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METHYLATION ANALYSIS OF NEURAMINIC ACIDS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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ABSTRACT

The permethylated methyl glycoside methyl esters of *N*-acetyl- and *N*-glycolyl-neuraminic acids and of *N*-acetylneuraminic acid 8-acetate have been analyzed by g.l.c.–mass spectrometry. Fragmentation of the neuraminic acid derivatives in electron-impact mass spectrometry has been studied by deuterium labelling. The results were applied in the methylation analysis of neuraminic acids from gangliosides of brain and kidney.

INTRODUCTION

Analysis of methylated sugar derivatives by g.l.c.–m.s. is a valuable tool in the structural study of glycolipids, glycoproteins, and polysaccharides[1–5]. However, methylation analysis has not been applied for analysis of the linkages of neuraminic acids. For this purpose, periodate oxidation is commonly used, but all possible substitutions cannot be ascertained by this method. Moreover, analysis of substituent positions on neuraminic acids by periodate oxidation can give erroneous results[6,7].

In the present paper, the possibility of applying methylation analysis to neuraminic acids of brain and kidney gangliosides is studied. The characteristic features, which should allow identification of the methyl substitution pattern, are presented for the fragmentation of three methylated neuraminic acid derivatives in electron-impact mass spectrometry.

RESULTS AND DISCUSSION

Analysis of the neuraminic acid derivatives by g.l.c. is shown in Fig. 1. For peaks A and B (Fig. 1*a*), obtained from an equimolar mixture of the permethylated derivatives of *N*-acetyl- and *N*-glycolyl-neuraminic acids, the ratio of the peak areas A:B was 1.0:0.9. Peaks A and C (Fig. 1*b*) were obtained from the permethylated mixture of rat-brain gangliosides after methanolysis and acetylation. By g.l.c.–m.s., peak A was identified as the permethylated methyl glycoside methyl ester of *N*-acetyl-neuraminic acid, and peak C as the respective derivative in which the methoxyl group at C-8 is replaced by an acetoxyl group. These two compounds were also identified by

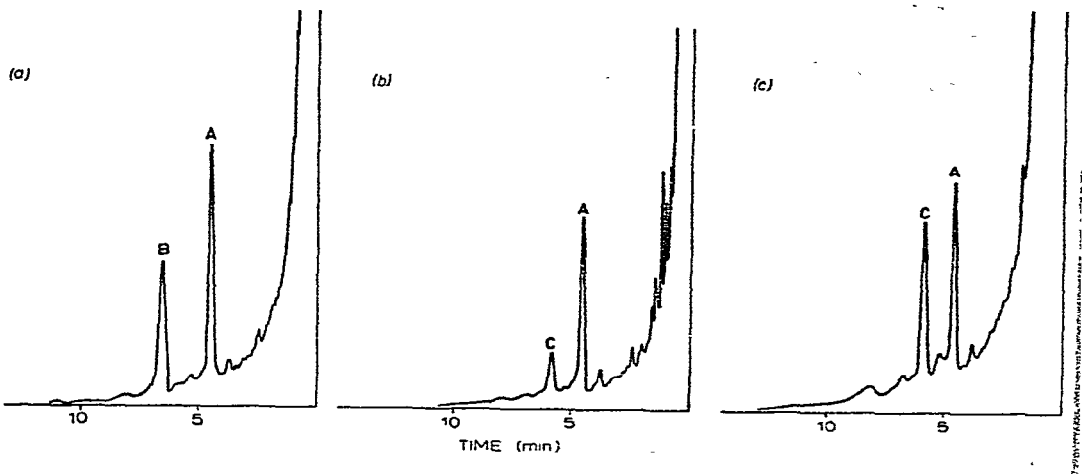
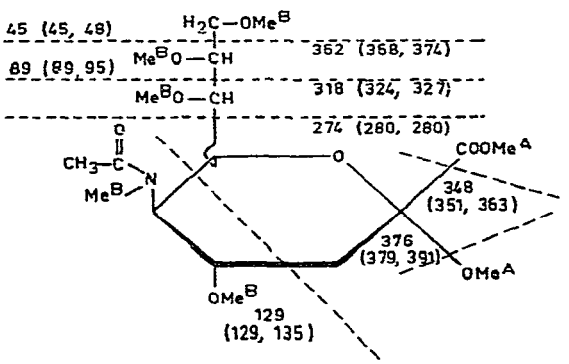


Fig. 1. G.l.c. of methylated neuraminic acid derivatives from standard sugars (*a*), from a mixture of rat-brain gangliosides (*b*), and from di-*N*-acetylneuraminyllactosylceramide of human kidney (*c*). Peak A, permethylated methyl glycoside methyl ester of *N*-acetylneuraminic acid; B, permethylated methyl glycoside methyl ester of *N*-glycolylneuraminic acid; C, 8-*O*-acetyl-4,7,9-tri-*O*-methyl derivative of the methyl glycoside methyl ester of *N*-acetylneuraminic acid. Conditions: 2.2% of SE-30, 230°.



