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DETERMINATION OF BINDING POSITIONS IN OLIGOSACCHARIDES AND GLYCOSPHINGOLIPIDS BY FAST-ATOM-BOMBARDMENT MASS SPECTROMETRY

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ABSTRACT

Application of f.a.b.-m.s. to the products obtained from glycoconjugates upon periodate oxidation followed by borohydride reduction and methylation gives the positions of binding of the monosaccharide residues on the basis of the sequences of the primary and secondary ions.

INTRODUCTION

The introduction of f.a.b.-m.s. has provided new opportunities for analysing biological compounds which could not be dealt with by conventional e.i.-m.s. F.a.b.-m.s. can be used for derivatised and underivatised compounds in both positive and negative ion modes. Analysis of underivatised glycoconjugates gives information about the molecular weight and, after acetylation or methylation, the sequence of monosaccharides can be deduced. The positions of binding can sometimes be obtained after methylation, for example, the positions of substitution of a 2-acetamido-2-deoxy sugar unit can be determined by the secondary fragments formed after a primary cleavage of the 2-acetamido-2-deoxyhexosyl (HexNAc) linkage[1]. However, in general, the positions of binding cannot be determined by f.a.b.-m.s. We now describe a simple procedure for determining, from an f.a.b. mass spectrum, the positions of linkages between the monosaccharide residues in methylated oligosaccharides and glycosphingolipids.

EXPERIMENTAL

The oligosaccharides and glycosphingolipids were obtained from BioCarb (Lund). Ozonolysis[2] and trifluoroacetolysis[3] of glycosphingolipids and methylation

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analysis[4] were carried out according to published procedures. All reagents used were of analytical grade.

Periodate oxidation[5]. — Solutions of oligosaccharides (2 mg) and glycosphingolipids (2 mg) in 0.1M acetate buffer (5 mL, pH 5.5) containing 8mM sodium periodate were stored at 4° in the dark for 48 h. The excess of periodate was then reduced with ethylene glycol (25 μL) and the sample was left at 4° overnight. The pH of the solution was adjusted to 7.0 with 0.1M NaOH, $NaBD_4$ (25 mg) was added, and the mixture was stored at 4° overnight. Acetic acid was added to pH 4.5, the mixture was concentrated to dryness, and boric acid was removed by distillation of methanol (3 × 3 mL) from the residue. The product was desalted by elution from a column (20 × 1 cm) of G-25 Sephadex with water. Fractions were monitored for hexose by the anthrone method[6].

Methylation. — Samples were methylated as previously described[7] and each product was purified on a SepPak C18 reverse-phase cartridge[8], previously rinsed with chloroform (20 mL), methanol (10 mL), acetonitrile (4 mL), and water (4 mL). A solution of each product in methyl sulfoxide was diluted with water (1 vol.) and applied to the cartridge. For oligosaccharides, the following procedure was used: washing with water (8 mL), aqueous 15% acetonitrile (4 mL), and elution of methylated oligosaccharides with acetonitrile (4 mL). The procedure for purification of methylated glycosphingolipids involved washing with water (8 mL), acetonitrile (4 mL), and methanol (2 mL), and the methylated products were recovered by elution with chloroform (4 mL).

Mass spectrometry. — F.a.b.-m.s. (positive ion mode) was performed on a VG ZAB SE instrument. Solutions of samples in thioglycerol (1-thio-2,3-propanediol) were loaded on the stainless-steel target which was bombarded with xenon atoms with a kinetic energy of 8 keV. G.l.c.–e.i.-m.s. was carried out on a VG 12-250 quadrupole instrument fitted with an SE-30 W.C.O.T. capillary column. Spectra were recorded at 70 eV with an ion-source temperature of 200°.

RESULTS

Oligosaccharides. — F.a.b.-m.s. of methylated oligosaccharides gives information about the monosaccharide sequence and the molecular weight. The sequences of ions are formed by a primary cleavage of the glycosidic bonds. For each hexosyl residue, 204 mass units (m.u.) are added to the sequence ions. Additional structural information can be obtained after applying, in sequence, periodate oxidation, borodeuteride reduction, methylation, and f.a.b.-m.s. In order to identify the products formed and the fragmentation pattern, model compounds were used. The oxidised products, formed from internal hexopyranosyl residues, are shown in Fig. 1 together with the increments that should be added to the sequence ions. For a 2- or 4-substituted hexosyl residue, 208 m.u. are added to the sequence ions. A secondary fragment, formed by elimination of methanol, is seen for a 2- but not for a 4-substituted residue. The product formed from a 6-sub-

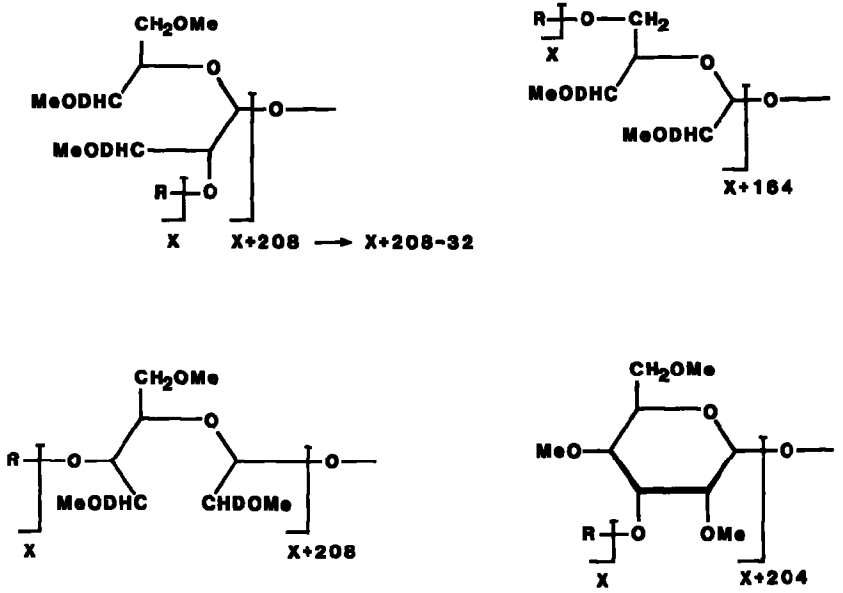


Fig. 1. Products formed after periodate oxidation, borodeuteride reduction, and methylation of a variously substituted, internal Hex residue. Fragments formed by a primary cleavage are indicated.

stituted residue results in an addition of 164 m.u. 3-Substituted residues are resistant to periodate and, as mentioned above, 204 m.u. are added to the sequence ions.

