DETERMINATION OF GLYCOSPHINGOLIPID STRUCTURES BY MASS SPECTROMETRY[†]

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Summary. Mass spectrometry is useful for the determination of oligosaccharide structures in complex glycosphingolipids. A partial mass spectrum (scanned from m/e 60 to m/e 800) of fully trimethylsilylated intact glycosphingolipid yields information regarding the positions of substitution of monosaccharide units, the nature of sphingolipid bases, and the relative amounts of constituent fatty acids.

A major difficulty in structural studies of glycosphingolipids and glycoproteins on the microscale is in the determination of the positions of glycosidic linkages and the arrangement of sugar moieties in the oligosaccharide chain. Extensive use has been made of techniques such as combined permethylation and gas chromatography (1 - 4), periodate oxidation coupled with colorimetric analysis (5) or gas chromatography (6), and specific glycosidases (7). These methods are somewhat tedious, depend on careful standardization, and require relatively large amounts of material.

A recent study on the mass spectrometry of trimethylsilyl (TMSi) derivatives of monosaccharides (8) showed the effect of various substituents on the fragmentation patterns of glucose (Glu), galactose (Gal) and N-acetylgalactosamine (GalNAc) and suggested that it might be possible to deduce the position of glycosidic linkages in an oligosaccharide unit from mass spectral data. However, since it was not possible to distinguish aldohexoses by mass spectrometry, it would be necessary to establish the

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composition of the oligosaccharide by an independent method such as gas chromatography (9 - 11). Other information might also be obtained by direct mass spectrometry of a glycosphingolipid. It has been shown, for example, that the trimethylsilyl derivatives of ceramides are sufficiently volatile for gas chromatography and can be identified by mass spectrometry (12,13).

In the present preliminary study an examination was made of the mass spectra of a number of glycosphingolipids as TMSi derivatives, ranging in molecular weight from approximately 1100 (TMSi glucosyl ceramide) to approximately 2800 (TMSi monosialoganglioside, G_{m1}). A summary is given of the mass spectral ions that are important for the identification of sphingosine bases, fatty acids, the number of monosaccharide residues, and the position of substitution of neutral and acetamido hexoses.

Materials and Methods.

All of the glycosphingolipids were isolated and purified in this laboratory with the exception of Tay-Sachs (G_{m2}) and monosialo (G_{m1}) gangliosides, which were generous gifts from Saul Roseman. The instrument used for these direct probe mass spectral analyses was the LKB Model 9000 mass spectrometer, operated at 3500 volts and 70 eV electron energy, 60 μamp electron current and an ion source temperature of 290°.

Bis-trimethylsilyltrifluoroacetamide (BSTFA) was obtained from Applied Science (State College, Pa.) and was used within 6 hr after opening a small vial. A mixture of this reagent (100 μl) and pyridine (50 μl) was added to a small amount (from 20 to 200 μg) of purified glycosphingolipid in a small capped vial and the solution was heated at 60° for about 30 minutes. An aliquot containing 10 to 20 μg of the trimethylsilyl glycosphingolipid was evaporated to dryness, under nitrogen, in a probe tube and the samples were volatilized in the ion source at temperatures ranging from 100° to 180°, depending on the complexity of the oligosaccharide unit.

Monosialoganglioside (1 mg) was incubated with commercial neuraminidase from influenza virus (General Biochemicals, Chagrin Falls, Ohio) at pH 5.0

for 48 hr at 37°, after which 5 ml of chloroform-methanol (2:1) was added to stop the reaction and partition the products. Asialoganglioside was isolated from the lower phase and was converted to the trimethylsilyl derivative as described above.

Results and Discussion.