Scheme 1. Fragmentation of permethylated derivatives of *N*-acetylneuraminic acid
(the methyl groups introduced in methanolysis are designated Me^A, those introduced by methylation are designated Me^B):

1 $Me^A = CH_3$, $Me^B = CH_3$, M = 407;
2 $Me^A = CD_3$, $Me^B = CH_3$, M = 413;
3 $Me^A = CH_3$, $Me^B = CD_3$, M = 422.

g.l.c.–m.s. from the permethylated di-*N*-acetylneuraminyllactosylceramide of human kidney[8] (Fig. 1*c*); the ratio of the peak areas A:C was 1.0:1.1.

The three derivatives of the permethylated *N*-acetylneuraminic acid, and some fragments of importance in the mass-spectral analysis are shown in Scheme 1*.

The molecular ion (407, 413, 422) and the ions formed by elimination of methyl (15) or trideuteriomethyl (18) groups were observed as weak signals (usually below 1%) in the mass spectra of **1**, **2**, and **3**. The molecular weight is clearly indicated by ions formed by elimination of methoxyl (31, 34, 31) and methoxycarbonyl (59, 62, 59) groups (Scheme 1; Fig. 2). On the basis of deuterium labelling, MeO is eliminated mainly either from the methoxycarbonyl group or the glycosidic site. The elimination of MeO is in agreement with previous work, which has shown that the glycosidic methoxyl group can be eliminated from methyl ethers of pyranoses[1,2].

The method used for methylation leads to *N*-methylation of amino sugars[9,10]. The prominent signal *m/e* 274 (280,280) indicates that the ring is fully methylated (Scheme 1; Fig. 2). Methylation at position 4 and *N*-methylation are also indicated by the ion *m/e* 129 (129,135), which arises by cleavage of the C-3–C-4 and C-5–C-6 bonds. This signal is very intense in the mass spectra of **1**, **2**, and **3** (Fig. 2). The analogous ion is also one of the most intense signals in the mass spectrum of the peracetylated derivative of *N*-acetylneuraminic acid[11]. Elimination of ketene (42) from this fragment gives rise to an intense signal at *m/e* 87 (87,93). The prominent ion at *m/e* 142 (142,148) containing the C-4–C-6 fragment also indicates methylation at C-4 and *N*-methylation. The fragment C-1–C-4 is indicated by *m/e* 159 (165,162). In the mass spectrum of the fully methylated *N*-glycolylneuraminic acid, the ion at *m/e* 159 is formed mainly in a different way (Scheme 2). The ion at *m/e* 198 (198,204) is formed from the fragment of 318 m.u. (Scheme 1) by elimination of CO_2Me and MeO from the glycosidic site plus one MeO group. The fragment *m/e* 201 (207,207) arises from *m/e* 274 (Scheme 1) through elimination of NMeAc. Elimination of methanol from this ion gives rise to *m/e* 169 (172,172).

The substituents of neuraminic acid are usually located in the side chain (one of the most usual substitutions is that formed by the (2→8)-ketosidic linkage from another neuraminic acid). Therefore, fragmentation of the side chain deserves special attention. A strong peak at *m/e* 45 is formed from compounds having a primary methoxyl group[12], and suggests that position 9 is methylated. Most, but not all of this signal was shifted to *m/e* 48 by trideuteriomethylation. A similar situation occurs in the mass spectrometry of methylated alditols: a minor proportion of the intensity at *m/e* 45 is from some unknown origin[12,13]. The ion at *m/e* 89 (89,95) is obtained from compounds having vicinal primary and secondary methoxyl groups[12,13], and indicates that position 8 is also methylated. The ion at *m/e* 362 (368,374) indicates methylation at positions 7 and 8. Methylation at 7 is also evident from the intense

*Three *m/e* numbers are given in several cases throughout the text: the first *m/e* number was obtained after methylation and methanolysis (compounds **1**, **4**, and **7**), the second after methylation and tetradeuteriomethanolysis (**2**, **5**, and **8**), and the third after trideuteriomethylation and methanolysis (**3**, **6**, and **9**).

