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SIALIC ACIDS IN PERMETHYLATION ANALYSIS: PREPARATION AND IDENTIFICATION OF PARTIALLY *O*-METHYLATED DERIVATIVES OF METHYL *N*-ACETYL-*N*-METHYL-β-D-NEURAMINATE METHYL GLYCOSIDE*

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ABSTRACT

A set of partially *O*-methylated derivatives of methyl *N*-acetyl-*N*-methyl-β-D-neuraminate methyl glycoside has been prepared as reference compounds for the incorporation of acylneuraminic acids into methylation analysis. G.l.c.–m.s. data for the *O*-trimethylsilyl and *O*-acetyl derivatives of these compounds are described in detail. The various substances give rise to highly characteristic mass-spectrometric fragmentation-patterns.

INTRODUCTION

In glycoconjugates, acylneuraminic acids have been detected as terminal units of the carbohydrate chain, coupled *via* α-glycosidic linkage. The amino group of the acylneuraminic acids is acetylated or glycoloylated, whereas the hydroxyl groups can be acylated (acetylated[1,2], lactoylated[3,4], or glycoloylated[5]), methylated[6,7], or sulphated[8]. Sometimes, small oligomeric chains of acylneuraminic acids are attached to the carbohydrate backbone of the glycoconjugate[9,10]. Acylneuraminic acids are also found as constituents of homo- and hetero-polysaccharides[11–13].

Linkage analysis of internal acylneuraminic acid residues is based mainly on periodate oxidation or Smith degradation (after saponification of possible ester groups); in some cases, ^{13}C-n.m.r. spectroscopy has also been used[11,12]. Until now, methylation analysis was not applicable, because of the lack of suitable references

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(partially methylated acylneuraminic acid derivatives). We now describe a number of partially *O*-methylated derivatives of methyl *N*-acetyl-*N*-methyl-β-D-neuraminate methyl glycoside, which can be analysed by g.l.c.–m.s. on the basis of a system[2–4] for the identification of the number, type, and position of the *O*-acyl groups, and of the type of the *N*-acyl group, in naturally occurring, free and bound acylneuraminic acids.

TABLE I

PARTIALLY *O*-METHYLATED DERIVATIVES (6–12) OF METHYL *N*-ACETYL-*N*-METHYL-β-D-NEURAMINATE METHYL GLYCOSIDE, TOGETHER WITH THE G.L.C. DATA FOR THE CORRESPONDING Me_3Si AND Ac DERIVATIVES

Compound[a]		R_N[b] *Me_3Si derivative*	*Ac derivative*
5	4,7,8,9-Tetra-*O*Me-*N*Me-*N*AcNeu	1.00	
9	4,8,9-Tri-*O*Me-*N*Me-*N*AcNeu	1.07	1.08
10	4,7,9-Tri-*O*Me-*N*Me-*N*AcNeu	1.14	1.25
11	4,7,8-Tri-*O*Me-*N*Me-*N*AcNeu	1.30	1.47
8	4,9-Di-*O*Me-*N*Me-*N*AcNeu	1.27	1.26
12	4-*O*Me-*N*Me-*N*AcNeu	1.70	1.70
7	9-*O*Me-*N*Me-*N*AcNeu	1.43	1.63
6	*N*Me-*N*AcNeu	1.89	2.17

[a]All compounds are in the form of their methyl ester methyl β-D-glycosides. *N*Me-*N*AcNeu = *N*-acetyl-*N*-methylneuraminic acid. [b]The R_N values (3.8% of SE-30 at 220°) are given relative to methyl *N*-acetyl-*N*-methyl-4,7,8,9-tetra-*O*-methyl-β-D-neuraminate methyl glycoside (**5**).

