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FORMATION AND IDENTIFICATION OF TWO NOVEL ANHYDRO COMPOUNDS OBTAINED BY METHANOLYSIS OF *N*-ACETYLNEURAMINIC ACID AND CARBOXYL-REDUCED, MENINGOCOCCAL B POLYSACCHARIDE

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ABSTRACT

Methanolysis of *N*-acetylneuraminic acid gives, in addition to the methyl ester methyl β-ketoside, 10–15% of a product identified by g.l.c.–m.s. as methyl 5-acetamido-2,7-anhydro-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosonate (**3**). Only the major product was formed on methanolysis of the capsular, sialic acid polysaccharides from *Neisseria meningitidis* serogroup B and C and *Escherichia coli* K1 (colominic acid). Methanolysis of carboxyl-reduced, meningococcal B polysaccharide affords a major product, identified by g.l.c.–m.s. as methyl 5-acetamido-1,7-anhydro-3,5-dideoxy-β-D-*glycero*-D-*galacto*-nonulopyranoside (**7**). Mass-spectral data for **3** and **7** together with those of known sialic acid derivatives correlate well with previously observed fragmentation patterns.

INTRODUCTION

Sialic acids, commonly present as terminal non-reducing components of glycoproteins and glycolipids, play a vital role in the control and recognition mechanisms of cell–cell interactions[1]. In oligo- and poly-meric form, sialic acids also occur in brain gangliosides[2], glycoproteins[3,4], and the capsular polysaccharides of *Neisseria meningitidis*[5], *Escherichia coli*[6,7], and *Streptococcus pneumoniae*[8], organisms that cause meningitis in humans.

During studies of meningococcal homopolymers[9] of sialic acid, i.r. spectroscopy was employed to quantify the degree of internal esterification that occurs in B polysaccharide, by measuring the ester C=O stretching band near 1750 cm^{-1}. However, this band is obscured for polysaccharides containing *O*-acetyl and/or carboxylic acid groups, which have C=O stretching bands near 1725 cm^{-1}. Therefore, the degree of internal esterification was quantified by carboxyl-reduction of the

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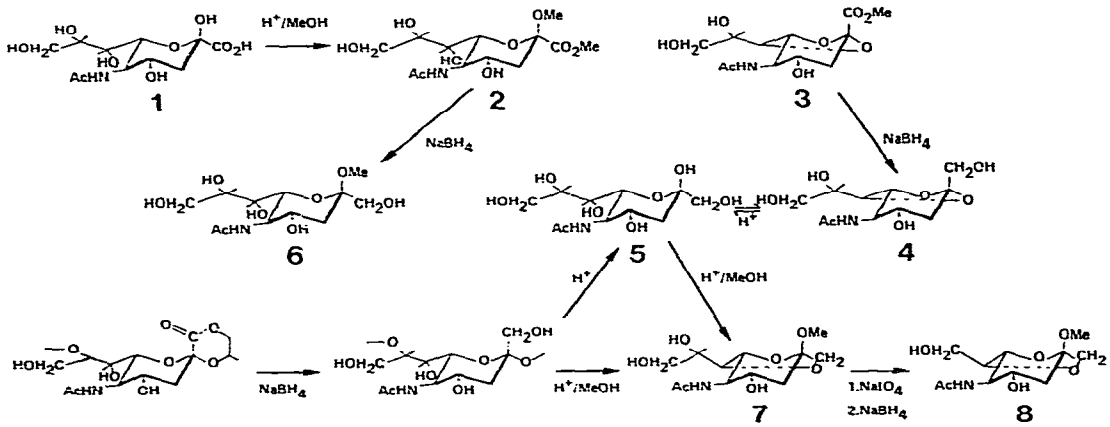
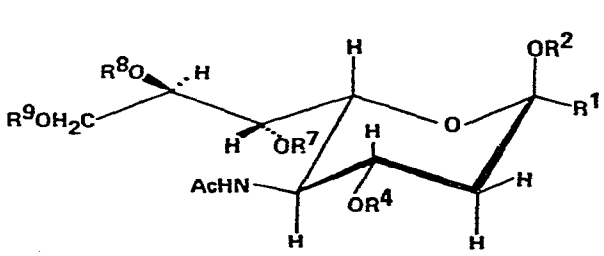
esterified residues followed by methanolysis and g.l.c. of the products. The expected derivatives, **2** (from non-esterified residues) and **6** (from esterified residues), were synthesised as standards. However, during this procedure, the formation was noted of two unexpected sialic acid derivatives that have been identified as anhydro compounds.

