Characterization of gangliosides by direct inlet chemical ionization mass spectrometry

Toshio Argia,[1,*] Robert K. Yu,[2,] and Tadashi Miyatake[***]**

Department of Biochemistry and Metabolism, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan,* Department of Neurology, Yale University School of Medicine, New Haven, CT 06510,** and Department of Neurology, Brain Research Institute, Niigata University, Niigata 951, Japan***

Abstract Permethylated derivatives of N-acetylneuraminic acid-containing G_{M3}, N-glycolylneuraminic acid-containing G_{M3}, G_{D1a}, and G_{D1b}, were analyzed by direct inlet ammonia chemical ionization (CI) mass spectrometry. These compounds were found to yield simple fragmentations, prominent fragment ions in the high mass region, and characteristic fragment ions corresponding to the cleavage of glycosidic linkages. This method also provides detailed information on the carbohydrate sequence and lipophilic composition in gangliosides.—**Ariga, T., R. K. Yu, and T. Miyatake.** Characterization of gangliosides by direct inlet chemical ionization mass spectrometry. *J. Lipid Res.* 1984. **25:** 1096–1101.

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Chemical ionization (CI) mass spectrometry, which is a type of soft-ionization mass spectrometry, is based on the ion-molecular reaction between sample molecules and reactant ions and has long been recognized as a powerful tool for the structural analysis of thermally labile compounds, including glycosphingolipids. In analyzing complex glycosphingolipids, the method has the advantage over conventional electron-impact ionization (EI) mass spectrometry in that it provides more information about their molecular weight, carbohydrate sequence, and lipophilic constituents (1–7). Both trimethylsilyloxy and permethylated derivatives of simple (3) and complex glycolipids, including gangliosides (4–7), have been subjected to CI–mass spectrometric analysis with success. Thus, in our initial attempt to analyze the completely methylated derivative of G_{M1} ganglioside by CI–mass spectrometry, we could demonstrate the presence of prominent molecular ions and characteristic fragment ions in the high mass region (5). This allowed us to deduce the structure of molecular species G_{M1} with a considerable degree of certainty. In contrast, the EI mass spectrum of the same compound failed to reveal the molecular ions, and the fragment ions in the high mass region were not sufficiently intense to be useful for structural analysis (8, 9). In order to generate algorithms for the structural analysis of various gangliosides by CI–mass spectrometry, we have recently analyzed several complex gangliosides with similar structures. The present paper describes the characterization of G_{M3}, which contains either N-acetyl- or N-glycolylneuraminic acid (NeuNAc or NeuNGc), G_{D1a} and G_{D1b}. The latter two gangliosides differ structurally in the position of the sialic acid moieties.

MATERIALS AND METHODS

G_{M3} gangliosides were isolated from bovine adrenal medulla by DEAE-Sephadex A-25 and Iatrobeads column chromatography as described previously (10). G_{D1a} and G_{D1b} were isolated from adult bovine brain in the same manner. Neutral sugars, sialic acids, fatty acids, and long-chain bases were analyzed by gas–liquid chromatography (GLC) as previously described (10). The sialic acid species were determined by the GLC method of Yu and Ledeen (11). Permethylation was done by the method of Ando et al. (6) with slight modifications (5). Purification of the permethylated gangliosides was achieved by preparative thin-layer chromatography with a solvent system of chloroform–methanol–n-hexane 40:10:20 (v/v/v) (5).

A Shimadzu-LKB gas chromatograph–mass spectrometer, 9000 A, equipped with a CI source was used. Ammonia was used as the reagent gas. The temperature of the ion source was kept at 220°C. The mass spectra were obtained at an electron energy of 500 eV, an emission current of 400 μA, and an accelerating voltage of 1.75 KV. Several microliters of a 0.5–1% sample

Abbreviations: CI, chemical ionization; EI, electron-impact ionization. Ganglioside nomenclature is based on the system of Svennerholm (22).

[1] Present address: Department of Neurology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

[2] To whom reprint requests should be addressed.

solution in chloroform were injected into a Pyrex direct probe glass cell (10 mm × 1 mm ID) and the solvent was evaporated at room temperature in a desiccator. The temperature of the direct probe inlet was programmed from 100° to 450°C at the rate of about 50°C/min. This temperature did not represent the temperature of the glass cell itself, which was placed 5 cm away from the heater, so that the actual temperature of the glass cell was probably 150°–160°C lower. The mass marker was calibrated by measuring tri(perfluorononyl)-S-triazine and ultra marker (PCR Research Chemicals, USA). We obtained about ten spectra using probe temperatures ranging from 270° to 330°C for each sample.