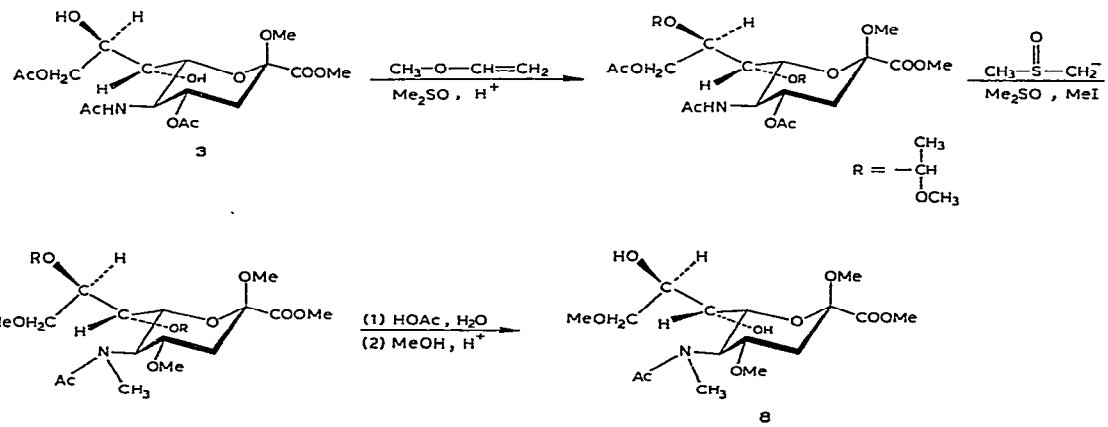


Fig. 1. Reaction scheme for the preparation of methyl *N*-acetyl-*N*-methyl-4,9-di-*O*-methyl-β-D-neuraminate methyl glycoside (**8**).

RESULTS

For the preparation of the methylated *N*-acetylneuraminic acid derivatives **5–12** (Table I), the methylation procedures of Hakomori[14] or Kuhn *et al.*[15] were used. Both methylations gave complete *N*-methylation, as shown by g.l.c.–m.s.

Compounds **6–9** were prepared from **1–4**, respectively. The synthesis of **8** is shown in Fig. 1, as a typical example of this derivatization. The starting compounds were treated with methyl vinyl ether to convert the free hydroxyl groups into the base-stable 1-methoxyethyl groups[16], and subsequently permethylated according to Hakomori. In the latter reaction, *O*-acetyl groups are replaced by *O*-methyl groups. The 1-methoxyethyl groups were then removed by treatment with acetic acid. Any partial de-esterification at C-1 was corrected by treatment with methanol–Dowex 50 (H^+) resin.

For the preparation of **10**, colominic acid, a homopolymer of (2 → 8)-linked *N*-acetylneuraminic acid residues[11,13,17], was permethylated[14]. The methylated polysaccharide was then methanolyzed and re-*N*-acetylated.

To obtain **11**, **1** was 9-*O*-tritylated, methylated[15], and finally detritylated with acetic acid. Compound **12** was isolated as a by-product, demonstrating some undermethylation of the trityl derivative.

To illustrate the highly characteristic mass-spectra that are obtained from acylneuraminic acid derivatives, the spectrum of **5** is given in Fig. 2. Table II contains assignments for the most relevant fragment-ions present in this mass spectrum. The interpretation is based on exact mass measurements, together with information deducible from the spectra of **6–12** and from data of acylneuraminic acid derivatives reported earlier[2]. For the analysis of **6–12** by g.l.c.–m.s., the free hydroxyl groups were trimethylsilylated or acetylated. In Table I, the retention times R_N of the various substances are given relative to **5**. The mass-spectrometric analysis of the acetylated, partially methylated neuraminic acids was carried out as described for the identification of the trimethylsilylated, naturally occurring, *O*-acetylated *N*-acylneuraminic acids[2] (fragments *A–G*). For the determination of the trimethylsilylated, partially

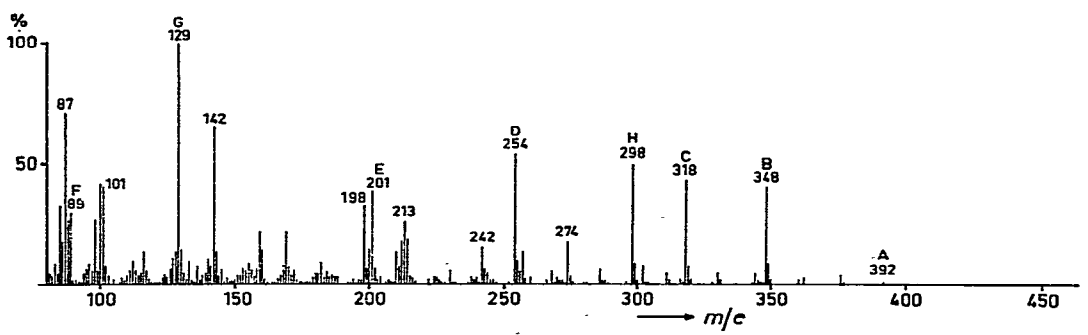


Fig. 2. Mass spectrum of methyl *N*-acetyl-N-methyl-4,7,8,9-tetra-*O*-methyl-β-D-neuraminate methyl glycoside (**5**); only values *m/e* > 80 and intensities ≥ 1% are given.

methylated neuraminic acids, fragment *H* is used in addition to *A–G*. In Table III, the *m/e* values of the fragment ions *A–H* of **5–12** are presented.