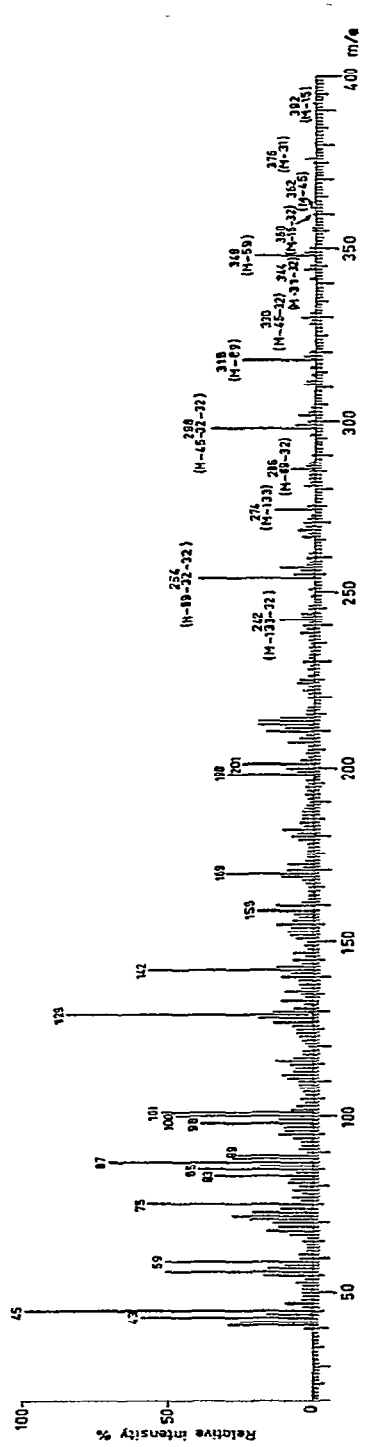
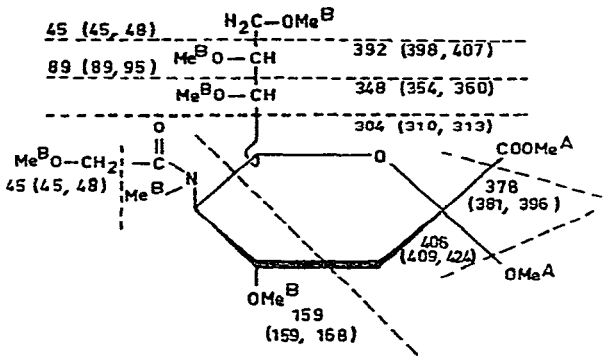


Fig. 2. Mass spectrum of permethylated methyl glycoside methyl ester of *N*-acetylneuraminic acid (1).

signal at *m/e* 318 (324,327). These two ions, as well as the ion 274 (280,280), give rise to several intense peaks, mainly by single or consecutive eliminations of methanol (32), as shown in Fig. 2.

The fragmentation of the permethylated derivative of *N*-glycolylneuraminic acid was similar to that of *N*-acetylneuraminic acid, except that the fragments containing the methylated *N*-glycolyl group (Scheme 2) were shifted by 30 m.u. towards larger mass, and the shifts of ions caused by trideuteriomethylation differed accordingly (*cf.* Scheme 1). Low intensity at *m/e* 43 indicates the absence of OAc and NAc groups.



Scheme 2. Fragmentation of permethylated derivatives of *N*-glycolylneuraminic acid:

4 $Me^A = CH_3$, $Me^B = CH_3$, M = 437;
5 $Me^A = CD_3$, $Me^B = CH_3$, M = 443;
6 $Me^A = CH_3$, $Me^B = CD_3$; M = 455.

The methyl substitution pattern of **4**, **5**, and **6** can be deduced in a way similar to that described above. This derivative can probably eliminate *m/e* 45 (45,48) from two sites (Scheme 2), and therefore high intensity at this *m/e* number may not be a reliable indication for methylation at position 9. However, the prominent signal at *m/e* 89 (89,95) indicates that this derivative contains vicinal primary and secondary methoxyl groups, suggesting methylation at positions 8 and 9. The fragments arising by partial or total elimination of the side chain eliminate mainly methanol, as in the fragmentation of the fully methylated derivative of *N*-acetylneuraminic acid (Figs. 2 and 3).