Mass spectra of the trimethylsilyl derivatives of glucosyl ceramide (GL-1), lactosyl ceramide (GL-2), galactosylgalactosylglucosyl ceramide (GL-3), globoside (GL-4) (Fig. 1), monosialoganglioside (G_{m1}) (Fig. 2), asialoganglioside and Tay-Sachs ganglioside (G_{m2}) were examined. Although the mass range for these spectra extended beyond m/e 1500 (upper limit of the instrument), all significant ions for structural deductions were found below m/e 850. The relative intensities of fragment ions characteristic of monosaccharides (8) and N-acyl sphingosines (12 - 14) are given in Table 1. A ratio of the relative intensities of m/e 204 $[TMSiOCH\text{-}CHOTMSi]^+$ from the hexose moieties and m/e 311 $[CH_3(CH_2)_{12}CH{=}CH\text{-}CHOTMSi]^+$ from sphingosine can be correlated with the number of neutral aldohexose residues in the glycolipid, provided they are not substituted at C-3. Thus, the ratio of intensities of these ions is about 2 for GL-1, for GL-2 it is about 4 and for GL-3 it is about 6. In the mass spectrum of the GL-1 derivative m/e 361 was the most intense ion; it was derived by loss of trimethylsilanol (90 amu) from m/e 451 which is a major ion from the hexose moiety.

In mass spectra of trimethylsilyl acetamido sugars an ion from C-2 and C-3 is always observed at m/e 173 (8). This ion is analogous to m/e 204 from glucose and galactose and can be used to detect the presence of N-acetylhexosamine unsubstituted at C-3. The significant peak at m/e 173 in the mass spectrum of trimethylsilyl globoside (Fig. 1) confirms the presence of an N-acetylhexosamine unit that is not substituted on C-3. The ratio of about 4 found for the intensities of m/e 204 to m/e 311 corresponded to that for a dihexosyl ceramide, suggesting that the formation of m/e 204 from one of the three hexose units in globoside is blocked by glycosidic

