Carbohydrate Research, 239 (1993) 35–50
Elsevier Science Publishers B.V., Amsterdam

An improved approach to the analysis of the structure of small oligosaccharides of glycoproteins: application to the *O*-linked oligosaccharides from human glycophorin A

Hubert Krotkiewski [a], Elwira Lisowska [a], Göran Nilsson [b], Gunnar Grönberg [b,1] and Bo Nilsson [b]

[a] *Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw (Poland)*
[b] *BioCarb Technology AB, S-223 70 Lund (Sweden)*

(Received April 21st, 1992; accepted July 14th, 1992)

ABSTRACT

Treatment of purified human glycophorin A with alkaline borohydride cleaved the oligosaccharide side chains to yield alditol derivatives that were separated by gel filtration into three mixtures of low molecular weight compounds. Each mixture was oxidised with periodate, and the products were reduced with borohydride and analysed after acetylation or methylation by GLC–MS and FABMS. The resulting data allowed the monosaccharide sequence and linkage positions to be assigned to each component of the mixtures. The anomeric configuration was determined by ^{1}H NMR spectroscopy of the intact fractions. The structures of a disialylated tetrasaccharide, two monosialylated trisaccharides, and five other minor products were defined.

INTRODUCTION

Mass spectrometry (MS) plays an important role in the analysis of the structure of glycoconjugates but, although its sensitivity is superior to that of other spectroscopic methods, complete structures cannot be determined. Tandem MS, which allows the analysis of derivatised and underivatised glycoconjugates, can increase the information on structure. However, this technique requires advanced and costly instruments. An alternative approach to increased information on structure is by mass spectrometry of derivatives.

We have shown that periodate oxidation–borohydride reduction can be used to modify glycoconjugates specifically for analysis by FABMS after methylation[1] or acetylation[2,3]. Due to the specificity of periodate oxidation, the positions of the

Correspondence to: Assistant Professor B. Nilsson, Juryvägen 62, S-226 57 Lund, Sweden.
[1] Present address: Carlsberg Research Center, Department of Chemistry, Gamle Carlsberg Vej 10, D-2500 Valby, Denmark.

linkages can be deduced from the masses of the primary and secondary sequence ions. This method is particularly useful for the analysis of *O*-linked and *N*-linked glycoprotein oligosaccharides[4,5] that contain branches with internal 2-acetamido-2-deoxyhexosyl (HexNAc) residues, which give rise to intense sequence ions. Oligosaccharides with branches lacking HexNAc residues, for example, high-mannose structures, give rather weak sequence ions in FABMS and are therefore best analysed in the EI mode[6,7].

In order to determine the structures, the oligosaccharides have to be released from the protein and then purified. This procedure can be tedious and there may be losses of compounds present in small quantities. Therefore, an improved general approach to study low molecular weight glycoprotein oligosaccharides, without extensive fractionation, has been developed.

Glycophorin A is one of the most extensively studied glycoproteins[8,9] and the structure of the major *O*-glycan has been elucidated[10] as α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc. Several structures which contain fragments of this tetrasaccharide have been reported[10–12] and considered as degradation products formed during the isolation procedure. Moreover, several other minor structures (fucose-containing and trisialylated) have been reported to be present in the human erythrocyte glycophorin preparations[11,13]. However, in these studies, either the method of isolation of the *O*-linked oligosaccharides could account for their partial degradation or the glycophorin preparations used were not checked for purity. So far, no publication has summarised all of the possible structures present in human glycophorin A.

The objective of this study was to define the array of *O*-linked structures from human glycophorin A by using (*a*) a purified glycophorin A preparation, (*b*) conditions for alkaline degradation safe enough to avoid "peeling" reactions, and (*c*) improved methodology for determination of structure.

EXPERIMENTAL

Preparation of glycophorin.—A crude preparation of glycophorin, obtained by phenol–water extraction of membranes isolated from outdated O,M erythrocytes[14], was fractionated on a column (1.6 × 100 cm) of Sephadex G-200 in the presence of SDS[15]. The elution profile was checked by SDS-PAGE and with PAS staining, and appropriate fractions were combined to give, after dialysis first against aq 50% EtOH and then water, a purified preparation of glycophorin A.

Degradation with alkaline borohydride.—Purified glycophorin A (120 mg) was degraded[16] with alkaline borohydride in the presence of cadmium acetate. The excess of borohydride was decomposed with aq 50% acetic acid and the products were fractionated on a column (3.5 × 56 cm) of Sephadex G-15 by elution with 50 mM acetic acid. The fraction containing *O*-linked oligosaccharide-alditols was rechromatographed on the above column and then fractionated further on a column (2.5 × 43 cm) of Bio-Gel P-4 by elution with water. In some experiments,

sialylated oligosaccharides were removed by a Dowex 1-X2 (AcO^-) resin (400 mesh).

Analytical methods.—Fractions from the gel filtration were analysed for salts by measurement of the conductivity, for neutral sugars by the phenol–H_2SO_4 method[17], and for sialic acid by the method of Jourdian et al.[18]. Monosaccharides were analysed by GLC as the alditol acetates[19] after hydrolysis in 4 M trifluoroacetic acid for 4 h at 100°C. Methylation[20] and acetylation[3] were carried out as described. *O*-Deacetylation was carried out in MeOH–aq 25% NH_4OH (4:1, 5 mL) at room temperature overnight. Oligosaccharide-alditols were oxidised with periodate[21] in 0.1 M acetate buffer (pH 5.5) containing 8 mM sodium periodate for 48 h at 4°C; the pH was adjusted to 7.0 prior to reduction with $NaBD_4$. The products were analysed by GLC–MS and FABMS after acetylation or methylation. For GLC, a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector was used. Separations were performed on an SE-30 capillary column (0.25 mm × 30 m).

Spectroscopic methods.—GLC–MS was carried out with a VG ZAB SE spectrometer fitted with an SE-30 capillary column (0.25 mm × 15 m). Spectra were recorded at 70 eV with an ion-source temperature of 200°C. For FABMS in the positive-ion mode, the same instrument was used with thioglycerol (3-mercapto-1,2-propanediol) as the matrix and Xe with a kinetic energy of 8 keV. Ions were accelerated using a potential of 10 kV. The molecular weight (*M*) was determined from the $[M + 1]^+$ ions or the $[M + Na]^+$ ions after the addition of NaI. ^1H NMR spectra were recorded at 25°C with a Bruker AM-500 Fourier-transform spectrometer. The oligosaccharide-alditols were exchanged with D_2O, lyophilised, and dissolved in D_2O (99.95%). The chemical shifts are expressed relative to that of internal 4,4-dimethyl-4-silapentane (DSS), but measured relative to that for internal acetone (2.225 ppm).