RESULTS AND DISCUSSION

*Methyl 5-acetamido-2,7-anhydro-3,5-dideoxy-α-*D*-glycero-*D*-galacto-nonulopyranosonate* (**3**). — Methanolysis of *N*-acetylneuraminic acid (**1**), either free or ketosidically bound, gives[10,11] the methyl ester methyl β-ketoside **2**. However, when the trimethylsilylated, methanolysis products of *N*-acetylneuraminic acid were subjected to g.l.c. on OV-101, two major peaks [R_{NeuNAc} 0.71 (14%) and 1.00 (76%)] and four minor peaks [R_{NeuNAc} 0.57 (<2%), 0.79 (2%), 0.90 (3%), and 1.15 (<2%)] were observed (Fig. 1). These components were identified by g.l.c.–m.s. The nomenclature employed by van Halbeek *et al.*[12] is used in the following discussion (see formula **9**).



Scheme 1. Formation of derivatives (**2**–**8**) of *N*-acetylneuraminic acid (**1**).



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A = $M^+-\dot{C}H_3$
B = M^+-R^1
C = $M^+-(CH_2OR^9-\dot{C}HOR^8)$
D = C-R^2OH-R^4OH
E′ = $M^+-(CH_2OR^9-CHOR^8-\dot{C}HOR^7)$
E = E′ -NH_2COCH_3
F = $CH_2OR^9-CH=\overset{\ominus}{O}R^8$
G = $CH_3CO-NH-\dot{C}H-CH-\overset{\ominus}{O}R^4$
H′ = $M^+-\dot{C}H_2OR^9$
H = H′ -R^4OH-R^7OH

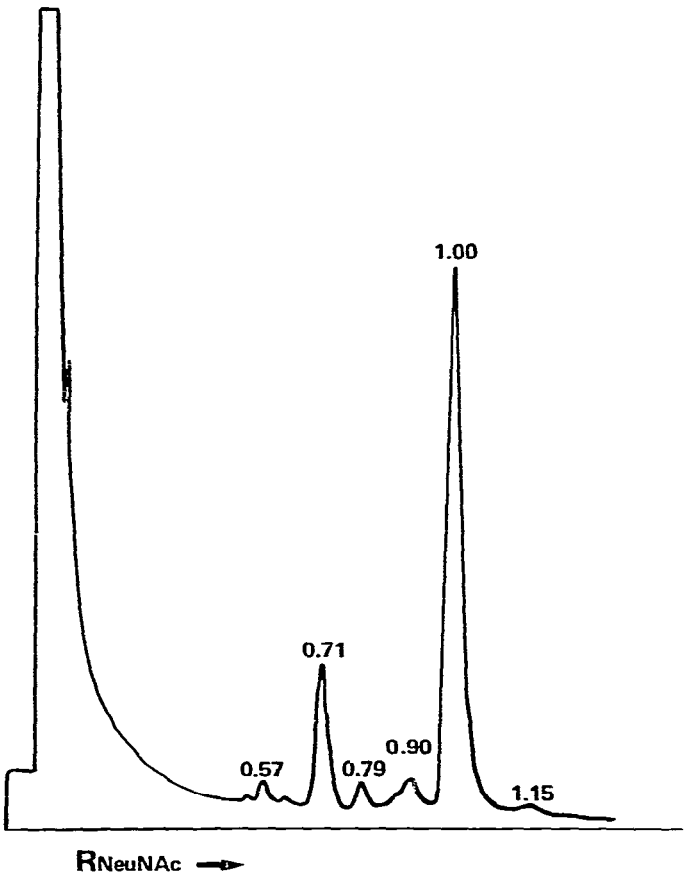


Fig. 1. G.l.c. on 3% OV-101 of the methanolysis products of *N*-acetylneuraminic acid (**1**). Retention times (R_{NeuNAc}) are given relative to that of the methyl ester methyl β-ketoside (**2**) of **1**: *N*-deacetylated derivative of **2**, 0.57; **3**, 0.71; 9-*O*-methyl derivative of **2**, 0.79; α anomer of **2**, 0.90; 9-*O*-acetyl derivative of **2**, 1.15.