In the mass spectra of the *O*-trimethylsilyl (Me_3Si) derivatives of **6–12**, fragment *A* (M − CH_3) can be formed by elimination of a methyl group from a Me_3Si group or from the *N*-acetyl-*N*-methyl group. The low abundance of fragment *A* in the mass spectra of **5** and the acetyl (Ac) derivatives of **6–12** suggests that, in the Me_3Si derivatives of **6–12**, the main contribution to the abundance of the ion [M − CH_3] stems from the elimination of a methyl group from a Me_3Si group.

Fragment *B* (M − $COOCH_3$) is formed only by elimination of the C-1 part of the molecule, which is in accordance with the observation of Kochetkov *et al.*[7] that, in the peracetyl derivative of **1**, the elimination of an acetoxy radical from the molecular ion does not occur.

As was demonstrated earlier[2,18], fragments *C* (M − $CHOR^8CH_2OR^9$) and *D* (M − $CHOR^8CH_2OR^9$ − CH_3OH − R^4OH) have only significant abundance if

TABLE II

INTERPRETATION OF SOME IMPORTANT FRAGMENT IONS PRESENT IN THE MASS SPECTRUM OF **5**

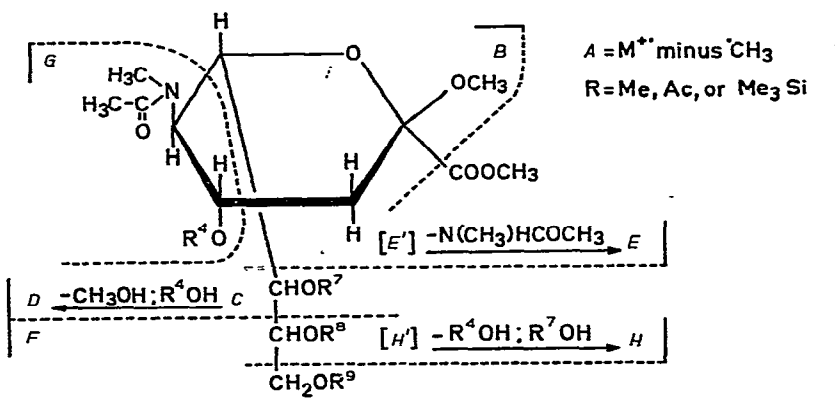
m/e	*Formula*	*Fragment*
392 (*A*)	$C_{17}H_{30}NO_9$	M − CH_3
376	$C_{17}H_{30}NO_8$	M − OCH_3
362 (*H*[1])	$C_{16}H_{28}NO_8$	M − CH_2OCH_3
348 (*B*)	$C_{16}H_{30}NO_7$	M − $COOCH_3$
344	$C_{16}H_{26}NO_7$	M − OCH_3−CH_3OH
330	$C_{15}H_{24}NO_7$	M − CH_2OCH_3−CH_3OH
318 (*C*)	$C_{14}H_{24}NO_7$	M − $CHOCH_3CH_2OCH_3$
311	$C_{15}H_{21}NO_6$	M − 3 × CH_3OH
302	$C_{14}H_{22}O_7$	M − CH_3OH−$N(CH_3)HCOCH_3$
298 (*H*)	$C_{14}H_{20}NO_6$	M − CH_2OCH_3−2 × CH_3OH
286	$C_{13}H_{20}NO_6$	M − $CHOCH_3CH_2OCH_3$−CH_3OH
274 (*E*[1])	$C_{12}H_{20}NO_6$	M − $CHOCH_3CHOCH_3CH_2OCH_3$
254 (*D*)	$C_{12}H_{16}NO_5$	M − $CHOCH_3CH_2OCH_3$−2 × CH_3OH
242	$C_{11}H_{16}NO_5$	M − $CHOCH_3CHOCH_3CH_2OCH_3$−CH_3OH
230	$C_{11}H_{20}NO_4$	CH_3CO–$N(CH_3)$ = CH–CH = $C(OCH_3)$–$CHOCH_3$–CH_2OCH_3
213	$C_{10}H_{13}O_5$	M − $CHOCH_3CH_2OCH_3$−CH_3OH−$N(CH_3)HCOCH_3$
201 (*E*)	$C_9H_{13}O_5$	M − $CHOCH_3CHOCH_3CH_2OCH_3$−$N(CH_3)HCOCH_3$
198	$C_{10}H_{16}NO_3$	CH_3CO–$N(CH_3)$ = CH–CH = $C(OCH_3)$–CH = $CHOCH_3$ CH_3CO–$N(CH_3)$ = CH–CH = $C(OCH_3)$–$C(OCH_3)$ = CH_2
169	$C_8H_9O_4$	M − $CHOCH_3CHOCH_3CH_2OCH_3$−$N(CH_3)HCOCH_3$−CH_3OH
142	$C_7H_{12}NO_2$	CH_3CO–$N(CH_3)$ = CH–CH = $CHOCH_3$ CH_3CO–$N(CH_3)$ = CH–$C(OCH_3)$ = CH_2
129 (*G*)	$C_6H_{11}NO_2$	CH_3CO–$N(CH_3)$–CH–CH = OCH_3
101	$C_5H_9O_2$	CH_2 = $C(OCH_3)$–CH = OCH_3 $CH(OCH_3)$ = CH–CH = OCH_3
100	$C_5H_{10}NO$	$N(CH_3)H$ = CH–CH = $CHOCH_3$ $N(CH_3)H$ = CH–$C(OCH_3)$ = CH_2
89 (*F*)	$C_4H_9O_2$	CH_2OCH_3–CH = OCH_3
87	C_4H_9NO	$N(CH_3)H$–CH–CH = OCH_3

