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# DETERMINATION OF THE LINKAGES IN SOME METHYLATED, SIALIC ACID-CONTAINING, MENINGOCOCCAL POLYSACCHARIDES BY MASS SPECTROMETRY*

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ABSTRACT

The application of gas–liquid chromatography–mass spectrometric (g.l.c.–m.s.) analysis to a number of sialic acid-containing polysaccharides of meningococcal origin has been studied. Methylation of these polysaccharides by the Hakomori conditions resulted in both *O*- and *N*-methylation. Methanolysis of the methylated polysaccharides from serogroup C [(2→9)-linked], colominic acid [(2→8)-linked], and serogroups Y and W-135 [both (1→4)-linked], yielded the respective 4,7,8-, 4,7,9-, and 7,8,9-tri-*O*-methyl derivatives of methyl *N*-acetyl-*N*-methyl-β-D-neuraminate methyl glycoside. As model compounds, methyl *N*-acetyl-4,7,8,9-tetra-*O*-methyl-α-D-neuraminate methyl glycoside and its *N*-methyl derivative were also synthesized. All of the methylated derivatives could be identified on the basis of their typical fragmentation-patterns, indicating that this method is applicable to the determination of the position of linkages to sialic acid residues in biopolymers.

## INTRODUCTION

Sialic acid occurs as the sole component in the capsular polysaccharides isolated from *N. meningitidis* serogroups B and C[1] and in colominic acid[2] isolated from *E. coli*. It is also present[3] as a major component in the polysaccharides of *N. meningitidis* serogroups Y and W-135. Sialic acids also occur in animal tissues as constituents of glycoproteins and glycolipids[4]. The structures of the capsular polysaccharides have been previously established by $^{13}C$ n.m.r. spectroscopy[3,5]. The linkage to the sialic acid residues was homogeneous in each polysaccharide; to C-9 in that of C, to C-8 in that of B (identical in structure to colominic acid), and to C-4 in those of Y and W-135. Methylation of these polysaccharides, with subsequent acidic scission of the methylated products, should yield three of the four possible trimethyl ethers of sialic acid. These model trimethyl ethers could prove useful in ascertaining the position of linkages to sialic acid in other biopolymers. Previous methylation studies have been confined to glycoproteins, where sialic acid is situated

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TABLE I

RETENTION TIMES OF METHYLATED SIALIC ACID DERIVATIVES AND THE MASSES OF THEIR CHARACTERISTIC FRAGMENT-IONS

| *No.* | *Compound*[a] | *Retention time (Ts)*[b] | *Fragment masses* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *A* | *B* | *C* | *D* | *E* | *F* | *G* | *H* | *I* | $M-CH_3$ | *M* |
| **1** | 4,7,8,9-tetra-OMe-NAcNeu | 1.6 | 334 | 304 | 284 | 240 | 196 | 201 | 169 | 115 | 73 | | 393 |
| **2**[c] | 4,7,8,9-tetra-OMe-NMe-NAcNeu | 1.7 | 348 | 318 | 298 | 254 | 210 | 201 | 169 | 129 | 87 | | 407 |
| **3** | 7,8,9-tri-OMe-NMe-NAcNeu | 3.1 | 334 | 304 | 298 | 254 | 210 | | 155 | 115 | 73 | | 393 |
| **4** | 7,8,9-tri-OMe-4-OAc-NMe-NAcNeu | 3.0 | 376 | 346 | 298 | 254 | 210 | | 197 | 157 | 115 | | 435 |
| **5** | 7,8,9-tri-OMe-4-$OSiMe_3$-NMe-NAcNeu | 2.0 | 406 | 376 | 298 | 254 | 210 | 259 | 227 | 187 | 145 | 450 | 465 |
| **6** | 4,7,9-tri-OMe-NMe-NAcNeu | 2.6<br>1.8[d] | 334 | 318 | 284 | 254 | 210 | 201 | 169 | 129 | 87 | | 393 |
| **7** | 4,7,9-tri-OMe-8-OAc-NMe-NAcNeu | 3.0 | 376 | 318 | 326 | 254 | 210 | 201 | 169 | 129 | 87 | | 435 |
| **8** | 4,7,8-tri-OMe-NMe-NAcNeu | 2.9<br>2.2[d] | 334 | 318 | 298 | 254 | 210 | 201 | 169 | 129 | 87 | | 393 |
| **9** | 4,7,8-tri-OMe-9-OAc-NMe-NAcNeu | 3.6 | 376 | 318 | 298 | 254 | 210 | 201 | 169 | 129 | 87 | | 435 |