When unreduced oligosaccharides are oxidised with periodate, the products from a reducing hexose vary with the position of substitution and the ring size (pyranose or furanose). However, ions containing the modified reducing hexose have *m/z* values that are too low to be identified unambiguously. The most abundant sequence ions are formed from the non-reducing terminal. Therefore, the linkage to a reducing hexose is determined best by subtracting the sequence ion with the highest *m/z* value from the molecular weight (*M*) which can always be determined. If the mass of the remainder is 121, the reducing hexopyranose is 2-substituted; if 208, 3-substituted; if 164, 4-substituted; and if 120, a reducing 6-substituted hexopyranose is present. When the reducing hexose residue is furanoid, other products have to be considered.

In order to confirm that an ion is consistent with the molecular weight (determined from the $[M + 1]^+$ species) and not a fragment ion, sodium iodide is added and $[M + 23]^+$ is observed for ions representing the molecular weight but not for fragment ions. In the spectra presented below, both $[M + 1]^+$ and $[M + 23]^+$ ions are seen. In some spectra, $[M + 18]^+$ is observed; this often occurs when thioglycerol is used as the matrix. For ions with $m/z > 1000$, the decimals of the atom masses are important. The contribution of the isotopes affects ions with $m/z > 1500$ and gives rise to several clusters of ions.

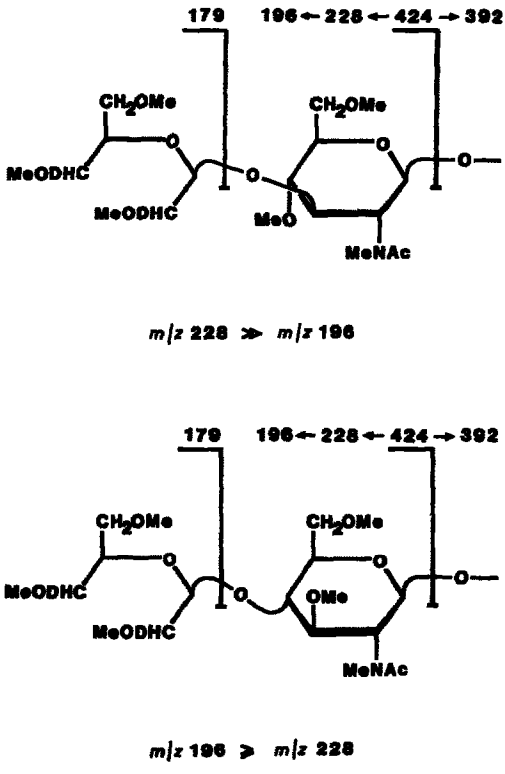


Fig. 2. Primary and secondary fragments formed from a 3- and 4-substituted HexNAc residue.

The position of substitution of a HexNAc residue can be determined by comparing the intensities of the secondary fragments formed after a primary cleavage of the HexNAc linkage (Fig. 2). The fragment with *m/z* 179 is the sequence ion for the product of periodate oxidation of a non-reducing terminal Hex residue. The next sequence ion, *m/z* 424, gives secondary fragments of *m/z* 392 (by elimination of methanol), 228 (by elimination of the oxidised hexose residue), and 196 (by elimination of both methanol and the oxidised hexose). The relative intensities of the ions with *m/z* 196 and 228 determine the position of substitution of the HexNAc residue (Fig. 2). For 3-substituted HexNAc, the abundance of the ion with *m/z* 228 is much greater than that with *m/z* 196; for 4-substituted HexNAc, the abundance of these ions is about the same. The actual intensities of these ions are seen in the spectra presented below.

In order to determine the positions of substitution of a 3,4-disubstituted HexNAc unit, the following human-milk oligosaccharides were chosen as model compounds: β-Gal-(1→3)[α-Fuc-(1→4)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (lacto-*N*-fucopentaose II, LNF-II) and β-Gal-(1→4)[α-Fuc-(1→3)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (lacto-*N*-fucopentaose III, LNF-III). The f.a.b. mass spectra and the products formed after periodate oxidation, reduction, and methylation are

Fig. 3. F.a.b. mass spectrum of lacto-*N*-fucopentaose II (LNF-II) after periodate oxidation, borodeuteride reduction, and methylation.

