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Carbohydrate Research 273 (1995) 27–40

CARBOHYDRATE RESEARCH

Structural elucidation of underivatized gangliosides by electrospray-ionization tandem mass spectrometry (ESIMS/MS)

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Received 21 November 1994; accepted 14 February 1995

Abstract

The structural characterization of intact polysialogangliosides by electrospray-ionization mass spectrometry (ESIMS) and tandem mass spectrometry (MS/MS) is described. The negative-ion ESI mass spectra of gangliosides [1], such as a tetrasialoganglioside GQ1b, showed molecularly related ions [2] with high sensitivity and accuracy. Collision-induced dissociation (CID) MS/MS provided adequate information to characterize the number and positions of sialic acids. ESIMS and CIDMS/MS techniques were applied successfully also in the positive-ion mode for characterization of intact gangliosides. Two isomeric disialogangliosides, GD1a and GD1b, are distinguishable from one another by CIDMS/MS in both negative- and positive-ion modes. The technique is readily applicable to structural analyses such as determining the positional isomers of glycoconjugates.

Keywords: Mass spectrometry, tandem, electrospray; ESIMS/MS; Gangliosides.

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[1] The nomenclature used for gangliosides follows the system of Svennerholm [L. Svennerholm, *J. Neurochem.*, 10 (1963) 613–623] and the recommendations of the IUPAC IUB commission [IUPAC-IUB Commission on Biochemical Nomenclature, *Lipids*, 12 (1977) 455–468].

[2] Molecularly related ions, i.e., those with H^+ or $metal^+$ species associated with the M, are sometimes referred to as "pseudomolecular ions" or "quasimolecular ions."

SSDI 0008-6215(95)00101-8

1. Introduction

Gangliosides, sialic acid(s)-containing glycosphingolipids, are located on the surface of animal cell membranes and are particularly abundant in nervous tissues [1,2]. They are often present in very small amounts, diversified in their structure, and play important roles individually, cooperatively or competitively among other biological molecules, functioning in important membrane-mediated processes in cell physiology [3,4]. Thus, structural characterization of these compounds, particularly of biologically active minor components, is important.

Mass spectrometry is one of the most useful methods for structural analysis. It is especially so if the information is complemented with NMR spectrometry or radio-immunoassays. However, when small sample size precludes NMR spectral analysis, mass spectrometry is the only practical means of analysis for glycolipids.

Fast atom bombardment mass spectrometry (FABMS) has made it possible to analyze underivatized, non-digested glycolipids as whole molecules. This technique, now considered to be invaluable for glycoconjugate biochemists, has been widely used. Methodologies in FABMS for glycoconjugates have been intensively studied for the past few years. Structurally more challenging glycoconjugates are now amenable to FABMS, and the information thus obtained is much more extensive so as to make it the method of choice [5–12]. However, FABMS analyses of both derivatized [5,6] and underivatized [7–12] gangliosides generally require microgram quantities of the samples, and the mass range is limited to several thousand daltons depending on the instrument used and information needed. Another problem of FABMS for samples that show molecular related ions with poor intensity is that fragment ions diagnostic of the structure in the low-mass region are often smeared by matrix ions, while the tandem mass spectrometry (MS/MS) technique cannot be applied to ions of low abundance in the molecular region.

Electrospray-ionization mass spectrometry (ESIMS) overcomes these problems to a great extent, and has been shown to be applicable to biological samples with high molecular weights [13], including intact gangliosides [14–17]. Huang and Henion demonstrated that negative-ion ESIMS was useful to characterize disialoganglioside GD1b [15] and oligosaccharides containing three sialic acids [16]. We have also reported [17] that the negative-ion ESIMS of disialoganglioside GD2 gives molecularly related ions with high sensitivity, and collision-induced dissociation (CID)MS/MS of the double-charged ion provides clear structural information.

In the ESI analyses, as is the case for soft ionization in general, acidic compounds such as carboxylates and sulfates, show higher sensitivity in the negative-ion mode because the acidic groups readily dissociate to lose proton(s). However, we have applied positive-ion ESIMS in addition to the more anticipated negative-ion mode to characterize structures of underivatized acidic glycolipids with up to four sialic acid or three sulfate groups (unpublished).