RESULTS AND DISCUSSION

Chemical ionization–mass spectrometry of hematosides

Ledeen, Salsman, and Cabrera (12) previously reported that bovine adrenal medulla contained hematoside G_{M3} (92% of total gangliosides) which contained both NeuNAc and NeuNGc in a ratio of 2:3. The most abundant fatty acid in these species was behenic acid (C22:0). Significant amounts of C16:0, C18:0, C23:0, C24:0, and C24:1 fatty acids were also present (12). The most prominent long-chain base was C18-sphingenine (13).

Fig. 1 and **Fig. 2** show the ammonia CI–mass spectra of the completely methylated G_{M3} gangliosides containing NeuNAc and NeuNGc, respectively. The fragmentation diagram of G_{M3} containing nervonic acid and C18-sphingenine is also shown in each of the two figures. In both mass spectra five ion groups are clearly discernible which arise from ceramide and ceramide-bearing fragments and are of diagnostic value in determining the structure of G_{M3} gangliosides. The prominent ion groups in the molecular ion region, $(MH)^+$ and $(MH - 32; MH - CH_3OH)^+$, show a difference in mass number of 30 between G_{M3} (NeuNAc) and G_{M3} (NeuNGc) because of the difference between the *N*-acetyl, *N*-methyl and *N*-(*O*-methyl-glycolyl), *N*-methyl groups of the sialic acid moieties. For NeuNAc-containing C_{M3} (Fig. 1), the protonated molecular ion groups ranging from m/z 1346 to 1462 and the ion groups produced by the loss of methanol ranging from m/z 1314 to 1430 $(MH - 32)^+$, correspond to G_{M3} (NeuNAc) molecular species containing fatty acids with chain lengths ranging from C16:0 to C24:0 and provide their molecular weight information. For NeuNGc-containing G_{M3} (Fig. 2), These