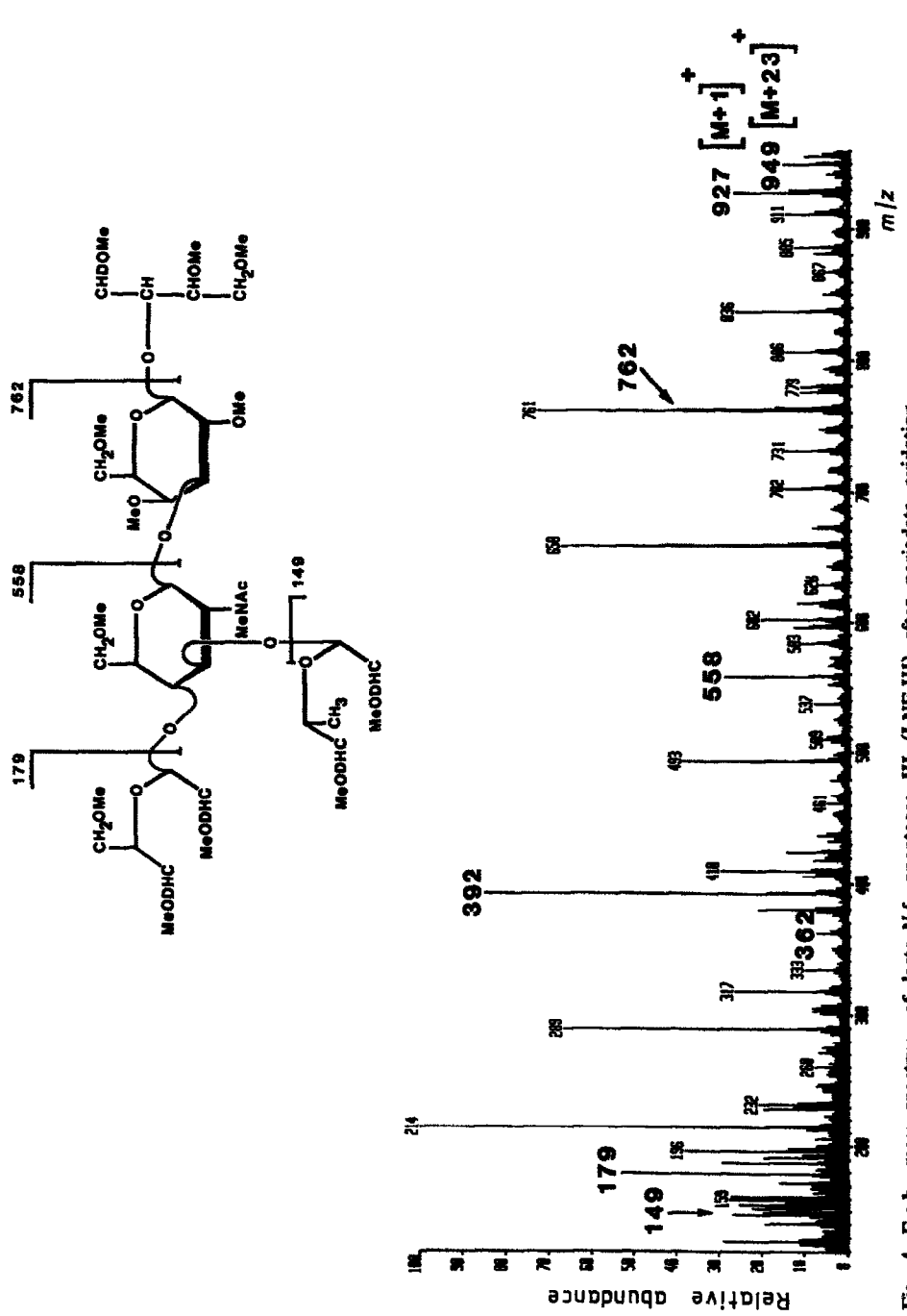


Fig. 4. F.a.b. mass spectrum of lacto-*N*-fucopentaose III (LNF-III) after periodate oxidation, borodeuteride reduction, and methylation.

shown in Figs. 3 and 4. The fragments with *m/z* 149 and 179 represent the products formed after oxidation of the non-reducing terminals of 6-deoxyhexose and hexose, respectively. These two residues are linked to a HexNAc, as shown by the ion with *m/z* 558. The next sequence ion, *m/z* 762, is formed by adding 204 m.u. consistent with a 3-substituted Hex. The ions with *m/z* 927 ($[M + 1]^+$) and 949 ($[M + 23]^+$) are consistent with this structure. By subtracting the fragment ion with the highest *m/z* value (762) from the molecular weight of 926, the value 164 is obtained,

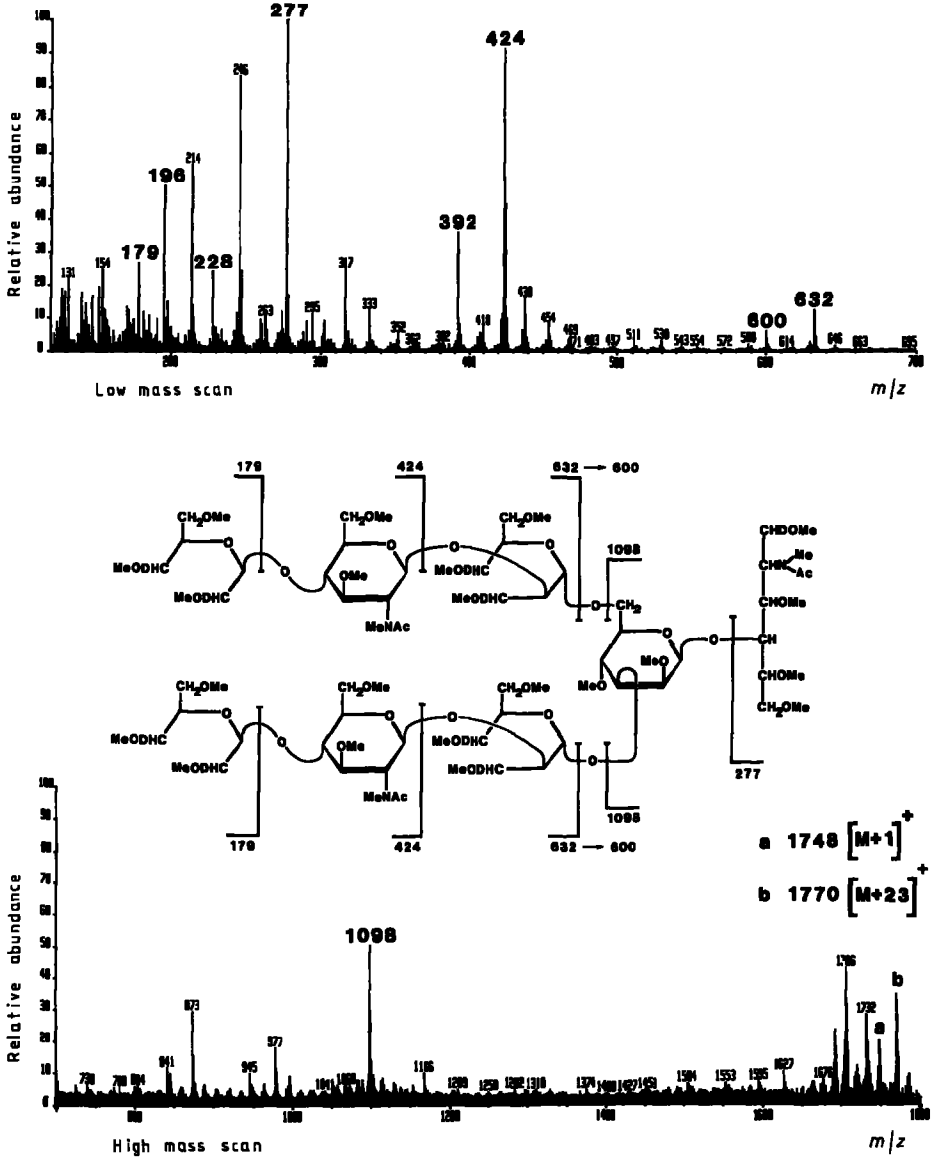
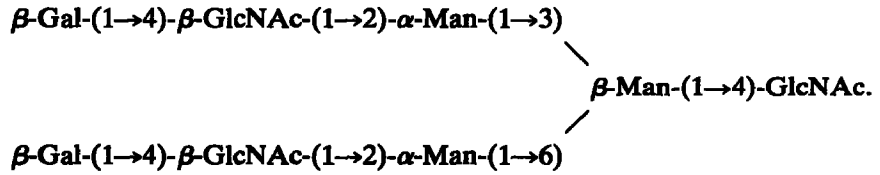