Derivatization, such as permethylation, with or without periodate oxidation and reduction, tremendously increases ion intensities and linkage or branching information, not only in FAB but also in ESI. However, when the sample size is in the submicrogram range, it is rather risky to run a few steps of derivatization reactions. Therefore, we have

Table 1
Gangliosides examined

Compound	R^1	R^2
GM1	–	Neu5Acα2
GD1a	Neu5Acα2	Neu5Acα2
GD1b	–	Neu5Acα2 → 8Neu5Acα2
GT1b	Neu5Acα2	Neu5Acα2 → 8Neu5Acα2
GQ1b	Neu5Acα2 → 8Neu5Acα2	Neu5Acα2 → 8Neu5Acα2

$$R^1 \rightarrow 3Gal\beta 1 \rightarrow 3GalNAc\beta 1 \begin{matrix} \searrow {}^{4}_{3}Gal\beta 1 \rightarrow 4Glc\beta 1 \rightarrow 1'Cer \\ R^2 \nearrow \end{matrix}$$

attempted to establish methodology for underivatized standard samples. These methods should allow one to analyze trace amounts of unknown samples from patient specimens.

Now we report characterization of gangliosides based on the negative- and positive-ion ESI and CIDMS/MS as the first report of the most efficient structure characterization of large, intact polysialogangliosides up to the tetrasialoganglioside, GQ1b, using ESIMS.

2. Experimental

Materials.—Gangliosides examined are listed in Table 1. GM1 and GT1b were purchased from Funakoshi Co. (Tokyo, Japan), GD1a and GD1b from Wako Pure Chemical Industries (Tokyo, Japan) and GQ1b from Iatron Co. (Tokyo, Japan). These commercial samples were prepared from bovine brain and used without further purification.

Mass spectrometry.—Mass spectra were recorded on a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (Analytica of Branford). At least twenty scans were averaged to obtain each spectrum. Samples were typically dissolved in methanol at a concentration of 10 pmol/μL and introduced into the electrospray needle by a mechanical infusion through a microsyringe at a flow rate of 1 μL/min. A potential difference of 3 keV was applied between the electrospray needle, which was kept at the ground potential, and the interior of the ion source. Hot nitrogen gas was used to evaporate the solvent from the charged droplets. CID-MS/MS spectra were taken using a 1.0–1.3 mtorr argon as the collision gas at 30–35 eV.

3. Results and discussion

Negative-ion ESI mass spectra of gangliosides.—The negative-ion ESI mass spectrum of a tetrasialoganglioside GQ1b is shown in Fig. 1 as an example. Figure 1 exhibits intense peaks in two distinctive regions, that is, from m/z 800 to 850 and from m/z 1200 to 1300, corresponding to the regions of triple-charged and double-charged ions, respectively. Ions at m/z 813, 818.5, 822.5 and 828 correspond to $[M_1 + Na - 4H]^{3-}$,

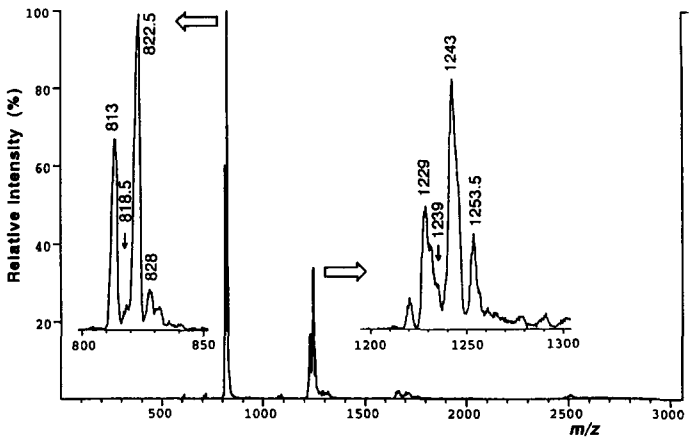

Fig. 1. Negative-ion ESI mass spectrum of GQ1b.

$[M_1 + K - 4H]^{3-}$, $[M_2 + Na - 4H]^{3-}$ and $[M_2 + K - 4H]^{3-}$ ions, respectively, where M_1 and M_2 represent the molecular mass of the free acids having sphingenine (d18:1) and icosashingenine (d20:1), respectively. Molecular masses are calculated as the exact masses throughout this article. On the other hand, ions at m/z 1229, 1239, 1243 and 1253.5 correspond to $[M_1 + 2Na - 4H]^{2-}$, $[M_1 + Na + K - 4H]^{2-}$, $[M_2 + 2Na - 4H]^{2-}$ and $[M_2 + Na + K - 4H]^{2-}$, respectively. No ions indicating dissociation of all four sialic acids were observed. The spectral features indicate that triple-charged ions are more abundant than double-charged ions for this tetrasialoganglioside, but that four negative charges within the limited space probably destroy stability owing to the Coulomb repulsion. This is the first example of ESIMS being successfully applied to the intact tetrasialonganglioside GQ1b.

