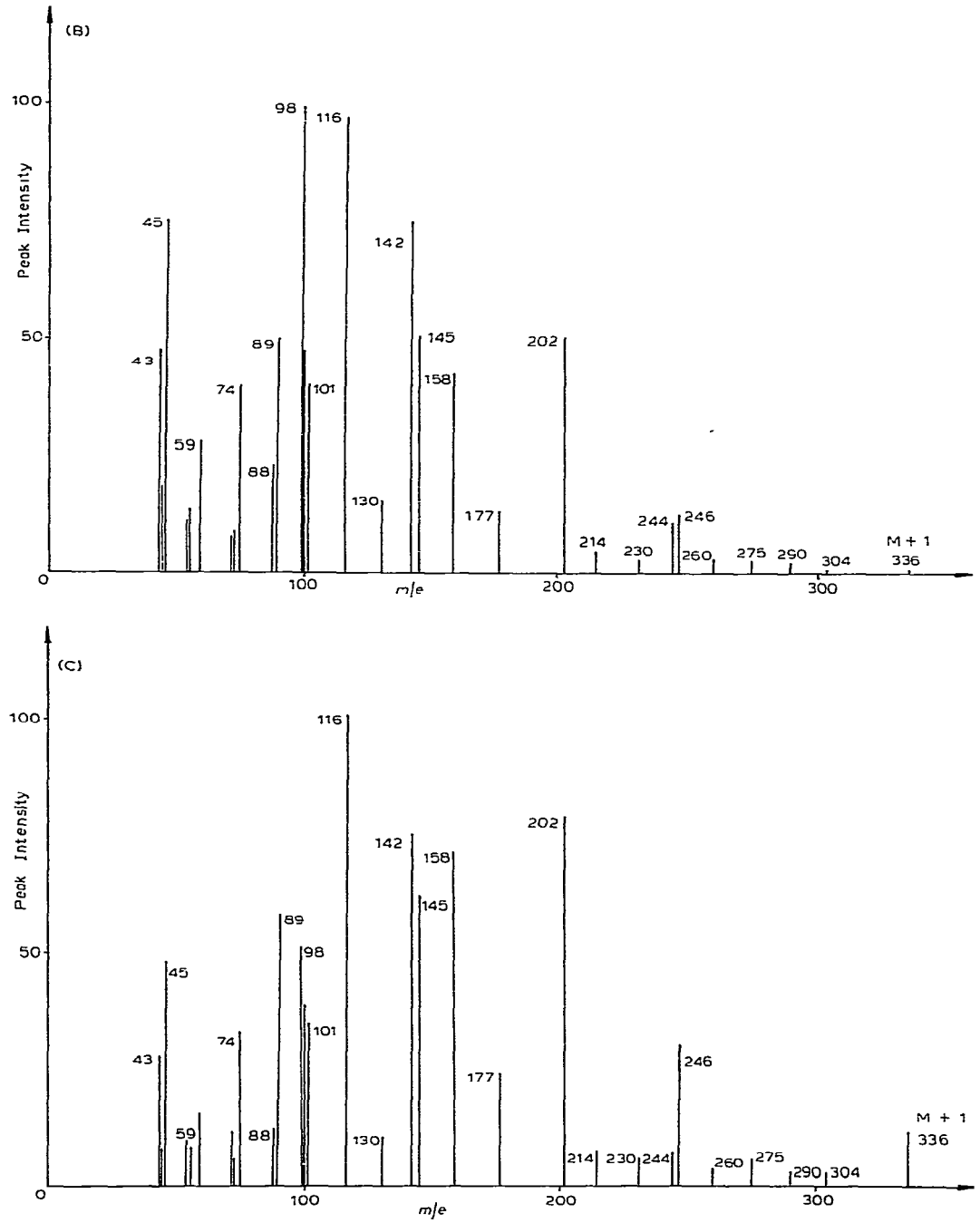


Fig. 1. Variation of the mass spectrum of 1-*O*-acetyl-2-deoxy-3,4,5,6-tetra-*O*-methyl-2-(*N*-methyl-acetamido)-D-glucitol (7) as a function of the g.l.c. column used: (A) 3% SE-30 on Varaport 30 (100–120 mesh), 140–220°, 2°/min. The other parameters are given under Materials and Methods. (B). 3% OV-225 on Gas Chrom Q (100–120 mesh), 190–240°, 1°/min. See legend to Fig. 1(A). (C). 3% OV-1 on Gas Chrom Q (100–120 mesh), 190–240°, 1°/min. See legend to Fig. 1(A).