Fig. 5. F.a.b. mass spectrum of a biantennary glycoprotein fragment after periodate oxidation, borodeuteride reduction, and methylation.

indicating a 4-substituted Hex as previously indicated. The secondary fragments formed from the ion with *m/z* 558 by elimination determine the positions of substitution of the HexNAc residue. Secondary fragments are formed by preferential elimination of the 3-substituent, to give the ions with *m/z* 362 (Fig. 3) and 392 (Fig. 4). For LNF-II (Fig. 3), the intensity of the ion with *m/z* 362 is greater than of *m/z* 392, whereas, for LNF-III (Fig. 4), the opposite is observed. The spectra of LNF-II and LNF-III also contain other ions which will not be discussed since they provide no further information on the structure.

The method is further demonstrated by an oligosaccharide from a bi-antennary structure of a glycoprotein with the structure



The f.a.b. mass spectrum of the product is shown in Fig. 5. The sequence ions *m/z* 179 and 424 and the determination of the position of substitution of the HexNAc from the secondary fragments from *m/z* 424 (Fig. 2) have been discussed above. The sequence ion *m/z* 632 is derived by adding 208 m.u. to the ion with *m/z* 424, which means a 2- or 4-substituted Hex residue (Fig. 1). A secondary fragment is formed by elimination of methanol, to give the ion with *m/z* 600, which is observed for a 2- but not for a 4-substituted oxidised Hex residue (see below). The ion with *m/z* 277 is typical of a borodeuteride-reduced HexNAc, which should be preserved since the periodate oxidation was carried out on the unreduced oligosaccharide. The ions with *m/z* 1748 ($[M + 1]^+$) and 1770 ($[M + 23]^+$) support the structure. The position of substitution of the reducing HexNAc cannot be determined from the spectrum, but this linkage could also have been determined if the periodate oxidation had been carried out on the reduced oligosaccharide. The linkages to the branched D-mannosyl residue cannot be deduced from the spectrum.

The example described above demonstrates that the primary fragment from an internal, 2-substituted, oxidised Hex residue gives a secondary fragment by elimination of methanol. An example of an internal 4-substituted Hex residue is given by globo-*N*-tetraose, β-GalNAc-(1→3)-α-Gal-(1→4)-β-Gal-(1→4)-Glc, obtained from the glycolipid globoside after trifluoroacetolysis[3]. Figure 6 shows the f.a.b. mass spectrum of the oxidised, reduced, and methylated product. The primary and secondary fragments *m/z* 264 and 232 (elimination of MeOH) are significant for a periodate-oxidised product of a non-reducing terminal HexNAc. The next sequence ion, *m/z* 468 (addition of 204 m.u.), adds a 3-substituted Hex residue to the sequence. The fragment *m/z* 676 is formed by adding 208 m.u. to *m/z* 468, indicating a 2- or 4-substituted oxidised Hex residue. In contrast to the previous example, no elimination of methanol is seen from the primary sequence