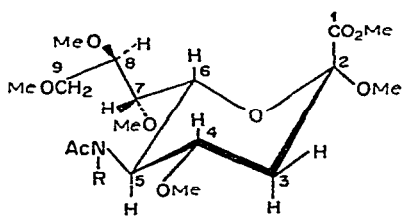
[a]All of the compounds were in the form of their methyl ester methyl glycosides. NMe-NAcNeu = *N*-acetyl-*N*-methylneuraminic acid. [b]With column (*a*). [c]The methyl $\beta$-anomer formed by the equilibration of *2* in 0.1M methanolic HCl gave a mass spectrum identical to that of *2*. [d]As their respective 8- and 9-$OSiMe_3$ derivatives.

as a nonreducing end-group[4]. Related studies on sialic acid derivatives have dealt with the per-*O*-acetyl[6], *O*-acetyl[7] and per-*O*-(trimethylsilyl)[8] derivatives.

## RESULTS AND DISCUSSION

Three individual trimethyl ethers (7,8,9-, 4,7,8-, and 4,7,9-) of *N*-acetylneuraminic acid were prepared by the methylation and subsequent methanolysis of sialic acid-containing polysaccharides of previously determined structures[2,3,5]. Except for the W-135 polysaccharide, which was fully methylated by using methyl iodide, tetrahydrofuran and solid sodium hydroxide[9], it was necessary to resort to the Hakomori reagents[10] to achieve complete methylation of most of the polysaccharides. Even then, the C polysaccharide resisted complete methylation under these conditions, but the corresponding 4,7,8-trimethyl ether could be readily identified by g.l.c. analysis (Table I). It would appear that both of these methods of methylation[9,10] result in *N*-methylation[11–15]. Although none of the polysaccharides were directly methylated by the Hakomori technique, it is obvious from studies on the monomer (methyl *N*-acetyl-α-D-neuraminic acid) that this method also results in *N*-methylation. The trimethyl ethers (**3**, **6**, and **8**) had one hydroxyl group corresponding to the point of linkage in the original polysaccharide. G.l.c. analysis with column (*a*) of **3**, **6**, and **8** as their *O*-acetyl and *O*-trimethylsilyl derivatives enabled all of these trimethyl ethers to be separated.

In order to study the methylation of these polysaccharides, and to assist in the interpretation of the structures of the fragment-ions produced by the mass spectrometry of their methanolyzed products, two model compounds were synthesized from methyl *N*-acetyl-α-D-neuraminic acid. Treatment of the methyl ester of the foregoing methyl glycoside, under conditions previously shown not to result in *N*-methylation[16], gave crystalline methyl *N*-acetyl-4,7,8,9-tetra-*O*-methyl-α-D-neuraminate methyl glycoside (**1**). An attempt to prepare the *N*-methyl derivative (**2**)




1 R=H
2 R=Me

by direct treatment of **1** with the Hakomori reagents[10] resulted in extensive degradation, and this also occurred when these conditions were used on the methyl ester of methyl *N*-acetyl-α-D-neuraminic acid. However, under identical conditions the free acid gave the required *N*-acetyl-*N*-methyl-4,7,8,9-tetra-*O*-methylneuraminic acid

derivative (**2**). These properties can be attributed to the enhanced susceptibility to the strongly basic medium of the carboxyl ester groups in comparison with the relative stability of the free acid, as previously observed with uronic acid residues[12]. Although **2** was not isolated pure, its structure could readily be determined by g.l.c.-m.s. analysis. The mass spectrum of **2** is shown in Fig. 1 and is typical of all of the methylated derivatives studied. The major characteristic fragment-ions are illustrated on the spectrum (Fig. 1) and their mass units (m.u.) are also listed in Table I, together with those of **1** and the other trimethyl ethers studied. It may be seen that a number of major fragment-ions from **2** are 14 m.u. larger than the corresponding fragments from **1**. This is only consistent with the introduction of an *N*-methyl group into **1**.