The negative-ion ESI mass spectra of other gangliosides examined are summarized in Table 2, which shows single-, double- and/or triple-charged ions without ambiguity, thus providing accurate molecular weights. Spectra of GM1, GD1a and GD1b alike showed ions with single- and double-charged states, while GT1b showed ions with single-, double- and triple-charged states. The second negative charge in GM1 must be derived from the dissociation of an amide or alcoholic proton in the molecule. It is concluded that the maximum charge-state can not be predicted directly from the number of the acidic functional groups such as sialic acid. Multiplicity of the negative charge is also affected by the presence of weak acids like alcoholic OH groups or amide protons. A maximum charge-state smaller than the expected from the number of formal acidic groups may be a reflection of the length or units of the sugar chains in the particular gangliosides. Our results suggest that the negative-ion ESIMS provides a new, rapid method for the analysis of native gangliosides.

Moreover, if the sample molecule, which has only one carboxylate such as GM1, is dissolved in a common solvent (e.g., chloroform–methanol), the ESI spectrum in the negative-ion mode creates ions that have a different type of extra charge(s) in addition to the expected normal ion and the over-deprotonated negative ion mentioned above. The

Table 2
Results of the negative-ion ESIMS of various gangliosides

Compound		MW (free acid) [a]	Ions observed (m/z: assignment)		
			Single-charged ions	Double-charged ions	Triple-charged ions
GM1	(d18:1/c18:0)	1546	1546 $[M_1-H]^-$	772 $[M_1-2H]^{2-}$	
	(d20:1/c18:0)	1574	1574 $[M_2-H]^-$	786 $[M_2-2H]^{2-}$	
GD1a	(d18:1/c18:0)	1837	1859 $[M_1+Na-2H]^-$	917.5 $[M_1-2H]^{2-}$	
	(d20:1/c18:0)	1865	1886.5 $[M_2+Na-2H]^-$	932 $[M_2-2H]^{2-}$	
GD1b	(d18:1/c18:0)	1837	1859 $[M_1+Na-2H]^-$	917.5 $[M_1-2H]^{2-}$	
	(d20:1/c18:0)	1865	1886.5 $[M_2+Na-2H]^-$	932 $[M_2-2H]^{2-}$	
GT1b	(d18:1/c18:0)	2128	2150 $[M_1+Na-2H]^-$	1075 $[M_1+Na-3H]^{2-}$	708 $[M_1-3H]^{3-}$
			2166 $[M_1+K-2H]^-$	1083 $[M_1+K-3H]^{2-}$	
	(d20:1/c18:0)	2156	2179 $[M_2+Na-2H]^-$	1089 $[M_2+Na-3H]^{2-}$	718 $[M_2-3H]^{3-}$
			2193.5 $[M_2+K-2H]^-$	1096.5 $[M_2+K-3H]^{2-}$	
GQ1b	(d18:1/c18:0)	2420		1229 $[M_1+2Na-4H]^{2-}$	813 $[M_1+Na-4H]^{3-}$
				1239 $[M_1+Na+K-4H]^{2-}$	818.5 $[M_1+K-4H]^{3-}$
	(d20:1/c18:0)	2448		1243 $[M_2+2Na-4H]^{2-}$	822.5 $[M_2+Na-4H]^{3-}$
				1253.5 $[M_2+Na+K-4H]^{2-}$	828 $[M_2+K-4H]^{3-}$

[a] M_1 and M_2 represent the molecular mass of the free acids having sphingenine (d18:1) and icosashingenine (d20:1), respectively. Molecular masses are calculated as the exact masses.

unusual negative ions produced under ESI conditions in the negative-ion mode are Cl^--adduct $[M - H + Cl]^{2-}$ and $C_4H_9O_2^-$-adduct $[M - H + 89]^{2-}$ observed in the double-charged ion region. They are the same type of negative ions as we have previously reported [18] for neutral glycolipids in the negative-ion ESI.

Negative-ion ESI CIDMS / MS spectrum of GM1.—The ESI CIDMS/MS of ganglioside GM1 having $[M - H]^-$ as the precursor ion showed a spectrum similar to the FAB CIDMS/MS spectrum induced by low-energy collisions [8]. Both of them mainly yielded product ions which retain the ceramide moiety. The CIDMS/MS spectrum of the $[M_1 - 2H]^{2-}$ ion of GM1 at m/z 772 yielded fragment ions of the single-charge state at higher masses than the precursor ion [17]. As shown in Fig. 2a, the spectrum of $[M_1 - 2H]^{2-}$ of GM1 showed peaks at m/z 290, 308, 835, 997 and 1254. These were derived from glycosidic bond cleavages where all but the last ion retained the sialic acid residue.

