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CHEMICAL-IONIZATION MASS SPECTRA OF THE PERMETHYLATED SIALO-OLIGOSACCHARIDES LIBERATED FROM GANGLIOSIDES

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ABSTRACT

Permethylated mono- and di-sialo-oligosaccharides liberated from several parent gangliosides have been examined by chemical-ionization mass spectrometry with ammonia as the reagent gas in order to elucidate their structures. Several major fragment-ions, in addition to both the protonated and ammonium adduct molecular-ions, may be readily assigned without interference from the ceramide moiety. Sialic acid-containing di-, tri-, and tetra-saccharide ions can be clearly observed and used to determine the sugar residue to which the sialic acid residue is attached. The neutral-sugar skeletons produced by the loss of sialic acid give rise to both the protonated and the ammonium adduct ions; in the case of tetrasaccharides, these are further degraded to produce di- and tri-saccharide ions. These characteristic ions are useful for the determination of the number of sugar residues and their sequence in an oligosaccharide structure. The chemical-ionization mass spectra of G_{M3}- and G_{M1}-oligosaccharides with isobutane show the ions corresponding to each monosaccharide residue. These results indicate that chemical-ionization mass spectrometry is highly useful in determining the complete sugar-sequence of gangliosides.

INTRODUCTION

Mass spectrometry has been shown by many investigators[1–9] to be a powerful tool for the structural analysis of complex glycosphingolipids*. Gangliosides, a

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*Abbreviations: e.i., electron impact ionization; c.i., chemical ionization; Gal, galactose; GalNAc, 2-acetamido-2-deoxygalactose; Glc-ol, glucitol; NeuAc, sialic acid; *int*Gal, galactose internally located; *ext*Gal, galactose located externally or at non-reducing end. Abbreviations for gangliosides follow the nomenclature system of Svennerholm (see ref. 30): G_{M3}, (II^3NeuAc-LacCer); G_{M2}, (II^3NeuAc-$GgOse_3$Cer); G_{M1}, (II^3NeuAc-$GgOse_4$Cer); G_{D1a}, (IV^3NeuAc, II^3NeuAc-$GgOse_4$Cer); G_{D1b}, ($II^3(NeuAc)_2$-$GgOse_4$Cer).

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family of glycosphingolipids that contain one or more sialic acid residues, have previously been analyzed by mass spectrometry because of the great interest in their presumed biological function. They have been most extensively studied by mass spectrometry as their permethylated, trimethylsilylated, or peracetylated derivatives[10–13]. Although conventional electron-impact (e.i.) ionization gives useful structural information, it sometimes produces molecular ions and many fragment peaks of such low intensity that definite assignment of these peaks is often difficult[10–13]. Chemical-ionization (c.i.) mass spectrometry, however, alleviates these difficulties by minimizing the secondary ions and increasing the intensities of heavier ions because of its inherent milder ionization process than e.i. This advanced method has recently been successfully employed in the structural studies of a number of complex glycosphingolipids[14–19]. We have also tested the c.i. method with ammonia as the reagent gas and obtained a simple spectrum of permethylated G_{M1} ganglioside[20]. The c.i. mass spectrum shows a more prominent ion-peak correlating with the molecular weight than does the corresponding e.i. spectrum[10]. The c.i. method, however, seems inadequate for deducing the complete saccharide structure from the spectrum when the intact G_{M1} molecule is examined. No disaccharide ion containing sialic acid is detected; hence, the site of attachment for the sialic acid residue is not discernible. In addition, it is inherent even in the c.i. mass spectrometry of glycosphingolipids that spectra of the intact molecules always reveal complicated fragmentation patterns contributed by both the saccharide and ceramide portions.

To secure more-reliable and detailed structural information on the oligosaccharide components of gangliosides, we have examined the sialo-oligosaccharides derived from several species of gangliosides by ammonia c.i. and in part by isobutane c.i. mass spectrometry. We describe here the identification of mono- and disialo-oligosaccharides, including their positional isomers, to show the usefulness of the c.i. mass-spectrometric method.

EXPERIMENTAL

Preparation of oligosaccharides from gangliosides. — Sialo-oligosaccharides were liberated by ozonolysis[21] (average yield, 65–70%) from individual gangliosides isolated from human brain[22]. Asialo-oligosaccharides were obtained from asialo-G_{M1} and asialo-G_{M2}, which were prepared from $G_{M1}$and G_{M2} by hydrolysis with formic acid[23]. Briefly, each ganglioside fraction (~5 mg) was dissolved in 10 mL of methanol and ozone gas was slowly bubbled into the solution until an excess of ozone emerged. The solvent was evaporated and the residue dissolved in 5 mL of 0.2M sodium carbonate with the pH maintained at 10.5 or higher. After the addition of a small amount of sodium borohydride, the solution was kept overnight at room temperature. The sample was passed through a column of Dowex 50W (H^+ form) resin and the effluent partitioned with 1:1:0.9, (v/v) chloroform–methanol–water . The aqueous layer was lyophilized to give the sialo-

oligosaccharide whose glucose residue at the reducing end had been converted into glucitol. The purity of the various sialo-oligosaccharides was >95%, as estimated by densitometric scanning of the silica gel t.l.c. plates developed with 7:1:2 (v/v) 1-propanol–pyridine–water.