The mass spectrum of the unknown component having R_{NeuNAc} 0.71 is shown in Fig. 2, and the interpretation of some of the important mass-fragments is given in Table I. The molecular ion at *m/z* 521 was not observed, but was deduced from ions at *m/z* 506 (fragment A) and 462 (fragment B); therefore, the structure is consistent with the loss of Me_3SiOMe from trimethylsilylated **2** (R_{NeuNAc} 1.00). Although a β-1,7-lactone derivative of *N*-acetylneuraminic acid has previously been identified[10] by g.l.c.–m.s., it is not a plausible structure here, because of the formation of fragment B (elimination of the C-1 group) in the mass spectrum of the unknown compound. The data are consistent with an anhydro derivative of **2**. Formation of ions at *m/z* 316 (fragment C) and 205 (fragment F) indicated that elimination of the C-8–C-9 part of the molecule occurred with retention of the anhydride structure. However, ions at *m/z* 307 and 217 (loss of C-7–C-9 side-chain) were absent, consistent with the formation of methyl 5-acetamido-2,7-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-

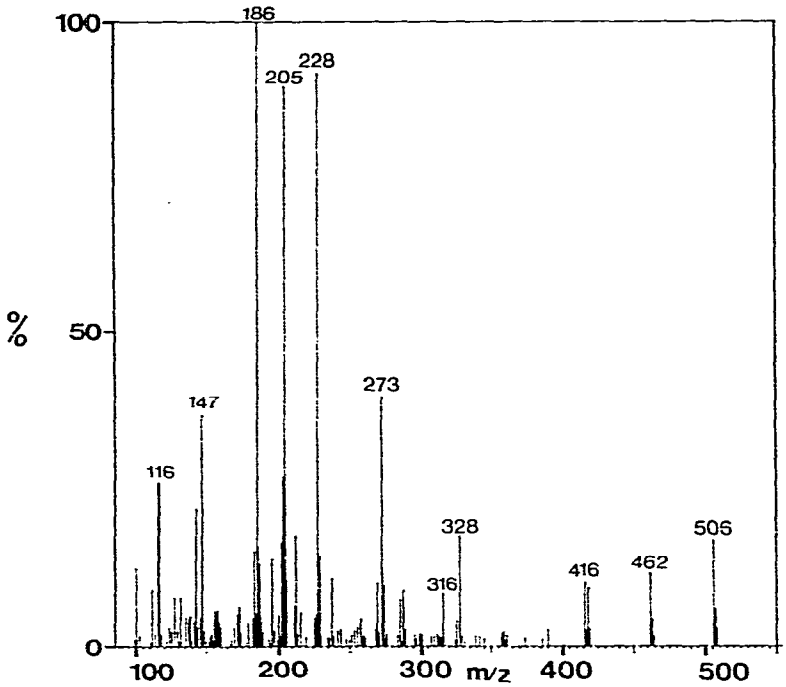


Fig. 2. Mass spectrum of the trimethylsilylated derivative of 3.

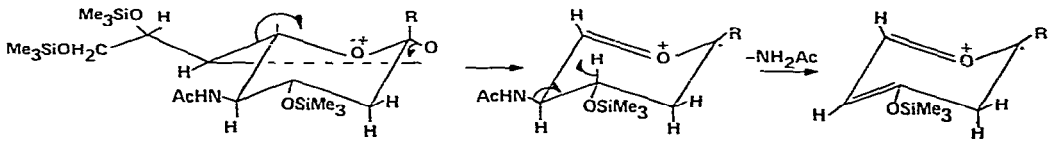
TABLE I

IMPORTANT FRAGMENT IONS OF THE TRIMETHYLSILYLATED DERIVATIVE HAVING R_{NeuNAc} 0.71 ON 3% OV-101 AT 230°

m/z	*Fragment*	*Pathway*
506	$M^+ - Me$	A
462	$M^+ - CO_2Me$	B
418	$M^+ - CH_2OSiMe_3$	H′
416	$M^+ - Me - Me_3SiOH$	
328	$M^+ - CH_2OSiMe_3 - Me_3SiOH$	
316	$M^+ - (CH_2OSiMe_3\text{–}CHOSiMe_3)$	C
238	$M^+ - CH_2OSiMe_3 - 2Me_3SiOH$	
228	$M^+ - (CH_2OSiMe_3\text{–}CHOSiMe_3\text{–}CHO) - NH_2Ac$	
205	$CH_2OSiMe_3\text{–}CH{=}O^+SiMe_3$	F
186	$AcN^+H{=}CH\text{–}COSiMe_3{=}CH_2$	
173	$AcNH\text{–}\dot{C}H\text{–}CH{=}O^+SiMe_3$	G