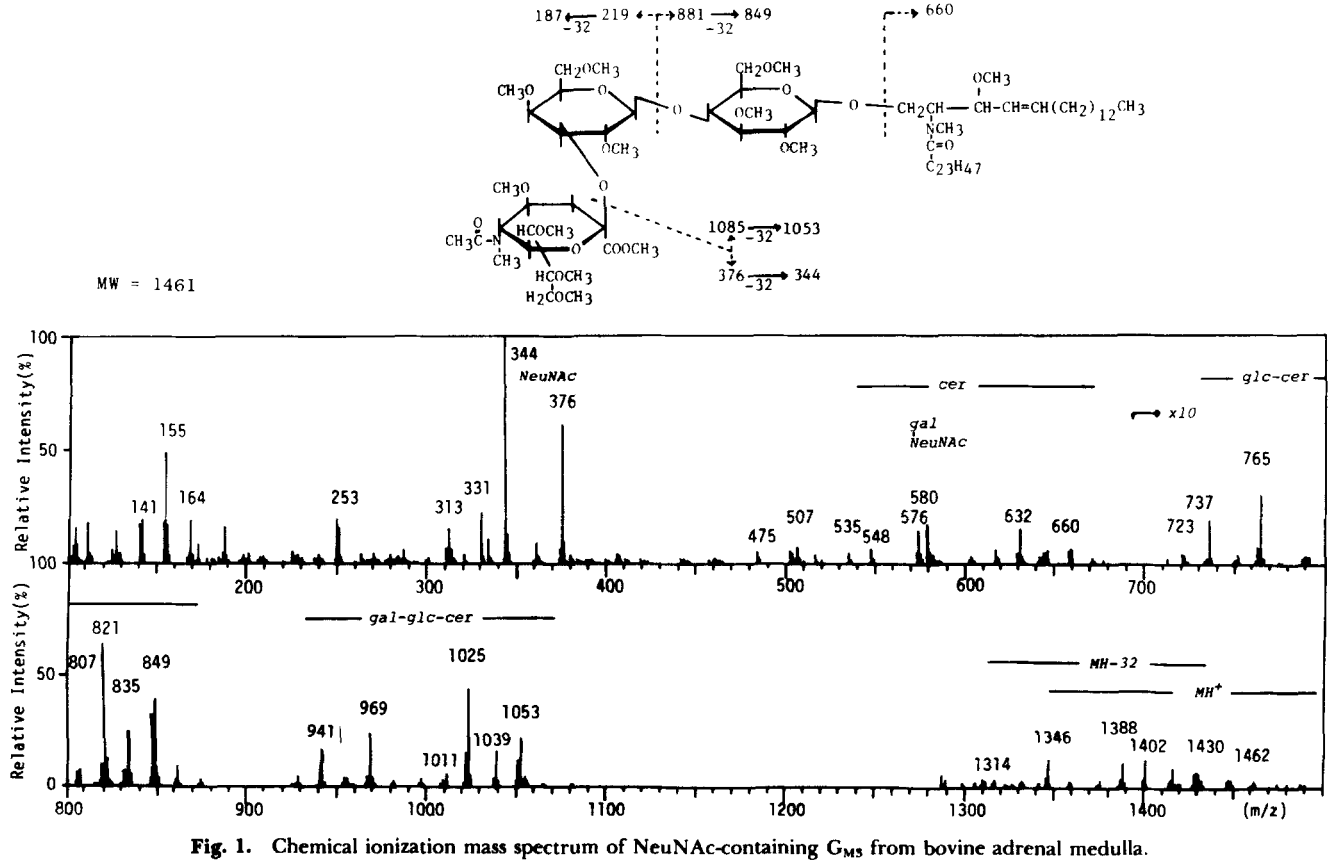

Fig. 1. Chemical ionization mass spectrum of NeuNAc-containing G_{M3} from bovine adrenal medulla.