Permethylation of oligosaccharides. — A sample of reduced sialosyloligosaccharide (~100 μg) was placed in a Reactivial (1 mL, Pierce Chemical Co., Rockford, IL), and acetylated with 10 μL of 1:4 (v/v) acetic anhydride–pyridine for 60 min at room temperature. The reagent was evaporated off after the addition of a small amount of toluene under a stream of nitrogen. The residue was dried *in vacuo* over P_2O_5 and then dissolved with sonication in 10 μL of anhydrous *N,N*-dimethylformamide. Permethylation was carried out according to Imanari and Tamura[24]. A suspension (20 μL) of sodium hydride in *N,N*-dimethylformamide (20 mg/mL) was added to the sample solution at 0°, followed by 5 μL of methyl iodide at room temperature. After 30 min, an additional 5 μL of methyl iodide was added, and the reaction was continued for 3 h. The mixture was freed of methyl iodide by evaporation, and treated again with 15 μL of acetic anhydride–pyridine for 1 h. The mixture was diluted with 0.6 mL of chloroform, and washed 5 times with water. The chloroform was removed and the residue subjected again to the methylation procedure. The product was applied to a t.l.c. plate (silica gel 60, E. Merck, Darmstadt, West Germany), and the plate developed with 94:6 (v/v) chloroform–methanol. The permethylated sialo-oligosaccharide was obtained by extracting the corresponding band on the plate with 9:1 (v/v) chloroform–methanol. The purified derivative, dissolved in chloroform, was introduced into a mass spectrometer via a stainless-steel rod.

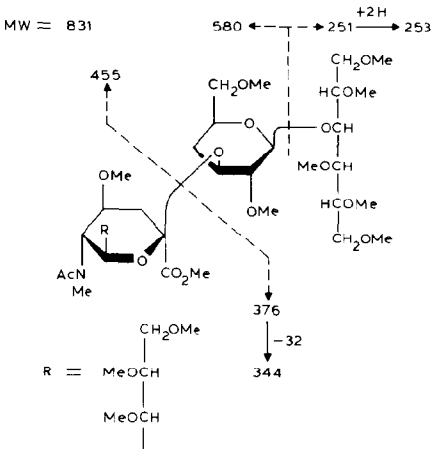
Mass spectrometry. — The sample (10–50 μg) was applied to a stainless-steel rod (10 × 2.5 mm, with a hemispheric top), which was attached to a direct-inlet probe. The probe temperature was programmed at 40°/min from 100 to 400°. Different temperatures were used for evaporation of different compounds as follows: 150–180° for G_{M3}-oligosaccharide, 280–310° for G_{M2}-oligosaccharide, 320–350° for G_{M1}-oligosaccharide, and 350–380° for G_{D1a}- and G_{D1b}-oligosaccharides. The mass spectrometer, a Shimadzu-LKB 9000B model equipped with a chemical-ionization source, was operated under the following conditions: acceleration voltage, 1750 or 3500 V; ionization energy, 300 eV; box current, 300 μA; ion-source temperature, 310°; resolution with c.i. mode, 1500 (10% valley); reagent gases, ammonia or isobutane. The flow rate of reagent gas into the ion source was adjusted by maximization of the intensity of the ion at *m/z* 18 $[NH_4]^+$ for ammonia c.i. or *m/z* 57 $[C_4H_9]^+$ for isobutane c.i. A mass marker was calibrated based on authentic spectra of perfluorotriazine derivatives and of perfluorokerosene.

RESULTS

G_{M3}-oligosaccharide (N-*acetylneuraminyllactose, II^{3}NeuAc-Lac*). — In the ammonia c.i. mass spectrum of permethylated G_{M3}-oligosaccharide (Fig. 1), the

ammonium adduct molecular ion, $[M + NH_4]^+$, at *m/z* 849 was the most intense peak. The fragment ions at *m/z* 457 $[455 + 2\,H]^+$ and *m/z* 474 $[455 + H + NH_4]^+$ arose from loss of sialic acid from the parent molecule. Specific ions for the sialic acid residue appeared[3,10–13,20] at *m/z* 344 $[376 - CH_3OH]^+$, 376, and 393 $[376 + OH]^+$. The ions at *m/z* 580 and 597 indicated the terminal Gal-NeuAc structure. As reported by Chizhov *et al.*[26], the glucitol moiety at the reducing end gave characteristic ion series at *m/z* 253 (AOH_2 ion according to Chizhov *et al.*), *m/z* 235 and 222.

G_{M2}-oligosaccharide (II³NeuAc-gangliotriaose). — Permethylated G_{M2}-oligosaccharide gave the protonated molecular ion, $[M + H]^+$, and the $[M + NH_4]$ ion at *m/z* 1077 and 1094, respectively, in its ammonia c.i. spectrum (Fig. 2). The presence of an *N*-acetylhexosamine residue at the non-reducing end was revealed[2,27,28] by a group of ions at *m/z* 260 (base peak), 246 $[260 - CH_2]^+$, and 228 $[260 - CH_3OH]^+$. Ions attributable to the gangliotriaose without sialic acid appeared at *m/z* 702 $[700 + 2H]^+$ and 719 $[700 + H + NH_4]^+$. The ions corresponding to $[M - GalNAc]^+$ and $[M - Glc\text{-}ol]^+$ were detected at *m/z* 817 $[816 + H]^+$ and 825, respectively. Ions at *m/z* 450 $[701 - Glc\text{-}ol + H]^+$ and 442 $[701 - GalNAc + H]^+$ were assigned to GalNAc-Gal and Gal-Glc-ol, respectively. An ion



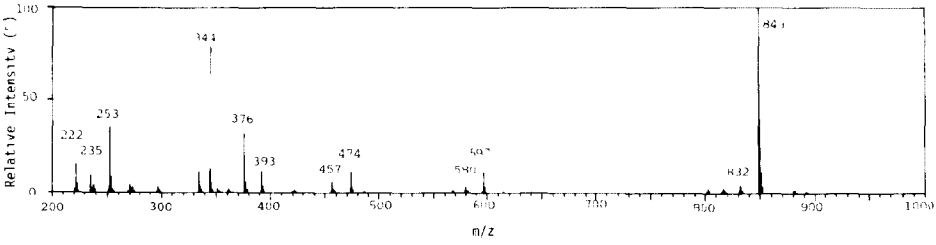


Fig. 1. Ammonia c.i. mass spectrum of permethylated G_{M3}-oligosaccharide (II^3NeuAc-Lac).