TABLE III

CHARACTERISTIC FRAGMENT IONS, *A–H*, USED FOR THE IDENTIFICATION OF THE *O*-METHYLATED DERIVATIVES (**5–12**) OF METHYL *N*-ACETYL-*N*-METHYL-β-D-NEURAMINATE METHYL GLYCOSIDE

Compound	*OMe Groups*	A	B	C	D	E	F	G	H	*Derivative*
5	1,2,4,7,8,9	392	348	318	254	201	89	129	298	—
9	1,2,4,..,8,9	450	406	376	312	201	89	129	298	Me_3Si
		420	376	—[a]	—	201	89	129		Ac
10	1,2,4,7,..,9	450	406	318	254	201	147	129	356	Me_3Si
		420	376	318	254	201	—	129		Ac
11	1,2,4,7,8,.	450	406	318	254	201	147	129	298	Me_3Si
		420	376	318	254	201	117	129		Ac
8	1,2,4,..,..,9	508	464	376	312	201	147	129	356	Me_3Si
		448	404	—	—	201	—	129		Ac
12	1,2,4,..,..,.	566	522	376	312	201	205	129	356	Me_3Si
		476	432	—	—	201	—	129		Ac
7	1,2,.,.,.,9	566	522	434	312	259	147	187	356	Me_3Si
		476	432	—	—	—	—	157		Ac
6	1,2,.,.,.,.	624	580	434	312	259	205	187	356	Me_3Si
		504	460	—	—	—	—	157		Ac

[a]— = absent or hardly observable (see text).



C-7 bears an ether group. In the mass spectra of the Ac derivatives of **6–9** and **12**, these fragment ions are absent, or hardly observable (≤1% of the base peak).

Fragment *E* (M − $CHOR^7CHOR^8CH_2OR^9$ − $N(CH_3)HCOCH_3$) is not observed if an *O*-acetyl group is attached[2] at C-4, as is the case in the Ac derivatives of **6** and **7**. This illustrates that the transition state in the McLafferty rearrangement, which is necessary to form fragment *E*, is more favoured when the substituent at C-4 is an ether group rather than an ester group.

Fragment *F* ($CH_2OR^9CHOR^8$) can only be formed readily[2,18] if an ether

group is attached to C-8. Therefore, in the Ac derivatives of **6–8**, **10**, and **12**, this fragment ion is almost absent (≤3% of the base peak). In the mass spectrum of the Ac derivative of **9**, fragment *F* is detected at *m/e* 89; the peak present at *m/e* 117 arises from the fragment ion $C_6H_{13}O_2$.

Two different ions contribute to the intensity of the peak at *m/e* 89 in the Me_3Si derivative of **9**, namely, CH_2OCH_3–$CHOCH_3$ (*F*; $C_4H_9O_2$) and OMe_3Si (C_3H_9OSi). The latter fragment always occurs in the mass spectra of Me_3Si carbohydrates, including the Me_3Si derivatives of **6–8** and **10–12**.