RESULTS

Many glycoproteins contain carbohydrate chains which, after cleavage from the protein, are small enough to be analysed by GLC–MS as the methylated derivatives. In order to extend the information obtainable by MS on structure beyond the monosaccharide sequence, the following procedure was applied to the oligosaccharide-alditols formed from the *O*-linked oligosaccharides when human glycophorin A was treated with alkaline borohydride in the presence of cadmium acetate. The smaller oligosaccharide-alditols were isolated by gel filtration on Sephadex G-15. The salt-free fractions were analysed for monosaccharide composition, after acid hydrolysis, by GLC and GLC–MS of the derived alditol acetates. Low molecular weight species present in fractions containing salt were acetylated, analysed by GLC, then hydrolysed, and re-analysed in order to identify those fractions that contained di- and/or oligo-saccharides. FABMS was then used in order to determine the monosaccharide sequence and molecular weight of the species in

the various fractions after acetylation or methylation. The results of FABMS indicate if analysis by GLC–MS is possible and the choice of derivative.

The various fractions were then subjected to periodate oxidation, borodeuteride reduction, and acetylation. The acetylated samples were analysed by GLC–MS directly or after *O*-deacetylation and methylation, and the linkage positions were deduced. The periodate-generated fragments were quantified by GLC. In order to check that periodate oxidation was complete and that structures not sufficiently volatile for GLC–MS were absent, the samples were analysed by FABMS which also provided supporting data on sequence and molecular weight.

The above improved approach is applicable in general to low molecular weight oligosaccharides.

Isolation of human glycophorin A.—A crude preparation of human glycophorin A was obtained from packed blood-group O,M erythrocytes (200 mg from 1 L). After gel filtration on Sephadex G-200 in the presence of SDS, glycophorin (120 mg) was obtained, the purity of which was checked by SDS-PAGE, followed by staining or immunoblotting with various mouse monoconal antibodies (anti-glycophorin A and B, anti-glycolipid structures, and anti-band 3). The purified glycophorin A contained minor proportions of glycophorins B and C, but was free of other proteins, SDS-stable aggregates, and glycolipids.

Fractionation of the released oligosaccharide-alditols.—Glycophorin A (120 mg) was treated with alkaline borohydride in the presence of cadmium acetate, and the products were fractionated on Sephadex G-15 (Fig. 1) to give carbohydrate-containing fractions I (36.4 mg) and II (38.9 mg), and a salt fraction. The components of fraction I contained Man, Fuc, Gal, GlcNAc, and GalNAc-ol, suggestive of *N*-linked structures and larger *O*-linked oligosaccharide-alditols. This fraction will be reported on elsewhere. Fraction II was rechromatographed on Sephadex G-15 and then fractionated on Bio-Gel P-4 to give subfractions II-1 (32.7 mg) and II-2 (6.2 mg) (Fig. 2).

Analysis of subfraction II-1.—Acid hydrolysis of II-1 followed by borodeuteride reduction and acetylation gave (GLC and GLC–MS) acetylated Gal-ol-*1-d*, GalNAc-ol, and a trace of Fuc-ol-*1-d*, indicative of terminal GalNAc-ol and the composition shown in Table I. Subfraction II-1 also contained sialylated structures and the absence of Man confirmed the absence of *N*-linked structures.

After methylation of II-1, FABMS gave the spectrum shown in Fig. 3. A primary sequence ion of m/z 376 and a secondary ion of m/z 344 (376 − MeOH) indicated a non-reducing terminal sialic acid moiety. No other sequence ions are seen. Three ions were shifted by 22 mass units to m/z 650, 895, and 1257 when sodium iodide was added before FABMS, and are assigned to be $[M + 23]^+$ ions. The ion of m/z 873 is the $[M + 1]^+$ ion corresponding to the $[M + 23]^+$ ion of m/z 895. A Fuc-containing species is consistent with an $[M + 1]^+$ ion of m/z 1047. The monosaccharide composition computed from these pseudo-molecular ions includes one disialylated and three monosialylated species, as indicated in Fig. 3. The absence of the ion with m/z 737, indicative of the sequence Neu5Ac–

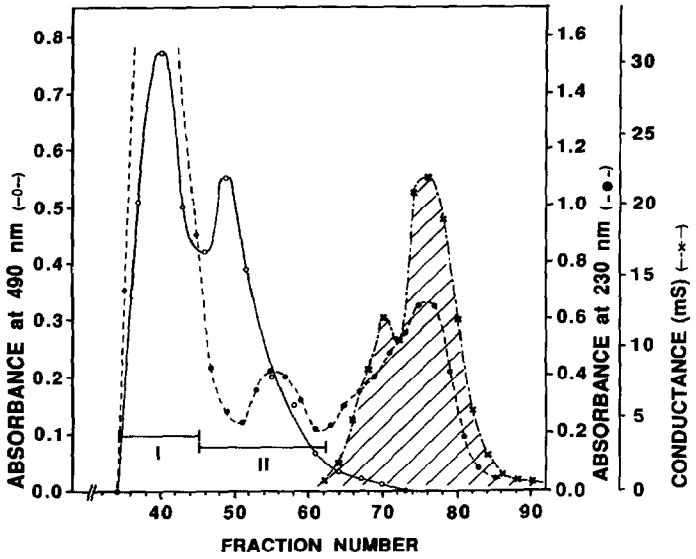

Fig. 1. Gel filtration of alkaline-borohydride-degraded human glycophorin A on Sephadex G-15. Fractions (3 mL) were assayed for hexose (A 490) and protein (A 230), and combined as indicated by I and II. The salt fraction is indicated by the hatched area.

Neu5Ac, shows that the disialylated oligosaccharide-alditol must have a branched structure. Some of these $[M + 23]^+$ ions may include several isomeric compounds as discussed below. The $[M + 23]^+$ ion of m/z 650 is in agreement with a disaccharide-alditol of Neu5Ac–Gal-ol, which suggests that this structure is present in the products of β-elimination.