The origins of some important fragments from the derivative of *N*-acetylneuraminic acid methylated at positions 4, 7, and 9, but acetylated at position 8, are shown in Scheme 3.

The molecular weight is indicated by the weak signals of M^+ and M−Me and clearly by M−MeO and M−COOMe (Fig. 4). That the ring is fully methylated can

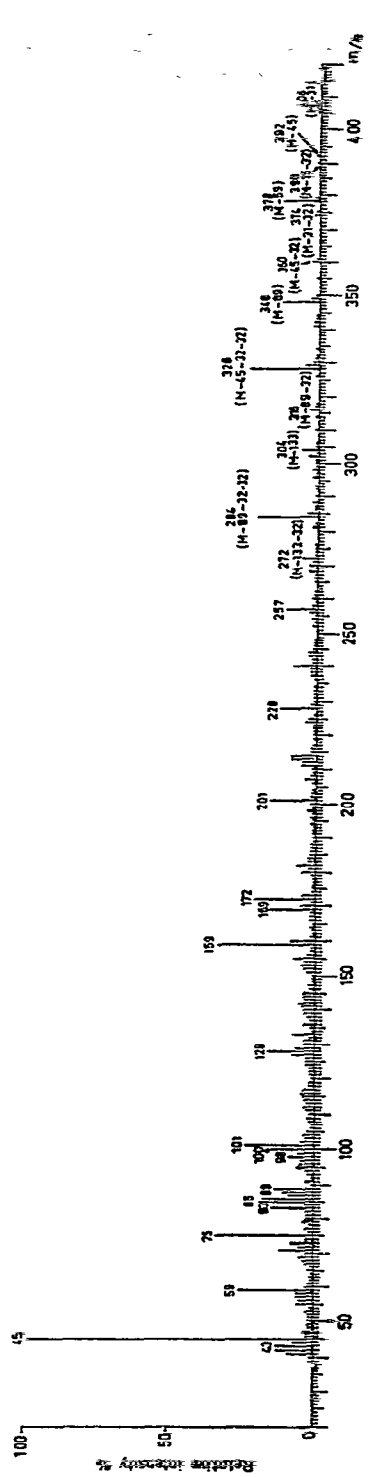


Fig. 3. Mass spectrum of permethylated methyl glycoside methyl ester of *N*-glycolylneuraminic acid (4).