Two different fragment ions contribute to the intensity of the peak at *m/e* 147 in the Me_3Si derivatives of **7** and **8**, namely, CH_2OCH_3–$CHOSiMe_3$ (*F*; $C_6H_{15}O_2Si$) and $Me_3SiOSi(CH_3)_2$ ($C_5H_{15}OSi_2$). The latter fragment is always present in the mass spectra of Me_3Si carbohydrates having more than one Me_3Si group. In the Me_3Si derivatives of **6** and **12**, the peak at *m/e* 147 stems only from the fragment ion $C_5H_{15}OSi_2$.

Fragment *G* [$CH_3CON(CH_3)CH$–$CHOR^4$] is found at *m/e* 129 when R^4 is CH_3, and is the base peak in the region *m/e* > 80 (**5**, and the Me_3Si and Ac derivatives of **8–12**; $C_6H_{11}NO_2$; 129.0790). The mass spectra of Me_3Si carbohydrates always show a peak at *m/e* 129 with low intensity. De Jongh *et al.*[19] found two formulae for this peak, namely, $C_5H_9O_2Si$ (129.0372) and $C_6H_{13}OSi$ (129.0736).

In the Me_3Si derivatives of **6** and **7**, the peak at *m/e* 129 is less important, as R^4 is Me_3Si (Found: $C_5H_9O_2Si$ and $C_6H_{13}OSi$). The ions $C_6H_{13}OSi$ and $C_6H_{11}NO_2$ cannot be distinguished, but comparison of the intensities of the peak at *m/e* 129 in the mass spectra of the Me_3Si derivatives of **8–12** with those of **6** and **7**, and further with the Ac derivatives of **8–12** and compound **5**, makes it highly probable that the main contribution to the peak at *m/e* 129 in the Me_3Si derivatives of **8–12** stems from $C_6H_{11}NO_2$ and not from $C_6H_{13}OSi$. The fragment ion $C_5H_9O_2Si$ was detected in the Me_3Si derivatives of **8**, **9**, and **12**, in addition to $C_6H_{11}NO_2$ (and $C_6H_{13}OSi$). In the Ac derivatives of **8–11**, the peak at *m/e* 129 consists mainly of the contribution from fragment *G* ($C_6H_{11}NO_2$), together with a small contribution from a fragment ion $C_6H_9O_3$ (129.0552). The latter ion originates from the C-7,8,9 part of the molecule after elimination of CH_3OH or CH_3COOH[18], as appropriate.

Fragment *H* (M − CH_2OR^9 − R^4OH − R^7OH) is useful for discriminating between an OMe_3Si group at C-8 or C-9 in the Me_3Si derivatives. For Ac derivatives, fragment *H* can be neglected, since the occurrence of fragment *F* is already indicative for an OAc group at C-8 or C-9. In the mass spectra of the mono-OAc derivatives, fragment *H* is present, even when the acetyl group is attached to C-8. However, this fragment ion is hardly detectable when two or more acetyl groups are present in the side chain.

DISCUSSION

Methylation analysis is frequently used for the determination of the position of glycosidic bonds in glycoconjugates, and in oligo- and polysaccharides[14,18,20].

After permethylation, the compound is solvolysed (*e.g.*, hydrolysed or methanolysed) and the resulting mixture of partially methylated monomers analysed.

Partially *O*-methylated derivatives of methyl *N*-acetyl-*N*-methyl-D-neuraminate methyl glycoside, obtained by methanolysis and re-*N*-acetylation of a permethylated compound containing acylneuraminic acid(s), can be analysed by g.l.c.–m.s. after trimethylsilylation or acetylation of the free hydroxyl groups. The equilibrium methanolysis mixture contains predominantly the methyl β-D-glycoside of each acylneuraminic acid derivative; only a few percent of the corresponding α-D anomers are present[17,21]. The mass spectra of the intact ring-forms are highly characteristic with respect to the substitution pattern of *O*-methyl groups.

