METHYLATION ANALYSIS DETERMINATION OF ACYLNEURAMINIC ACID RESIDUE TYPE 2→8 GLYCOSIDIC LINKAGE

Application to GT_{1b} ganglioside and colominic acid

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1. Introduction

Acylneuraminic acids are known to occur in carbohydrates and glycoconjugates in different positions: at the non-reducing end (in general), at the reducing end (disaccharides [1,2] bacterial polysaccharides [1,3–5]) and as inter-chain residues (polysaccharides [1,3–5], glycoproteins [6], gangliosides [7–9]). Permethylation analysis of compounds containing acylneuraminic acids has been reported in literature [7,9–11]. However, these studies inform only about the structure of the asialo-part of the molecules, as only partly methylated neutral and amino sugars have been investigated. Incorporation of neuraminic acid derivatives into methylation analysis is of great value for the elucidation of the complete structure of carbohydrates and glycoconjugates containing neuraminic acid.

In this paper the analysis is described of (partly) methylated acylneuraminic acids, obtained on methanolysis and subsequent re-*N*-acetylation from the methylated GT_{1b} ganglioside Neu*N*Ac-α(2→3)-Gal-β(1→3)-Gal*N*Ac-β(1→4)-[Neu*NA*-α(2→8)-Neu*N*Ac-α(2→3)]-Gal-β(1→4)-Glc-β(1→1)-ceramide [7,8] and the Neu*N*Ac-polymer colominic acid [4], respectively. After trimethylsilylation or acetylation of the free hydroxyl groups, the neuraminic acid derivatives were identified by gas–liquid chromatography–mass spectrometry (GLC–MS). For GT_{1b} as well as colominic acid the 2→8 type of glycosidic linkage between the *N*-acetylneuraminic acid residues could be confirmed.

Abbreviations: Gal, galactose; Glc, glucose; Gal*N*Ac, *N*-acetylgalactosamine; Neu*N*Ac, *N*-acetylneuraminic acid; Me, methyl; TMS, trimethylsilyl; Ac, acetyl; 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me), 4,7,8,9-tetra-*O*-methyl-*N*,*N*-acetyl, methyl-neuraminic acid methyl ester β-D-methyl glycoside. Analogous compounds are abbreviated in a similar way. The shorthand nomenclature of Svennerholm [17] is used for gangliosides

2. Materials and methods

GT_{1b} was isolated from bovine brain [12]. To increase the solubility of colominic acid (from *Escherichia coli*, Koch Light Lab. Ltd) the compound was applied to a small column of Dowex-50 (H^+ form), eluted with water and subsequently lyophilized. Both compounds (5 mg) were methylated according to Hakomori [13]. Methylated GT_{1b} was purified by partition between water and chloroform, whereas methylated colominic acid was dialysed against water. The products were methanolysed in 1 ml of 0.5 M HCl in methanol at 85°C for 24 h. The reaction mixtures were neutralized, re-*N*-acetylated and dried [14]. One part of the material was trimethylsilylated [15] the other part acetylated in acetic acid anhydride–

pyridine (1:1, v/v) at 95°C for 20 min, followed by evaporation to dryness. For GLC and GLC–MS the derivatives were dissolved in 0.5 ml of chloroform.

GLC was carried out on a Varian Aerograph 2740-30-01 gas chromatograph (dual flame ionization detector, glass columns 2.00 m × 4.0 mm packed with 3.8% SE-30 on Chromosorb W-AW DMCS H.P. 80–100 mesh, N_2 flow rate 40 ml/min, column-oven temperature 220°C) and GLC–MS on a Jeol JGC-1100/JMS-07 combination (glass columns as described above, column-oven temperature 200°C, ion-source temperature 250°C, electron energy 75 eV, trap current 300 μA, accelerating voltage 3.0 kV). Retention times (R_N) are given relative to that of 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me).

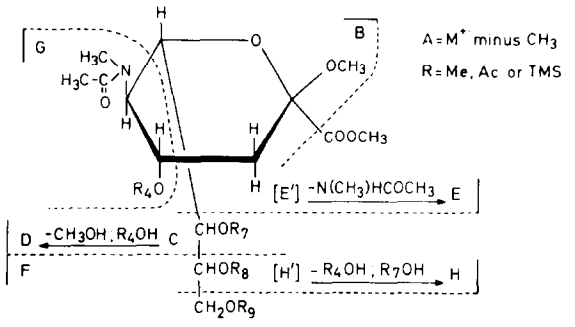

Fig.1. The characteristic fragment ions used for the mass spectrometric determination of the positions of Me, TMS and Ac substituents in the neuraminic acid derivatives mentioned in table 1.