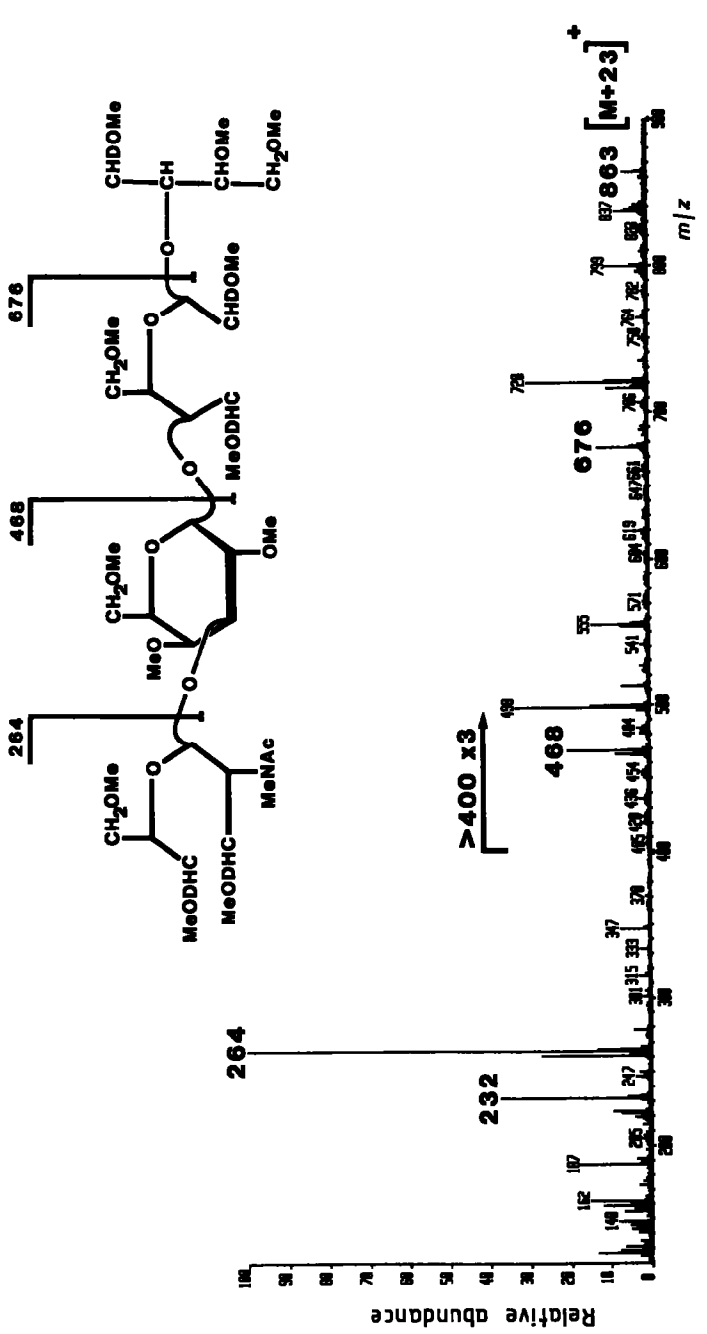


Fig. 6. F.a.b. mass spectrum of globo-*N*-tetraose after periodate oxidation, borodeuteride reduction, and methylation.

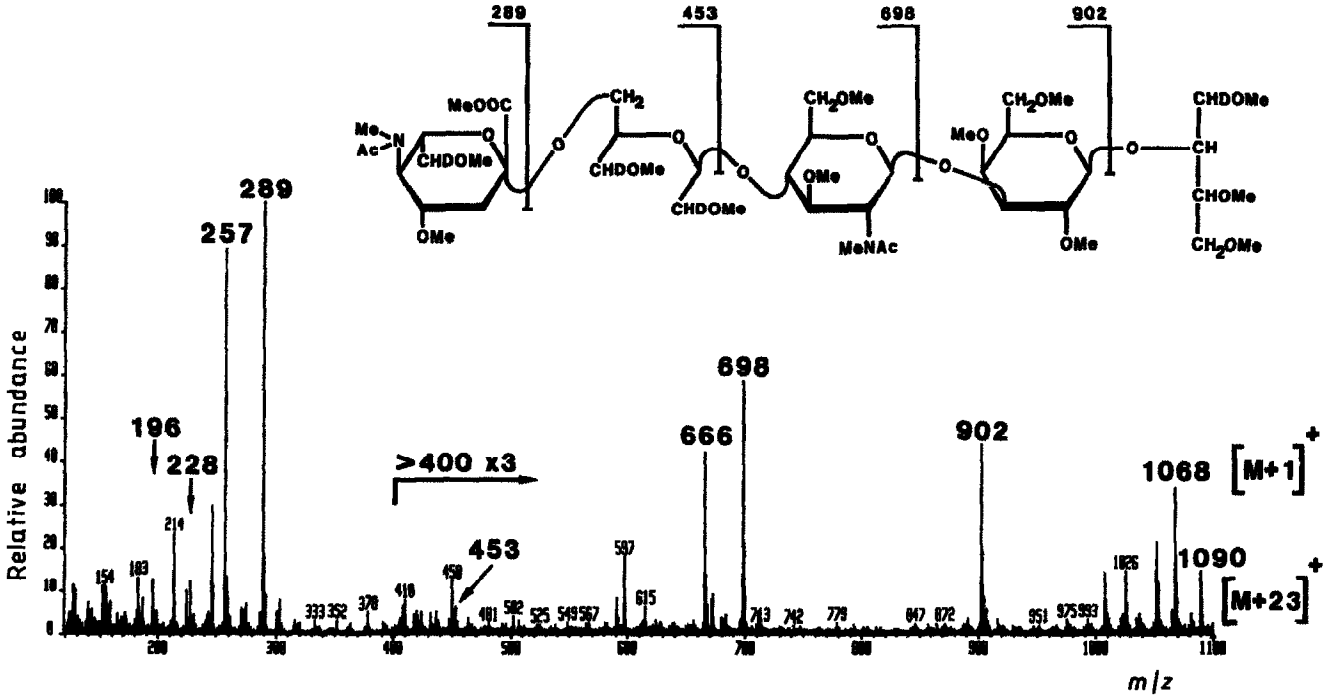


Fig. 7. F.a.b. mass spectrum of a sialic acid-containing oligosaccharide after periodate oxidation, borodeuteride reduction, and methylation.

