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DETERMINATION (BY METHYLATION ANALYSIS) OF THE SUBSTITUTION PATTERN OF 2-AMINO-2-DEOXYHEXITOLS OBTAINED FROM *O*-GLYCOSYLIC CARBOHYDRATE UNITS OF GLYCOPROTEINS

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ABSTRACT

The derivatives obtained by permethylation of unsubstituted 2-amino-2-deoxyhexitols and of these compounds monosubstituted at C-3, C-4, or C-6, and disubstituted at C-3 and C-6, have been analysed by g.l.c.–m.s. Each derivative can be identified on the basis of retention time and mass spectrum. In methylation analysis, methanolysis gave one derivative of each hexitol, whereas a mixture of products was formed when degradation was effected by acetolysis followed by hydrolysis. An application in the analysis of amino-sugar linkages in alkali-labile *O*-glycosylic oligosaccharides from rat-brain glycoproteins is described.

INTRODUCTION

Methylation analysis is widely used in the structural analysis of complex carbohydrates. Improved permethylation methods[1] and the use of g.l.c.–m.s. for the identification of the methylated sugars have made possible the linkage analysis of complex carbohydrates containing neutral sugars[2–5], amino sugars[6–10], and neuraminic acids[11].

The *O*-glycosylic carbohydrate chains of glycoproteins are conveniently isolated by treatment[12] with $NaOH–NaBH_4$, whereby the amino sugar linked to the protein is reduced. Analysis of the substitution pattern of the reduced amino sugars by methylation has revealed difficulties. In addition to *N*-methylacetamido derivatives, *N*-acetylacetamido derivatives are also formed[13] if the methylated saccharide is degraded by acetolysis followed by acid hydrolysis and acetylation. Although a fully *O*-methylated 2-amino-2-deoxyhexitol gives almost exclusively the latter derivative, the extent of its formation from other methylated derivatives is dependent on the pattern of substitution. However, this complication is avoided if degradation is effected by methanolysis.

RESULTS

Analysis of unsubstituted 2-amino-2-deoxyhexitols

When 2-acetamido-2-deoxy-D-galactitol or 2-acetamido-2-deoxy-D-glucitol were permethylated (Hakomori) and the products subjected to g.l.c. (Fig. 1*A*), only one peak was observed, and the mass-spectral data (Table I) indicated the expected structures, namely, 2-deoxy-1,3,4,5,6-penta-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol and -D-glucitol[13] (**1**). When each of these compounds was subjected to acetolysis–acid hydrolysis[7], a new major product was observed (Fig. 1*B*), the mass-spectral data of which (Table I) revealed a molecular weight of 251, as indicated by the ions at *m/e* 220 (M − 31), 188 (M − 31 − 32), and 174 (M − 45 − 32). The low intensity of the peak at *m/e* 43 indicates the absence of acetyl groups, and the strong peak at *m/e* 74 reflects an unsubstituted amino group. In samples treated with CH_3COOD, this ion was shifted to *m/e* 75 and 76, thus confirming the presence of an unsubstituted amino group. The fragments at *m/e* 45, 89, and 133 are also consistent with the structures 2-amino-2-deoxy-1,3,4,5,6-penta-*O*-methyl-D-galactitol and -D-glucitol (**2**). Thus, both *N*-methyl and *N*-acetyl groups are cleaved during the acetolysis–acid hydrolysis procedure.

After acetylation of these amino derivatives, one peak was observed in g.l.c. (Fig. 1*C*), and the mass-spectral data (Table I) indicated the products to be 2-(*N*-acetylacetamido)-2-deoxy-1,3,4,5,6-penta-*O*-methylhexitols[13] (**3**).

Since the acetolysis–acid hydrolysis procedure cleaves the *N*-methyl group from methylated 2-amino-2-deoxyhexitols and their 4- and 6-*O*-derivatives[13] to various extents, the interpretation of methylation analysis data is difficult. Therefore, we have studied degradation by methanolysis. The products obtained from permethylated 2-acetamido-2-deoxy-D-galactitol and the D-glucitol analogue, after methanolysis and acetylation, were identified as the *N*-methylacetamido derivatives (**1**) from their relative retention times and mass spectra, indicating that the *N*-methyl and the *N*-acetyl groups were both resistant to methanolysis.