3. Results and discussion

For the identification of methylated neuraminic acid derivatives by GLC–MS use is made of a recently developed mass spectrometric method for the analysis of partially *O*-acetylated *N*-acylneuraminic acid derivatives [15] and of reference compounds as given in table 1. Details of the preparation of the reference compounds will be published elsewhere. The characteristic fragment ions used for determination of the positions of Me, TMS and Ac substituents are given schematically in fig.1. The fragments A–G are used to identify the acetylated compounds, whereas an additional fragment H is necessary for the trimethylsilylated compounds in order to discriminate between an *O*TMS group at C-8 or C-9.

The gas chromatogram of the mixture of methyl glycosides obtained after methylation, methanolysis, re-*N*-acetylation and trimethylsilylation of GT_{1b} is presented in fig.2a. Two main peaks occur in the area of the acylneuraminic acids, with R_N-values of 1.00 and 1.14, respectively (c.f. table 1). GLC–MS pointed out that the peak at R_N 1.00 corresponds to 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me) and the peak at R_N 1.14 to 1,2,4,7,9-*O*Me-8-*O*TMS-Neu*N*(Ac,Me). The presence of 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me) and 1,2,4,7,9-*O*Me-Neu*N*(Ac,Me) in the mixture after methanolysis of GT_{1b} is confirmed by analysis of the acetylated sample which consisted of 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me)

Table 1
GLC data of reference partly methylated *N,N*-acetyl,methyl-neuraminic acid methyl ester β-D-methyl glycosides, analysed as the trimethylsilyl and the acetyl derivatives

Compound	R_N	
	TMS derivative	Ac derivative
1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me)	1.00	1.00
1,2,4, 8,9-*O*Me-Neu*N*(Ac,Me)	1.07	1.08
1,2,4,7, 9-*O*Me-Neu*N*(Ac,Me)	1.14	1.25
1,2,4,7,8, -*O*Me-Neu*N*(Ac,Me)	1.30	1.47
1,2,4, 9-*O*Me-Neu*N*(Ac,Me)	1.27	1.26
1,2,4 -*O*Me-Neu*N*(Ac,Me)	1.70	1.70
1,2, 9-*O*Me-Neu*N*(Ac,Me)	1.43	1.63
1,2 -*O*Me-Neu*N*(Ac,Me)	1.89	2.17

R_N values on 3.8% SE-30 at 220°C are given relative to 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me) (R_N 1.00)