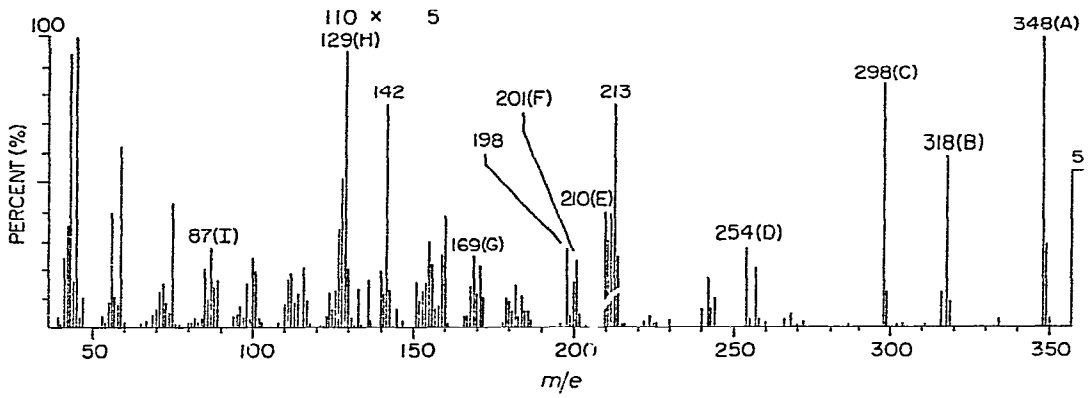
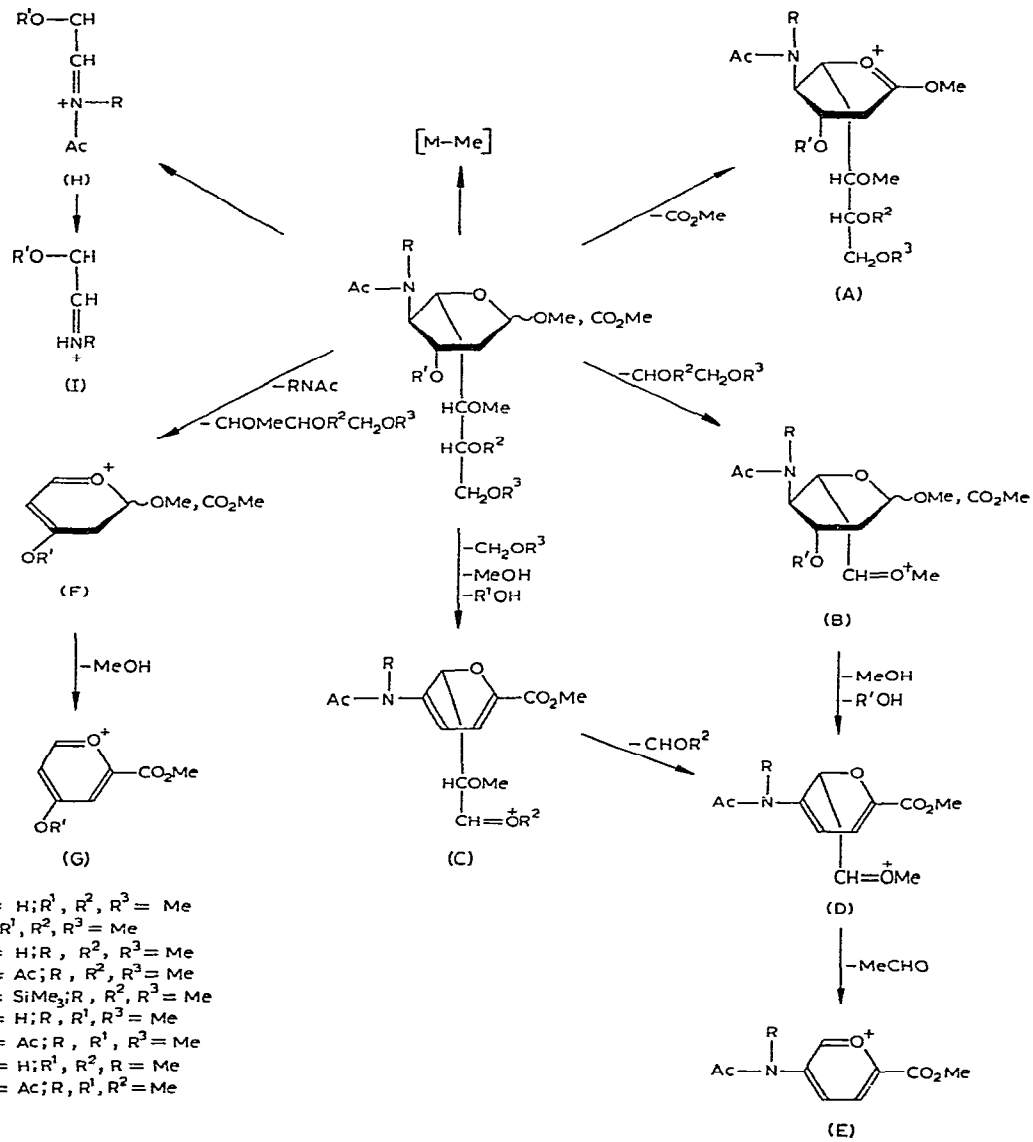