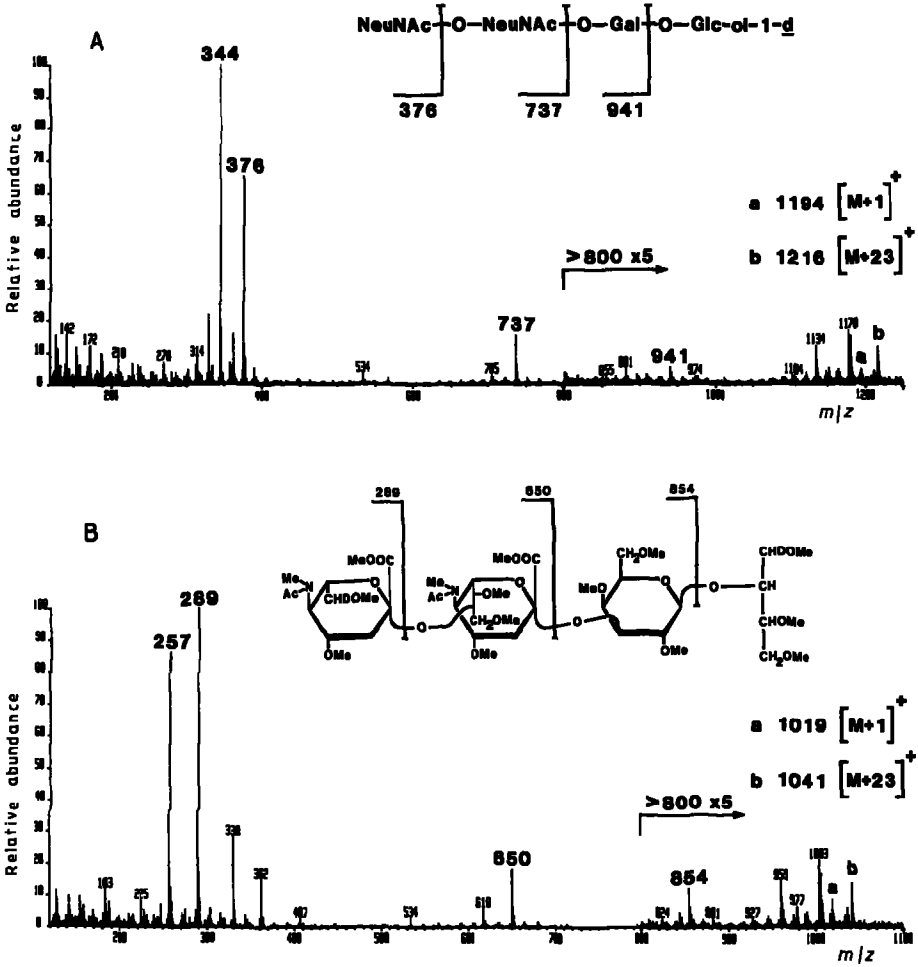


Fig. 8. F.a.b. mass spectra of the oligosaccharide from G_{D3}-ganglioside: A, as the methylated alditol; B, after periodate oxidation, borodeuteride reduction, and methylation.

ion of a 4-substituted oxidised Hex residue. The linkage to the reducing terminal is determined as described above for LNF-II and LNF-III. The structure of the product is verified by the ion *m/z* 863, which is $[M + 23]^+$.

The method can also be applied to sialic acid-containing oligosaccharides, as demonstrated with the oligosaccharide α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc isolated from human milk. When subjected to periodate oxidation, borodeuteride reduction, and methylation, the product gave the f.a.b. mass spectrum shown in Fig. 7. The primary fragment *m/z* 289 and the secondary fragment *m/z* 257 (formed by elimination of MeOH) indicate a periodate-degraded residue of sialic acid. The next sequence ion, *m/z* 453, which has an increase of 164 m.u., shows that sialic acid is (2→6)-linked to a Hex residue. The intensity of the

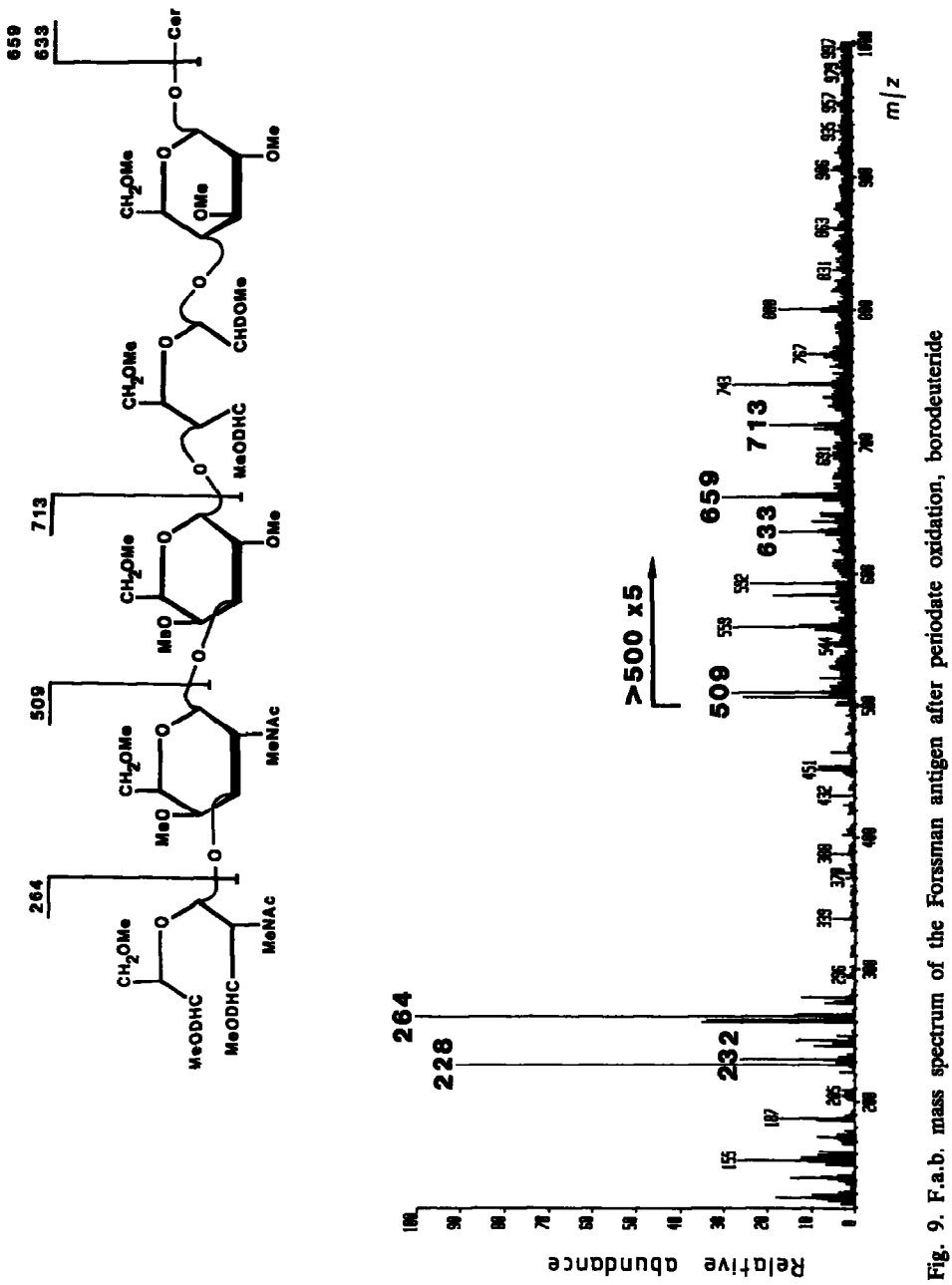


Fig. 9. F.a.b. mass spectrum of the Forssman antigen after periodate oxidation, borodeuteride reduction, and methylation.

ion *m/z* 453 is rather weak, but the next primary sequence ion *m/z* 698 and the secondary ion *m/z* 666 give additional support for the Neu5Ac-(2→6)-Hex linkage. The ion *m/z* 902 adds a 3-substituted Hex residue to the sequence. The 4-substitution of the HexNAc residue is determined by comparing the intensities of the secondary fragments *m/z* 196 and 228 formed from *m/z* 698 by elimination as previously described. The position of substitution of the reducing hexose was determined as discussed above. The structure is further supported by the ions of *m/z* 1068 ($[M + 1]^+$) and 1090 ($[M + 23]^+$).