TABLE I

MASS SPECTRA (*m/e*) OF **1–7**[a]

Compound **1**	**2**	**3**	**4**	**5**	**6**	**7**
43	43	43	43	43	43	43
(11)	(25)	(12)	(31.6)	(99.3)	(26.4)	(12.2)
45	45	45	45	45	45	45
(25)	(100)	(47)	(63.8)	(80)	(29.6)	(14.8)
56	59	59	48	59	59	56
(9)	(20)	(18.7)	(15.6)	(23)	(18.7)	(5.8)
59	74	60	56	60	60	57
(10.3)	(50.3)	(5)	(20.6)	(14.8)	(11)	(5.8)
71	86	61	59	71	71	59
(6.5)	(16)	(3)	(15.5)	(20)	(9)	(11)
75	89	71	71	74	73	70
(6.5)	(38.7)	(8.3)	(13.5)	(40)	(9.6)	(5)
88	101	73	74	84	77	71
(84)	(57.4)	(5.8)	(11.6)	(46.4)	(22.5)	(5)
89	116	75	88	89	89	74
(35)	(43.8)	(8.3)	(29)	(60)	(38.7)	(9.7)
98	128	79	89	101	101	88
(2.6)	(99.3)	(3.2)	(47)	(73.5)	(100)	(7)
99	133	88	91	102	102	89
(9.6)	(16.7)	(6.5)	(100)	(21)	(33.5)	(24)
100	142	89	99	116	119	98
(5.8)	(12.5)	(27)	(22.5)	(22.5)	(67.7)	(100)
101	145	90	101	128	133	99
(31)	(47)	(5.8)	(41.3)	(100)	(11.6)	(27)
130	160	91	104	129	145	101
(100)	(66.4)	(72)	(10.3)	(18.7)	(93.5)	(11.6)
142	172	92	130	133	161	116
(56.8)	(9.6)	(5.8)	(15)	(13.5)	(45.8)	(40)
145	174	94	133	142	177	130
(58.7)	(16)	(5.8)	(98.7)	(23.2)	(14)	(7)
174	177	101	142	145	205	142
(57)	(16)	(33.5)	(74)	(40.6)	(50.3)	(28)
177	184	102	145	160	217	145
(11)	(28.3)	(14.8)	(65.8)	(19.3)	(11.6)	(26.4)
198	204	103	148	166	247	157
(6.5)	(75)	(8.3)	(10.3)	(10.3)	(12.2)	(5.8)
218	216	133	174	170	249	158
(24)	(37.4)	(94.8)	(8.4)	(11.6)	(19.3)	(26.6)
230	248	134	177	173	293	177
(18)	(31.6)	(6.5)	(73.4)	(13)	(1.3)	(7)
244	262	136	221	177	307	202
(5.8)	(1.3)	(5)	(23.8)	(13.5)	(0.6)	(26.4)
262	294	145	230	184	339	214
(20)	(3.2)	(100)	(20.6)	(21)	(1.3)	(11.6)
276		146	246	188		230
(1.3)		(6.45)	(3.2)	(38)		(3.2)
308		147	262	200		244
(1.3)		(5)	(24.5)	(11)		(14)
		148	265	202		246

TABLE I *(continued)*

Compound			
3	4	5	7
(5)	(3.2)	(16)	(10.3)
157	311	205	258
(4.5)	(1.9)	(33.5)	(1.3)
158		216	260
(1.9)		(26.4)	(3.8)
159		232	275
(4.5)		(53.5)	(7.8)
177		244	290
(55.4)		(16)	(1.9)
178		276	304
(5.8)		(16)	(1.3)
180		290	336
(7)		(1)	(1.3)
189		322	
(1.3)		(1.9)	
191			
(1.3)			
201			
(2.6)			
221			
(24.5)			
233			
(14.2)			
246			
(5.8)			
265			
(25)			
279			
(1.9)			
311			
(1.3)			