ulopyranosonate (3). Molecular models of 3 can be constructed in the α configuration only. Our conclusions are similar to those of Gross *et al.*[13] in the identification of 5-acetamido-2,7-anhydro-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranose (4), and comparison of the mass spectra of trimethylsilylated 3 and 4 reveals many

similarities. A major peak in each spectrum occurred at ($M^+ - 293$). Exact mass measurements, however, indicated that the fragmentation pathways were different. The formula for this peak in **4** was $C_{12}H_{24}O_3Si_2$ (272.1238; $C_{12}H_{22}D_2O_3Si_2$ in the deuterated analogue), which was consistent with fragmentation **10**→**11**. The exact mass of this peak in **3** was 228.0974, from which no suitable fragmentation pathway can be deduced. It is probable that two or more different fragment-ions contributed to the intensity of the peak at *m/z* 228. Evidence for this came from the spectrum of **4**, in which a significant peak at *m/z* 228 occurred, unaltered in the deuterated derivative, indicating that C-1 was eliminated. It is likely that this peak was also present in **3**.



10

R = CO_2Me or CH_2OSiMe_3(CD_2OSiMe_3)

($M^+ - 293$)

11

Further proof of the structure and configuration of **3** was obtained following methanolysis of *N*-acetylneuraminic acid and subsequent reduction with $NaBH_4$ (or $NaBD_4$). After trimethylsilylation, three peaks were observed by g.l.c. on OV-225 (Fig. 3), with R_{NeuNAc} 0.50 (11%), 0.57 (17%), and 0.64 (67%). A mass spectrum of the major peak (R_{NeuNAc} 0.64) was consistent with methyl 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranoside (**6**). The two minor peaks at R_{NeuNAc} 0.50 and 0.57 had retention times and mass spectra identical to those of authentic samples of 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-nonulopyranose (**5**) and its 2,7-α-anhydride **4**, respectively (see Scheme 1). The presence of **4** (the reduction product of **3**) was expected, although it is not certain why **5** was also a product of the reduction process. However, **4** and **5** are in equilibrium under mildly acidic conditions[13], and it may be that passage through Dowex 50 (H^+) resin during purification was sufficient for this equilibrium to occur.

Although **3** was obtained after methanolysis of *N*-acetylneuraminic acid, it was not formed on methanolysis of the sialic acid polysaccharides from *Neisseria meningitidis* serogroups B and C or *Escherichia coli* K1.

Of the four minor components from the methanolysis of *N*-acetylneuraminic acid identified by g.l.c.–m.s. (see Fig. 1), the component having low retention time (R_{NeuNAc} 0.57) had properties consistent with those of *N*-deacetylated **2**. The mass spectrum was similar to that for **2**, allowing for a shift of 42 m.u. in many fragments. The component having R_{NeuNAc} 0.90 was the α anomer of **2**. The peaks at R_{NeuNAc} 0.79 and 1.15 were readily identifiable as the respective 9-*O*-methyl- and 9-*O*-acetyl derivatives of **2**. The latter had a mass spectrum identical to the literature spectrum of an authentic sample[14]. These two components were presumably artefacts caused by the methanolysis and *N*-reacetylation procedures.