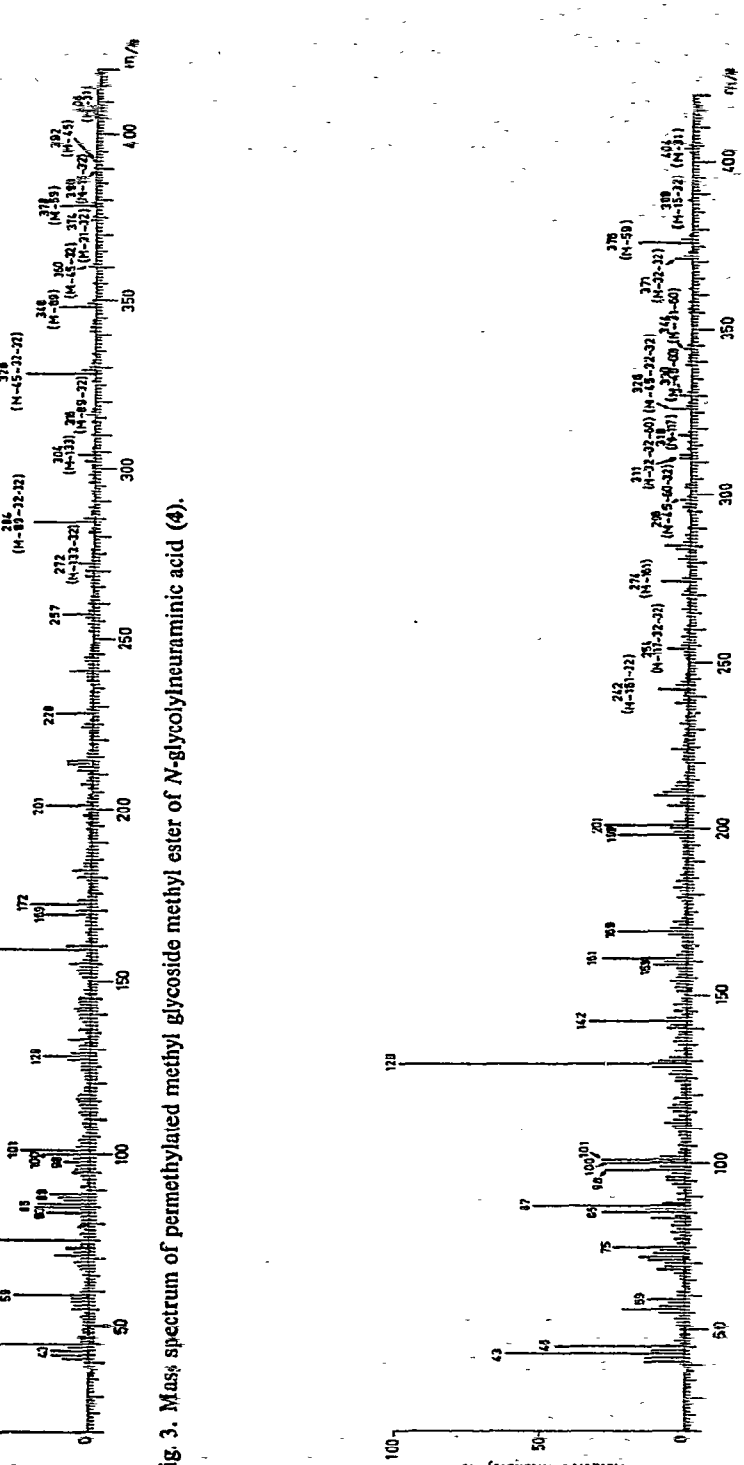
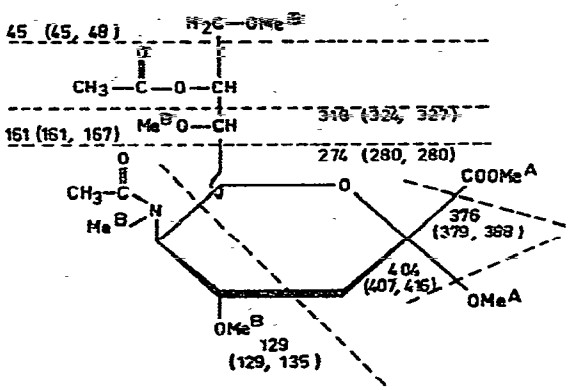


Fig. 4. Mass spectrum of 8-*O*-acetyl-4,7,9-tri-*O*-methyl derivative of the methyl glycoside methyl ester of *N*-acetylneuraminic acid (7).



Scheme 3. Fragmentation of permethylated derivatives of *N*-acetylneuraminic acid 8-acetate:

7 $Me^A = CH_3$, $Me^B = CH_3$, M = 435;
8 $Me^A = CD_3$, $Me^B = CH_3$, M = 441;
9 $Me^A = CH_3$, $Me^B = CD_3$, M = 447.

be concluded as for **1**, **2**, and **3**. Methylation at position 9 is indicated by an intense peak at *m/e* 45 (45,48). An intense signal at *m/e* 89 (89,95) is not found, which suggests that position 8 is not methylated[12,13]. The ion at *m/e* 318 (324,327) shows that position 7 is methylated. This signal is of much lower intensity than in the mass spectra of **1**, **2**, and **3** (Figs. 2 and 4). This finding is expected on the basis of the rule that the fission between two methoxylated carbons is favoured over the fission between a methoxylated and an acetoxylated carbon[12]. The intense signal at *m/e* 161 (161,167) gives evidence for one acetoxyl group in the side chain. This peak is expected to be high if the acetoxyl group is located at C-8, but it should be low if the acetoxyl group were located at C-7 or C-9[12,13]. Taking into account the previous findings, the acetoxyl group is located at C-8. Other significant ions from this derivative include fragments arising by partial (M−45 and M−117) or total (M−161) elimination of the side chain followed by eliminations of methanol (32) and/or acetic acid (60), as seen in Fig. 4.