Analysis of 2-amino-2-deoxyhexitols monosubstituted at C-3, C-4, or C-6

Disaccharides containing a 2-amino-2-deoxy-D-galactitol or 2-amino-2-deoxy-D-glucitol moiety, with substitution at C-3, C-4, or C-6, were subjected to permethylation, methanolysis, and acetylation. Each disaccharide gave only one hexitol derivative. In the mass spectra of all these compounds (Table I), the intense peak at *m/e* 130 indicates the presence of an *N*-methylacetamido group at C-2. The base peak of these compounds is formed by elimination of ketene (42) from the *N*-acetyl moiety of this fragment. Thus, the stability of the *N*-methyl group in these compounds towards methanolysis is not affected by the site of *O*-substitution.

The fragmentation of the methylated 2-amino-2-deoxyhexitols can be generally explained by the rules established for neutral hexitols[14,15], and are shown in Scheme 1. The mono-*O*-acetyl derivatives **4**–**6** are all characterised by a prominent ion at *m/e* 290 produced by cleavage of a primary methoxylated carbon. This ion is

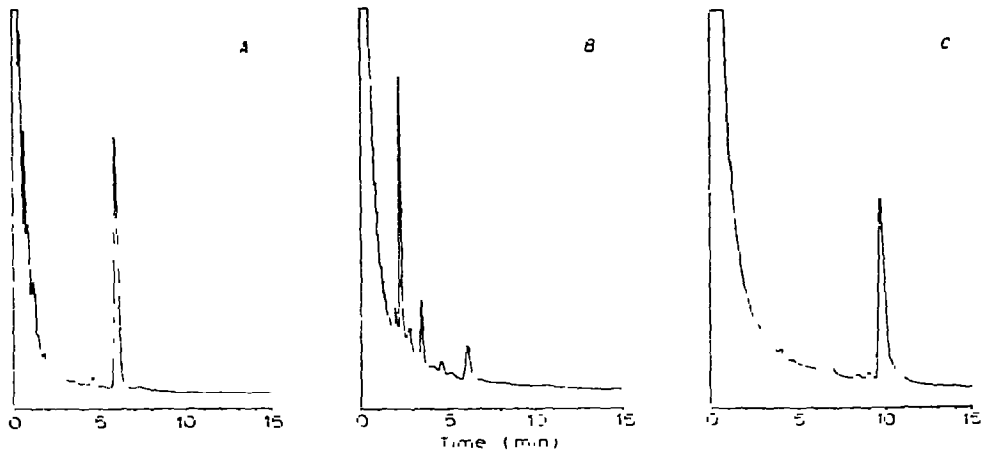


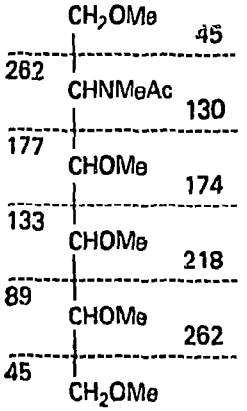
Fig. 1. Gas chromatogram (2.2% of SE-30, 180°) of methylated galactosaminitols *A*, permethylated 2-acetamido-2-deoxy-D-galactitol; *B*, after acetolysis and acid hydrolysis of *A*; *C*, after acetylation of *B*.

TABLE I

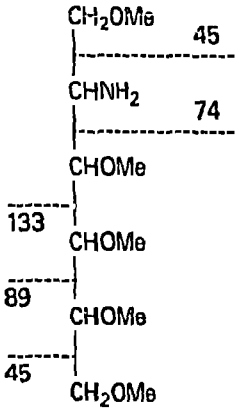
MASS-SPECTRAL DATA FOR THE 2-AMINO-2-DEOXYHEXITOL DERIVATIVES (1–7) SHOWN IN SCHEME 1[a]

m/e	*Relative intensities (% of base peak)*						
	1	**2**	**3**	**4**	**5**	**6**	**7**
43	17	7	29	47	58	62	100
45	47	40	34	71	35	20	17
74		100	30				
88	100	19	16	100	100	100	73
89	36	6	39	14	38	8	10
101	45	49	31	31	47	61	60
116			100				
117						23	15
130	87		10	68	91	89	52
133	8	6	5	17			
142	35		34	20	16	26	10
145	29		35				
158			43				
161						19	14
174	27	2				22	
177	15		22				
188		3					
202	2		26	4			
205					39		
218	14					12	
220		13					
246			16	31			18
262	15						
290			4	30	13	7	
318							20

[a]Partial data, including fragments relevant to the identification of the compounds.