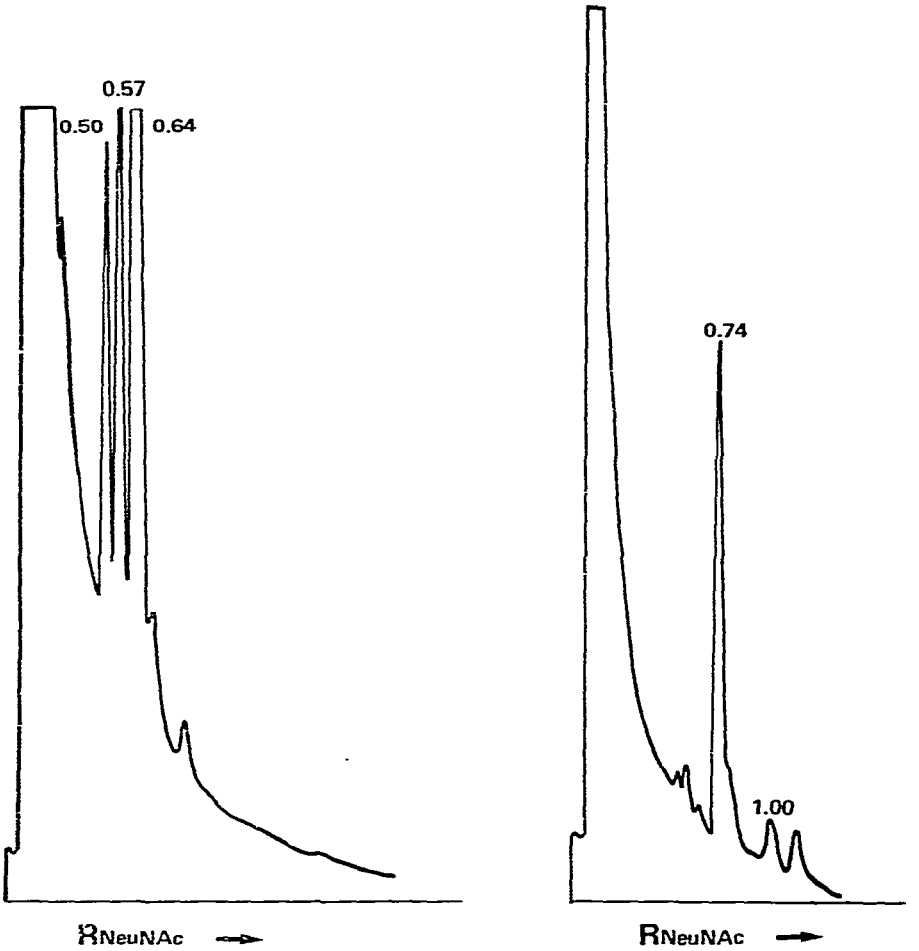


Fig. 3. G.l.c. on 3% OV-225 following methanolysis of *N*-acetylneuraminic acid (**1**) and subsequent reduction with $NaBH_4$. R_{NeuNAc} values: **5**, 0.50; **4**, 0.57; **6**, 0.64.

Fig. 4. G.l.c. on 3% OV-225 after methanolysis of carboxyl-reduced, meningococcal B polysaccharide: **7**, R_{NeuNAc} 0.74.

Methyl 5-acetamido-1,7-anhydro-3,5-dideoxy-β-D-glycero-D-galacto-*nonulopyranoside* (**7**). — (2→8)-α-Linked homopolymers of sialic acid readily undergo internal esterification upon mild treatment with acid or by reaction with a carbodi-imide[9]. Subsequent reduction of the fully esterified polysaccharide with $NaBH_4$ (or $NaBD_4$) followed by mild methanolysis (see Scheme 1) gave one major product in g.l.c. of the trimethylsilylated derivative (R_{NeuNAc} 0.74 on OV-225; Fig. 4), which was different from the methyl ketoside of the *N*-acetylnonulosamine (**6**; R_{NeuNAc} 0.64). The mass spectrum of the unidentified component is shown in Fig. 5 and the interpretation of some of the important mass-fragments is given in Table II. Ions at *m/z* 507 (M^+) and 492 (fragment A) indicated that the molecular weight was 162 lower than that