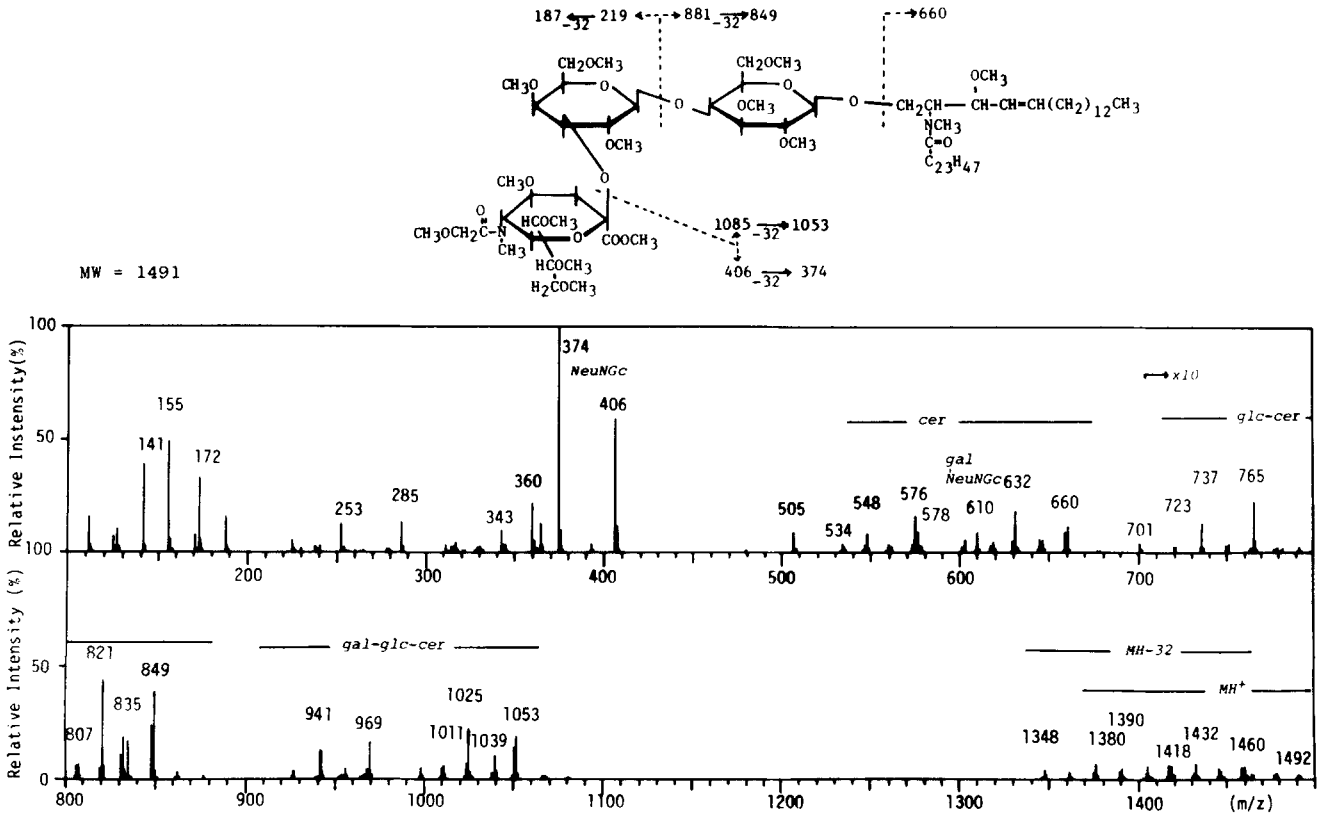

Fig. 2. Chemical ionization mass spectrum of NeuNGc-containing G_{M3} from bovine adrenal medulla.

ion groups are shifted by 30 mass units from these observed in G_{M3} (NeuNAc). In these spectra, the ions m/z 1402 and 1432 are for $(MH - 32)^+$ of G_{M3} (NeuNAc) and G_{M3} (NeuNGc), respectively, which contains C22:0 fatty acid. Other ion groups, which arise from ceramide, glucosylceramides, and lactosylceramide moieties, are nearly identical for G_{M3} (NeuNAc) and G_{M3} (NeuNGc). In both mass spectra, the ion groups ranging from m/z 548 to 660, m/z 737 to 849, and m/z 941 to 1053 are for ceramide, glucosylceramide, and lactosylceramide moieties, respectively. In each of these groups, molecular species containing fatty acids with chain lengths ranging from C16:0 to C24:0 can be detected and quantitatively estimated. We have previously reported that direct inlet CI–mass spectrometry can provide quantitative information on molecular species of cerebroside (3). Thus, the three groups of ions should provide the fatty acid compositional data for G_{M3}. For example, the major ions at m/z 548, 576, 632, and 660 correspond to ceramide with C16:0, C18:0, C22:0, and C24:0 fatty acids, respectively. **Table 1** shows the relative intensities of various ions used to estimate the fatty acid composition in each ion group. The fatty acid composition of the parent hematosides as determined by GLC is also shown for comparison. It is clear that the CI–mass spectrometric method and

TABLE 1. Percent distribution of fatty acids of hematosides from bovine adrenal medulla

	Mass Spectrometry				GLC	
Fatty acids	Cer	Glc-Cer	Lac-Cer	Average[a]	This Study	Reference 13
G_{M3} (NeuNAc)						
16:0	9.7	7.1	9.0	8.6 ± 1.3	10.0	14.4
18:1	1.8	1.6	2.5	2.0 ± 0.5	2.8	2.4
18:0	21.9	15.5	15.9	17.8 ± 3.6	17.2	15.9
20:0	8.9	5.2	5.1	6.4 ± 2.2	3.8	4.4
21:0	4.1	2.5	2.5	3.0 ± 0.9	1.2	
22:1	1.0	0.8	2.5	1.4 ± 0.9	0.3	
22:0	26.0	26.2	29.2	27.1 ± 1.8	27.6	28.9
23:0	5.9	11.4	10.8	9.4 ± 3.0	8.4	5.6
24:1	8.9	13.7	8.2	10.3 ± 3.0	13.8	12.0
24:0	11.8	16.6	14.1	14.2 ± 2.4	14.7	12.8
G_{M3} (NeuNGc)						
16:0	8.8	6.5	9.8	8.4 ± 1.7	11.1	16.1
18:1	3.1	2.2	1.5	2.3 ± 0.8	2.5	2.3
18:0	18.9	12.7	15.6	15.7 ± 3.1	13.5	12.3
20:0	5.7	4.7	5.1	5.2 ± 0.5	3.6	3.3
21:0	4.4	3.2	5.5	4.4 ± 1.2	1.0	0.4
22:1	2.2	2.4	1.1	1.9 ± 0.7	0.4	trace
22:0	21.6	24.6	20.4	22.2 ± 2.3	24.8	22.6
23:0	7.0	10.8	9.5	9.1 ± 1.9	10.4	9.5
24:1	10.1	11.9	13.5	11.8 ± 1.7	15.4	15.6
24:0	18.1	21.1	18.2	19.1 ± 1.7	17.1	14.6

[a] Average ± SD (n = 3).