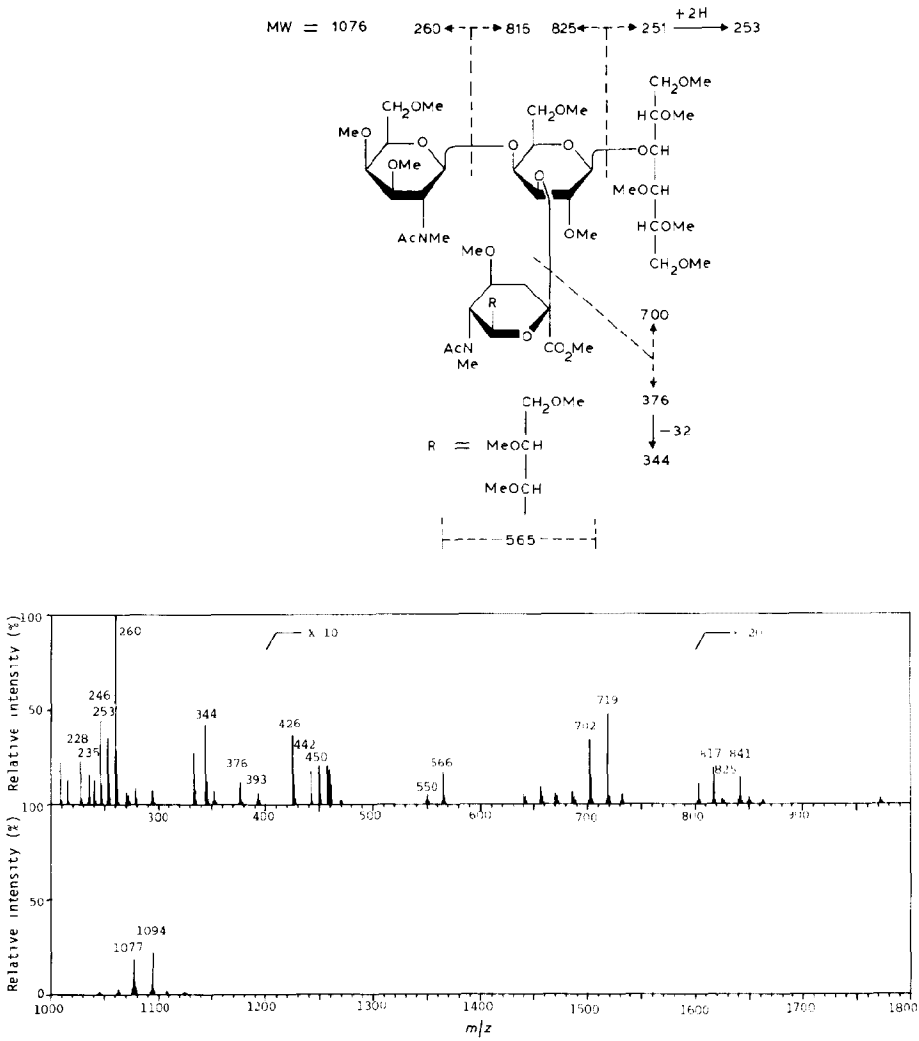


Fig. 2. Ammonia c.i. mass spectrum of permethylated G_{M2}-oligosaccharide (II^3NeuAc-gangliotriaose).

at *m/z* 566 $[565 + H]^+$ was attributable to the branching structure, sialosylgalactose. Ions corresponding to glucitol (*m/z* 253, 235, and 222) and *N*-acetylneuraminic acid (*m/z* 393, 376, and 344) were also observed.

G_{M1}-oligosaccharide (II^3NeuAc-gangliotetraose). — As shown in fig. 3, $[M + H]^+$ ion (*m/z* 1281) and $[M + NH_4]^+$ ion (*m/z* 1298) were both observed in the ammonia c.i. spectrum of permethylated G_{M1}-oligosaccharide. the ions corresponding to sialic acid-containing tetrasaccharides were recognized in the range of *m/z* 1015 and 1094. The fragments of (M − Glc-ol) and (M − *ext*Gal) were presumed to give the ion series of *m/z* 1029, 1044 $[1029 + 15]^+$, and 1061 $[1029 + 32]^+$, and the ion series of *m/z* 1062, 1077 $[1062 + 15]^+$, and 1094 $[1062 + 32]^+$,