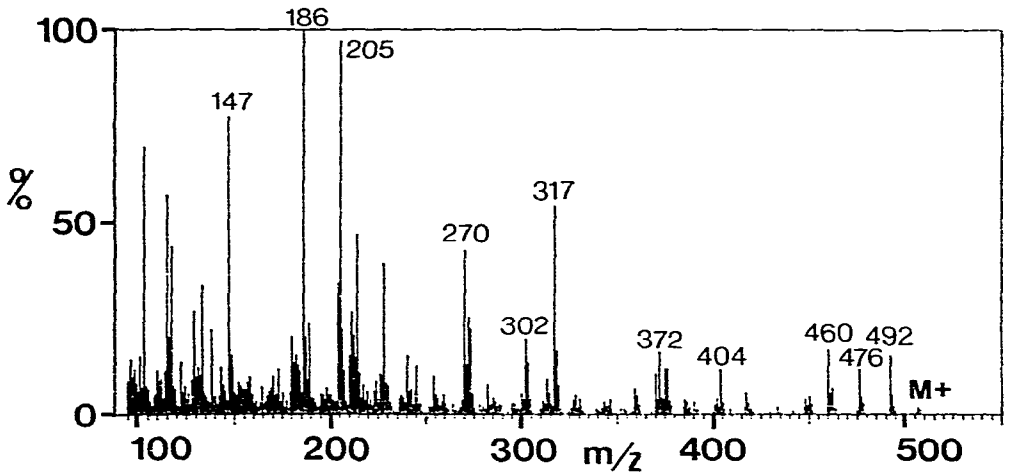


Fig. 5. Mass spectrum of the trimethylsilylated derivative of 7.

TABLE II

IMPORTANT FRAGMENT IONS OF THE TRIMETHYLSILYLATED DERIVATIVE HAVING R_{NeuNAc} 0.74 ON 3% OV-225 AT 230°

m/z	*Fragment*	*Pathway*
507	M^+	
492	$M^+ - Me$	A
476	$M^+ - OMe$	
460	$M^+ - Me - MeOH$	
417	$M^+ - Me_3SiOH$	
404	$M^+ - CH_2OSiMe_3$	H′
372	$M^+ - CH_2OSiMe_3 - MeOH$	
370	$M^+ - Me - MeOH - Me_3SiOH$	
317	$M^+ - (CH_2OSiMe_3\text{–}CH_2OSi(Me)(=CH_2))$	
302	$M^+ - (CH_2OSiMe_3\text{–}CHOSiMe_3)$	C
282	$M^+ - CH_2OSiMe_3 - MeOH - Me_3SiOH$	
270	$M^+ - (CH_2OSiMe_3\text{–}CHOSiMe_3) - MeOH$	
205	$CH_2OSiMe_3\text{–}CH{=}O^+SiMe_3$	F
186	$AcN^+H{=}CH\text{–}COSiMe_3{=}CH_2$	
180	$270 - Me_3SiOH$	
173	$AcNH\text{–}\dot{C}H\text{–}CH{=}O^+SiMe_3$	G

for trimethylsilylated **6**, corresponding to loss of $Me_3SiOSiMe_3$ (*i.e.*, the component is an anhydro derivative of **6**). The absence of fragment B (loss of the C-1 part of the molecule) suggested that the C-1 group was involved in the anhydro structure, whereas the presence of ions at *m/z* 302 (fragment C) and 205 (fragment F) indicated

that C-8–C-9 did not participate in the linkage. However, the absence of ions at *m/z* 307 and 217 was consistent with structure **7**. Molecular models can be formed only of the β anomer, and **7** is also formed by methanolysis of the equilibrium mixture of **4** and **5** (Scheme 1).

Confirmation of the 1,7-anhydro structure was obtained by periodate oxidation and subsequent $NaBH_4$ (or $NaBD_4$) reduction. A mass spectrum of the major peak (R_{NeuNAc} 0.60 on OV-225) contained the molecular ion at *m/z* 405, fragment A at 390, and (M^+ — OMe) at 374, consistent with periodate cleavage of **7** between C-8 and C-9. Overall, the spectrum was compatible with structure **8** (methyl 5-acetamido-1,7-anhydro-3,5-dideoxy-D-*galacto*-octulopyranoside).

Mass spectra of **2–8**. — Trimethylsilylated derivatives of **2–8** were prepared, of which four (**4–7**) were also prepared deuterated at C-1 and one (**8**) deuterated at C-8, and the mass spectra were obtained. The fragment ions A–H are shown in **9**. The molecular ion was present as a weak fragment in the spectra of **4**, **7**, and **8** only, but fragment A (M^+ — Me) was abundant for all the compounds.