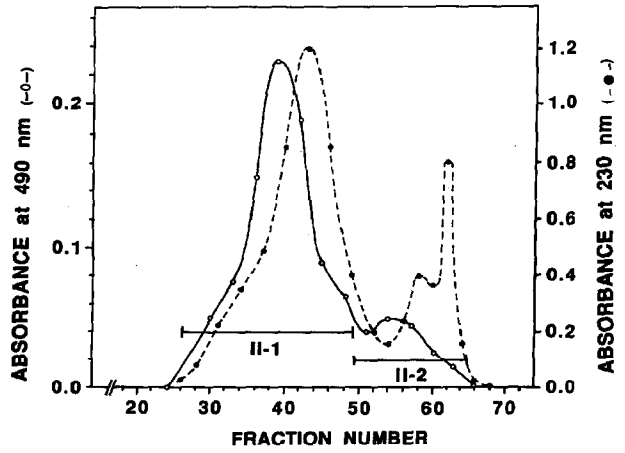

Fig. 2. Gel filtration of fraction II (Fig. 1) on Bio-Gel P-4. Fractions (3 mL) were assayed for hexose (A 490) and protein (A 230), and combined as indicated by II-1 and II-2.

TABLE I

Monosaccharide composition of fractions and subfractions obtained after alkaline-borohydride degradation of human glycophorin A and gel filtration of the products

Sugar	Relative molar proportions			
	Fraction I	Subfraction II-1	Subfraction II-2	Salt fraction
Fuc	1.8	< 0.05	0.2	0
Man	3.0	0	0	0
Gal and Gal-ol	6.2	1.2	2.1	[a]
GlcNAc	5.3	0	0	0
GalNAc-ol	4.1	1.0	1.0	0
Neu5Ac	n.d. [b]	1.4	0.8	0

[a] Identified by GLC–MS but not quantified. [b] Not determined.

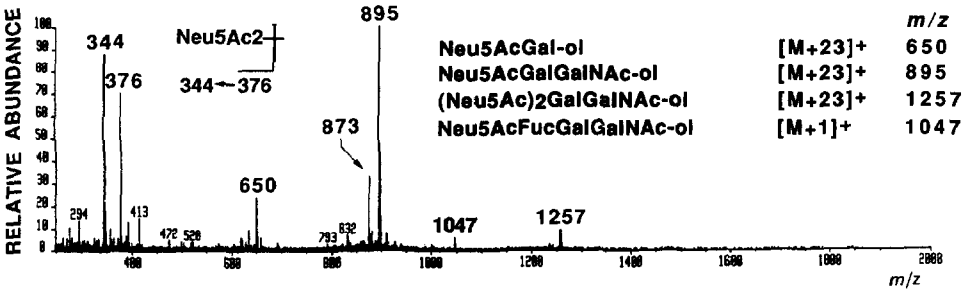

Fig. 3. FAB-mass spectrum of methylated subfraction II-1.

Subfraction II-1 was subjected in sequence to periodate oxidation, borodeuteride reduction, acetylation, *O*-deacetylation, and methylation. GLC–MS of the products gave the total-ion-current (TIC) chromatogram shown in Fig. 4. The mass spectrum of the component associated with scan 231 is shown in Fig. 5. The ion of

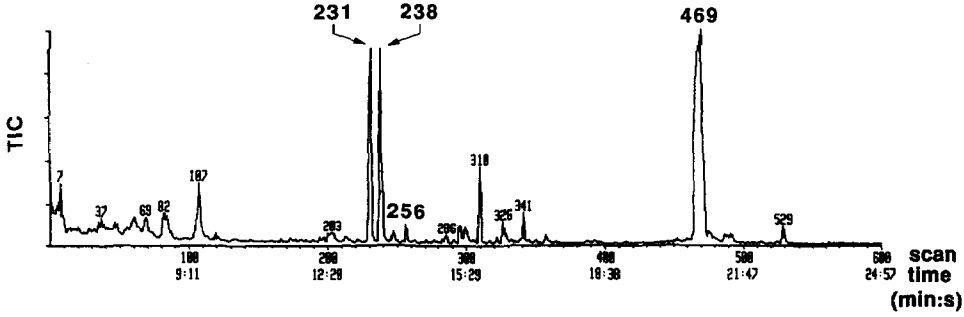

Fig. 4. TIC chromatogram from GLC–MS of subfraction II-1 after periodate oxidation, borodeuteride reduction, and methylation. Carbohydrate-containing peaks are indicated by scan numbers. The temperature of the GLC column was programmed from 200 to 300°C at 8°C/min.

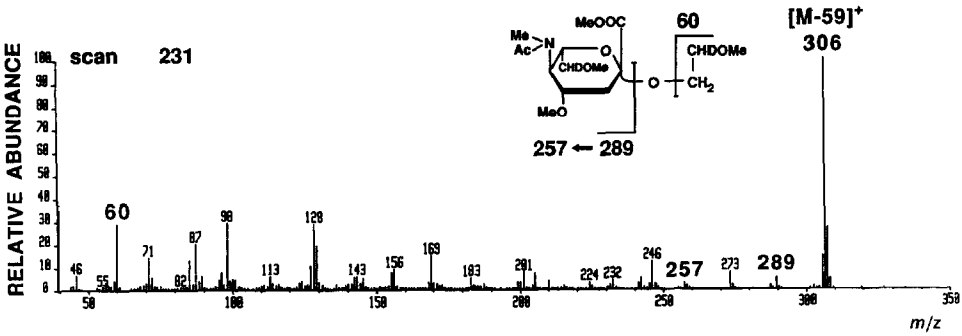

Fig. 5. GLC–mass spectrum of the component in scan 231 from Fig. 4.

m/z 60 indicates an ethylene glycol moiety. A periodate-degraded non-reducing terminal sialic acid moiety is indicated by the primary and secondary sequence ions of *m/z* 289 and 257. Therefore, a sialic acid–ethylene glycol structure can be proposed which is supported by an $[M - 59]^+$ ion of *m/z* 306, formed by loss of a methoxycarbonyl radical (·COOMe) that involves C-1 of the sialic acid residue. This periodate-generated fragment is derived from Neu5Ac-(2 → 6)-GalNAc-ol and/or Neu5Ac-(2 → 6)-Gal-ol. The former alternative must represent an element of a larger structure since no evidence for a disaccharide-alditol with this composition was seen in Fig. 3. The latter alternative is most unlikely for the reasons discussed below.