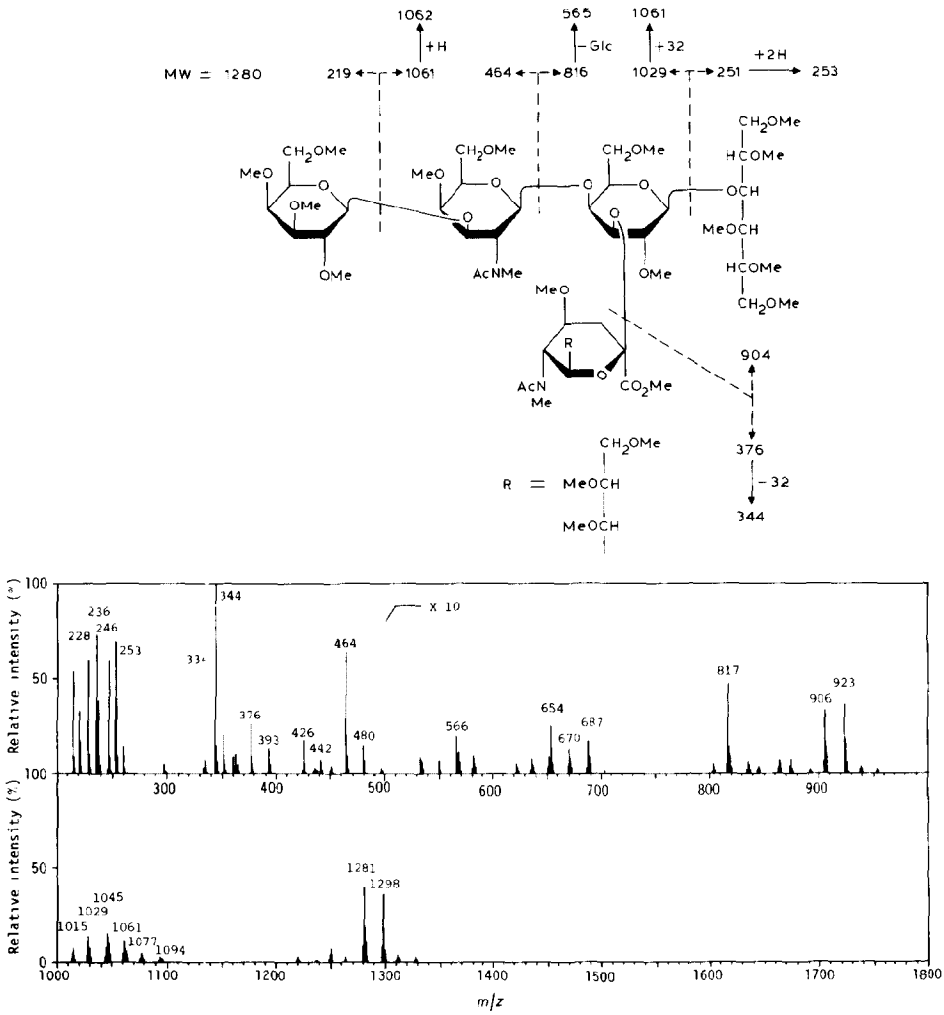


Fig. 3. Ammonia c.i. mass spectrum of permethylated G_{M1}-oligosaccharide (II^3NeuAc-gangliotetraose).

respectively. Intense ions corresponding to the sialic acid-containing di- and tri-saccharide were detected at *m/z* 566 (NeuAc-*int*Gal) and 817 (NeuAc-*int*Gal-Glc-ol), respectively.

The tetrasaccharide backbone without the sialic acid residue (gangliotetraose) appeared as ions at *m/z* 906 $[904 + 2H]^+$ and 923 $[904 + H + NH_4]^+$. The fragment ions at *m/z* 654 and 687 were assigned to trisaccharides produced by the loss of the terminal glucitol or galactose from the reduced gangliotetraose, respectively. A disaccharide ion derived from the non-reducing end, *ext*Gal-GalNAc, gave[3,20] a relatively intense peak at *m/z* 464, and another from the reducing end, *int*Gal-Glc-ol, gave a fragment ion at *m/z* 442.