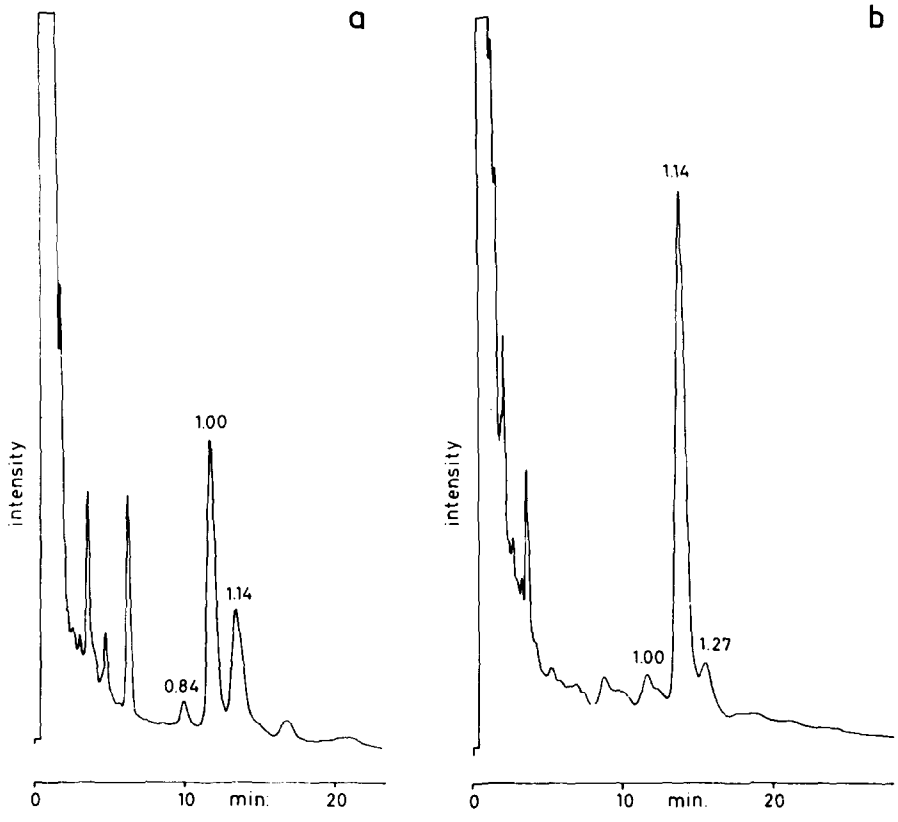

Fig.2. GLC on 3.8% SE-30 of neuraminic acid derivatives obtained from methylated GT_{1b} (a) and colominic acid (b). Peaks, identified by GLC–MS of the trimethylsilylated samples: R_N 0.84, 4,7,8,9-tetra-*O*-methyl-*N*,*N*-acetyl, methyl-neuraminic acid methyl ester α-D-methyl glycoside; R_N 1.00, 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me); R_N 1.14, 1,2,4,7,9-*O*Me-8-*O*TMS-Neu*N*(Ac,Me); R_N 1.27, 1,2,4,9-*O*Me-7,8-*O*TMS-Neu*N*(Ac,Me).

(R_N 1.00) and 1,2,4,7,9-*O*Me-8-*O*Ac-Neu*N*(Ac,Me) (R_N 1.25). In the gas chromatogram of the trimethylsilylated as well as the acetylated sample a small peak is observed with R_N 0.84 (4% of the peak at R_N 1.00) representing 4,7,8,9-tetra-*O*-methyl-*N*,*N*-acetyl, methyl-neuraminic acid methyl ester α-D-methyl glycoside. In view of the large preponderance of the β-D over the α-D glycosides formed upon methanolysis only the β-anomers are taken into account for determination of the linkage type.

The mass spectra of 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me), 1,2,4,7,9-*O*Me-8-*O*TMS-Neu*N*(Ac,Me) and 1,2,4,7,9-*O*Me-8-*O*Ac-Neu*N*(Ac,Me) are given in fig.3. It has to be noted that in the acetylated derivative, fragment A stems only from the elimination of a Me group from the *N*(Ac,Me) function, whereas in the trimethylsilylated derivative the Me group can also be eliminated from the TMS group. The 8-*O*Ac group (fig.3c) is responsible for the absence of fragment F [15].

1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me) stems from the terminal Neu*N*Ac residues in GT_{1b}, whereas the presence of 1,2,4,7,9-*O*Me-Neu*N*(Ac,Me) indicates the occurrence of internal Neu*N*Ac residues which were linked at position 8. It can be concluded from the GLC analysis of the trimethylsilylated as well as the acetylated sample that both compounds were present in a ratio of 2:1. These results are in agreement with the proposed structure for GT_{1b} [7,8].

The gas chromatogram of the trimethylsilylated mixture of neuraminic acid derivatives obtained from methylated colominic acid is given in fig.2b. The main peak at R_N 1.14 was identified by GLC–MS as 1,2,4,