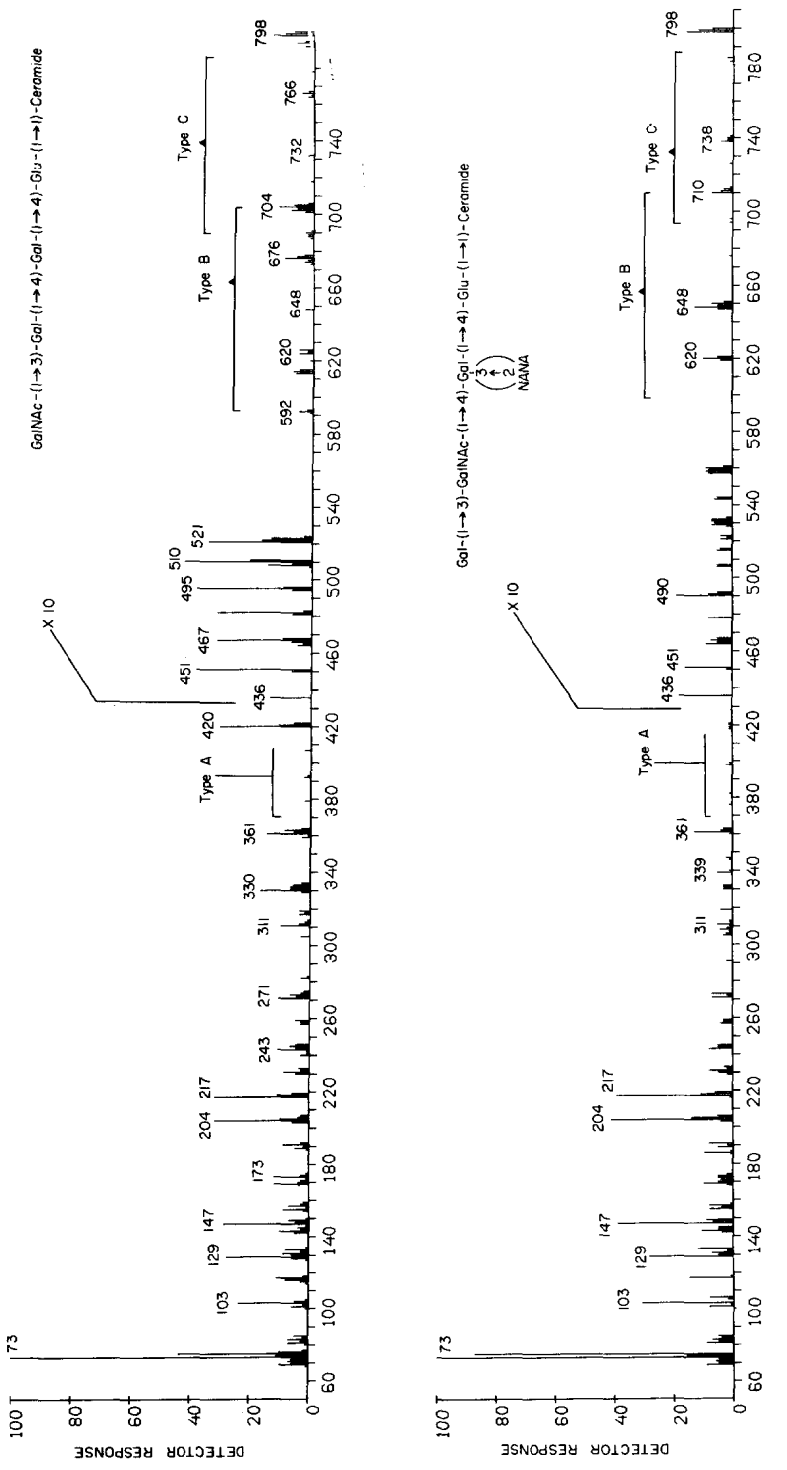

FIG. 1. Partial mass spectrum of trimethylsilyl globoside.
FIG. 2. Partial mass spectrum of trimethylsilyl monosialoganglioside (G_{m1}).

TABLE 1

Relative intensities of ions in mass spectra of trimethylsilyl glycosphingolipids.

m/e	Glycosphingolipid						
	GL-1	GL-2	GL-3	GL-4	G_{m1}	ASG_{m1}	G_{m2}
169	24.8	20.8	16.9	36.1	24.7	10.9	54.2
173	2.4	1.4	1.9	35.1	10.3	2.9	30.9
204	100	100	100	100	100	100	100
205	26.8	27.2	23.2	30.9	35.3	23.6	65.2
217	101	52.1	45.6	98.7	98.0	44.6	63.2
243	18.6	17.2	14.8	33.2	20.6	5.8	59.4
271	27.3	17.8	17.0	36.5	23.7	7.2	41.3
308	0.2	0.2	0.3	0.9	6.7	8.9	19.3
311	56.2	25.0	15.0	27.4	12.8	6.7	109
339	0.1	0.2	0.1	0.1	12.8	6.9	14.2
361	271	80.0	53.8	47.3	30.8	16.8	20.6
420	0.6	0.5	0.6	90.0	1.7	1.9	34.2
451	10.7	10.9	12.0	9.5	3.4	2.8	3.2
490	5.6	0.1	0.2	0.3	5.1	8.7	1.9
204/311*	1.8	4.0	6.6	3.7	4.0*	7.5*	0.9*

*Ratio obtained from sum of ions for bases when more than one present.