The component associated with scan 238 gave the mass spectrum shown in Fig. 6. A periodate-oxidised non-reducing terminal Hex, which, according to the sugar analysis, should be Gal, is indicated by the primary sequence ion of *m/z* 179 and an ion of *m/z* 104 (see Fig. 6). An alditol ion of *m/z* 189 corresponds to 2-acetamido-2-deoxythreitol-*4-d* and is an oxidation product of a 3-substituted GalNAc-ol[4]. 3-Substitution is supported further by a cleavage ion of *m/z* 264 from the non-reducing terminal. An ion of *m/z* 130 is formed by cleavage of the C-2–C-3 bond in the alditol. No information on molecular weight is obtained from

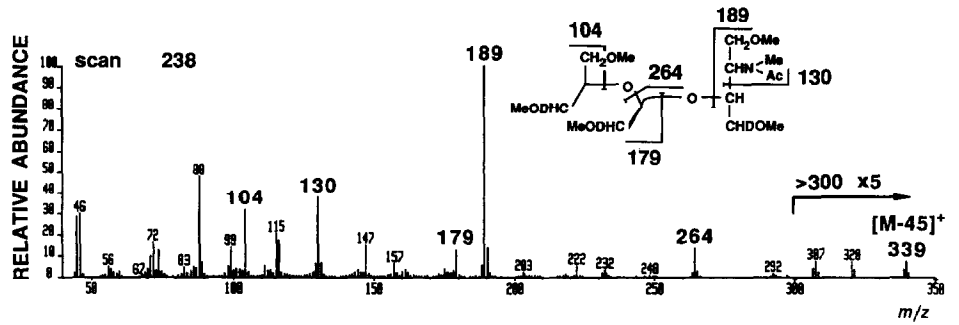

Fig. 6. GLC–mass spectrum of the component in scan 238 from Fig. 4.

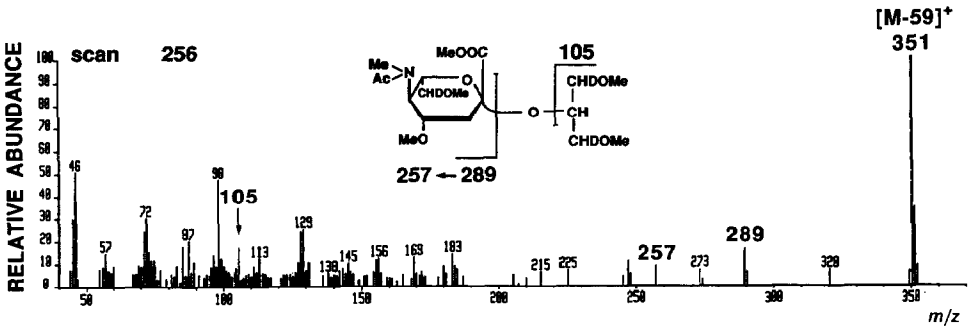

Fig. 7. GLC–mass spectrum of the component in scan 256 from Fig. 4.

the spectrum but can be deduced from an $[M - 45]^+$ ion of m/z 339. Therefore, it is concluded that this component is derived from Gal-(1 → 3)-GalNAc-ol. However, no $[M + 23]^+$ ion for this disaccharide was found in the methylated sample, so that it must be an element of a larger structure (Fig. 3).

The products derived by periodate oxidation were quantified by GLC. The components associated with scans 231 and 238 were in the ratio 1.01:1, which demonstrates that they were formed from the branched trisaccharide-alditol Gal-(1 → 3)-[Neu5Ac-(2 → 6)]-GalNAc-ol and that the alternative structure Neu5Ac-(2 → 6)-Gal-ol noted above is probably not present. This branched structure corresponds to the $[M + 23]^+$ ion of m/z 895 (Fig. 3).

A minor component associated with scan 256 gave the mass spectrum shown in Fig. 7. A terminal sialic acid moiety is indicated by the primary and secondary sequence ions of m/z 289 and 257. This residue is linked to glycerol-d_2 (ion at m/z 105) which means a 3- or 4-substituted Gal-ol. Loss of a ·COOMe from M^+, giving an $[M - 59]^+$ ion of m/z 351, is consistent with this interpretation. Since the periodate oxidation was carried out on the reduced compound, 3- and 4-substitution cannot be differentiated, but the former is the most likely alternative according to what is known generally about the substitution of galactose by sialic acid. Therefore, the most probable structure of the parent compound is Neu5Ac-(2 → 3)-Gal-ol, which corresponds to the $[M + 23]^+$ ion of m/z 650 (Fig. 3).

The mass spectrum of the component associated with scan 469 is shown in Fig. 8. The primary sequence ions of m/z 289 and 493 indicate the sequence Neu5Ac-(2 → 3)-Gal. The ion of m/z 493 (289 + 204) represents a periodate-resistant Hex, which therefore must be 3-substituted[1]. An alditol ion of m/z 189 indicates a 3-substituted GalNAc-ol (see Fig. 6). As with the previous sialylated compounds, an intense ion is formed by loss of ·COOMe to give an $[M - 59]^+$ ion of m/z 639. The structure of the present component is therefore Ne5Ac-(2 → 3)-Gal-(1 → 3)-GalNAc-ol. The $[M + 23]^+$ ion of m/z 895 (Fig. 3) is also compatible with this linear structure.