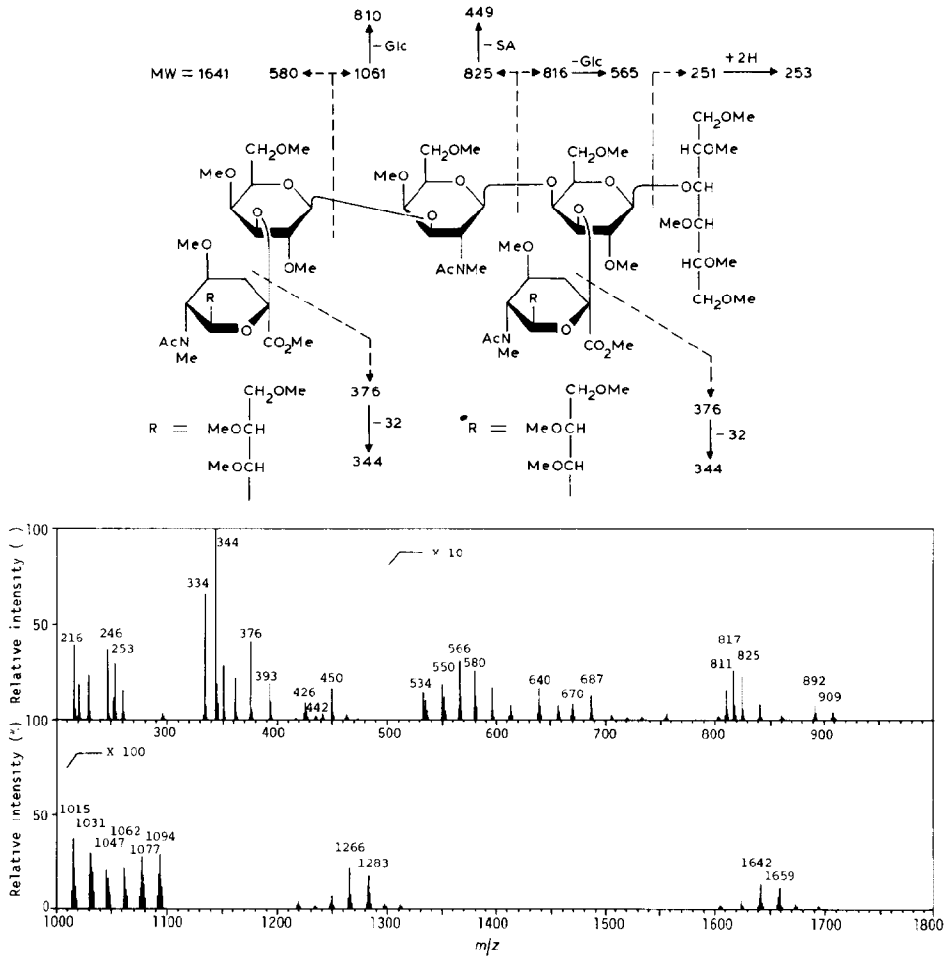
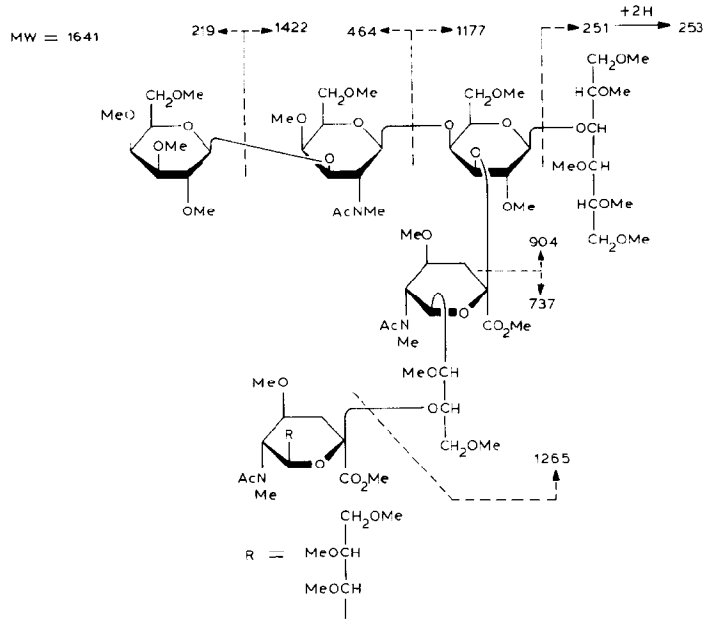


Fig. 4. Ammonia c.i. mass spectrum of permethylated G_{D1a} -oligosaccharide (IV^3NeuAc, II^3NeuAc-gangliotetraose).

G_{D1a}-oligosaccharide (IV^3NeuAc-gangliotetraose). — In the ammonia c.i. spectrum of permethylated G_{D1a}-oligosaccharide (Fig. 4), the $[M + H]^+$ and $[M + NH_4]^+$ ions were detected at *m/z* 1642 and 1659, respectively. Different kinds of fragment ions containing sialic acid arose from the disialosyl oligosaccharide. The ions at *m/z* 1266 $[1265 + H]^+$ and 1283 $[1265 + NH_4]^+$ were produced by the elimination of one mole of sialic acid from the molecular ion. A cluster of ions located between *m/z* 1015 and 1094 appeared to correspond to sialic acid-containing tetrasaccharides. Losses of glucitol and one mole of sialic acid gave the ion peaks at *m/z* 1015, 1030 $[1015 + 15]^+$, and 1047 $[1015 + 32]^+$. An ion series of *m/z* 1062, 1077, and 1094 arose from loss of the terminal sialosylgalactose. The fragment ions of sialic acid-containing trisaccharide appeared at *m/z* 825 (NeuAc-*ext*Gal), 817 $[816 + H]^+$ (NeuAc-*int*Gal-Glc-ol), and 811 $[810 + H]^+$ (GalNAc-*int*Gal-NeuAc).



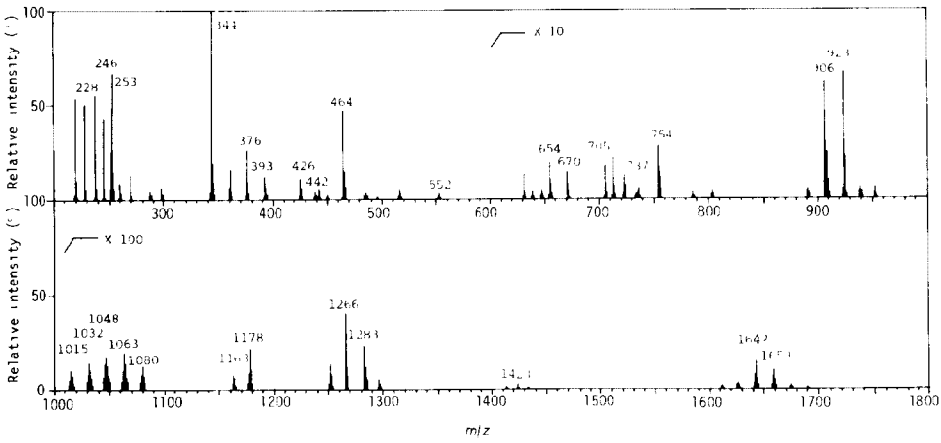


Fig. 5. Ammonia c.i. mass spectrum of permethylated G_{D1b} -oligosaccharide ($II^3(NeuAc)_2$-gangliotetraose).

The ions at *m/z* 580 and 566 indicated the presence of the terminal NeuAc-*ext*Gal and the internal NeuAc-*int*Gal structure, respectively.

