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STRUCTURAL ANALYSIS OF THE NEURAMINIC ACID LINKAGES IN DEAMINATED TRISACCHARIDE-ALDITOLS BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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ABSTRACT

The separation and structural analysis of four deaminated sialotrisaccharide alditols as their permethyl ethers by gas–liquid chromatography (g.l.c.) and gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) is described. For the assignment of (2→3) and (2→6) linkages between sialic acid and D-galactose, trisaccharides having two different kinds of alditol units and also isotopic labelling of the sialic acid moiety have been used. The analysis by m.s. of the sialic acid moiety liberated is also presented. It is shown that the alditols may be separated from one another by g.l.c. with the SE-30 stationary phase. The position of the terminal glycosidic linkage may be established by m.s., without deuterium labelling of the molecule, in a similar way to that described for the corresponding alditols composed of simple hexoses. The use of g.l.c.–m.s. in studies of oligosaccharide mixtures produced by *N*-deacetylation and deamination of sialic acid-containing glycopeptides is discussed.

INTRODUCTION

The acidic type of carbohydrate chains in *N*-glycosylic glycopeptides contain a core composed of D-mannose, 2-acetamido-2-deoxy-α-D-glucopyranose, and occasionally L-fucose, to which various numbers of sialic acid–D-galactose–2-acetamido-2-deoxy-α-D-glucopyranose branches are attached[1]. By affinity chromatography with concanavalin A, a mixture of acidic type glycopeptides may be resolved into two fractions: one containing glycopeptides having three and four peripheral branches, and another containing those with two branches[2]. Except for the promising results obtained by n.m.r. spectroscopy, no direct method has been available for further characterization of the peripheral branches, and the position of the sialic acid linkages has usually been deduced indirectly through analysis of the substitution-mode of D-galactose as the partially methylated alditol acetate[3].

Deamination of 2-amino-2-deoxy-α-D-hexopyranoses with nitrous acid is commonly used for the selective cleavage of their glycosidic linkages, leading to the formation of oligosaccharides having 2,5-anhydro-D-hexose residues at the reducing

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end[4]. The method has usually been applied to desialylated glycopeptides to obtain small oligosaccharides for further analysis.

According to our recent studies, glycosidically linked sialic acid may also be *N*-deacetylated and deaminated without cleavage of the glycosidic linkage of the sialic acid residue. The oligosaccharides formed from the terminal branches of acidic *N*-glycosylic glycopeptides are composed of sialic acid, D-galactose, and 2,5-anhydro-D-mannose residues and may be isolated for further analysis by ion-exchange chromatography from the neutral deamination products[5]. This procedure allows a specific method for the fractionation and further analysis of peripheral, sialic acid-containing structures of biological importance.

Deamination of the free methyl ester methyl glycoside of *N*-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulopyranosonic acid) by nitrous acid was recently described[6]. The main deamination product was characterized by subsequent g.l.c.–m.s. and n.m.r. studies[7] as 3-deoxy-D-*glycero*-β-D-*galacto*-nonulopyranosidonic acid methyl ester methyl glycoside.

The present work describes the g.l.c. and g.l.c.–m.s. analysis of permethylated, sialic acid-containing trisaccharides formed by *N*-deacetylation and deamination of acidic glycopeptides and *N*-acetylneuraminolactitol, with special emphasis on the differentiation between (2→3) and (2→6) linkages. In order to clarify the effect of the reducing end on the fragmentation, two different kinds of permethylated alditols were tested, and isotopic labelling of sialic acid was used.

EXPERIMENTAL

Compounds. — The following trisaccharides were studied: *O*-3-deoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucitol (**1**), *O*-3-deoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-2,5-anhydro-D-mannitol (**2**), *O*-3-deoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-D-glucitol (**3**), and *O*-3-deoxy-α-D-*glycero*-D-*galacto*-nonulopyranosylonic acid-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-2,5-anhydro-D-mannitol (**4**).

Compounds **2** and **4** were prepared from α_1-acid glycoprotein obtained as a gift from Dr. Erkki Raaska, the Finnish Red Cross Transfusion Service, SF-00310 Helsinki 31, Finland. Compounds **1** and **3** were prepared from *N*-acetylneuraminolactose obtained as a gift from Dr. Jaakko Parkkinen, in this laboratory. Before *N*-deacetylation, *N*-acetylneuraminolactose was reduced to the corresponding alditol with sodium borohydride, and the glycopeptides of α_1-acid glycoprotein were isolated[2].