linkage of the N-acetylhexosamine to C-3 on that sugar. These results agree with the previously proposed structure for human globoside (15), containing the sequence GalNAc(1 → 3)Gal(1 → 4)Gal(1 → 4)-Glu. Asialoganglioside contains the same monosaccharides as globoside but the sequence is Gal(1 → 3)GalNAc(1 → 4)Gal(1 → 4)Glu. The ratio of m/e 204 to m/e 311 should

therefore be like that of trihexosyl ceramide since all three hexose units are unsubstituted on C-3, and m/e 173 should be absent since the N-acetylgalactosamine unit (GalNAc) is substituted on C-3. The results shown in Table I confirm this reasoning and the proposed method for interpretation of the mass spectral data. If the N-acetylgalactosamine had been substituted at another position such as C-2 or C-6 there would theoretically have been a much larger peak at m/e 173. Evidence for a terminal position of N-acetylhexosamine can be found in the presence of a strong ion at m/e 420 (analogous to m/e 451 in a terminal aldohexose), which was 90% of m/e 204 in the mass spectrum of the globoside derivative but was nearly absent (1.7%) in that of asialoganglioside where the aminosugar is not terminal.

The mass spectrum of trimethylsilyl G_{m1} is shown in Fig. 2. Since the internal galactose residue in this lipid is substituted by sialic acid (NANA) at C-3 there are only two hexose units that can form m/e 204 and the observed ratio of 4 for m/e 204 to m/e 311 was exactly the same as that of dihexosyl ceramide. In this case, in which the long-chain base consisted of a mixture of C_{18} and C_{20} sphingosine, it was necessary to compare m/e 204 with the sum of contributions from the two bases (m/e 311 and m/e 339). The peak at m/e 173 was much smaller than that for N-acetylhexosamine unsubstituted at C-3; its presence has been attributed to the formation of $[TMSiOCH\text{-}CHNHCOCH_3]^+$ from the sialic acid in G_{m1}. Although we have not made a confirming study, it should be possible to distinguish N-acetylneuraminic acid, in which this ion is located at m/e 173, and N-glycolylneuraminic acid, which would give an analogous ion $[TMSiOCH\text{-}CHNHCOCH_2OTMSi]^+$ at m/e 261.

Tay-Sachs ganglioside (G_{m2}) contains an oligosaccharide with the structure GalNAc (1 → 4)Gal(1 → 4)Glu substituted with a sialic acid residue at C-3 on the galactose unit (5). The mass spectrum should accordingly have an intense ion at m/e 173 for terminal N-acetylgalactosamine, and the ratio of m/e 204 to m/e 311 should correspond to that of a monohexosyl ceramide; the observed values given in Table I were in close agreement with theory.

More detailed studies are being made of these mass spectra to determine whether it will be possible to distinguish between 1 → 2 and 1 → 4 linkages and to evaluate the effect of C-6 substitutions.

Information about the mixture of sphingolipid bases in these glycosphingolipids was also obtained from the mass spectra. As previously observed with N-acetyl bases (14) and ceramides (12,13), an ion characteristic of each base $[RCHOTMSi]^+$ forms by cleavage between C-2 and C-3 in the base. The plasma glycosphingolipids (GL-1, GL-2, GL-3 and globoside) contained sphingosine (m/e 311) but there was no evidence for homologs (m/e 283, 297, 325, 339) or saturated analogs such as sphinganine (m/e 313). Phytosphingosine (4-hydroxy-sphinganine) was not found in plasma or spleen glycosphingolipids but dihexosyl ceramide from kidney showed a mass spectral ion at m/e 299 $[CH_3(CH_2)_{13}CHOTMSi]^+$ that would be formed from this base; this result agrees with the findings of Karlsson and Mårtensson on the distribution of phytosphingosine in mammalian sphingolipids (16). As expected, monosialoganglioside contained equal amounts of C_{18} (m/e 311) and C_{20} (m/e 339) sphingosine; in contrast, Tay-Sachs ganglioside contained much lower amounts of the C_{20} base (10-12%).