G.l.c.–m.s. analysis was applied for the five main gangliosides of human kidney, which comprise well over 95% of the total[8]. Of the two main gangliosides, *N*-acetylneuraminyllactosylceramide and di-*N*-acetylneuraminyllactosylceramide, the former gave only the fully methylated derivative of *N*-acetylneuraminic acid; the latter also gave the derivative acetylated at position 8 (Fig. 1). The g.l.c.–m.s. analysis of di-*N*-acetylneuraminyllactosylceramide is compatible with the suggestion that the two neuraminic acids in this ganglioside are linked to each other by a (2→8)-linkage[8]. The two glucosamine-containing gangliosides and the galactosamine-containing ganglioside[8] gave only the permethylated derivative. Only the permethylated derivative and the 8-*O*-acetyl 4,7,9-tri-*O*-methyl derivative of *N*-acetylneuraminic acid

were found in the analysis of the gangliosides from rat and pig brain, which is in agreement with previous work on the structure of brain gangliosides[14]. Only the permethylated derivative was found from the *N*-glycolylneuraminyllactosylceramide of bovine kidney, consistent with previous work[15]. The method is applicable for the analysis of $\sim 5\ \mu g$ of glycolipid (the di-*N*-acetylneuraminyllactosylceramide; the value corresponds to 2 μg of neuraminic acid).

The linkages between neuraminic acid residues are classically studied by periodate oxidation, in which the neuraminic acid substituted on C-8 is resistant. However, possible substitution at other sites of neuraminic acid cannot be confirmed by periodate oxidation. Neuraminic acids may contain *O*-acyl groups in several positions[6,7]. These are expected to be cleaved during the methylation procedure, and should be localized by additional techniques[6,7,16,17]. It has been shown that the oxidation of neuraminic acids substituted at C-9 by *O*-acyl groups is retarded, and can therefore give erroneous results in the determination of linkages between neuraminic acids by periodate oxidation[6,7]. In addition, glycolipids and glycoproteins are often isolated in extremely low quantities, and the oxidation with periodate therefore cannot always be reliably performed (for example, due to the risk of overoxidation of low amounts of sample). From the above analysis of the three methyl ether derivatives of neuraminic acid, and on the basis of previous knowledge on mass spectrometry of methyl ethers of sugars[1–5], it is suggested that the method described herein should be applicable for the identification of other derivatives of neuraminic acid obtained from permethylated glycolipids, glycoproteins, or polysaccharides.

EXPERIMENTAL

N-Acetyl- and *N*-glycolyl-neuraminic acids were purchased from Sigma Chemical Company. The mixture of pig-brain gangliosides was made available by Dr. P. Maury (Helsinki, Finland), and the mixture of rat-brain gangliosides by Dr. J. Finne (Helsinki, Finland). The *N*-glycolylneuraminic acid-containing hematoside[15] was a gift from Dr. K. Puro (Helsinki, Finland). Gangliosides I–V were isolated from human kidney as described earlier[8].

Methylation was carried out with methyl iodide in methyl sulphoxide in the presence of methylsulphinyl carbanion[18,19]. The reaction mixture was diluted with water and partitioned with an equal volume of chloroform. The chloroform phase was extracted 5 more times with water. The permethylated glycolipid was degraded in 0.5M HCl–methanol at 80° for 18–20 h. The methyl esters of fatty acids were extracted with hexane. The methanolic phase was evaporated to dryness under nitrogen, and the residue was acetylated[20] with pyridine–acetic anhydride (1/1, v/v) at 80–85° for 0.5 h. The mixture was evaporated to dryness after addition of an excess of toluene, and the residue was dissolved in chloroform and analyzed by g.l.c. and g.l.c.–m.s. For identification of the methylated derivative of *N*-glycolylneuraminic acid (4) from glycolipid, degradation was performed[21] in 0.05M HCl–methanol at

80° for 3 h. However, it was shown by g.l.c.–m.s. that, in contrast to the glycolyl group, the methylated glycolyl group is not detached significantly, even in 0.5M HCl–methanol (80°, 18–20 h). Therefore, the degradation was performed in 0.5M acid, to ensure quantitative liberation of the methylated neuraminic acid from glycolipid. For deuterium-labelling of the COOMe and of the glycosidic MeO groups (**2**, **5**, and **8**), the methylation product was methanolyzed in 0.5M HCl–tetradeuteriomethanol (E. Merck, CD_3OD, min. 99.7%) and acetylated as described before. For labelling of the methyl ether and the *N*-methyl groups (**3**, **6**, and **9**), methylation was carried out with trideuteriomethyl iodide (E. Merck, CD_3I, min. 99%), and the methylation product was methanolyzed in 0.5M HCl–methanol and acetylated as described above.

G.l.c. was performed on a column (2 m × 3 mm, i.d.) of 2.2% of SE-30 at 230°. 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. Mass spectra were recorded at 70 eV with an ionization current of 300 μamp from *m/e* 40 to *m/e* 600.

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