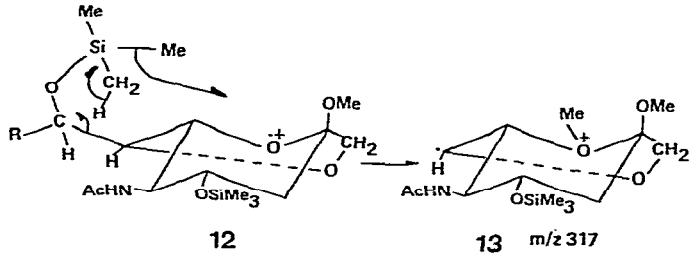
Fragment B (M^+ — R^1) could not be formed when C-1 was bound, and its absence was important for the identification of the 1,7-anhydro derivatives **7** and **8**. Formation of a 2,7-anhydride (**3** and **4**) did not impede the elimination of the C-1 fragment. Although fragment H′ (M^+ — CH_2OR^9) was present in the spectra of **3** and **7**, the relative abundance of fragments H′ and B could not be ascertained directly from the spectra of **4–6** since these ions have the same mass. It is apparent from the deuterated analogues, however, that fragment H′ is not significant, in accordance with previous observations[12,15].

Fragment C (M^+ — $CH_2OR^9CHOR^8$) was present in all spectra (except that of **8**), although the abundance was reduced in the spectra of **3**, **4**, and **7** where anhydro formation at C-7 occurred. Conversely, fragment F ($CH_2OR^9CHO^+R^8$), although present in all spectra (except that of **8**), had greater abundance in those of **3**, **4**, and **7**. Fragment D (M^+ — $CH_2OR^9CHOR^8$ — R^2OH — R^4OH), formed from fragment C, was absent from the spectra of **3** and **4**, consistent with the 2,7-anhydro structure, and had only low abundance in the spectrum of **7**; fragment E (M^+ — CH_2OR^9-$CHOR^8CHOR^7$ — NH_2COCH_3) was absent from the spectra of **3**, **4**, **7**, and **8** (which have anhydro linkages through C-7).

Fragment H (M^+ — CH_2OR^9 — R^4OH — R^7OH) was present in the spectrum of **2**, but had only low intensity in that of **6**. However, the latter had a significant ion at *m/z* 444 (M^+ — CH_2OR^9 — R^2OH — R^4OH and/or M^+ — CH_2OR^9 — R^2OH — R^7OH), which was also present in the spectrum of **5** but not in that of **2**; this peak may have arisen (in **5**) by fragmentation as for **6**, through the formation of fragment H or by a combination of all three pathways.

Ions at *m/z* 390 (weak) and 300 (strong), unaltered in the deuterated derivatives, were present only in the spectra of **2**, **5**, and **6** (*i.e.*, from those compounds not having anhydro linkages through C-7). Our observations accord with those of Kamerling *et al.*[16], who concluded that these fragments were formed from the C-5–C-9 part of the molecule.

Finally, a peak at *m/z* 317 was present in the spectra of **7** (*m/z* 319 in the deuterated derivative) and in **8** (unaltered in the deuterated derivative), suggesting that C-1 was retained (in **7**) and that C-8 was eliminated (in **8**). Exact mass measurement of this peak in both samples gave the formula $C_{14}H_{27}NO_5Si$ (317.1624), consistent with **12**→**13**.



12 **13** m/z 317

R = CH_2OSiMe_3 or H(D)

EXPERIMENTAL

N-Acetylneuraminic acid, colominic acid, and *N. meningitidis* serogroup B and C polysaccharides were obtained as previously described[9].

G.l.c. — A Perkin–Elmer F17 instrument was used equipped with dual flame-ionisation detectors and glass columns: *A*, 2 m × 3 mm i.d., packed with 3% of OV-225 on Chromosorb W-HP (100–120 mesh); and *B*, 4 m × 3 mm i.d., packed with 3% of OV-101 on Chromosorb W-HP (100–120 mesh). The oven temperature was 230°, and the carrier gas was nitrogen at 40 mL/min. Retention times and peak areas were determined with a Spectra-Physics 4000 data system. Samples (1 mg) were trimethylsilylated with 50 μL of pyridine, 30 μL of hexamethyldisilazane, and 20 μL of chlorotrimethylsilane in 0.3-mL Reacti-vials with stirring at room temperature for 30 min, and aliquots were analysed directly by g.l.c.–m.s.