[a]In parentheses, %.

its presence at O-1 was unambiguously established by the appearance of peaks at *m/e* 177 (73.4%; $CH_2OCD_3CHNMeAcCHOCH_3^+$) and 133 (98.7%; CH_2OCD_3-$CHNMeAc^+$), analogs of those at *m/e* 174 (57%) and 130 (100%) observed for the nondeuterated reference compound **1**.

Finally, conservation of the *N*-methyl group during treatment with acid was unambiguously established. 2-Deoxy-1,3,4,5,6-penta-*O*-methyl-2-[*N*-($^2H_3$)methyl-acetamido]-D-glucitol (**3**) was treated with 4M, hydrochloric acid, and then acetylated. In the mass spectrum (see **6**, Table I) of the compound obtained, the fragments $AcOCH_2CHNMeAc^+$ and $CH_2{=}CNMeAc$, which in the nondeuterated compound **7** have *m/e* 158 and 98, respectively (Table I), were shifted to 161 and 101, thus proving the retention of the trideuteriomethyl group in those fragments. The observa-

tion that loss of a methyl group also took place upon treatment with acid of 2-acetamido-2-deoxy-1,3,4,5,6-penta-*O*-methyl-D-glucitol, a compound that cannot undergo *N*-demethylation, confirmed that per-*O*-methylated 2-amino-2-deoxy alditol derivatives are susceptible to *O*-demethylation.

Because of the importance of *O*-methyl derivatives of 2-acetamido-2-deoxy-D-galactitol for the structural analysis of glycoproteins of animal origin, 2-deoxy-1,3,4,5,6-penta-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol (**8**) was also submitted to treatment with acid; the results (see Table II) were very similar to those obtained for the D-glucitol derivative.

Thus, it appears that the 1-*O*-methyl group in 2-deoxy-2-(*N*-methylacetamido)-hexitol derivatives is quite labile; it is quantitatively removed under conditions often used in the analysis of glycoproteins (Table II). Cleavage of the 1-*O*-methyl group is much faster when the hexitol bears a secondary amine group; under conditions that quantitatively *O*-demethylate at C-1 2-deoxy-per-*O*-methyl-*N*-(methylacetamido)-hexitols, only 30% of *O*-demethylation is observed for per-*O*-methylated *N*-acetamido-2-deoxy-D-glucitol.

The apparently selective *O*-demethylation at C-1 is rather unexpected. As OCH_3-1 and -3 may assume the same steric position with respect to NH_2-2, at least partial *O*-demethylation at C-3 would also be expected. While the values given for **7** and **6** (Table I) leave no doubt that the main site of *O*-demethylation is C-1, it could not be established by the present study whether *O*-demethylation at C-3 also occurred. Indeed, it is well known[10] that partially methylated alditol acetates are difficult to separate by gas–liquid chromatography unless efficient capillary columns (not available to us) are used. It is, therefore, possible that 3-*O*-acetyl-2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol was in fact present, but was not separated from the isomeric 1-acetate.

TABLE II

PROPORTION (%) OF ACID-CATALYZED *O*-DEMETHYLATION OF *N*-ACETAMIDO- AND *N*-METHYLACETAMIDO-PER-*O*-METHYLHEXITOLS AT 100°

Compound	*Acid conditions*	*Time (h)*			
		1	*2*	*4*	*6*
1	M HCl	5	8	41	
	2M HCl	13	26	65	
	4M HCl	52	83	100	
	6M HCl in MeOH[a]		50		
2	4M HCl				30
8	4M HCl	40	81	100	
1[b]	Acetolysis	100			
8[b]	Acetolysis	100			

[a]At 80°. [b]See conditions in ref. 5.