These ions are all single-charged negative ions. Contrary to positive product ions, which may lose one of the precursor's charges by deprotonation from the precursor ion in the unimolecular dissociation process, reduction of a negative charge-state in MS/MS can be brought about only if one negative charge has been carried away by the leaving moiety. The presence of a pair of complementary ions at m/z 290 and 1254 supports this assumption. The peaks at m/z 438 and 460 are attributable to the double-charged ions resulting from the sugar ring openings. These observations suggest that the CIDMS/MS having a double-charged ion as the precursor is as useful as the technique utilizing a single-charged precursor ion for the structural characterization of gangliosides.

Negative-ion ESI CIDMS / MS of trisialoganglioside GT1b and tetrasialoganglioide GQ1b.—The CIDMS/MS of trisialoganglioside and tetrasialoganglioside, GT1b and GQ1b, each having their double-charged ion as their respective precursor, also gave information diagnostic of their structures. The negative-ion CIDMS/MS spectrum of $[M_2 + K - 3H]^{2-}$ (m/z 1096.5) of GT1b as shown in Fig. 2b, exhibits single-charged ions at m/z 1902, 1573 and 1246, which correspond to the ions produced by eliminations of the negative ions of sialic acid (NeuAc), NeuAc–NeuAc or two NeuAcs (one of them being the K-salt), and both NeuAc and NeuAc–hexose (Hex)–hexosamine (HexNAc) units, respectively. Fragment ions indicating presence of a terminal NeuAc and NeuAc–NeuAc moiety are also apparent (m/z 290, 328 and 619).

The CIDMS/MS spectrum of $[M_2 + 2Na - 4H]^{2-}$ (m/z 1243 [3]) of GQ1b is shown in Fig. 3a. No ions were observed above m/z 2000. Features of this spectrum are similar to those of GT1b. Assignments of the fragment ions are illustrated in Fig. 3a. These results indicate that CIDMS/MS can be used for structure identification of GQ1b.

The CIDMS/MS of the triple-charged ion $[M_2 + Na - 4H]^{3-}$ (m/z 822.5) of GQ1b is also rich in information of the structure as shown in Fig. 3b. Ions were not detected above m/z 2000. Product ions were detected again at m/z 1886 and 1573, as elucidated for Fig. 3b. It is of interest that daughter ions corresponding to the

[3] Ambiguity in mass comes from inadequate resolution of the quadrupole instrument for a large molecule with isotope distributions.