1



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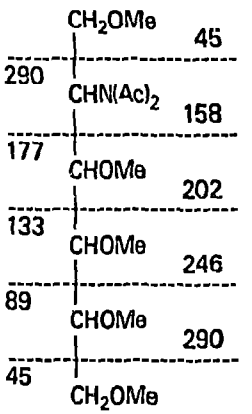

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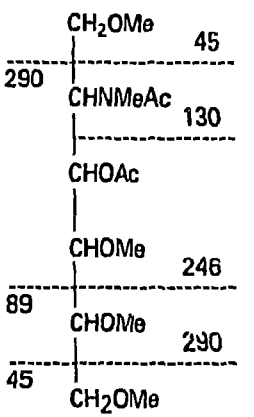

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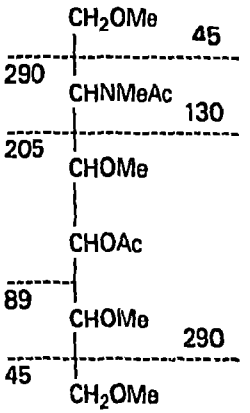

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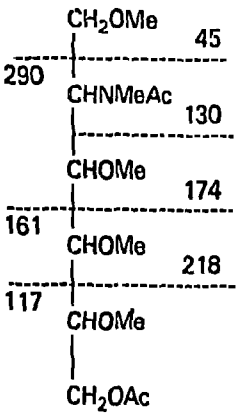

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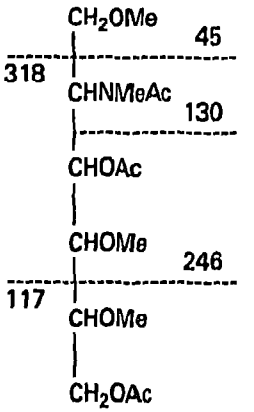

7

Scheme 1. Fragmentations of some 2-amino-2-deoxyhexitol derivatives.

analogous to that at *m/e* 262 from the fully methylated 2-amino-2-deoxyhexitols **1**. The location of the acetyl groups in **4–6** can be established from the following, prominent primary fragments: *m/e* 246, 3-acetate; *m/e* 205, 4-acetate; *m/e* 117, 161, 174, and 218, 6-acetate. Secondary fragments arise through single or consecutive eliminations of methanol (32), ketene (42), and acetic acid (60). The relative retention times of the methylated 2-amino-2-deoxyhexitols are listed in Table II.

TABLE II

RELATIVE RETENTION TIMES OF SOME 2-AMINO-2-DEOXYHEXITOL DERIVATIVES

Compound	*Stationary phase*	
	2.2% of SE 30 (180°)	*1% of OV-225 (190°)*
2-Deoxy-2-(N-methylacetamido)-D-glucitol		
1,3,4,5,6-Penta-*O*-methyl-	1.00	1.00
3-*O*-Acetyl-1,4,5,6-tetra-*O*-methyl-	1.76	2.39
4-*O*-Acetyl-1,3,5,6-tetra-*O*-methyl-	1.58	1.97
6-*O*-Acetyl-1,3,4,5-tetra-*O*-methyl-	1.75	2.17
2-Deoxy-2-(N-methylacetamido)-D-galactitol		
1,3,4,5,6-Penta-*O*-methyl-	1.21	1.27
3-*O*-Acetyl-1,4,5,6-tetra-*O*-methyl-	1.69	2.29
4-*O*-Acetyl-1,3,4,5-tetra-*O*-methyl-	2.11	2.93
3,6-Di-*O*-acetyl-1,4,5-tri-*O*-methyl-	3.16	6.83

In order to study the extent of *N*-deacetylation during methanolysis, the samples were treated with hexadeuterioacetic anhydride after methanolysis. For the permethylated 2-amino-2-deoxyhexitols and their 6-*O*-substituted derivatives, there was no shift of the ion at *m/e* 130, reflecting the stability of the *N*-acetyl groups towards methanolysis. In contrast, the ion at *m/e* 130 was replaced by one at *m/e* 133 in the 3-*O*- and 4-*O*-substituted derivatives, indicating cleavage of the *N*-acetyl groups. This behaviour does not, however, influence the results of methylation analysis, as the samples are always acetylated after methanolysis and only one product is obtained from each methylated 2-amino-2-deoxyhexitol.

Analysis of brain oligosaccharides

Rat-brain glycopeptides were treated with $NaOH–NaBH_4$, and the liberated oligosaccharides were purified[16] by gel filtration, ion-exchange chromatography, and t.l.c. The neuraminic acid-containing oligosaccharides III, IV, and V were subjected to permethylation, methanolysis, and acetylation. Because of the very small amounts of material available, the relative retention times on SE-30 and OV-225 columns were determined by using mass fragmentography and monitoring of the characteristic ion at *m/e* 130.