GLC method yield similar fatty acid compositional data, which agree well with those previously reported by Ledeen and Salsman (13).

In G_{M3} (NeuNAc), the NeuNAc residue gives rise to characteristic ions at m/z 376 and 344 (376 − 32; 376 − $CH_3OH)^+$ with the m/z 344 peak being the most abundant. The ion at m/z 580 indicates the disaccharide moiety galactose-NeuNAc (Fig. 1). In G_{M3} (NeuNGc), these ions are shifted by 30 mass units, namely m/z 406 and 374 for NeuNGc and m/z 610 for galactose-NeuNGc (Fig. 2).

Chemical ionization–mass spectrometry of G_{D1a} and G_{D1b}

G_{D1a} and G_{D1b} were isolated from adult bovine brain; they contain about equal amounts of C18- and C20-sphingenine, and less than 5% of other bases. The prominent fatty acid is stearic acid (about 90%) (14, 15). Therefore, the most prominent ceramide-bearing ion species originating from these gangliosides should contain C18- and C20-sphingenine.

Fig. 3 and **Fig. 4** show the ammonia CI–mass spectra and the fragmentation diagrams of permethylated, C20-sphingenine containing G_{D1a} and G_{D1b}, respectively. Ions in the molecular ion region were not detected, because of the limit of mass numbers of this instrument. However, the fragment ions produced by the loss of a sialic acid molecule from the protonated molecular ions are recorded at m/z 1839 and 1807 (1839 − 32; 1839 − $CH_3OH)^+$, which represent a monosialoganglio-N-tetraosylceramide structure containing C20-sphingenine. The ions corresponding to ceramide, glucosylceramide, lactosylceramide, and N-acetyl galactosaminyl lactosylceramide structures are recorded between m/z 576 and 604, m/z 765 and 793, m/z 954 and 982, and m/z 1199 and 1227, respectively, which indicate that they all contain C18- and C20-sphingenine and provide information on their molecular weights. These fragment ions were also observed for the CI–mass spectra of permethylated G_{M1} ganglioside (5). In both spectra, the fragment ions corresponding to the loss of two moles of sialic acid are quite different for G_{D1a} and G_{D1b}. In