The characteristic ions for the gangliotetraose structure without sialic acid occurred at *m/z* 892 $[889 + 3H]^+$, and 909 $[889 + 2H + NH_4]^+$. The ions corresponding to *ext*Gal-GalNAc-*int*Gal, GalNAc-*int*Gal-Glc-ol, *ext*Gal-GalNAc, and *int*Gal-Glc-ol were detected at *m/z* 640, 687, 450, and 442, respectively.

G_{D1b}-oligosaccharide ($II^3(NeuAc)_2$-gangliotetraose). — In the ammonia c.i.

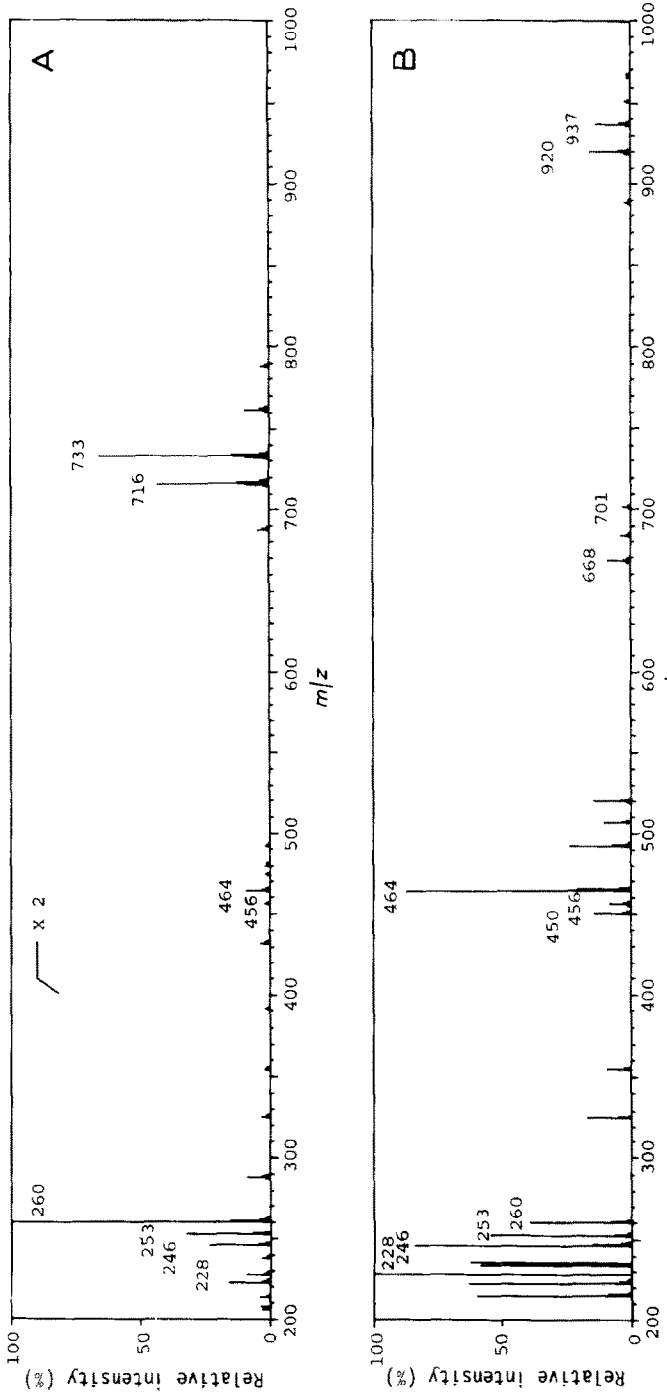


Fig. 6. Ammonia c.i. mass spectrum of permethylated asialo-G_{M2}- (A) and asialo-G_{M1}- oligosaccharide (B)