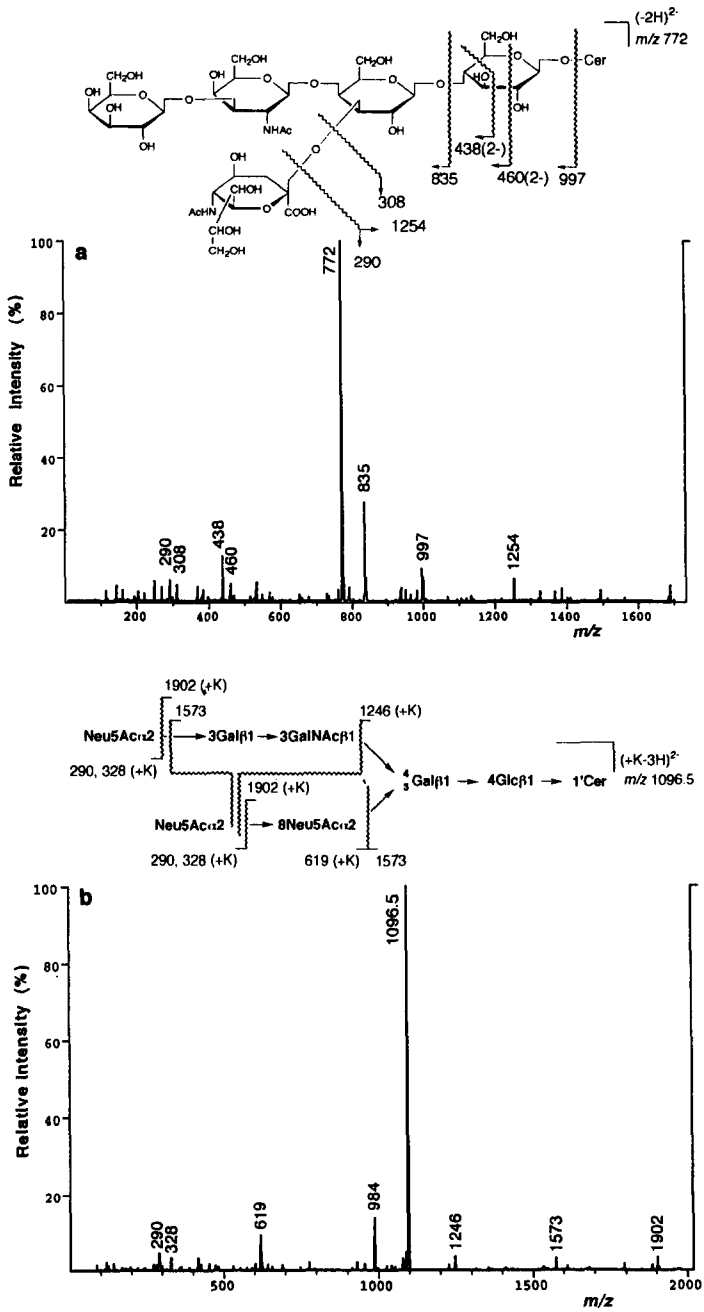

Fig. 2. Negative-ion ESI CIDMS/MS spectra of double-charged ions, its precursor being (a) the $[M_1 - 2H]^{2-}$ (m/z 772) of GM1, and (b) the $[M_2 + K - 3H]^{2-}$ (m/z 1096.5) of GT1b.

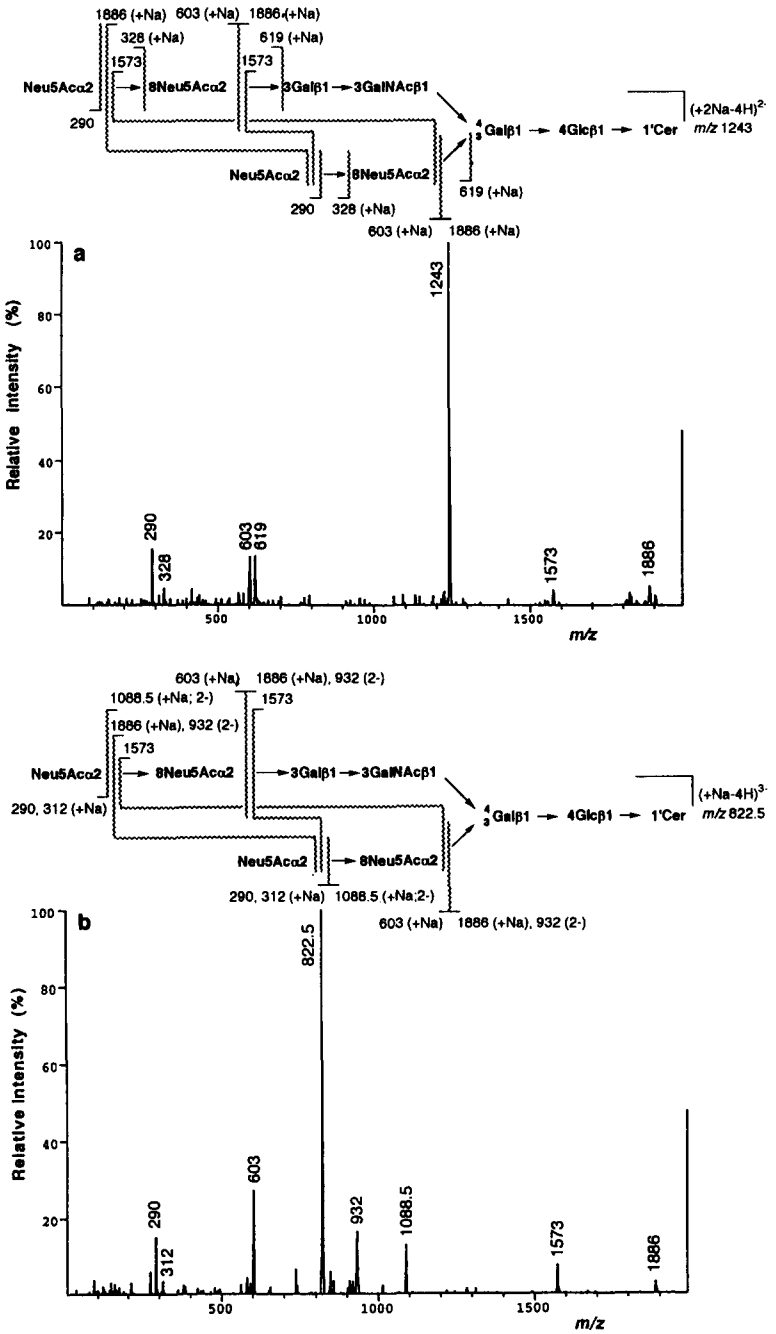

Fig. 3. Negative-ion ESI CIDMS/MS spectra of GQ1b. (a) The $[M_2 + 2Na - 4H]^{2-}$ ion (m/z 1243) as the precursor ion; (b) the $[M_2 + Na - 4H]^{3-}$ ion (m/z 822.5) as the precursor ion.

elimination of single-charged negative ions, that is, negative ions of NeuAc and NeuAc–NeuAc or two NeuAcs (one of NeuAcs being the Na-salt), appeared as double-negative (2−) ions at m/z 1088.5 and 932, respectively.