For the identification of the partially *O*-methylated derivatives of methyl *N*-acetyl-*N*-methyl-β-D-neuraminate methyl glycoside, the availability of a number of reference compounds is required. To obtain a reliable system for the mass-spectrometric characterization of the substances, mass spectra were recorded for both the Me_3Si and Ac derivatives. Owing to the presence of only ether substituents, the mass spectra of the Me_3Si derivatives contain each of the selected fragment-ions *A–H*. The molecular weight, and therefore the number of Me_3Si groups, can be deduced from the fragments *A* and *B*. The fragments *C–H* provide information about the positions of the Me_3Si groups. On the other hand, the Ac derivatives give mass spectra wherein some selected fragment-ions may be absent, depending on the

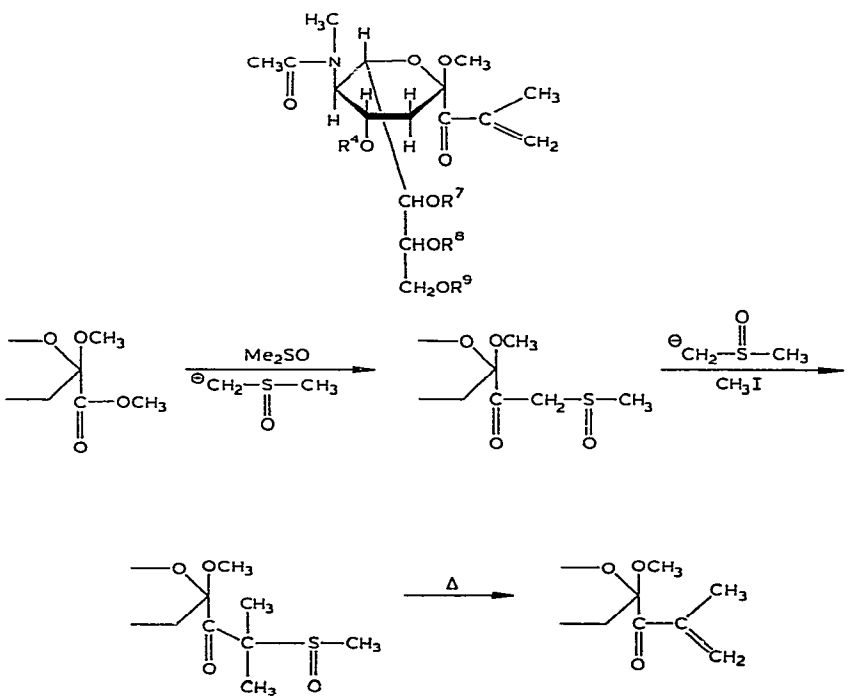


Fig. 3. Structure of the by-product and its formation.

presence of Ac groups at C-4, C-7, or C-8. The molecular weight, and therefore the number of Ac groups, is evident from the fragments *A* and *B*, whereas the positions of the latter groups follow from the fragments *C-G*.

Methylation of a methyl acylneuraminate with sodium methylsulphinylmethanide as base yields a by-product (~50% yield) in addition to the expected derivative. The structure of the by-product, given in Fig. 3, was deduced from comparative studies of the mass spectra of **5–9** with those of the corresponding alternative substances, high-resolution mass spectrometry, and labelling experiments with CD_3I and $^{13}CH_3I$. During the reaction with sodium methylsulphinylmethanide, the methyl ester group (C-1) is transformed to a β-ketosulphoxide[22]. After addition of CH_3I, the methylene group is di-*C*-methylated, and finally methylsulphenic acid is eliminated[23,24]. Hakomori methylation of compounds containing acylneuraminic acid does not give rise to this side-reaction[17]. However, the finding of this type of product in methylation analysis gives a direct indication of the presence of (naturally occurring) neuraminate esters.

The partially *O*-methylated derivatives of methyl *N*-acetyl-*N*-methyl-β-D-neuraminate methyl glycoside can also serve as model compounds for the analysis of naturally occurring, *O*-acylated *N*-acylneuraminic acids, to establish the position of the *O*-acyl substituents. So far *O*-acyl groups have been detected[1–4] at C-4, C-7, and/or C-9. However, the occurrence of acyl-migrations during the isolation procedures cannot be completely excluded. To define the position of *O*-acyl groups in native glycoconjugates, the following procedure can be used: The compound is treated with methyl vinyl ether[16] to block the free hydroxyl groups, and subsequently the *O*-acyl groups are replaced by stable *O*-methyl groups (see Fig. 1).

The suitability of the mass-spectrometric method of identification described herein has recently been demonstrated for the confirmation of the (2 → 8)-type of linkage in the ganglioside G_{T1b} and in colominic acid[17].

EXPERIMENTAL

General methods. — G.l.c. was carried out on a Varian Aerograph 2740-30-01, equipped with dual flame-ionization detectors, and glass columns (2.00 m × 4.0 mm i.d.) packed with 3.8% of SE-30 on Chromosorb W/AW-DMCS (HP, 80–100 mesh). The oven temperature for the column was 220°, and the nitrogen flow-rate was 40 ml/min. Retention times were determined with a Varian CDS 101 integrator.