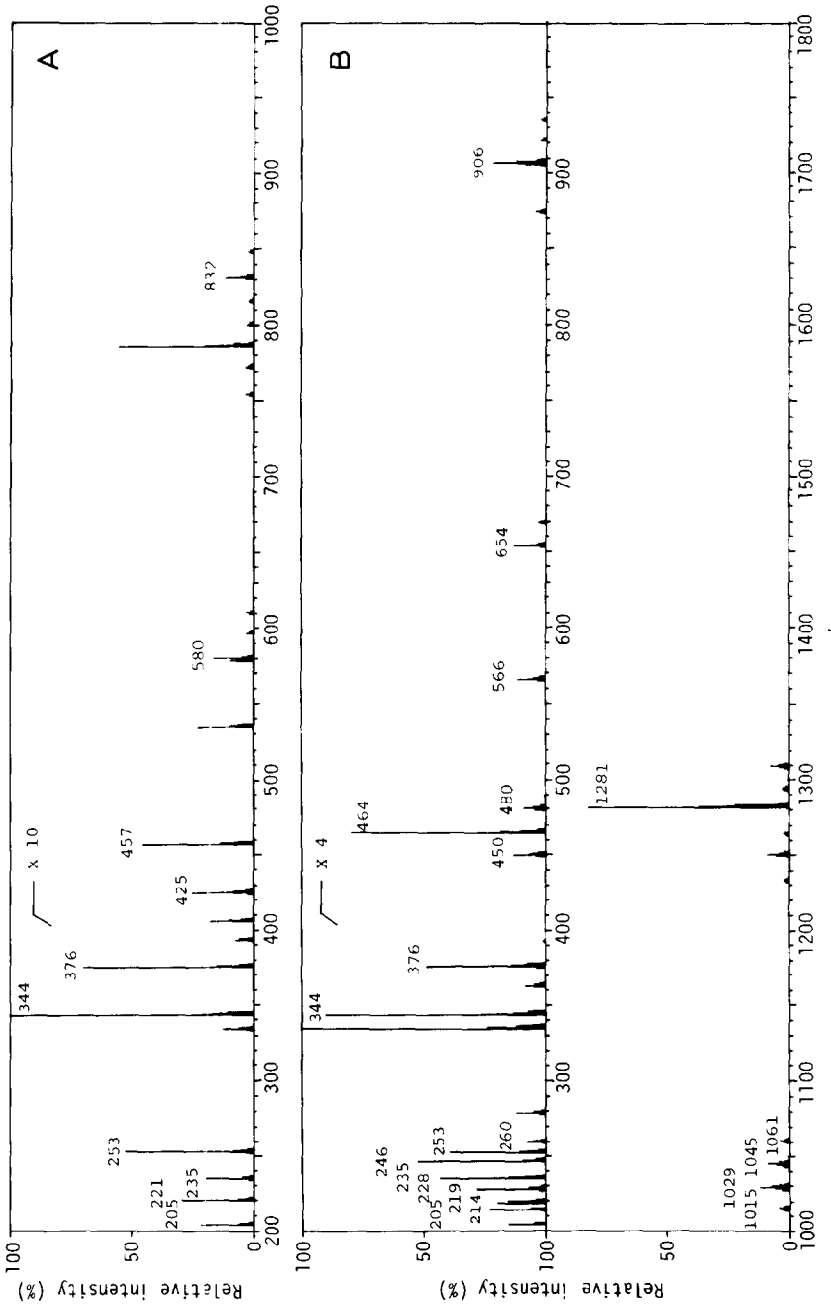


Fig. 7. Isobutane c.i. mass spectra of permethylated G_{M3}- (A) and G_{M1}-oligosaccharides (B).

mass spectrum of G_{D1b}-oligosaccharide (Fig. 5), the $[M + H]^+$ ion (*m/z* 1642), $[M + NH_4]^+$ ion (*m/z* 1659), and ions corresponding to [M − one sialic acid]$^+$ (*m/z* 1266 and 1283) were all observed. Ions representing the structure containing the sialyl-sialyl linkage that was characteristic for G_{D1b} were detected at *m/z* 1178 $[1177 + H]^+$, 1163 $[1177 - CH_2]^+$ (NeuAc-NeuAc-*int*Gal-Glc-ol), and *m/z* 754 $[737 + OH]^+$, 737, 705 $[737 - 32]^+$ (NeuAc-NeuAc). On the other hand, only minor ions corresponding to monosialosyl fragments were detected in the spectrum of G_{D1b}-oligosaccharide. In the mass range from *m/z* 1015–1080, which corresponded to the sialic acid-containing tetrasaccharide region as in G_{M1}- and G_{D1a}-oligosaccharides, ions at *m/z* 1048 $[1266 - ext\text{Gal} + H]^+$ and 1015 $[1266 - \text{Glc-ol}]^+$ appeared. A fragment ion of relatively low intensity corresponding to the monosialosylgalactose structure, NeuAc-*int*Gal, was detected at *m/z* 522.

Fragment ions derived from disaccharide (*m/z* 464 for *ext*Gal-GalNAc, 442 for *int*Gal-Glc-ol), trisaccharide (*m/z* 654 for *ext*Gal-GalNAc-*int*Gal), and tetrasaccharide lacking sialic acid (*m/z* 906 and 923) were the same as those observed in the spectrum of the G_{M1}-oligosaccharide.

Asialo-G_{M2}- and asialo-G_{M1}-oligosaccharide (gangliotriaose and ganglio-tetraose). — In the ammonia c.i. mass spectrum of asialo-G_{M2}-oligosaccharide (Fig. 6A), the $[M + H]^+$ and $[M + NH_4]^+$ ions were detected at *m/z* 716 and 733, respectively. Two different ion-peaks corresponding to disaccharide units were observed at *m/z*464 (GalNAc-Gal) and 456 (Gal-Glc-ol). Fragment ions attributable to nonreducing-end 2-acetamido-2-deoxygalactose appeared at *m/z* 260 (base peak), 246, and 228. The glucitol residue gave the ion at *m/z* 253.

Asialo-G_{M1}-oligosaccharide gave the $[M + H]^+$ and $[M + NH_4]^+$ ions at *m/z* 920 and 937, respectively (Fig. 6B). The loss of either terminal glucitol or galactose from the parent molecule produced trisaccharide ions at *m/z* 668 (*ext*Gal-GalNAc-*int*Gal) or 701 (GalNAc-*int*Gal-Glc-ol). Fragment ions corresponding to disaccharide components were detected at *m/z* 464 (*ext*Gal-GalNAc), 456 (*int*Gal-Glc-ol) and 450 (GalNAc-*int*Gal). The penultimate 2-acetamido-2-deoxygalactose residue yielded the base peak at *m/z* 228 and smaller ions at 246 and 260.

Isobutane c.i. mass spectrometry. — We have also examined G_{M3}- and G_{M1}-oligosaccharides by c.i. mass spectrometry employing isobutane as the reagent gas (Fig. 7A and 7B). The fragmentation patterns bear some resemblance to those obtained with ammonia gas. Notable differences, however, were observed; for instance, ions of NeuAc-*int*Gal-Glc-ol and GalNAc-*int*Gal-Glc-ol (*m/z* 817 and 687 in Fig. 3, respectively) were missing in the isobutane c.i. spectrum of G_{M1}-oligosaccharide (Fig. 7B). On the other hand, ion peaks corresponding to every monosaccharide unit were clearly detected in the isobutane c.i. mass spectra. The fragment ions at *m/z* 219 and 205 in Fig. 7B indicated the presence of nonreducing-end galactose and internally located galactose, respectively. Other components, 2-acetamido-2-deoxygalactose, glucitol, and sialic acid, gave the same ions as those that appeared in the ammonia c.i. spectra.