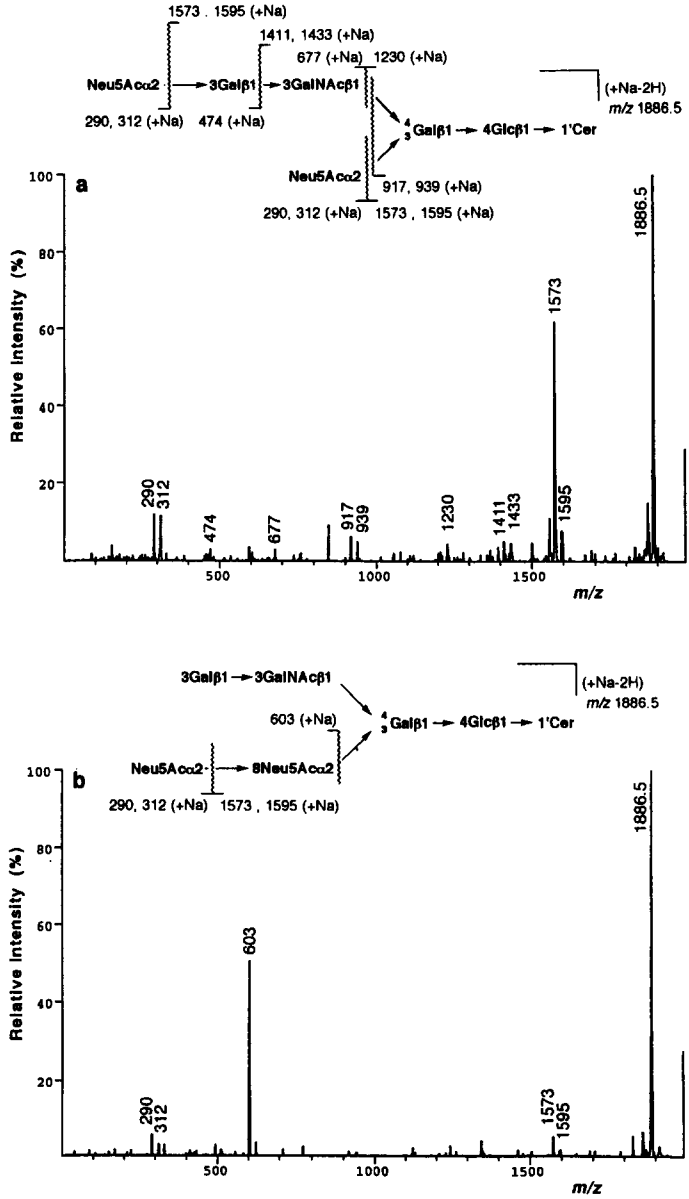

Fig. 4. Negative-ion ESI CIDMS/MS spectra of disialogangliosides having $[M_2 + Na - 2H]^-$ ions (m/z 1886.5) as the precursor ion. (a) GD1a; (b) GD1b.