The methylated 2-amino-2-deoxyhexitol from oligosaccharide IV was identified as 3-*O*-acetyl-2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)galactitol (**4**) from its relative retention times and mass spectrum, as expected from the reported

structure[16] *O*-(*N*-acetylneuraminyl)-(2→3)-*O*-β-D-galactosyl-(1→3)-2-acetamido-2-deoxy-D-galactitol.

The mass-spectral data for the derivative obtained from oligosaccharide V are given in Table I and are consistent with the structure 7. Thus, the intense peaks at *m/e* 130 and 88 (130−42) indicate the presence of an *N*-methylacetamido group. The intense ion at *m/e* 318 (M−45), arising by cleavage of the primary methoxylated carbon, indicates the presence of two *O*-acetyl groups. This inference is also supported by the high intensity of the peak at *m/e* 43 (CH_3CO^+). The peak at *m/e* 246 is obtained only from 3-acetyl derivatives (see Table I). The low intensity of the ion at *m/e* 45 and the peak at *m/e* 117 indicate that the second *O*-acetyl group is at C-6. These data confirm the reported structure[16] of oligosaccharide V as *O*-(*N*-acetylneuraminyl)-(2→3)-*O*-β-D-galactosyl-(1→3)-*O*-[*N*-acetylneuraminyl-(2→6)]-2-acetamido-2-deoxy-D-galactitol.

One hexitol product was also obtained from oligosaccharide III. The relative retention times were the same as for the corresponding product 7 obtained from oligosaccharide V (Table II), and the mass spectra were identical. Oligosaccharide III has been reported[16] to comprise *O*-β-D-galactosyl-(1→3)-2-acetamido-2-deoxy-D-galactitol and one *N*-acetylneuraminic acid residue of unknown location. The result of the methylation analysis established the structure as *O*-β-D-galactosyl-(1→3)-*O*-[*N*-acetylneuraminyl-(2→6)]-2-acetamido-2-deoxy-D-galactitol.

DISCUSSION

In methylation analysis of complex carbohydrates, the methylated sugars are often analysed as alditol acetates after degradation of the permethylated saccharide by acetolysis followed by acid hydrolysis. Only one derivative is usually obtained from each neutral and amino sugar, but two products (*N*-methylacetamido and *N*-acetylacetamido derivatives) may arise from 2-amino-2-deoxyhexitol moieties as a result of *N*-demethylation.

However, when degradation is effected by methanolysis, only one product is obtained from each methylated 2-amino-2-deoxyhexitol, which differs clearly from other methylated, amino-sugar derivatives in g.l.c.–m.s.

If the oligosaccharide to be analysed contains neuraminic acid residues, their location can be determined from the same sample as that used for investigation of the 2-amino-2-deoxyhexitols, as the preparation methods for both analyses are identical[11]. Also, the neutral[2,3,5] and amino sugars[9,10,17] can be analysed as their methyl glycosides, although the interpretation of the results may be more difficult than for the alditol derivatives.

In the methylation analysis of glycopeptides containing an *O*-glycosylic carbohydrate unit, difficulties may arise because of cleavage of the alkali-labile carbohydrate–peptide linkage and subsequent detachment of substituents at C-3 of the galactosamine moiety[5,18]. It may therefore be more convenient to deliberately cleave the alkali-labile linkages by treatment[12] with $NaOH–NaBH_4$ and then

determine the substitution pattern of the resulting 2-amino-2-deoxyhexitol derivative as described above.

Comparison of the mass-spectral data in Table I with those presented by Hase and Rietschel[13] reveals marked differences in the relative intensities of the larger fragments. The fragments M−45, *i.e.*, 262 and 290, were not detected, in contrast to the present work, and several other fragments in the higher mass region gave much weaker peaks. There are also comparable differences in the mass spectra of amino sugars[7,8]. The spectra with the low intensities in the high *m/e* region were obtained with a quadrupole instrument, whereas the other spectra were recorded with a magnetically scanning mass spectrometer. Thus, the type of instrument used should be taken into consideration in the interpretation and comparison of mass spectra. For the identification of the methyl substitution pattern of hexoses, the presence of signals over 10% of the base peak[14] is widely used. Due to drastic differences in the intensities of many important ions when different instruments are used, this approach cannot be recommended for amino sugar derivatives.