In oligosaccharides having sialic acid linked to sialic acid, the position of attachment is often difficult to determine. The oligosaccharide, α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc, isolated after ozonolysis[2] of the ganglioside G_{D3}, was analysed by f.a.b.-m.s. as the methylated alditol-*1-d* (Fig. 8A). The monosaccharide sequence from the non-reducing terminal was determined by the ions *m/z* 376, 737, and 941. The ion of *m/z* 1216 corresponds to $[M + 23]^+$. The oligosaccharide was oxidised, borodeuteride reduced, and methylated, and the product gave the f.a.b. mass spectrum shown in Fig. 8B. The corresponding sequence ions are *m/z* 289, 650, and 854, and $[M + 23]^+$ is *m/z* 1041. The sequence ion *m/z* 650 is formed by adding 361 m.u. (which represents a periodate-resistant sialic acid residue) to *m/z* 289, indicating a Neu5Ac-(2→8)-Neu5Ac linkage. If this linkage had been (2→4), (2→7), or (2→9), the internal sialic acid should have been oxidised and the fragment *m/z* 650 could not be formed. The next sequence ion, *m/z* 854, is derived by adding 204 m.u. to *m/z* 650, indicating a 3-substituted Hex residue. The substitution of the reducing hexose is determined as described above. With this method, all linkages in the oligosaccharide could be determined from the f.a.b. mass spectrum.

Glycosphingolipids. — The method described above can also be applied to glycosphingolipids. The f.a.b. mass spectrum of the Forssman antigen, α-GalNAc-(1→3)-β-GalNAc-(1→3)-α-Gal-(1→4)-β-Gal-(1→4)-Glc-Cer, is shown in Fig. 9. The sequence ions *m/z* 264, 509, and 713 define a sequence of HexNAc–HexNAc–Hex. The next sequence ion, which should have been *m/z* 921, is not seen. This also was the situation for the corresponding ion of the intact compound (data not shown). The relative intensities of the secondary fragments *m/z* 196 and 228 formed from *m/z* 509 determine also the position of substitution of the internal HexNAc residue, using the same arguments as before (Fig. 2). The ions at *m/z* 633 and 659 give information about the ceramide moiety. The molecular weight of the compound could not be determined accurately due to the presence of several ions of low abundances in the molecular weight range. The f.a.b. spectrum of the intact and methylated compound revealed considerable heterogeneity in the ceramide moiety. This heterogeneity and the different reactivity of the ceramide residues towards periodate could explain the difficulties in determining an accurate molecular weight of the oxidised compound. In order to verify that all of the periodate-susceptible residues were oxidised, methylation analysis was carried out and showed that the inner 4-substituted D-glucosyl residue was resistant to periodate.