Fig. 1. Mass spectrum of methyl *N*-acetyl-*N*-methyl-4,7,8,9-tetra-*O*-methyl-α-D-neuraminate methyl glycoside (**2**). The peak heights are recorded as a percentage of the base peak at m.u. 45.

A fragmentation pattern can be proposed that is consistent with the major fragment-ions produced in the mass spectra of both **1** and **2**. Further evidence in support of this proposed fragmentation-pattern is that some of these proposed fragment-ions are similar in structure to those proposed for the analogous per-(trimethylsilyl) ethers of sialic acid by Kamerling and coworkers[7,8]. The other methylated derivatives studied (**3**–**9**) also have major fragment-ions that are consistent with this common fragmentation pattern (Fig. 2), and the mass units of the nine different fragments (A–I) are listed for each of the compounds in Table I. In comparison with the per(trimethylsilyl) ethers[7,8], the methyl ethers yielded a number of major, additional fragment-ions including C (Fig. 2), which proved to be crucial in the eventual differentiation of the three trimethyl ethers (**3**, **6**, and **8**) obtained by the methylation of the polysaccharides. Fragment A is formed by the elimination of C-1 and can be represented as $M-C^1O_2Me$, where M represents the intact molecule. Thus, this fragment is useful in determining the molecular weight (M) of the original methylated derivative. Fragment B ($M-CHOR^2CH_2OR^3$) enables the 4-linkage to be differentiated from that of 8-, and 9-, and fragment C ($M-R^1OH-$

$CH_2OR^3 - MeOH$) permits the differentiation of the 8- and 9-linkage. In fact, provided that this fragmentation pattern is maintained, even the remaining linkage (the hypothetical 7-linkage) of the four possible linkages to a sialic acid residue may also be differentiated from the others. The mass-units of the ions A, B, and C generated by the different trimethyl ethers from the four possible linkage-points are shown in Table II. Interestingly, fragment A of the tetra-*O*-methylated **1** has the same number of mass units as the trimethyl ethers (**3**, **6**, and **8**) obtained from the polysaccharides.




1 R = H; $R^1$, $R^2$, $R^3$ = Me
2 R, $R^1$, $R^2$, $R^3$ = Me
3 $R^1$ = H; R, $R^2$, $R^3$ = Me
4 $R^1$ = Ac; R, $R^2$, $R^3$ = Me
5 $R^1$ = $SiMe_3$; R, $R^2$, $R^3$ = Me
6 $R^2$ = H; R, $R^1$, $R^3$ = Me
7 $R^2$ = Ac; R, $R^1$, $R^3$ = Me
8 $R^3$ = H; $R^1$, $R^2$, R = Me
9 $R^3$ = Ac; R, $R^1$, $R^2$ = Me

Fig. 2. Structures of the individual fragment-ions (A–I) given by the methylated, sialic acid derivatives.