Procedures. — *N*-Deacetylation of *N*-acetylneuraminolactitol and the mixture of glycopeptides was accomplished in hot, alkaline dimethyl sulfoxide according to Erbing *et al.*[8]. The deamination was performed in aqueous acetic acid with sodium nitrite as described earlier[7], but without the use of ion-exchange resin, and the oligosaccharides of glycopeptide origin were reduced with sodium borohydride[5]. All samples were desalted as described earlier. Compounds **2** and **4** were isolated by ion-

exchange chromatography[9] on a column of DEAE-Sephadex A-25 and partially purified by t.l.c.[5]. Monosaccharides were quantitated as their trimethylsilyl ethers by g.l.c.–m.s.[6]. The trisaccharide samples (~200 μg) were methylated according to a modification of the Hakomori procedure[10] in the presence of methylsulfinyl carbanion and *tert*-butoxide anion[11], and analyzed by g.l.c. or g.l.c.–m.s. A portion of the permethylated trisaccharide alditols was converted into the corresponding trideuteriomethyl ester derivatives by a further methylation step with trideuteriomethyl iodide instead of methyl iodide. The methyl ester methyl β-glycoside of deaminated sialic acid was formed by methanolysis[6] of permethylated trisaccharides, and its trideuteriomethyl ester trideuteriomethyl β-glycoside was obtained after methanolysis in acidic trideuteriomethanol, respectively. A part of the sample was also analyzed as the partially methylated alditol acetates[3]. Details of the methods will be published elsewhere[5].

TABLE I

PARTIAL LOW-MASS RANGE MASS SPECTRA OF PERMETHYLATED, DEAMINATED NEURAMINIC ACID-CONTAINING TRISACCHARIDE-ALDITOLS

m/z	*Intensity*				*Symbol*
	1	**2**	**3**	**4**	
45	44	22	65	9.2	
59	27	4.8	5.0	1.7	
71	59	71	54	18	
75	36	34	38	15	J_1
88	17	18	47	100	H_1
89	52	18	54	10	
101	100	100	100	41	F_1
111	15	10	25	3.6	
115	30	19	29	6.5	C_2
127	25	40	23	13	
129	6.0	6.4	10	2.7	
133	25	35	17	8.0	
142	1.7	3.4	5.6	0.79	
157	9.0	29	12	29	
159	43	86	26	8.6	
169	14	27	9.0	2.7	
171	18	3.3	16	1.2	
183	34	14[a]	13	1.3	
187	13	8.9	17	6.6	
189	5.9	22	7.0	32	
247	8.8	4.0	3.8	—	
249	0.89	23	15	95	
304	7.0[a]	14[a]	2.0[a]	2.0[a]	
336	3.6[a]	4.0[a]	3.0[a]	6.1[a]	
377	4.2[a]	15[a]	1.4[a]	0.70[a]	
Relative retention times	2.05	2.59	2.65	2.88	

[a]In the deuterated derivative, >50% of the ion intensity is converted into *m/z* three mass units higher.

TABLE II

PARTIAL HIGH-MASS RANGE MASS SPECTRA OF PERMETHYLATED, DEAMINATED NEURAMINIC ACID-CONTAINING TRISACCHARIDE-ALDITOLS

Symbol	*Intensity (mass number)*							
	1		2		3		4	
cA_3	18	(171)	8.5	(125)	15	(171)	4.4	(125)
cA_2	3.0	(203)	29	(157)	2.1	(203)	29	(157)
cA_1	50	(235)	22	(189)	26	(235)	32	(189)
bcJ_1	1.7	(295)	23	(249)	2.4	(295)	95	(249)
aA_3	16	(271)	25[a]	(271)	5.1	(271)	3.2[a]	(271)
aA_2	43[a]	(303)	92[a]	(303)	15[a]	(303)	11[a]	(303)
aA_1	11[a]	(335)	6.7[a]	(335)	3.0[a]	(335)	5.2[a]	(335)
bcA_3	0.33	(375)	0.96	(329)	—	(375)	0.70	(329)
bcA_2	0.99	(407)	2.7	(361)	0.49	(407)	0.63	(361)
bcA_1	4.8	(439)	5.8	(393)	2.9	(439)	23	(393)
baA_3	—	(475)	0.01	(475)	0.47[a]	(475)	—	(475)
baA_2	0.27[a]	(507)	—	(507)	0.44[a]	(507)	—	(507)
baA_1	0.90[a]	(539)	0.73[a]	(539)	0.38[a]	(539)	—	(539)
M-133	0.44[a]	(657)	0.23[a]	(611)	0.34[a]	(657)	0.13[a]	(611)
M-89	0.37[a]	(701)	—	(655)	—	(701)	—	(655)
M-59	0.36	(731)	4.5	(685)	0.10	(731)	0.65	(685)
M-45	0.26[a]	(745)	—	(699)	—	(745)	—	(699)
M-31	0.14[a]	(759)	0.28[a]	(713)	—	(759)	0.16[a]	(713)
M-15	—	(775)	—	(729)	—	(775)	0.12	(729)
M*	—	(790)	—	(744)	—	(790)	0.14[a]	(744)