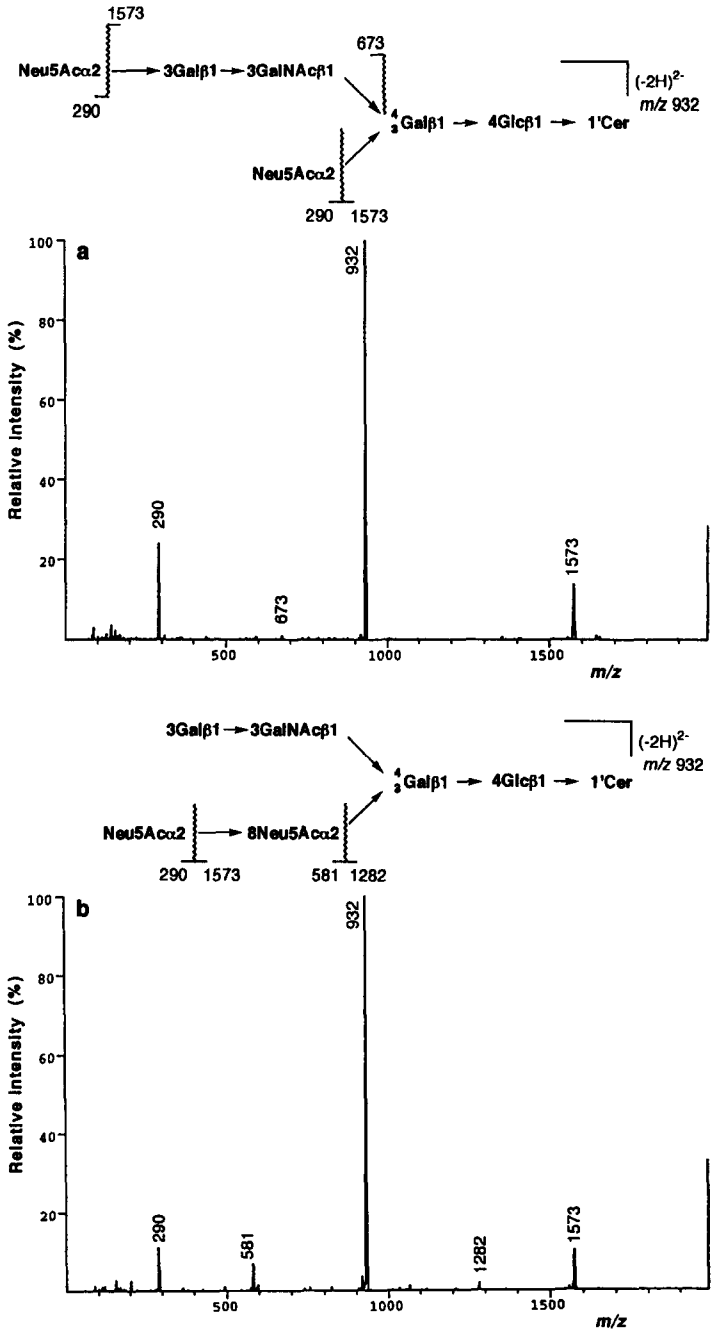

Fig. 5. Negative-ion ESI CIDMS/MS spectra of disialogangliosides having $[M_2 - 2H]^{2-}$ ions (m/z 932) as the precursor ion. (a) GD1a; (b) GD1b.

Although FABMS of acidic glycoconjugates such as polysialogangliosides gives clear spectra with abundant fragment ions, the intensities of molecularly related ions are not sufficient to perform CIDMS/MS with high sensitivity. ESI CIDMS/MS gives characteristic daughter ions that provide important structural information such as the number and positions of sialic acids. These are the first examples of CIDMS/MS being successfully applied to intact trisialoganglioside and tetrasialoganglioside, GT1b and GQ1b.

Negative-ion ESI CIDMS / MS of isomeric disialogangliosides GD1a and GD1b.— The two structurally isomeric gangliosides GD1a and GD1b have been studied. The product-ion spectrum of GD1a having $[M_2 + Na - 2H]^-$ ion (m/z 1886.5) as the precursor ion showed a dominant peak at m/z 1573 that may result from the elimination of either one of the terminal NeuAcs, as shown in Fig. 4a. However, an ion at m/z 677 reflects the non-reducing terminal structure as NeuAc-Hex-HexNAc. The ion, at m/z 1230, although weak, is complementary to m/z 677. Appearance of these two ions indicates that either one of two sialic acids can be a Na-salt, the Na^+ not being confined to a particular one. Other sequence ions are assigned as shown in the figure. On the other hand, in the case of CIDMS/MS of $[M_2 + Na - 2H]^-$ of GD1b, a diagnostic ion corresponding to the negative ion of NeuAc-NeuAc (Na-salt) was found at m/z 603 (Fig. 4b), in sharp contrast to the corresponding spectrum of GD1a. Domon and Costello reported the negative-ion FAB CIDMS/MS of GD1a and GD1b, having their individual $[M + Na - 2H]^-$ ions as the precursor and applying the high-collision energy (8 keV), and they succeeded in distinguishing these isomers [7]. Our CIDMS/MS spectra with low collision energy (30–35 eV) also provide an easy method to distinct the isomeric gangliosides, although the spectral features are naturally different from these of high-energy FAB CIDMS/MS.

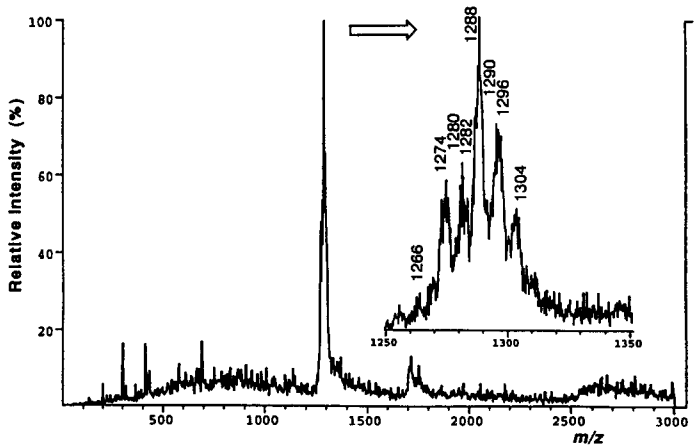

Fig. 6. Positive-ion ESI mass spectrum of GQ1b.