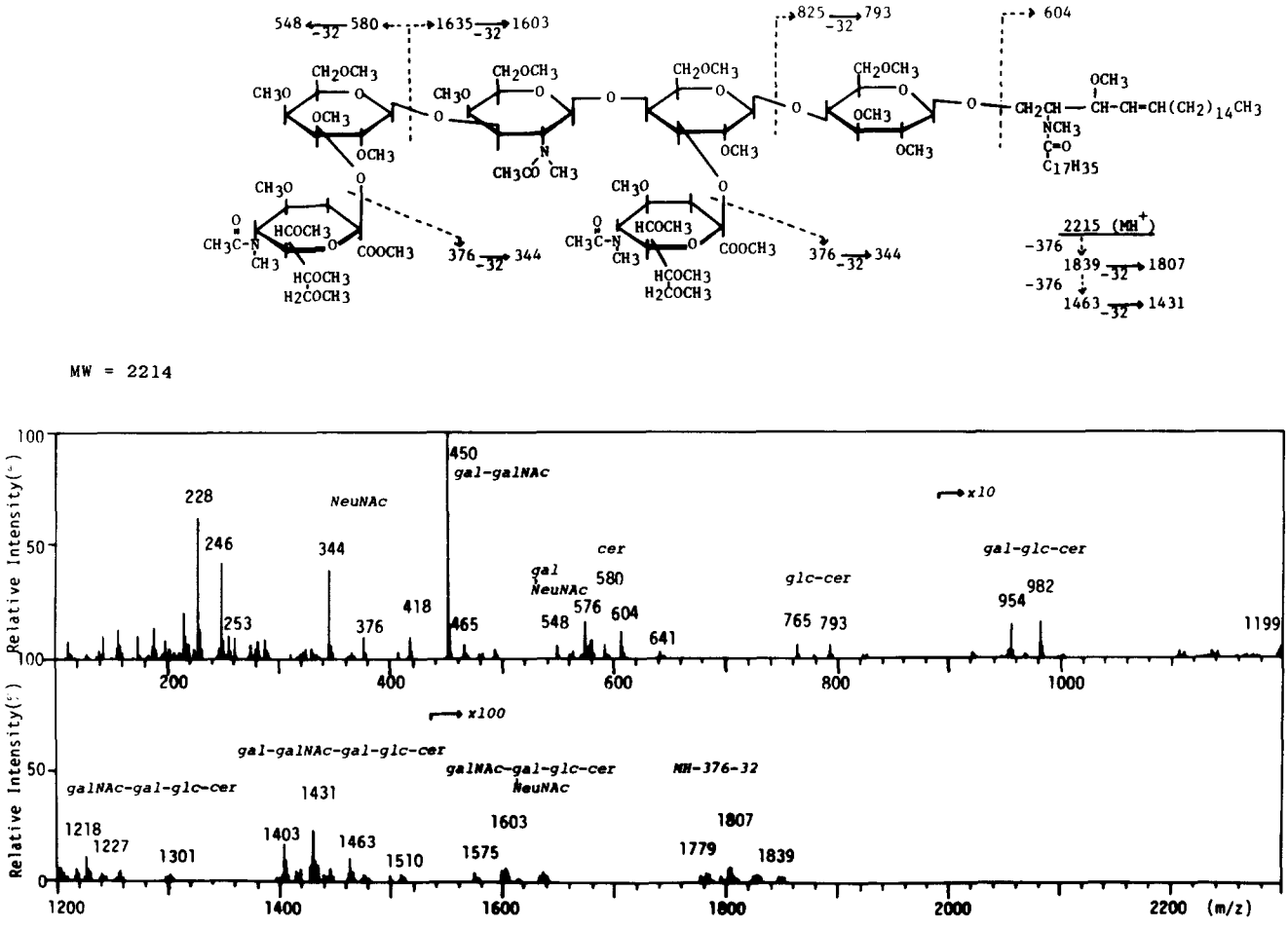

Fig. 3. Chemical ionization mass spectrum of G_{D1a} from bovine brain.