EXPERIMENTAL

Materials. — *O*-β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose, *O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose, and *O*-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucose were kindly supplied by Dr. A. Gauhe (Heidelberg, Germany). Disaccharide alditols, and 2-acetamido-2-deoxy-D-glucitol and -D-galactitol were prepared from the corresponding reducing sugars by reduction with $NaBH_4$. *O*-β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactitol was a gift from Dr. W. T. Shier (San Diego, California). *O*-α-*N*-Acetylneuraminyl-(2→6)-2-acetamido-2-deoxy-D-galactitol was prepared by NaOH–$NaBH_4$ treatment of bovine submaxillary mucin (Type I, Sigma), followed by DEAE-Sephadex chromatography[19].

Methylation analysis. — Methylation was carried out with methyl iodide in methyl sulphoxide in the presence of methylsulphinylmethyl carbanion[1]. The reaction mixture was diluted with water and partitioned with an equal volume of chloroform. The chloroform phase was extracted with water (5×).

The permethylated oligosaccharides were treated with 0.5M methanolic HCl at 80° for 18 h and the solution was concentrated to dryness under nitrogen. The residue was treated with pyridine–acetic anhydride (1:1, 0.5 ml) at 80° for 30 min. After the addition of toluene (1 ml), the mixture was concentrated to dryness, and a solution of the residue in chloroform was analysed by g.l.c.–m.s. For trideuterioacetylation, hexadeuterioacetic anhydride (Merck) was used.

Acetolysis and acid hydrolysis of the permethylated oligosaccharides were performed as described by Stellner *et al.*[7].

For deuteration of free amino groups, the samples were dissolved in CH_3COOD (Merck) and subjected to g.l.c.–m.s. in this solvent.

G.l.c.–m.s. — G.l.c. was performed with a Perkin–Elmer 900 instrument and

columns (2 mm × 2 m) of 2.2% of SE-30 at 180°, or 1% of OV-225 at 190°. G.l.c.–m.s. was performed with a Varian 1700 gas chromatograph coupled to a Varian MAT CH-7 mass spectrometer equipped with a SpectroSystem 100 MS data-processing system and an Altema AL 5 mutiple ion detector. Mass spectra were recorded (m/e 40–400) at 70 eV with an ionization current of 300 μA.

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REFERENCES

1 S.-I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
2 N. K. KOCHETKOV AND O. S. CHIZHOV, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
3 K. HEYNS, H. F. GRUTZMACHER, H. SCHARMANN, AND D. MULLER, *Fortsch. Chem. Forsch.*, 5 (1966) 448–490.
4 J. LONNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
5 J. MONTREUIL, *Pure Appl. Chem.*, 42 (1975) 431–477.
6 H. BJORNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem.*, 82 (1970) 643–651.
7 K. STELLNER, H. SAITO, AND S.-I. HAKOMORI, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
8 T. TAI, K. YAMASHITA, AND A. KOBATA, *J. Biochem (Tokyo)*, 78 (1975) 679–686.
9 S. K. KUNDU AND R. W. LEDEEN, *Carbohydr. Res.*, 39 (1975) 179–191.
10 S. K. KUNDU AND R. W. LEDEEN, *Carbohydr. Res.*, 39 (1975) 329–334.
11 H. RAUVALA AND J. KARKKAINEN, *Carbohydr. Res.*, 56 (1977) 1–9.
12 D. M. CARLSON, *J. Biol. Chem.*, 243 (1968) 616–626.
13 S. HASE AND E. T. RIETSCHEL, *Eur. J. Biochem.*, 63 (1976) 93–99.
14 H. BJORNDAL, B. LINDBERG, AND S. SVENSSON, *Carbohydr. Res.*, 5 (1967) 433–440.
15 H. BJORNDAL, B. LINDBERG, Å. PILOTTI, AND S. SVENSSON, *Carbohydr. Res.*, 15 (1970) 339–349.
16 J. FINNE, *Biochim. Biophys. Acta*, 412 (1975) 317–325.
17 A. STOFFYN, P. STOFFYN, AND J. C. ORR, *Carbohydr. Res.*, 23 (1972) 251–260.
18 A. NEUBERGER AND R. D. MARSHALL, in A. GOTTSCHALK (Ed.), *Glycoproteins*, Elsevier, Amsterdam, 1966, pp. 235–272.
19 J. FINNE AND T. KRUSIUS, *FEBS Lett.*, 66 (1976) 94–97.