DISCUSSION

Hakomori's permethylation method for carbohydrates[25] has been widely and successfully applied to glycosphingolipids. However, we have noted that the sialo-oligosaccharides are not always converted into the permethylated derivatives in high yields by this method. The major reason is probably their poor solubility in dimethyl sulfoxide. We, therefore, used Imanari and Tamura's procedure[23], which employs *N,N*-dimethylformamide as the solvent. This procedure has been successfully used for the methylation of neutral oligosaccharides[16]. In the present study, Imanari and Tamura's procedure was performed twice to ensure complete methylation. To further enhance the solubilization of sialosyloligosaccharides in *N,N*-dimethylformamide, an acetylation step was performed prior to the methylation. *O*-Acetyl groups were replaced by *O*-methyl groups under the strongly basic conditions employed later.

In introducing samples directly into the mass spectrometer, a small, tubular glass sample-container is usually employed. In this study, we have used a simple stainless-steel rod on which the sample is coated. Samples having relatively high molecular weights may be more readily evaporated, giving spectra showing stronger intensities in the high-mass region than when samples are introduced by a glass sample-container. Another advantage is that samples in solution may be readily applied to the rod, as the solvent is rapidly evaporated.

Protonated molecular ions and ammonium adduct molecular ions are always observed with appreciable intensities in the sialo-oligosaccharides studied by the present method (Figs. 1–5). This pair of ions immediately give information on the molecular weights. Other major fragment-ions, which are produced by cleavage of glycosidic bonds rather than by ring opening[16,29], are readily assignable because of the lack of interference from the ceramide moieties. The ions corresponding to di-, tri- and tetra-saccharides containing sialic acids are almost always recorded as prominent peaks, and they provide key information on the site of attachment of sialic acid. This has not been achieved by others[10–13,20,27], and appears to be a major advantage in structural studies by mass spectrometry. The fragment ions corresponding to the sialosylgalactose residues appear in various sizes, depending on different types of substitution. The ion at *m/z* 580 (NeuAc-*ext*Gal) is formed from G_{M3}- and G_{D1a}-oligosaccharides. The internally located sialosylgalactose (NeuAc-*int*Gal) in G_{M2}-, G_{M1}-, and G_{D1a}-oligosaccharides appear as the ion at *m/z* 566. Another ion at *m/z* 552 corresponding to the fragment (-NeuAc-*int*Gal) is observed in the spectrum of G_{D1b}-oligosaccharide. The presence of a sialic acid residue linked to an internal galactose residue may be further confirmed by other ions, such as *m/z* 817 (NeuAc-*int*Gal-Glc-ol) in the spectra of G_{M2}-, G_{M1}-, and G_{D1a}-oligosaccharides, or *m/z* 1178 (NeuAc-NeuAc-*int*Gal-Glc-ol) in the spectrum of G_{D1b}-oligosaccharide. These sialic acid-containing fragment-ions may be used to identify such positional isomers as G_{D1a} and G_{D1b} with respect to sialic acid linkages (Figs. 4 and 5).

Fragment ions corresponding to the neutral-sugar skeletons that arise in the course of complete elimination of sialic acids are always detected as a pair of ions to which 2H or H + NH_4 is added. They are smaller than the non-substituted gangliotetraose by 14 mass units per one substitution of sialic acid, as follows: *m/z* 920 and 937 for asialo-G_{M1}-oligosaccharide; *m/z* 906 and 923 for G_{M1}- and G_{D1b}-oligosaccharides; and *m/z* 892 and 909 for G_{D1a}-oligosaccharide. Thus, the ions representing the neutral core are useful for the determination of the number of neutral sugar and sialic acid residues.

Two different kinds of trisaccharide fragment produced from the gangliotetraose structure by loss of glucitol or loss of galactose at the nonreducing end are revealed in the spectra of asialo-G_{M1}-oligosaccharide (*m/z* 668 and 701, respectively), G_{M1}-oligosaccharide (*m/z* 654 and 687), and G_{D1a}-oligosaccharide (*m/z* 640 and 687). The fragment ions corresponding to the disaccharides, *ext*Gal-GalNAc and *int*Gal-Glc-ol, can be derived from the gangliotetraose backbone. The former ion is recorded at *m/z* 464 for asialo-G_{M1}-, G_{M1}-, G_{D1b}-oligosaccharides and at *m/z* 450 for G_{D1a}-oligosaccharide. These di-and tri-saccharide ions are useful for determining the sugar sequence and for further confirmation of the number of substitutions.

Such monosaccharide ions as 2-acetamido-2-deoxygalactose (*m/z* 260, 246, and 228), sialic acid (*m/z* 344, 376, and 393), and glucitol (*m/z* 253 and 222) are detected with relatively high intensities. The ion at *m/z* 344 corresponds to *N*-acetylneuraminic acid, and no appreciable peak due to *N*-glycolylneuramic acid was detected. Ions arising from the internal galactose residue of gangliotetraose is not seen in any ammonia c.i. spectra of the samples tested.

We have tried isobutane as an alternative reagent gas to ammonia, and obtained somewhat different spectra with G_{M3}- and G_{M1}-oligosaccharides (Fig. 7A and 7B). The isobutane c.i. spectra clearly show the ions corresponding to every monosaccharide unit, whereas some ions observed in the ammonia c.i. spectra are missing. Therefore, ammonia c.i. and isobutane c.i. are complementary in structural elucidation of oligosaccharides.

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