In the absence of authentic samples of both isomers, it was not feasible either to establish with certainty whether the mass spectra of the acetylated or trideuteriomethylated compounds obtained upon acid treatment of the permethylated *N*-(methylacetamido)-D-glucitol or -D-galactitol represent single isomers. It has been reported[6,10] that upon gas–liquid chromatography–mass spectrometry derivatives of amino sugars undergo uncontrolled decomposition and that, therefore, their mass spectra are not as simple as those of partially methylated alditol acetates. It was also observed, during the present study, that the mass spectrum of the same compound varied very considerably as a function of the column (SE-30 on Varaport 30, and OV-225 and OV 1 on Gas Chrom Q) used (Fig. 1). It is interesting to note that when gas–liquid chromatography was carried out with the OV-1 column, the base peak for **7** was at *m/e* 116, as found[7,8] for the "*N*-acetylacetamido" compound, whereas with our SE-30 and OV-225 columns, *m/e* 98 appeared as the base peak.

EXPERIMENTAL

General methods. — Mass spectra were recorded with a DuPont 21-492 B double-focussing, medium-resolution spectrometer coupled to a Varian Aerograph, Model 2700 gas–liquid chromatograph; the mass spectra presented are those of products introduced through the gas chromatograph. Gas–liquid chromatography was performed on a stainless-steel column (3.2 × 1500 mm) packed with 3% methyl silicone SE-30 on Varaport 30 (100–120 mesh) under a temperature gradient (140–220°, 2°/min); carrier gas, He (40 mL/min); injector at 220°, detector at 250°, and He separator (glass, jet type) at 220°; electron-beam energy, 75 eV; ionizing current, 250–300 μA; and source temperature, 240°.

2-Deoxy-1,3,4,5,6-penta-O-*methyl-2*-(N-*methylacetamido*)-D-*glucitol* (**1**). — 2-Acetamido-2-deoxy-D-glucitol (10 mg) was methylated with dimethylsulfinylsodium and methyl iodide[4] as described by Jansson *et al.*[11]; after addition of water (10 mL), the methylated product was extracted into dichloromethane (3 × 6 mL), the solvent was removed, and the residue dried from the frozen state. It was then dissolved in ethyl acetate and analyzed by g.l.c.–m.s.: single peak; mass spectrum: see Table I. Its retention time (~10 min) was used as reference throughout this work.

2-Acetamido-2-deoxy-1,3,4,5,6-penta-O-*methyl*-D-*glucitol* (**2**). — To 2-acetamido-2-deoxy-D-glucitol (6 mg) dissolved in dry *N,N*-dimethylformamide (1.4 mL) was added barium oxide (546 mg) and barium hydroxide octahydrate (158 mg), and the mixture was stirred for 2.5 h at 0°. Methyl iodide (30 μL) was added and stirring was continued for another 2.5 h at 0°. The mixture was diluted with chloroform (150 μL) and centrifuged, and the supernatant recovered. The sediment was washed three times with chloroform, the chloroform extracts were pooled, and the solvent was removed. The residual syrup was lyophilized; the dried residue was dissolved in a small amount of ethyl acetate and analyzed by g.l.c.–m.s.: single peak (R_1 0.7); mass spectrum: see Table I.

2-Deoxy-1,3,4,5,6-penta-O-*methyl-2*-[N-($^2H_3$)*methylacetamido*]-D-*glucitol* (**3**).

— Compound **2** (1 mg) was methylated as described for **1**, but with ($^2H_3$)methyl iodide. The product was analyzed by g.l.c.–m.s.: single peak (R_1 1); mass spectrum: see Tabie I.

O-Demethylation of **1**. — (a) *By hydrolysis with acid.* Compound **1** (0.5 mg) was treated with 4M hydrochloric acid for 6 h at 100° in a sealed tube. The solvents were removed and dry toluene was evaporated from the residue several times. The dried residue was then acetylated by treatment with acetic anhydride (0.5 mL) and sodium acetate (5–10 mg) for 1 h at 100°. After removal of volatile material (water-pump vacuum), toluene was evaporated several times from the residue, which was finally extracted with ethyl acetate. After evaporation, the residue was analyzed by g.l.c.–m.s.: single peak (R_1 1.26); mass spectrum: see Table I (7). The presence of a peak at *m/e* 275 (M — 60) strongly suggested the presence of an *O*-acetyl group in this compound.