The disialylated component, corresponding to an $[M + 23]^+$ ion of m/z 1257 (Fig. 3), did not give any new components after periodate oxidation, which suggests

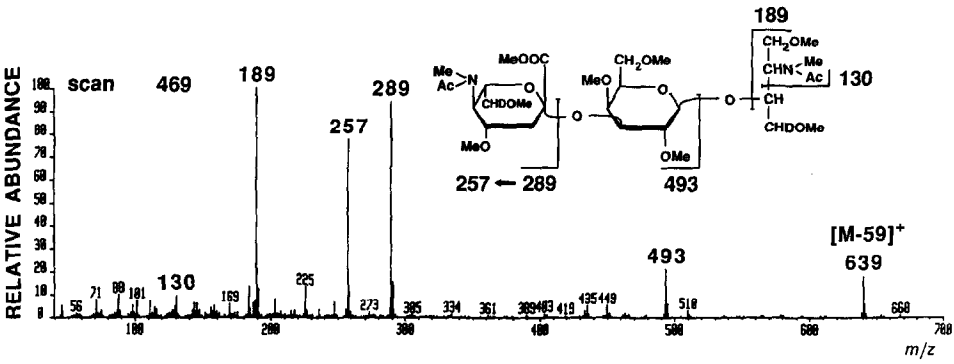

Fig. 8. GLC–mass spectrum of the component in scan 469 from Fig. 4.

a 3,6-disubstituted GalNAc-ol with the 3-substituent Neu5Ac-(2 → 3)-Gal and the 6-substituent Neu5Ac, i.e., Neu5Ac-(2 → 3)-Gal-(1 → 3)-[Neu5Ac-(2 → 6)]-GalNAc-ol. The Neu5Ac-(2 → 6)-GalNAc-ol moiety should contribute to the component in scan 231 (Fig. 4). However, the ratio of the components corresponding to the structural elements Neu5Ac-(2 → 6)-GalNAc-ol and Gal-(1 → 3)-GalNAc-ol is 1 : 1, as discussed above, which means that the disialylated structure is a minor component in this subfraction. The moiety Neu5Ac-(2 → 3)-Gal-(1 → 3)-GalNAc-ol is included in the component associated with scan 469 (Fig. 8). FABMS of methylated II-1 showed an $[M + 1]^+$ ion of m/z 1047, which was compatible with a sialylated and fucosylated tetrasaccharide-alditol (Fig. 3). Due to the small amount of this compound, no Fuc-containing product of periodate oxidation could be identified by GLC–MS.

In order to verify the structures of the products of periodate oxidation, the sample used for GLC–MS was also analysed by FABMS (Fig. 9). Two $[M + 23]^+$ ions of m/z 407 and 721 are consistent with structures or structural elements indicated. The corresponding $[M + 1]^+$ ions are m/z 385 and 699, respectively. An $[M + 1]^+$ ion of m/z 563 is compatible with the structural element Fuc-(1 → 2)-

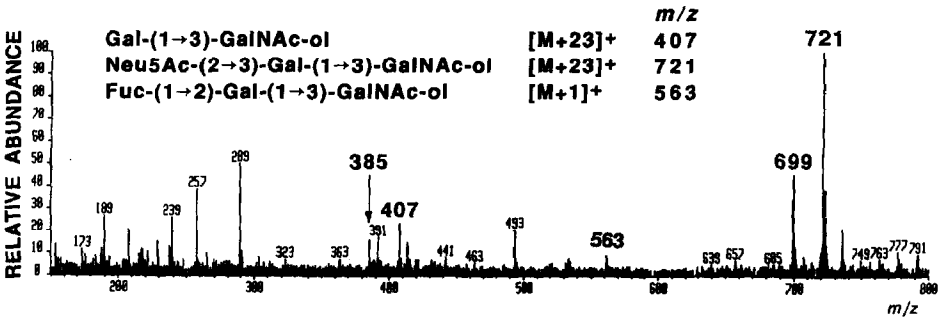

Fig. 9. FAB-mass spectrum of subfraction II-1 after periodate oxidation, borodeuteride reduction, and methylation.

Gal-(1 → 3)-GalNAc-ol or Fuc-(1 → 4)-Gal-(1 → 3)-GalNAc-ol. The former is most likely since this structure is also present in subfraction II-2 (see below). The sialic acid was cleaved from this structure by periodate oxidation, and therefore must be 6-linked to GalNAc-ol in the parent compound. No $[M + 1]^+$ or $[M + 23]^+$ ions for sialylated ethylene glycol and sialylated glycerol were observed.

The anomeric configurations of the sugar residues, except for those of fucosylated tetrasaccharide, were established by ^1H NMR spectroscopy and the chemical shift data accorded with those reported[22]. Thus, the components of subfraction II-1 and their methylated pseudo-molecular ions are as shown.

α-Neu5Ac-(2 → 3)-Gal-ol ($[M + 23]^+$ m/z 650)

1

α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-GalNAc-ol ($[M + 23]^+$ m/z 895)

2

α-Neu5Ac-(2 → 6)
β-Gal-(1 → 3) > GalNAc-ol ($[M + 23]^+$ m/z 895)

3

α-Neu5Ac-(2 → 6)
α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3) > GalNAc-ol ($[M + 23]^+$ m/z 1257)

4

α-Neu5Ac-(2 → 6)
α-Fuc-(1 → 2)-β-Gal-(1 → 3) > GalNAc-ol ($[M + 1]^+$ m/z 1047)

5

The relative molar proportions of these components (based on peak areas in GLC) are: **1** 1.8%, **2** 83.4%, **3** 14.8%, **4** < 1%, and **5** < 1%.

Analysis of subfraction II-2.—Subfraction II-2 (Fig. 2) contained some salt; hence, the components were acetylated. GLC of the products revealed no monosaccharides or monosaccharide-alditols. A portion of the acetylated sample was subjected in sequence to acid hydrolysis and borodeuteride reduction, and the products were analysed by GLC and GLC–MS. Derivatives of Fuc, Gal, and GalNAc were found in the relative proportions shown in Table I. Deuterium was incorporated into Fuc and Gal but not into GalNAc, showing the presence of oligosaccharides terminated by GalNAc-ol. Analysis for sialic acid, after *O*-deacetylation, showed that II-2 also contained sialylated compounds. The FAB-mass

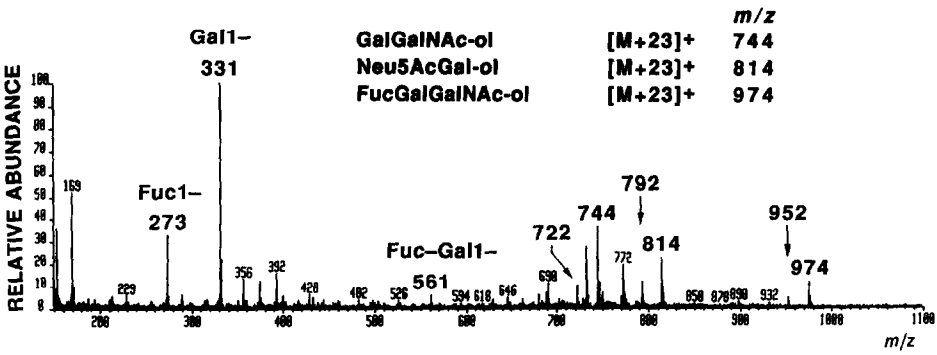

Fig. 10. FAB-mass spectrum of subfraction II-2 after acetylation.