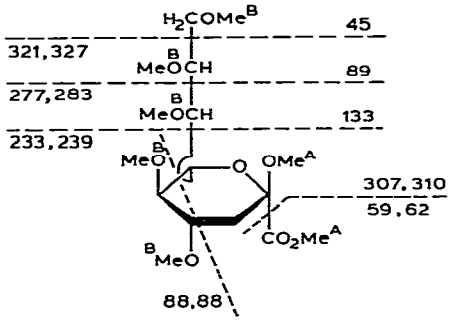
[a]In the deuterated derivative, >50% of the ion intensity is converted into *m/z* three mass units higher.

Symbols A–J, employed by Kochetkov *et al.*[12], further subdivided by lower-case letters a, b, and c to designate the monosaccharide residues[13] from the reducing end, are used in discussing mass-spectral fragmentations. The first lower-case letter indicates the fragmented part of the molecule. The second and the third lower-case letter indicate the intact part of the molecule. Thus, baA_1, stands for the first ion in the series following pathway A, and is formed through the cleavage of ring b substituted with ring a.

G.l.c.–m.s. was performed with a Varian MAT CH-7 instrument equipped with a Varian 1700 gas chromatograph and a SpectroSystem 100 MS data-processing system. The ionization potential and current were 70 eV and 300 μA, respectively. Glass columns (2 m × 2 mm, i.d.) filled with 2% of SE-30 were used for g.l.c. The peak intensities in Tables I and II are expressed as percentages of the base peak after subtraction of the background spectrum.

RESULTS AND DISCUSSION

Analysis by g.l.c. — The retention times of deaminated, permethylated neurami-



1 $Me^A = CH_3$; $Me^B = CH_3$; M = 366

2 $Me^A = CD_3$; $Me^B = CH_3$; M = 372

Scheme 1

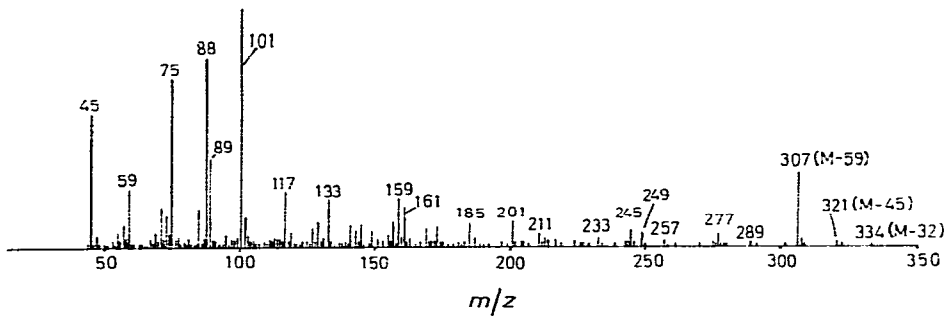


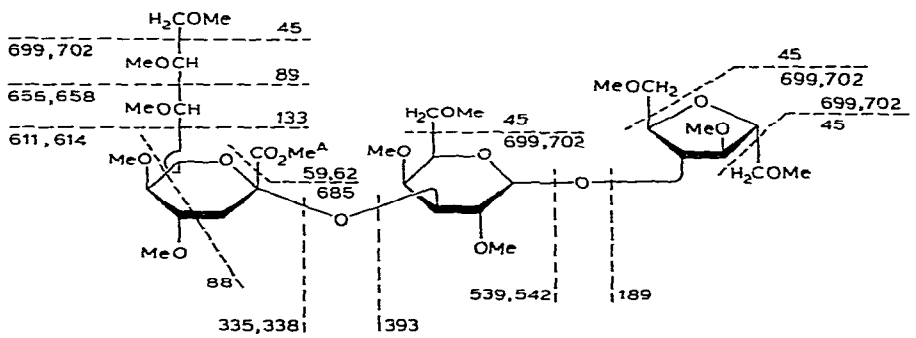
Fig. 1. The mass spectrum of the neuraminic acid residue liberated from deaminated, permethylated trisaccharide-alditols by methanolysis.