(b) *By acetolysis.* Compound **1** (0.5 mg) was subjected to the conditions of acetolysis described by Stellner *et al.*[6], except that the hydrolyzate was not percolated through an ion-exchange column but was neutralized (pH 5–6) by addition of barium carbonate to the vigorously stirred mixture. Solids were removed by centrifugation, the sediment was washed twice with methanol, and the supernatants were combined and dried by addition–evaporation of toluene. The residue was treated with acetic anhydride and sodium acetate, and the reaction mixture processed as described under (*a*). The ethyl acetate extract was analyzed by g.l.c.–m.s.: single peak (R_1 1.26); mass spectrum: identical to that of **7** (see Table I).

(*c*) *By methanolysis.* Compound **1** (0.5 mg) was treated with 6M hydrogen chloride in methanol for 2 h at 100° in a sealed tube. The mixture was processed as described under (*a*). Two peaks were observed by g.l.c.–m.s.: the first (R_1 1) showed the same mass spectrum as **1**; the second (R_1 1.26) showed a mass spectrum identical to that of **7** (see Table I). Both compounds were present in about equal amounts.

Proof of O*-demethylation occurring during the acid treatment of* **1** *with formation of* **4**. — Compound **1** (0.5 mg) was treated with 4M hydrochloric acid and acetylated as described earlier. The resulting compound was dissolved in a saturated solution of anhydrous ammonia in methanol, and the mixture was kept overnight at 0°. After removal of volatile material *in vacuo*, the residue was dried by addition and evaporation of toluene, and then treated overnight with [$^2H_3$]methyl iodide (1 g) and silver oxide (30 mg) with vigorous stirring at 45° in a Teflon-capped tube. The warm mixture was diluted with warm ethyl acetate and then cooled. Solids were removed by centrifugation, and the sediment was washed several times with ethyl acetate. The supernatants were combined, the solvent removed, the residue taken up in ethyl acetate (50 μL), and the solution was analyzed by g.l.c.–m.s.: single peak (R_1 1); mass spectrum: see Table I (4).

Proof of conservation of N*-methyl group in* **3** *during acid treatment.* — Compound **3** (0.5 mg) was treated with 4M hydrochloric acid and then acetylated as described for **1**. G.l.c.–m.s.: single peak (R_1 1.26); mass spectrum: see Table I (6).

O*-Demethylation of* **2** *with formation of* **5**. — Compound **2** (1 mg) was treated

with 4M hydrochloric acid for 6 h at 100°. After removal of volatile material, toluene was added to and evaporated from the residue several times; the residue was then acetylated, and the resulting material analyzed by g.l.c.–m.s. Two peaks were observed. The material corresponding to the first (70%) peak was unchanged **2** (R_1 and mass spectrum identical to those of **2**). The material corresponding to the second peak was **5** (R_1 1); mass spectrum: see Table I (**5**).

O-Demethylation of 2-amino-2-deoxy-per-O-methylhexitol derivatives under various conditions of acidity. — (*a*) Aliquots of **1** were treated with 4M, 2M, and M hydrochloric acid for 1, 2, and 4 h at 100°. The samples were dried, acetylated, and analyzed by g.l.c.

(*b*) A sample of **2** was treated with 4M hydrochloric acid for 6 h, at 100°, and the material analyzed as described earlier.

(*c*) A sample of **8** (prepared from 2-acetamido-2-deoxy-D-galactitol as described for the D-glucitol derivative) was treated with 4M hydrochloric acid for 1, 2, and 4 h at 100°, and the material obtained analyzed as described earlier.

(*d*) Samples of **1** and of the corresponding D-galactitol derivative were subjected to the conditions of acetolysis described by Stellner *et al.*[6] and the acetylated material analyzed. Results are given in Table II.

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