spectrum of acetylated II-2 is shown in Fig. 10. Among the several presumed $[M + 1]^+$ and $[M + 23]^+$ ions, some can be attributed to oligosaccharides. The $[M + 23]^+$ ions of m/z 744 and 974 are consistent with the structures GalGalNAc-ol and FucGalGalNAc-ol, respectively. The corresponding $[M + 1]^+$ ions are m/z 722 and 952, respectively. Neu5Ac–Gal-ol in the lactone form is indicated by the $[M + 23]^+$ ion of m/z 814 and the $[M + 1]^+$ ion of m/z 792. Some sequence ions are also seen in the FAB-mass spectrum; thus, the ion with m/z 331 indicates non-reducing terminal Gal and those with m/z 273 and 561 indicate the sequence Fuc–Gal.

Since the neutral compounds were of most interest, II-2 was *O*-deacetylated and the acidic components were removed by using an ion-exchange resin. The residual neutral components were then subjected in sequence to periodate oxidation, borodeuteride reduction, acetylation, and analysis by GLC–MS and FABMS. Two carbohydrate-containing compounds were detected by GLC–MS as indicated in the TIC chromatogram (Fig. 11). The mass spectra of oligosaccharides with periodate-oxidised terminal and internal residues are characterised[23] by intense

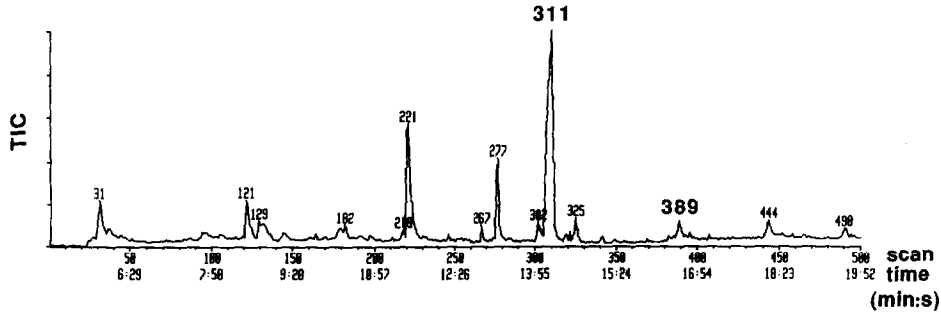

Fig. 11. TIC chromatogram from GLC–MS of the neutral subfraction II-2 after periodate oxidation, borodeuteride reduction, and acetylation. Carbohydrate-containing peaks are indicated by scan numbers. The temperature of the GLC column was programmed from 200 to 300°C at 8°C/min.

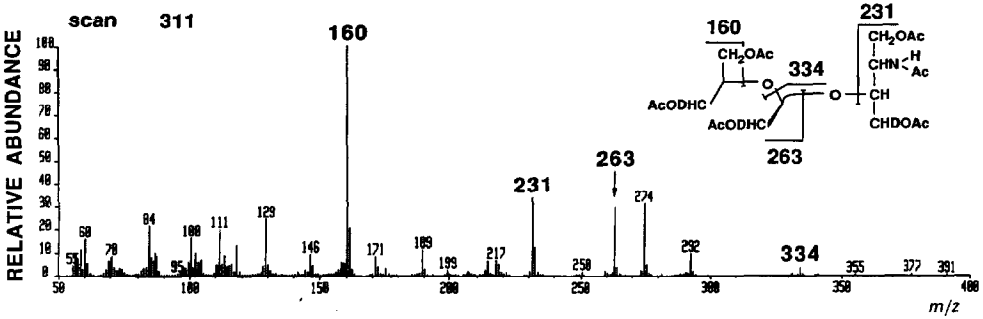

Fig. 12. GLC–mass spectrum of the component in scan 311 from Fig. 11.

ions formed by cleavage of the C-5–O-5 bond with retention of the charge on C-5. For internal residues, this type of ion gives information on the linkages. A major component associated with scan 311 gave the mass spectrum shown in Fig. 12. Non-reducing terminal Gal is indicated by the ions of m/z 160 and 263. The reduced terminal 2-acetamido-2-deoxythreitol-*4-d*, derived from a 3-substituted GalNAc-ol, is indicated by an alditol ion of m/z 231. An ion of m/z 334 formed by cleavage on the other side of the former ring oxygen indicates 3-substitution of the alditol. Therefore, the structure of the parent compound must be Gal-(1 → 3)-GalNAc-ol, which corresponds to the $[M + 23]^+$ ion of m/z 744 (Fig. 10).

A minor component associated with scan 389 gave the mass spectrum shown in Fig. 13. A non-reducing, terminal, periodate-degraded Fuc is indicated by the ions of m/z 102 and 205. That the Fuc is linked to Gal is indicated by the secondary sequence ion of m/z 455 formed after elimination of ketene from the primary sequence ion of m/z 497, which is not seen. It has been shown[6] that 2- and 4-substituted Hex residues give the same mass increment to the sequence ions after periodate oxidation. These residues can be differentiated by an ion formed by

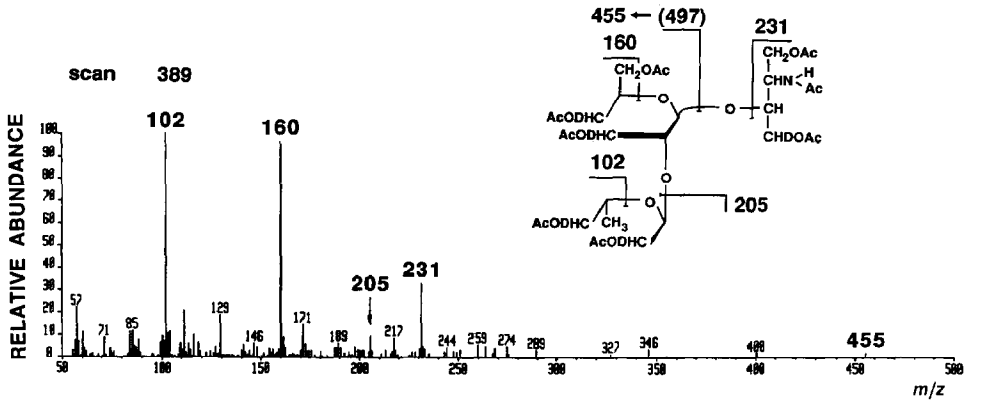

Fig. 13. GLC–mass spectrum of the component in scan 389 from Fig. 11.