nyl trisaccharide alditols relative to permethylated maltotriitol on SE-30 at a column temperature of 265° are given on the lowest line in Table I. 2,5-Anhydromannitol-containing trisaccharides showed somewhat longer retention-times than those carrying the glucitol moiety, and all were eluted from the column considerably later than the corresponding derivatives of trisaccharide-alditols composed of simple hexoses[14]. Trisaccharides having similar alditol residues were well resolved from one another on the basis of their different sialic acid linkages, and essentially no extra peaks were present in the chromatograms. G.l.c. seems therefore suitable for preliminary identification and quantitation of deaminated trisaccharide mixtures.

Mass spectrometry of the free, deaminated neuraminic acid residue. — In order to obtain a reference mass spectrum of the nonreducing part of the deaminated trisaccharides, the neuraminic acid residue was liberated both by methanolysis and trideuteriomethanolysis. The mass spectrum of the residue is shown in Fig. 1, and its major fragmentation in Scheme 1, where the fragments carrying the deuterium label are given as the second of two numbers. The molecular weight (366) was clearly ascertained by ions at *m/z* 334 (M — 32) and 307 (M — 59). Cleavages of the side

chain give rise to the fragments at *m/z* 321, 289 (321 — 32), 277, 245 (277 — 32), 233, and 201 (233 — 32). The gas-chromatographic and mass-spectrometric data for the neuraminic acid residue liberated were identical to those of the major product formed upon deamination with nitrous acid of the methyl 5-amino-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulopyranosidonic acid methyl ester[7]. These results demonstrate that deamination of *N*-deacetylated neuraminic acid with nitrous acid, gives the same product, no matter whether the molecule preceding the deamination procedure is combined to another monosaccharide and has a free carboxyl group, or is in the form of methyl ester methyl glycoside.

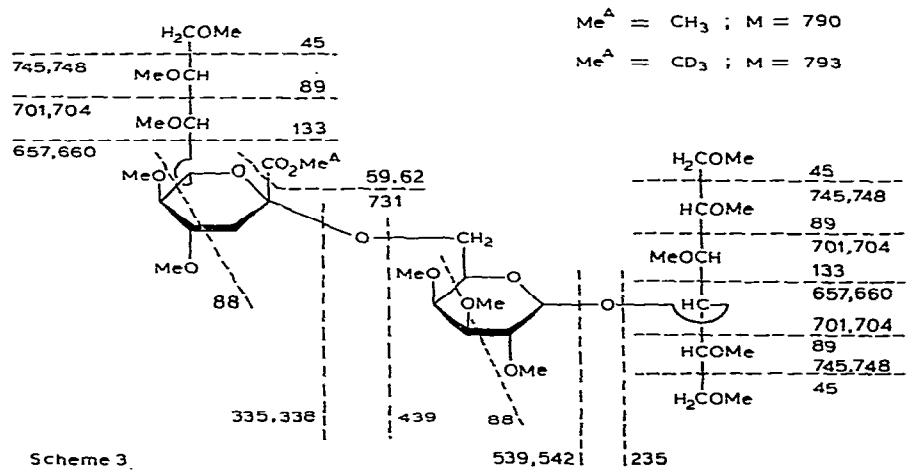
Characteristics of the mass spectra of the trisaccharide alditols. — Partial, low- and high-mass-range spectra of the trisaccharide alditols are shown in Tables I and II. Major fragmentations of compounds **2** and **3** are given in Schemes 2 and 3. They include the 10 highest peaks of each spectrum as well as other significant peaks



Me^A = CH_3 ; M = 744

Me^A = CD_3 ; M = 747

Scheme 2



Scheme 3

for structural discussion. The mass spectra of both trideuteriomethyl ester- and methyl ester-containing derivatives were recorded.