This is because, in contrast to **3**, **6**, and **8**, compound **1** is not *N*-methylated and therefore, despite its additional *O*-methyl group, it has the same overall number of methyl groups. This indicates the importance of knowing whether any methylated sialic acid derivative is *N*-methylated or not. Fragments B and C enable **1** to be differentiated from **3**, **6**, and **8** (Table I) and this may also be achieved using fragment D ($M-CHOR^2CH_2OR^3-R^1OH-MeOH$). However, fragment E could prove to be the key fragment in determining whether any of the methylated derivatives of sialic acid are in fact *N*-methylated. This is because fragment E is devoid of all *O*-methyl groups but still contains the 5-acetamido function (–NRAc).

TABLE II

MASSES OF THE LARGER FRAGMENT-IONS OF THE TRI-*O*-METHYLATED SIALIC ACID MONOMERS OBTAINED FROM THE METHYLATED POLYSACCHARIDES

| *Linkage to the sialic acid residues in the polysaccharides* | *Fragments* | | |
|---|---|---|---|
| | *A* | *B* | *C* |
| →4)-Linked (serogroups Y & W-135) | 334 | 304 | 298 |
| →8)-Linked (serogroup B) | 334 | 318 | 284 |
| →9)-Linked (serogroup C) | 334 | 318 | 298 |
| →7)-Linked (hypothetical) | 334 | 304 | 284 |

Fragment F ($M-CHOMeCHOR^2CH_2OR^3-RNAc$) represents a fragment-ion in which the substituent on C-4 remains intact. However, compounds **3** and **4**, having respectively hydroxyl and *O*-acetyl groups on C-4, failed to show the presence of this ion in their mass spectra. This situation was rectified with fragment G, which is formed by the elimination of the glycosidic methoxyl group from F. In this case, all of the compounds (including **3** and **4**) gave this fragment-ion, which differed by numbers of mass units corresponding to the masses of the substituents on C-4 of each compound. Fragment H is formed by scission of the ring between C-5 and C-6, and C-3 and C-4, to give $R^1OCHCHN^+RAc$, whereas fragment I is formed by the further loss of ketene ($CH_2=CO$) from H. These fragments are also very useful in determining the nature of substituents at C-4.

Additional evidence in support of the proposed fragmentation-pattern (Fig. 2) can be obtained by acetylating or (trimethylsilyl)ating the free hydroxyl groups of **3**, **6**, and **8**. This derivatization was the most informative with the compound (**3**) having the free hydroxyl group on C-4, because of the retention of this group in a larger number of fragments. The 4-*O*-acetyl (**4**) and 4-*O*-trimethylsilyl (**5**) derivatives gave fragments A, B, G, H, and I, all differing from **3** by 42 m.u. for **4** and 72 m.u. for **5**. In contrast, **7** (formed by 8-*O*-acetylation of **6**) gave only two fragments (A and C), differing from **8** by 42 m.u. Of all of the compounds analyzed by g.l.c.–m.s., only **5** gave a high molecular-weight ion of 450 m.u. arising from loss of a methyl group ($M-CH_3$) from the *O*-trimethylsilyl substituent of **5**. This characteristic mode of

fragmentation of *O*-trimethylsilyl ethers has been reported previously in studies on other *O*-trimethylsilyl derivatives of sialic acid[7,8].

## EXPERIMENTAL

*Materials and methods.* — Purified capsular polysaccharides from *N. meningitidis* serogroups C, Y and W-135 were obtained as previously described[3,5]. The serogroup C and Y polysaccharides were *O*-deacetylated also as previously described[5]. Colominic acid (*Escherichia coli*) was obtained from Koch-Light Laboratories, Colnbrook, England and *N*-acetylneuraminic acid was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Ill., U.S.A. Methyl *N*-acetyl-α-D-neuraminic acid was prepared by the method of Yu and Ledeen[17].