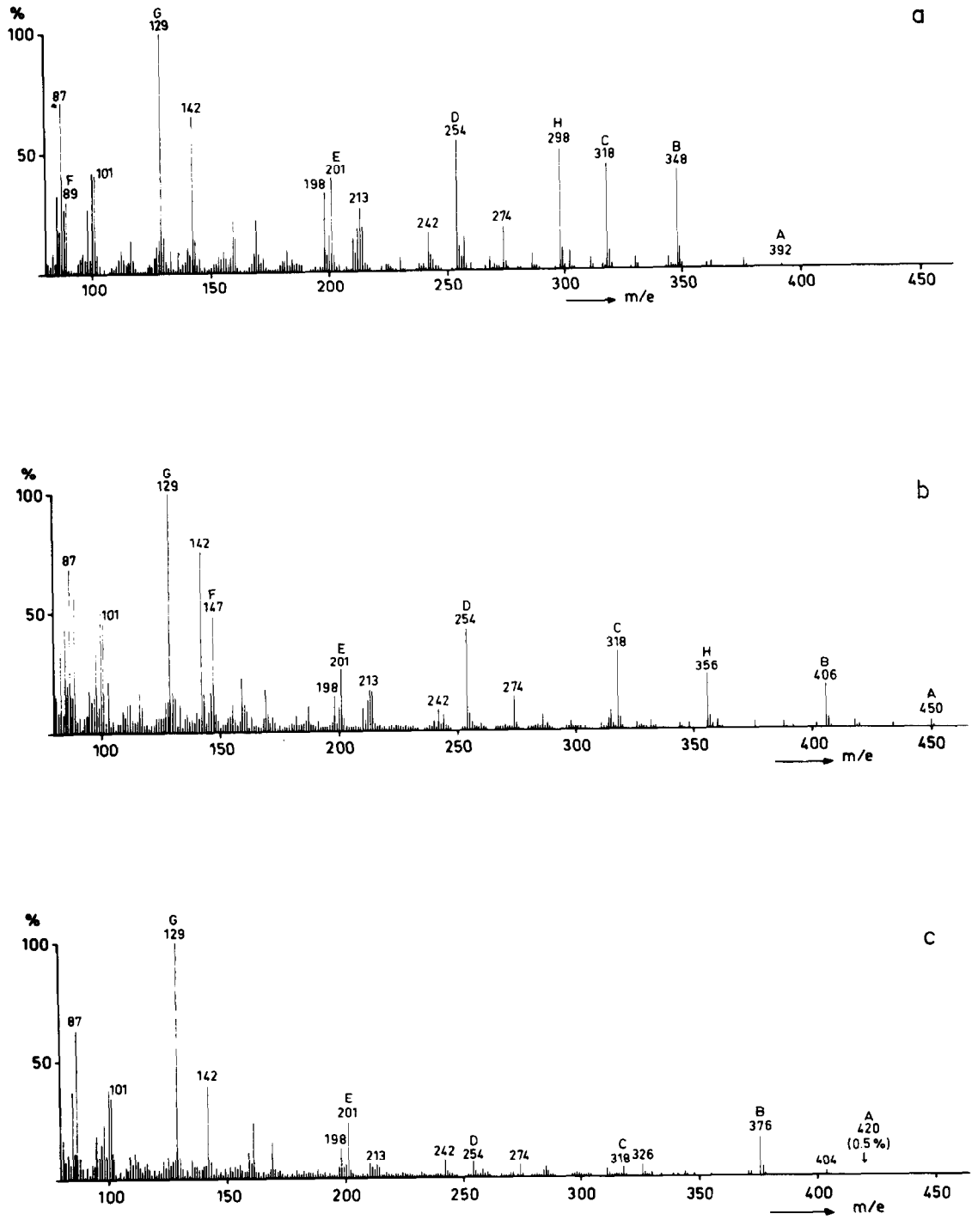

Fig.3. Mass spectra of 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me) (a), 1,2,4,7,9-*O*Me-8-*O*TMS-Neu*N*(Ac,Me) (b) and 1,2,4,7,9-*O*Me-8-*O*Ac-Neu*N*(Ac,Me) (c). Values > m/e 80 and intensities ≥ 1% of m/e 129 are given.

7,9-*O*Me-8-*O*TMS-Neu*N*(Ac,Me), and the peak at R_N 1.00 (3% of the main peak) as 1,2,4,7,8,9-*O*Me-Neu*N* (Ac,Me). The small peak at R_N 1.27 (10% of the main peak) corresponds to 1,2,4,9-*O*Me-7,8-*O*TMS-Neu*N* (Ac,Me) arising from undermethylation of the homopolymer. The possibility that 1,2,4,9-*O*Me-Neu*N*(Ac, Me) stems from branching points is unlikely, because in the ^{13}C magnetic resonance spectrum of colominic acid only 11 resonances occur for the Neu*N*Ac residues, the chemical shift for C-7, C-8 and C-9 being characteristic for a glycosidically linked C-8 atom (unpublished results, c.f. ref. [3]) GLC analysis of the mixture of acetylated compounds makes it impossible to detect this undermethylation as 1,2,4,7,9-*O*Me-8-*O*Ac-Neu*N*(Ac,Me) and 1,2,4,9-*O*Me-7,8-*O*Ac-NeuN(Ac,Me) have almost the same R_N value (see table 1).

The presented data from methylation analysis show that the Neu*N*Ac units in colominic acid are linked by 2→8 glycosidic bonds.

4. Concluding remarks

Until now, determination of the positions at which acylneuraminic acid residues are glycosidically linked is based mainly on periodate oxidation or Smith degradation after saponification of possible ester groups [3–5, 7,8]. The presence of a glycosidic bond at C-8 makes a non-reducing acylneuraminic acid residue insusceptible to periodate. However, substitution at C-9 hinders periodate oxidation [16] which may give rise to a misinterpretation of the oxidation data.

Recently, ^{13}C magnetic resonance spectroscopy has been applied to the structure elucidation of polysaccharides containing internal Neu*N*Ac units [1,3]. Glycosidic linkages at positions 4,8 and 9 of α-D-Neu*N*Ac residues could clearly be distinguished on the basis of specific chemical shift values. However, rather large amounts of (de-*O*-acylated) substances are required.

The method presented in this paper is a valuable alternative to the techniques mentioned above, especially if only small amounts of material are available. It can easily be incorporated into the usual procedures for methylation analysis.

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