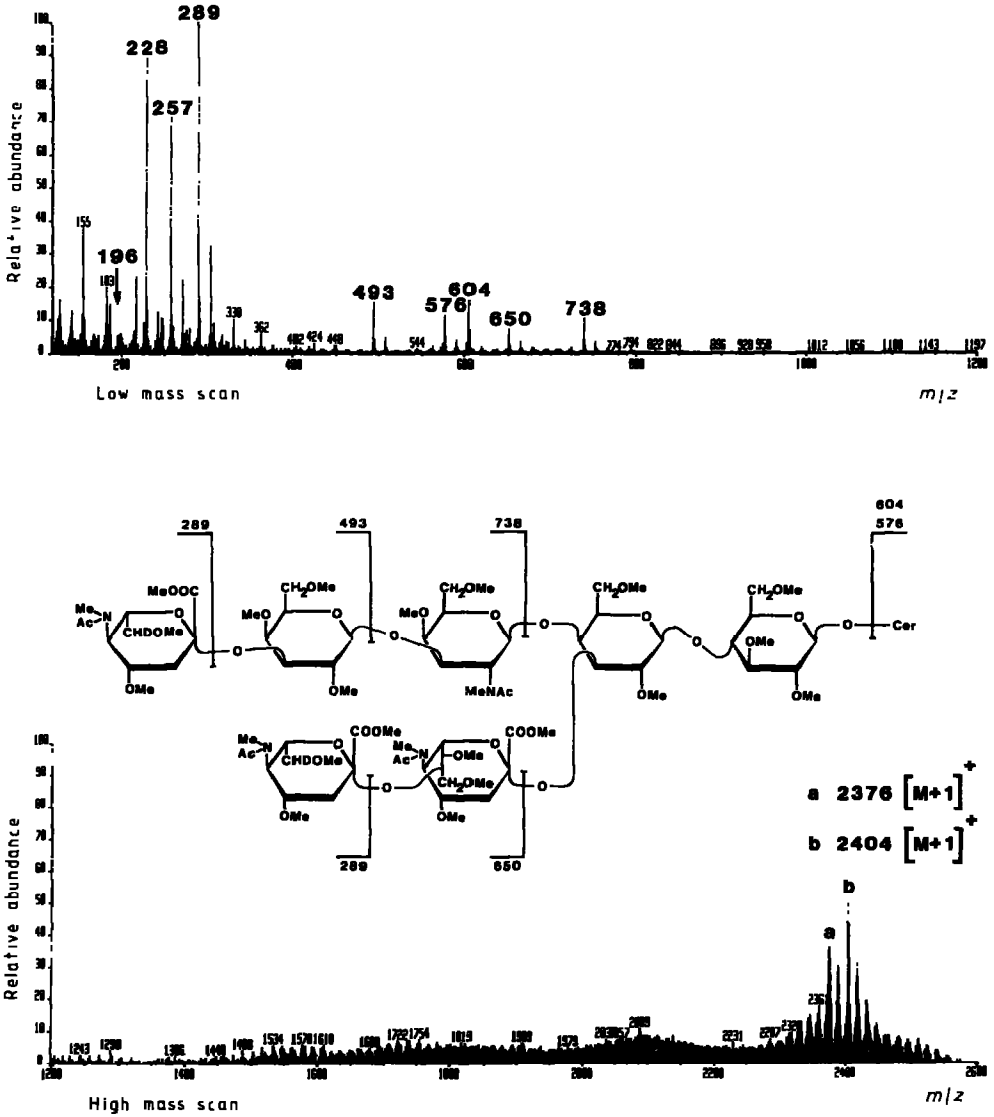


Fig. 10. F.a.b.-mass spectrum of G_{T1b}-ganglioside after periodate oxidation, borodeuteride reduction, and methylation.

The product formed from

α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4)
β-Gal-(1→4)-Glc-Cer
α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)

(G_{T1b} ganglioside) and its f.a.b. mass spectrum are shown in Fig. 10. The fragment *m/z* 493 is formed by adding 204 m.u. to *m/z* 289 and indicates a Neu5Ac-(2→3)-Hex-(1→ sequence. The fragment *m/z* 738 adds a HexNAc residue and, combined with the previous sequence ions, a sequence of Neu5Ac-(2→3)-Hex-(1→3)-HexNAc-(1→ is determined. Secondary fragments, formed from *m/z* 738 by elimination to give *m/z* 196 and 228, determine the Hex-(1→3)-HexNAc linkage by analogy with previous examples (Fig. 2). Another sequence of Neu5Ac-(2→8)-Neu5Ac-(2→ is represented by the fragments *m/z* 289 and 650, where the linkage is determined according to the previous discussion (Fig. 8). The positions of substitution of the branched D-galactosyl residue by these sequences cannot be inferred from the spectrum, as well as the linkage to the inner D-glucosyl residue which is resistant to periodate. The ceramide-containing fragments *m/z* 576 and 604 show two different ceramide residues. The clusters of ions in the molecular weight range are consistent with molecular weights of the two species.

DISCUSSION

F.a.b.-m.s. can be used on underivatised glycoconjugates, but very little fragmentation is seen. Fragmentation is enhanced by methylation, which is the method of choice since the molecular weight will be lower compared to that of other derivatives such as acetates.

F.a.b.-m.s. of methylated oligosaccharides and glycosphingolipids (decamicrogram amounts) can give valuable information on the primary structure. The fragmentation patterns are similar to those obtained by e.i.-m.s. and the spectra also give information on the molecular weight. Furthermore, the f.a.b. technique is not dependent on volatility of the compound and this allows analysis of high-molecular-weight glycoconjugates.

The fragmentation of the periodate-oxidised products is similar to that of the intact compounds. The primary fragments derived from cleavage of the oxidised residues show about the same abundance as those of the unoxidised residues. For a 2- or 4-substituted oxidised Hex residue, 208 m.u. are added to the sequence ions (Fig. 1). A secondary fragment, formed by elimination of methanol, discriminates between these residues. The e.i. mass spectra of methylated methyl glycopyranosides[9] show that the oxonium ion formed after a primary cleavage of the glycosidic bond preferentially loses MeO-3 and H-2 to form a 2,3-unsaturated oxonium ion. If this rule is applied to f.a.b. mass spectra of oxidised and methylated hexosyl residues, the secondary fragment for a 2-substituted residue should have the structure shown in Fig. 11. Such an ion cannot be formed for an oxidised 4-substituted hexosyl residue.

Several intense ions in the spectra presented have not been explained, but they add no further information to the sequence or the binding positions. However, further studies of the detailed fragmentation of the different oxidised residues will be reported elsewhere.

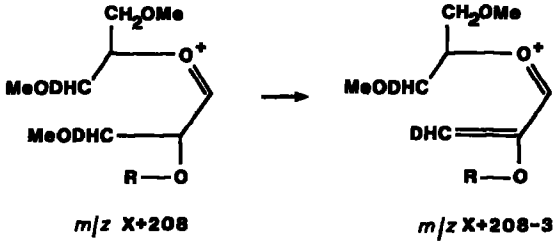


Fig. 11. Primary and secondary fragments formed from a periodate-oxidised, borodeuteride-reduced, and methylated 2-substituted Hex residue.

The method reported here is useful for oligosaccharides and glycosphingolipids containing sialic acid. Sialic acid, when attached to D-galactose, is either (2→3)- or (2→6)-linked and the primary sequence ions using this method would be *m/z* 493 and 453, respectively (Figs. 7 and 10). Derivatives of compounds containing sialic acid linked to sialic acid cannot be analysed readily by conventional methylation analysis. However, the f.a.b. mass spectrum of the methylated compound gives the monosaccharide sequence, and the disialyl linkage is determined from that of the oxidised and methylated material (Figs. 8 and 10). The method will also be helpful in locating such non-carbohydrate substituents as acetyl, sulfate, phosphate, and acetal groups. The completeness of periodate oxidation was established by methylation analyses.

F.a.b. mass spectra of methylated glycosphingolipids show only sequence ions from the outer residues, and the number of monosaccharides has to be calculated from the molecular weight. However, the molecular weight can sometimes be difficult to determine due to the variety of combinations of sphingosine bases and fatty acids. The molecular weights will therefore be divided up among several ions. Furthermore, the fragmentations of the ceramide moieties are poorly understood. The problems are also the same for oxidised glycosphingolipids. Oxidation of the sphingosine base can take place if vicinal hydroxyl groups are present, which can generate several additional components. The glycosphingolipids used in this study showed the 4-substituted inner D-glucosyl residue to be resistant to periodate, possibly due to steric hindrance by the ceramide moiety and micelle formation.

However, there are still some questions which cannot be answered by this method; for example, the positions of substitution of branched hexosyl residues require additional data from chemical analyses and n.m.r. spectrometry.

ACKNOWLEDGMENTS

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