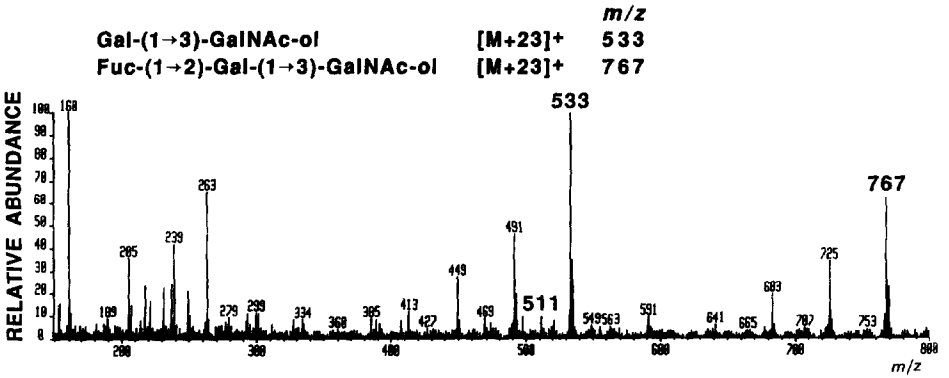

Fig. 14. FAB-mass spectrum of the neutral subfraction II-2 after periodate oxidation, borodeuteride reduction, and acetylation.

cleavage of the C-5–O-5 bond in the internal residue, as mentioned above. For a 2-substituted residue, this ion has m/z 160, whereas, for a 4-substituted residue, m/z depends on the mass of the substituent. The ion of m/z 160 in Fig. 13 shows that the above sequence is Fuc-(1 → 2)-Gal and not Fuc-(1 → 4)-Gal. That this sequence is 3-linked to GalNAc-ol is indicated by the alditol ion of m/z 231, giving the structure of Fuc-(1 → 2)-Gal-(1 → 3)-GalNAc-ol, which corresponds to an $[M + 23]^+$ ion of m/z 974 (Fig. 10).

FABMS (Fig. 14) of the above sample shows an $[M + 23]^+$ ion of m/z 533 with a corresponding $[M + 1]^+$ ion of m/z 511 and an $[M + 23]^+$ ion of m/z 767, all of which are in agreement with the above structures.

The anomeric configurations of the monosaccharide residues were determined by ^1H NMR spectroscopy of the intact subfraction II-2. Two components had chemical shift and coupling constant data in agreement with those reported[24]. Therefore, the neutral structures present in subfraction II-2 and their acetylated pseudo molecular ions are as shown.

β-Gal-(1 → 3)-GalNAc-ol $([M + 23]^+$ m/z 744)
6

α-Fuc-(1 → 2)-β-Gal-(1 → 3)-GalNAc-ol $([M + 23]^+$ m/z 974)
7

Quantification of the components by GLC gave the relative molar proportions: **6** 80%, **7** 20%.

Analysis of the salt fraction.—The salt fraction of low molecular weight obtained after fractionation on the Sephadex G-15 was acetylated and analysed by GLC and GLC–MS (Table I). The presence of Gal-ol suggested that Gal is linked to Ser/Thr of the polypeptide chain. Analysis after acid hydrolysis gave the same monosaccharide composition.

DISCUSSION

In previous publications[1–7], we have shown that information obtainable on the structure of oligosaccharides by FABMS can be extended to include linkage analysis by applying periodate oxidation then borohydride reduction. We now show that the products obtained are also amenable to GLC–MS as the methylated or acetylated derivatives. An advantage of GLC–MS is that the mass spectra are obtained from pure compounds and quantification of the products is possible. However, there is a limit to the size of the compounds that will pass through a GLC column. Therefore, the products were also analysed by FABMS. However, the methylated products of low molecular weight (sialic acid–ethylene glycol and sialic acid–glycerol) could not be detected by FABMS (Fig. 9), possibly because they are ionised less favourably in the presence of the other structures when thioglycerol was used as the matrix.

FABMS data on the products obtained from α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-GalNAc-ol after periodate oxidation, borodeuteride reduction, and methylation have been reported[4]. The FAB-mass spectrum showed a fragmentation in terms of sequence ions which was similar to that in the EI-mass spectrum (Fig. 8). However, there are differences which are connected with cleavage of carbon–carbon bonds; for example, the ion at m/z 130 and the $[M - 59]^+$ ion of m/z 639 are virtually absent from the FAB-mass spectrum. Loss of ·COOMe, which gives rise to an intense ion in the EI-mass spectrum, seems to be characteristic for periodate-oxidised, borohydride-reduced, sialylated and methylated compounds. Furthermore the EI-mass spectrum shows no molecular ion species, whereas they are prevalent in the FAB-mass spectrum.

The choice of derivatisation after periodate oxidation–borohydride reduction can be either methylation or acetylation. However, when larger structures are expected, methylation is preferred in order to increase the volatility. Acetylated samples can be *O*-deacetylated, methylated, and re-analysed if necessary. Acetylation is also convenient for isolating low molecular weight products which cannot be desalted readily by other means. Acetylated sialic acid-containing compounds form lactones and therefore give no specific sequence ion for this residue[2]. This situation also occurs in FABMS where the pseudo-molecular ion is found at 60 mass units lower than expected.

β-Gal-(1 → 3)-GalNAc-ol was analysed both as methylated and acetylated derivatives (Figs. 6 and 12). Both spectra give essentially the same structural information. However, alditol cleavage ions are not formed from the acetylated compound, but are quite abundant in the mass spectrum of the methylated compound.