G.l.c.–m.s. — A VG 7070F instrument was used interfaced to a VG Multispec data system using glass columns *A*, 1.52 m × 3 mm i.d., packed with 1% of OV-1 on Chromosorb W-HP (80–100 mesh); and *B*, 1.52 m × 3 mm i.d., packed with 3% of OV-225 on Chromosorb W-HP (80–100 mesh). The oven temperature was 220° and the carrier gas was helium at 40 mL/min. Mass spectra were obtained by electron impact at 75 eV at a source temperature of 200°, scan speed of 3 s/decade, and interface temperature of 250°. The injection size was adjusted to give 1–10 μg on column.

*Methyl (methyl 5-acetamido-3,5-dideoxy-β-*D*-glycero-*D*-galacto-nonulopyranosid)onate* (**2**) and *methyl 5-acetamido-2,7-anhydro-3,5-dideoxy-α-*D*-glycero-*D*-galacto-nonulopyranosonate* (**3**). — *N*-Acetylneuraminic acid (**1**, 5 mg) was treated with a boiling solution of M methanesulphonic acid in dry methanol (2 mL) with shaking for 15 h. The solution was neutralised with Dowex 2 (HCO_3^-) resin, decanted, and concentrated to dryness. The residue was *N*-acetylated[17], trimethylsilylated, and subjected to g.l.c. on OV-101. Major products were **2** (76%) and **3** (14%).

5-Acetamido-2,7-anhydro-3,5-dideoxy-α-D-glycero-D-galacto-*nonulopyranose* (**4**) and *5-acetamido-3,5-dideoxy-β*-D-glycero-D-galacto-*nonulopyranose* (**5**). — Compounds **4** and **5** were prepared by a modification of a previously described method[13]. A solution of *N. meningitidis* serogroup B polysaccharide (10 mg) in water (4 mL) was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide HCl (100 g), to form the fully esterified polymer. The water-insoluble product was reduced with $NaBH_4$ (or $NaBD_4$) (40 mg) at room temperature for 3 h, and the solution was dialysed for 48 h at 4° against distilled water (4 × 2 L) and freeze-dried. The carboxyl-reduced polysaccharide (2 mg) was hydrolysed in 0.05M H_2SO_4 (1 mL) at 80° for 1 h, and the solution was neutralised with Dowex 2 (HCO_3^-) resin, decanted, and concentrated to dryness. The residue was trimethylsilylated, and subjected to g.l.c. on OV-225. Major products were **4** (60%) and **5** (33%).

Methyl 5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-*nonulopyranoside* (**6**). — *N*-Acetylneuraminic acid (**1**, 5 mg) was methanolysed as described above, to give **2** and **3**. Reduction was performed with $NaBH_4$ (or $NaBD_4$) (20 mg) at room temperature for 3 h, followed by acidification of the solution to pH 6, passage through a short column of Dowex 50 (H^+) resin, and co-concentration with methanol to dryness. The residue was trimethylsilylated, and subjected to g.l.c. on OV-225. Major products were **6** (67%), **4** (17%), and **5** (11%).

Methyl 5-acetamido-1,7-anhydro-3,5-dideoxy-β-D-glycero-D-galacto-*nonulopyranoside* (**7**). — A mixture of carboxyl-reduced B polysaccharide (prepared as described above) (2 mg), Dowex 50 (H^+) resin (40 mg), and methanol (1 mL) was boiled under reflux and shaken for 15 h. After decantation, the solution was concentrated to dryness. The residue was trimethylsilylated, and subjected to g.l.c. on OV-225. Major products were **7** (80%) and **2** (9%).

Methyl 5-acetamido-1,7-anhydro-3,5-dideoxy-β-D-galacto-*octulopyranoside* (**8**). — Carboxyl-reduced B polysaccharide (non-deuterated derivative) (5 mg) was methanolysed as described for the preparation of **7**. A solution of the residue (3 mg) in 10mM $NaIO_4$ (3 mL) was stored at room temperature for 1 h. Excess of periodate was reduced by the addition of glycerol (10 μL), and then $NaBH_4$ (or $NaBD_4$) (12 mg) was added. After storage at room temperature for 3 h, the solution was neutralised to pH 6, passed through a short column of Dowex 50 (H^+) (upper half) and Dowex 2 (HO^-) resins (lower half), and concentrated to dryness. The residue was trimethylsilylated, and subjected to g.l.c. on OV-225. The major product was **8** (80%).

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