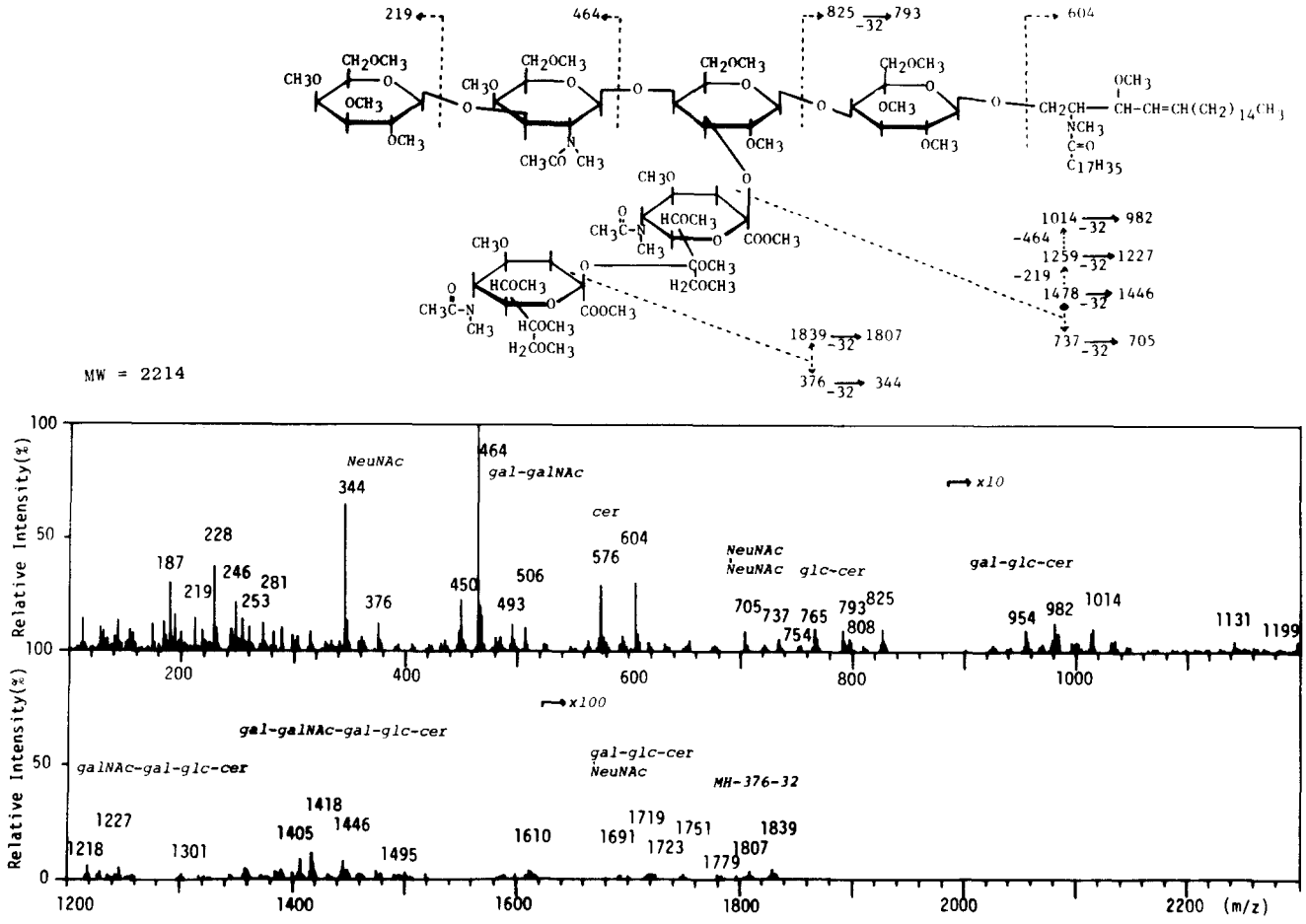

Fig. 4. Chemical ionization mass spectrum of G_{D1b} from bovine brain.

G_{D1b}, these ions are recorded at m/z 1418 and 1446, which represent a ganglio-N-tetraosylceramide structure containing C18- and C20-sphingenine, respectively. These ions have also been observed in the spectra of permethylated G_{M1} ganglioside. This finding suggests that the two sialic acids in G_{D1b} are sequentially linked to one of the two galactose moieties in the molecule. In G_{D1a}, these ions are recorded at m/z 1403 and 1431, which indicates a ganglio-N-tetraosylceramide structure containing C18- and C20-sphingenine, with the two sialic acids linked to two different galactose molecules. This finding is confirmed by the presence of the fragment ions having only monosialosyl residues. In G_{D1b}, the fragment ion at m/z 705 corresponds characteristically to a disialosyl moiety. In the case of G_{D1a}, the fragment ion at m/z 548 corresponds to a sialosylgalactosyl residue. The most striking difference between the two spectra is the fragmet ion corresponding to the galactosyl-N-acetylgalactosaminyl residue. This ion appears at m/z 450 in G_{D1a} and m/z 464 in G_{D1b}. The ions m/z 376 and 344 (376 − 32; 376 − $CH_3OH)^+$ are due to N-acetyl neuraminic acid (Figs. 3 and 4). The ion at m/z 288 is probably derived from N-acetylgalactosamine (16).

Recently, fast atom bombardment (FAB) and field desorption (FD) mass spectrometry have been applied to the structural studies of glycolipids, including gangliosides, and have been successfully used to determine their molecular weights (17–21). These methods have proved to be useful for structural studies of macromolecules without sample derivatization. However, in FAB and FD spectra the adduct ions, such as $(M + Na)^+$, $(M + matrix)^+$, and $(fragmentations + Na)^+$, are often observed. These adduct ions may complicate the interpretation of the molecular weights of glycolipid molecular species from natural sources (19, 20). Hence, the ability of CI–mass spectrometry to simultaneously provide detailed structural information on the oligosaccharide and lipophilic constituents of gangliosides makes this procedure well suited for analyzing their structures.

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