Gel filtration is sufficient to fractionate the released oligosaccharide-alditols and to remove *N*-linked and larger structures. Therefore, sugar analysis is carried out in order to verify the absence of Man. Fraction I (Fig. 1) also contained larger *O*-linked structures, previously shown[13] to be trisialylated derivatives of β-Gal-(1

→ 3)-GalNAc-ol. The preponderant *O*-linked structure[12] in human glycophorin A is the tetrasaccharide **4**, most of which must be present as the corresponding alditol in fraction I, as confirmed by FABMS.

The use of a well-known model of a membrane glycoprotein, namely human glycophorin A, allowed the improved method for the analysis of *O*-linked oligosaccharides to be checked. The method, based on GLC–MS and FABMS performed after methylation or acetylation of the native or periodate-oxidised sugar chains, is sensitive and gives unequivocal results. Since a purified glycophorin A preparation was used, the structures found must come mostly from that molecule. Minor proportions of glycophorin B and C, present in the glycophorin A preparation, should not influence the results significantly. Although studies of structure of the oligosaccharides isolated from purified glycophorin B and C have not been reported, indirect evidence obtained from their reactivity towards lectins and carbohydrate-specific monoclonal antibodies indicates the same glycosylation pattern in all glycophorins.

The structures **1**–**7** were established, all of which are noted in the literature on human glycophorin A, although some were given only as tentative structures. Depending on the sugar content and oligosaccharide heterogeneity, the amount of glycoprotein needed for the procedure described could be ten times lower than used here for glycophorin A.

α-Neu5Ac-(2 → 3)-Gal-ol and Gal-ol were identified among the products of alkaline degradation performed under conditions that minimise peeling reactions. Hitherto, these structures were considered as products of degradation of GalNAc-terminated oligosaccharides[10–12]. Our results raise the question as to whether glycophorin A contains a minor proportion of oligosaccharides with Gal linked to Ser/Thr residues or whether the structures that terminate in Gal-ol are products of peeling reactions. The first possibility could indicate that the GalNAc to Ser/Thr transferase shows a "trace" affinity for UDP-galactose, as shown for the blood-group A transferase[25]. GalNAc-ol and α-Neu5Ac-(2 → 6)-GalNAc-ol were not detected among the oligosaccharide-alditols formed from glycophorin A, although these structures were identified by Thomas and Winzler[10] in a crude preparation of glycophorin and their presence was suggested by the reaction of Tn-specific [α-GalNAc-(1-*O*)-Ser/Thr] lectin of *Helix pomatia* with asialoglycophorin[12]. Recent studies[26] of another Tn-specific lectin from *Moluccella laevis* showed a distinct preference of the lectin reaction with blood-group N type glycophorin A. Therefore, it is possible that a minor proportion of the *O*-linked chains, those containing GalNAc which are not galactosylated, is present only in blood-group N-type glycophorin A and glycophorin B, and absent (or undetecable by spectroscopic methods) in the M-type glycophorin A.

REFERENCES

1 A.-S. Angel, F. Lindh, and B. Nilsson, *Carbohydr. Res.*, 168 (1987) 15–31.
2 H. Krotkiewski, E. Lisowska, A-S. Angel, and B. Nilsson, *Carbohydr. Res.*, 184 (1988) 27–38.

3 A.-S. Angel and B. Nilsson, *Methods Enzymol.*, 193 (1990) 721–730.
4 A.-S. Angel and B. Nilsson, *Biomed. Environ. Mass Spectrom.*, 19 (1990) 721–730.
5 A.-S. Angel, G. Grönberg, H. Krotkiewski, E. Lisowska, and B. Nilsson, *Arch. Biochem. Biophys.*, 291 (1991) 76–88.
6 A.-S. Angel, P. Lipniunas, K. Erlansson, and B. Nilsson, *Carbohydr. Res.*, 221 (1991) 17–35.
7 P. Lipniunas, A.-S. Angel, K. Erlansson, F. Lindh, and B. Nilsson, *Anal. Biochem.*, 200 (1992) 58–67;
8 H. Furthmayr and V. Marchesi, *Methods Enzymol.*, 96 (1983) 268–280.
9 E. Lisowska, in A.M. Wu (Ed.), *The Molecular Immunology of Complex Carbohydrates*, Plenum Press, New York, 1988, pp 265–315.
10 D.B. Thomas and R.J. Winzler, *J. Biol. Chem.*, 244 (1969) 5943–5946.
11 S. Takasaki, K. Yamashita, and A. Kobata, *J. Biol. Chem.*, 253 (1978) 6086–6091.
12 E. Lisowska, M. Duk, and W. Dahr, *Carbohydr. Res.*, 79 (1980) 103–113.
13 M. Fukuda, M. Lauffenburger, H. Sasaki, M.E. Rogers, and A. Dell, *J. Biol. Chem.*, 262 (1987) 11952–11957.
14 E. Lisowska, L. Messeter, M. Duk, M. Czerwinski, and A. Lundblad, *Molec. Immunol.*, 24 (1987) 605–613.
15 H. Krotkiewski, E. Daniluk, G. Skibinski, and E. Lisowska, *Arch. Immunol. Ther. Exp.*, 32 (1984) 613–620.
16 L.M. Likhosherstov, O.S. Novikova, V.A. Derevitskaya, and N.K. Kochetkov, *Carbohydr. Res.*, 199 (1990) 67–76.
17 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
18 G.W. Jourdian, L. Dean, and S. Roseman, *J. Biol. Chem.*, 246 (1976) 430–435.
19 J.S. Sawardeker, J.H. Sloneker, and A.R. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.
20 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
21 I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
22 H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, A.-M. Fiat, and P. Jolles, *FEBS Lett.*, 133 (1981) 45–50.
23 A.-S. Angel and B. Nilsson, *Biol. Mass Spectrom.*, 21 (1992) 202–212.
24 H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, W.E. Hull, G. Lamblin, M. Lhermitte, A. Boersma, and P. Rousel, *Eur. J. Biochem.*, 127 (1982) 7–20.
25 A.D. Yates and W.M. Watkins, *Biochem. Biophys. Res. Commun.*, 109 (1982) 958–965.
26 M. Duk, D. Mitra, E. Lisowska, E.A. Kabat, N. Sharon, and H. Lis, *Carbohydr. Res.*, 236 (1992) 245–258.