Fatty acids that occur in glycosphingolipids commonly range from C_{16} to C_{24}. In all the mass spectra studied three sets of fragment ions were observed, characterized by families of ion species separated by 14 or 28 mass units. Peaks in the first series (m/e 370 to 482) were rather small and corresponded to the fragment $[RCONH(TMSi)CHCH_2O]^+$ which is indicated in Figs. 1 and 2 as Type A fragments. More intense ions were obtained from Type B fragments that were useful in determinations of fatty acid compositions. The structure of Type B ions, occurring at m/e 592 to 704, is presumed to be $[CH_3(CH_2)_{12}CH{=}CH{-}CH(OTMSi)CH(NHCOR)CH_2]^+$. Since it contains both the base and fatty acid moieties, shifts will occur with bases other than C_{18} sphingosine. In G_{m1} ganglioside, for example, stearic acid constituted nearly 90% of the total fatty acid mixture; two strong peaks were observed at m/e 620 (C_{18} sphingosine and stearic acid) and m/e 648 (C_{20} sphingosine and stearic acid).

In G_{m2} from Tay-Sachs brain, C_{18} sphingosine predominates and the peak at m/e 620 was the only one of significance in the Type B series. Fragment ions of Type C occur in a region of the mass spectrum from m/e 710 to m/e 822 and are probably represented by the structure $[CH_3(CH_2)_{12}CH{=}CH{-}CH(OTMSi)CH(NHCOR)CH_2OCHOTMSi]^+$, which is Type B with an additional OCHOTMSi group (118 mass units) In glycolipids such as the digalactosylceramide isolated from human kidney the presence of α-hydroxy fatty acids is evidenced by the occurrence of an additional series (Type D) from m/e 680 (α-hydroxypalmitate) to m/e 792 (α-hydroxylignocerate), which is the Type B ions with an additional OTMSi group (88 mass units).

It was anticipated that glycosphingolipids containing such a wide range of fatty acid chain lengths might have considerably different vapor pressures and that the fatty acid composition calculated from mass spectral data might vary with time as temperature is increased in the probe tube. This was verified by comparing mass spectra recorded at several times. As shown in Fig. 3, the proportion of lignoceric acid (C_{24}) was lowest at the low temperature and increased continuously as the sample was evaporated at

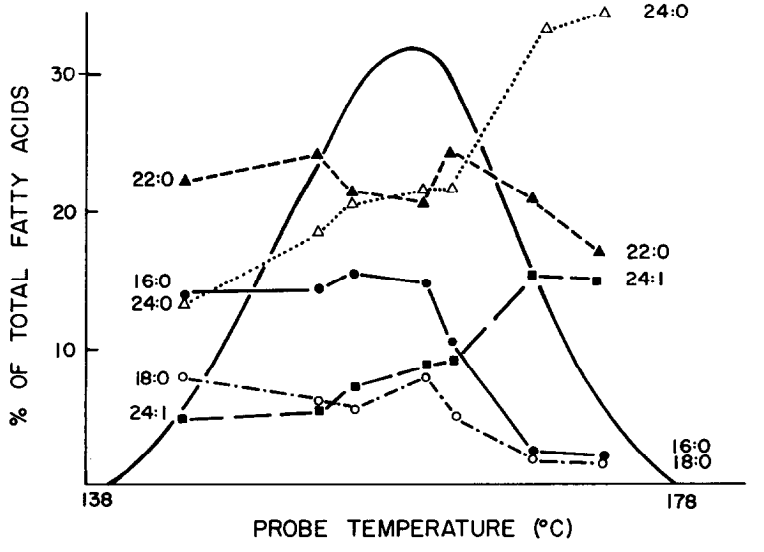

FIG. 3. Variation in fatty acid composition from mass spectra with probe temperature at which spectra were recorded.

higher temperatures, whereas the proportion of palmitic acid (C_{16}) dropped markedly at the higher temperatures. Compared with data obtained by gas chromatography of methyl esters from this lipid, the most accurate composition was that calculated from a mass spectrum recorded at the apex of the curve for total ion concentration (solid curve in Fig. 3).

Although the results are not as precise as those obtained by classical techniques, the sensitivity of this method for structural studies is such that analyses can be made of very small amounts of glycosphingolipids separated from complex mixtures by thin-layer chromatography.

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