Major ions between *m/z* 40 and 240 are the same as in the mass spectra of permethylated trisaccharide-alditols of simple hexoses[14]. The most intense specific ions for the deaminated sialic acid residue were observed at *m/z* 335 and 303. The most prominent ions in the mass range from *m/z* 350 to 550 were formed through the pathways baA_{1-3} and bcA_{1-3}. Relatively few ions were detected above *m/z* 539, and they were formed through the loss of 15, 31, 45, 59, 89, or 133 mass units from the molecular ion (Table II). Only the molecular ion of compound **4** was observable, and the molecular weights were deduced from the fragmented ions of high-mass range (Table II).

Assignment of the position of the neuraminic acid→galactose-(a→b) linkage. — The (2→3) linkage may be readily distinguished from the (2→6) linkage by the low intensity of *m/z* 88 and the high intensity (base peak) of *m/z* 101, if the reducing end carries a cyclic alditol. This is always the case, when neuraminic acid-containing trisaccharides are produced from the branches of *N*-glycosylic glycopeptides by the present method. For the (2→6) linkage, the intensities are reversed and the ion at *m/z* 88 appears as the base peak. This is because the (2→6) linkage also disposes the b unit to fragmentation through pathway H_1 (*m/z* 88) (Scheme 3). The straight-chain alditol residue at the reducing end of the molecule (**1** and **3**) favors the fragmentation through pathway F_1 (*m/z* 101), and the assignment of the position of the (a→b) linkage must be made in another way. An important feature of the (2→3) linkage is the high intensity of aA_{1-3} ions (Table II), regardless of the structure of the c unit. The most intense ions are formed through aA_2 cleavage.

In the case of permethylated, neutral trisaccharide-alditols, the (1→3) linkage in an (a→b) position showed the predominance of baA ions over bcA ions, whereas the fragmentation of the (1→6) linkage proceeded mainly through the bcA pathway[14]. In the present case, however, both types of linkage show increased intensities of the ions of that series.

Characteristic of a (2→3) linkage is also the increased intensity of the ions at *m/z* 159 and 377 (Table I). The former is probably composed[14] of the fragments of unit b, and the origin of the latter is unknown. However, it is completely converted into *m/z* 380 by the trideuteriomethyl ester group as well as *m/z* 336, which shows a moderate intensity only in the mass spectra of (2→6)-linked trisaccharides. Its origin is unknown. The ion bcJ_1 showed higher intensities to some degree in the spectra of (2→6)-linked trisaccharides than in the (2→3)-linked ones. Its intensity of 23% in the spectrum of the (2→3)-linked compound **2** confirms the earlier observations that various structural factors influence its generation[15], and the existence of bcJ_1 should not be used alone as an indicator for an (2→6) linkage in an (a→b) position, when permethylated trisaccharide-alditols containing the deaminated sialic acid residue are analyzed.

Consequently, the position of the (a→b) linkage in the mass spectra of deaminated, permethylated neuraminic acid-containing trisaccharide-alditols may be assigned

as follows: an ion ratio >1 of the intensities of *m/z* 159 and 303 (aA_2) to *m/z* 88 combined with a base peak at *m/z* 101, and a moderate intensity of the ion at *m/z* 377 indicate a (2→3) linkage. An ion ratio <1 of both *m/z* 159 and 303 (aA_2) to *m/z* 88 indicate a (2→6) linkage.

The combined results demonstrate that the trisaccharide-alditols containing the deaminated sialic acid residue may be analyzed by g.l.c.–m.s. as their permethyl ethers, and the position of the sialic acid linkage assigned on the basis of the mass spectra without hydrolyzing the molecules. The results also demonstrate that the sialic acid residue in the main oligosaccharides is 3-deoxy-D-*glycero*-D-*galacto*-nonulopyranosonic acid. This is in accord with the earlier results of deamination of the methyl ester methyl β-glycoside of neuraminic acid[7] and other glycosides carrying an equatorial amino group in the analogous position, for example, methyl 4-amino-4-deoxy-α-D-glucopyranoside, for which the main deamination product was characterized as methyl α-D-glucopyranoside[4]. Presumably, the deamination reaction is participated in by the ring-oxygen ion to give a cyclic oxonium ion, which then reacts with water to give this retained configuration[7].

During the editorial review of the present work, Strecker *et al.*[16] published results that are in disaccord with those presented here concerning the ring structure of the deaminated sialic acid residue. Their conclusion was that the main reaction-path of neuraminic acid under deamination is the change of the size of the pyranoid ring as in the case of 2-amino sugars[4]. Further studies are indicated to resolve this problem.

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