The 75-eV mass spectra were recorded on a Jeol JGC-1100/JMS-07 combination (column material, SE-30; oven temperature, 200°; ion-source temperature, 250°; accelerating voltage, 1.5 kV or 3.0 kV; ionizing current, 300 μA). High-resolution mass measurements were performed with a dynamic resolving power of 10.000 and a scan speed of 16 sec per mass decade by using an AEI MS-902 mass spectrometer (ion-source temperature, 100°; accelerating voltage, 8 kV; ionizing current, 500 μA) connected on-line with a Ferranti Argus 500 computer.

T.l.c. was performed on Kieselgel 60 F_{254} (0.5-mm DC-Fertigplatten; Merck)

with *A*, benzene–methanol[25] (96:4); *B*, benzene–ethanol–water–ammonia[26] (200:47:15:1, organic layer); *C*, chloroform–methanol (9:1); and *D*, hexane–acetone (3:2). Spots were detected with the orcinol–Fe^{3+}–HCl reagent[27] (*O*-acetylated neuraminic acid derivatives), or with a 1:1 mixture of 0.2% naphthoresorcinol in methanol and 20% H_2SO_4 in methanol (*O*-methylated neuraminic acid derivatives).

Compounds (0.5 mg) were trimethylsilylated in 1 ml of a mixture of hexamethyldisilazane–chlorotrimethylsilane–pyridine (2:1:10). After 2 h at room temperature, 2 ml of chloroform and 2 ml of water were added to the turbid mixture. The chloroform layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*.

Compounds (0.5 mg) were acetylated in 1 ml of a mixture of acetic anhydride–pyridine (1:1) for 30 min at 95°. The solvent was then removed by coevaporation to dryness with absolute ethanol.

Neuraminic acid derivatives **1–4**. — Methyl *N*-acetyl-β-D-neuraminate methyl glycoside (**1**) was prepared by the procedure of Yu and Ledeen[28].

The methyl β-D-glycosides of methyl *N*-acetyl-9-*O*-acetylneuraminate (**2**), methyl *N*-acetyl-4,9-di-*O*-acetylneuraminate (**3**), and methyl *N*-acetyl-4,8,9-tri-*O*-acetylneuraminate (**4**) were obtained by *O*-acetylation of **1** with *N*-acetylimidazole, as described by Haverkamp *et al.*[29]. By g.l.c.–m.s., it was shown that, besides **2**, **3**, and **4**, small proportions of the 8-*O*-acetyl and 4,8-di-*O*-acetyl derivatives were also formed, with retention values of 1.12 and 1.28 relative to the Me_3Si derivative of methyl *N*-acetyl-β-D-neuraminate, respectively.

Methyl N-*acetyl*-N-*methyl-4,7,8,9-tetra*-O-*methyl*-β-D-*neuraminate methyl glycoside* (**5**). — Compound **5** was prepared by methylation of **1** (10 mg) using either the procedure of Kuhn *et al.*[15] or that of Hakomori[14]. The title compound was purified by preparative t.l.c. in solvent *A* (R_F 0.24).

Methyl N-*acetyl*-N-*methyl*-β-D-*neuraminate methyl glycoside* (**6**), *methyl* N-*acetyl*-N-*methyl-9*-O-*methyl*-β-D-*neuraminate methyl glycoside* (**7**), *methyl* N-*acetyl*-N-*methyl-4,9-di*-O-*methyl*-β-D-*neuraminate methyl glycoside* (**8**), *and methyl* N-*acetyl*-N-*methyl-4,8,9-tri*-O-*methyl*-β-D-*neuraminate methyl glycoside* (**9**). — In a capped serum-flask of 25 ml, the dry compound (10 mg of **1**, **2**, **3**, or **4**, respectively) dissolved in dry methyl sulphoxide (5 ml) was treated with methyl vinyl ether[16] (2 ml); 1 mg of dry, recrystallized *p*-toluenesulphonic acid in 10 µl of methyl sulphoxide was added as catalyst. The solution was stirred magnetically at 18–20° until an intense yellow colour was obtained (3–7 h). The excess of methyl vinyl ether was removed by a stream of nitrogen at 30–40°. Methylation was performed by the Hakomori method (2 ml of 2M sodium methylsulphinylmethanide in methyl sulphoxide; 2 ml of methyl iodide). The methylated product was isolated by partition between water and chloroform. The residue of the organic layer was treated with 50% acetic acid (3 ml) for 1 h at 95°. After evaporation of the solvent, the residue was dried over P_2O_5. Subsequently, 3 ml of absolute methanol and 300 mg of Dowex 50 X8 (H^+) resin were added, and the mixture was stirred for 2 h at room temperature. After filtration of the solution, the resin was thoroughly washed with 50 ml of