*Methylation of* N-*acetyl*-α-D-*neuraminic acid methyl glycoside.* — The title glycoside (140 mg) was methylated by the method of Kuhn *et al.*[16] and the syrupy product was crystallized from 1:6 ethyl acetate–hexane to give crystals of methyl *N*-acetyl-4,7,8,9-tetra-*O*-methyl-α-D-neuraminate methyl glycoside (**1**, 70 mg), m.p. 144–145°, $[\alpha]_D^{23}$ +0.2° (*c* 1.04, methanol); $^1$H n.m.r. (100 MHz, $CDCl_3$): δ 3.79 ($CO_2Me$), 3.46 (double intensity), 3.38, 3.33, 3.22, and 1.98 (–NHAc) representing seven methyl signals in the compound.

*Anal.* Calc. for $C_{17}H_{31}NO_9$: C, 51.9; H, 7.89; N, 3.56. Found: C, 51.6; H, 7.97; N, 3.73.

To prepare the *N*-methyl derivative (**2**) of the foregoing compound, it was not possible to subject the methylated derivative directly to a Hakomori methylation[10] because of extensive degradation in the reaction. This degradation also occurred when the Hakomori methylation[10] was performed on the methyl ester of methyl *N*-acetyl-α-D-neuraminic acid. However, the free acid (methyl *N*-acetyl-α-D-neuraminic acid) could be methylated without extensive degradation using these conditions[12]. The mixture was applied to a column of Sephadex LH20, which was eluted with methanol to yield a syrup that contained one major component (80%), as revealed by g.l.c. and which had a retention volume almost identical to that of the *N*-acetyl compound (**1**) (Table I). The structure of this compound (**2**) was confirmed by g.l.c.–m.s. in comparison with the *N*-acetyl derivative (Table I). Attempted crystallization of the *N*-methyl derivative (**2**) was unsuccessful.

*Methylation of the polysaccharides.* — The serogroup C, Y and W-135 polysaccharides and colominic acid (100-mg quantities) were first reduced with sodium borohydride in aqueous solution to avoid alkaline degradation during methylation. The reduced polysaccharides were partially methylated by the method of Haworth[18], and the partially methylated polysaccharides were then dissolved in 4:1 tetrahydrofuran–water and methylated again by the same procedure. The products of these methylations were then methylated by the method of Falconer and Adams[9]. Only the W-135 polysaccharide was found to be completely methylated at this stage, yielding 90 mg of product. Incomplete methylation of these linear polymers could be readily ascertained by g.l.c. analysis. A small sample of the methylated polysaccharide was

methanolyzed, and the trimethylsilyl ethers of the products were made as described. Any incomplete methylation resulted in the formation of di- or tri(trimethylsilyl) ethers. These derivatives had characteristically faster retention-volumes on column (*a*) than those of the mono(trimethylsilyl) ethers (Table I). The remaining, partially methylated polysaccharides were further methylated by the Hakomori procedure[10] to yield approximately 60 mg of fully methylated polysaccharides, except in the case of the C polysaccharide, which could not be fully methylated.

*Identification of the monomer units of the methylated polysaccharides.* — The methylated polysaccharides were methanolyzed with methanolic hydrogen chloride. It was found to be sufficient to use 0.1M reagent for 3 h at 100° for sialic acid homopolymers (C and colominic acid), whereas the Y and W-135 polysaccharides required M reagent for 12 h at 100° for complete fission. The methanolyzed samples were re-*N*-acetylated with acetic anhydride. The methylated sugars were *O*-acetylated by treatment with acetic anhydride and pyridine. The trimethylsilyl derivatives were prepared by the method of Brobst and Lott[19].

The methylated derivatives of *N*-acetylneuraminic acid were examined by g.l.c. on glass column (*a*), 2% OV-17 on Chromosorb G (100–120 mesh), at 230° and a carrier gas flow-rate of ~60 ml/min on a Hewlett-Packard, Model 402 gas chromatograph. The results are shown in Table I and retention volumes are recorded relative to that of the trimethylsilyl derivative of the methyl ester methyl α-glycoside of *N*-acetylneuraminic acid (Ts).

The 70 eV mass spectra were recorded on a Finnigan 3100D gas chromatograph mass spectrometer (g.l.c.–m.s.) connected on-line to a computer. The gas chromatograph was used with a column of 3% SE-30 on Gas Chrom Q at 230°, and the ion-source temperature was 90°.

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