methanol. The combined methanol solution was evaporated and the residue dried over P_2O_5. The products **6**, **7**, **8**, and **9**, respectively, were purified by preparative t.l.c. in solvent *B* ($R_F \sim 0.30$). For g.l.c.–m.s. analysis, the compounds were trimethylsilylated or acetylated.

Methyl N-*acetyl*-N-*methyl-4,7,9-tri*-O-*methyl-β*-D-*neuraminate methyl glycoside* (**10**). — Colominic acid (25 mg) from *Escherichia coli* (Koch–Light Labs. Ltd.) was applied to a small column of Dowex 50 X8 (H^+) resin, and eluted with water. The eluate was lyophilized, and the residue was dried over P_2O_5 and subsequently dissolved in dry methyl sulphoxide (3.5 ml). The polysaccharide was methylated by the Hakomori method (3.5 ml of 2M sodium methylsulphinylmethanide in methyl sulphoxide; 3.5 ml of methyl iodide). After dialysis against water, lyophilisation, and drying of the material over P_2O_5, the resulting permethylated colominic acid was dissolved in 7 ml of methanolic 0.5M HCl. Nitrogen was bubbled through the solution and then the ampoule was sealed. The sample was heated for 24 h at 85°, the acid neutralized with Ag_2CO_3, and the product re-*N*-acetylated by the addition of 0.8 ml of acetic anhydride for 24 h at room temperature. The precipitate was triturated thoroughly and centrifuged, and the supernatant solution collected. The residue was washed twice with 1 ml of dry methanol. The combined supernatant solutions were evaporated under reduced pressure. Compound **10** was purified by preparative t.l.c. in solvent *C* (R_F 0.46). For g.l.c.–m.s. analysis, the compound was trimethylsilylated or acetylated.

Methyl N-*acetyl*-N-*methyl-4,7,8-tri*-O-*methyl-β*-D-*neuraminate methyl glycoside* (**11**) *and methyl* N-*acetyl*-N-*methyl-4*-O-*methyl-β*-D-*neuraminate methyl glycoside* (**12**). — Compound **1** (260 mg) was dissolved in dry pyridine (6 ml), and 226 mg of recrystallized chlorotriphenylmethane was added in small portions. The mixture was kept overnight at room temperature and subsequently poured into 100 ml of ice–water. After filtration, the precipitate was washed with cold water and dried over P_2O_5. The mixture containing the 9-*O*-trityl derivative of **1** and triphenylmethanol was then dissolved in 7 ml of dry *N,N*-dimethylformamide and methylated by the Kuhn procedure (2.4 g of Ag_2O; 2.4 ml of methyl iodide; 0.5 g of Drierite). The methylated product was isolated by partition between water and chloroform. For detritylation, the residue from the organic layer was dissolved in 60% acetic acid (10 ml) and heated for 1 h at 95°. Detritylation was followed by t.l.c. in solvent *D*. After the addition of 15 ml of H_2O, the mixture was extracted three times with 25 ml of hexane. The aqueous layer was evaporated and the residue dried over P_2O_5. Finally, the residue was dissolved in 8 ml of absolute methanol and stirred with 800 mg of Dowex 50 X8 (H^+) resin for 2 h. After filtration and washing of the resin with 100 ml of methanol, the solution was evaporated and the residue dried. G.l.c.–m.s. of the trimethylsilylated or acetylated sample showed the presence of two compounds **11** and **12**, which were isolated by preparative t.l.c. in solvent *C*; R_F values: **11**, 0.45; **12**, 0.21.

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NOTE

After the preparation of our manuscript, A. K. Bhattacharjee and H. J. Jennings [*Carbohydr. Res.*, 51 (1976) 253–261] reported on the determination (g.l.c.–m.s. analysis) of the linkages in some methylated, sialic acid-containing, meningococcal polysaccharides. However, the structure which they propose for fragment C (in the present paper, *H*) is incorrect: the substituent at C-7 is eliminated instead of that at C-2.

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