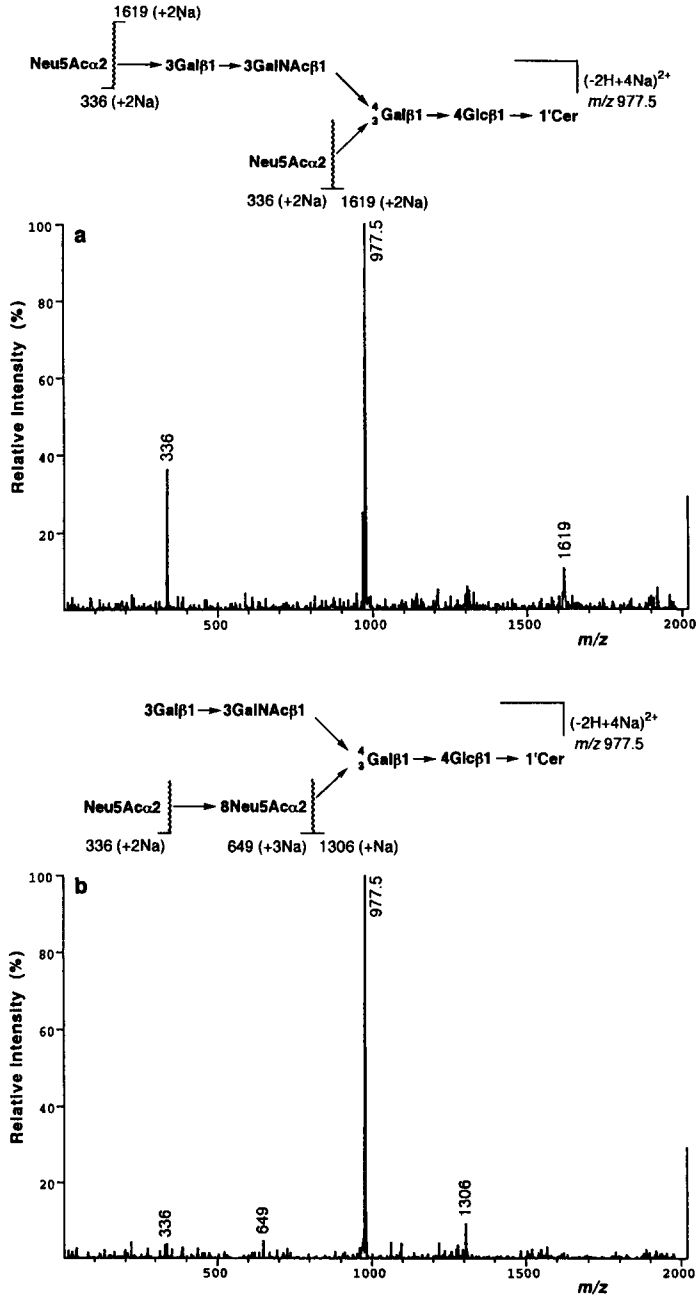

Fig. 7. Positive-ion ESI CIDMS/MS spectra of disialogangliosides having $[(M_2-2H+2Na)+2Na]^{2+}$ ions (m/z 977.5) as the precursor ion. (a) GD1a; (b) GD1b.

The CIDMS/MS was applied also to the double-charged ion $[M_2 - 2H]^{2-}$ (m/z 932) of the two isomeric gangliosides GD1a and GD1b, as shown in Figs 5a and 5b, respectively. For GD1a, a single-charged weak ion at m/z 673 represents the negative ion of NeuAc–Hex–HexNAc unit, while the complementary ions at m/z 581 and 1282 for GD1b represent a NeuAc–NeuAc unit at an end. Two peaks at m/z 290 and 1573, common to both isomers, are described above. These results distinguish positions of two sialic acids in GD1a and GD1b.

Positive-ion ESI mass spectra of gangliosides.—ESIMS was tried in the positive-ion mode for large intact gangliosides. Such a spectrum of GQ1b reproduced in Fig. 6 shows an intense peak, which is enlarged to reveal the fine structure in the region from m/z 1250 to 1350. These ions are double-charged. Intense ions at m/z 1280, 1288, 1296 and 1304 correspond to $[(M_2 - 3H + 3Na) + 2Na]^{2+}$, $[(M_2 - 3H + 2Na + K) + 2Na]^{2+}$, $[(M_2 - 3H + Na + 2K) + 2Na]^{2+}$ and $[(M_2 - 3H + 3K) + 2Na]^{2+}$, respectively, due to icosasphingenine as the long-chain base. Another similar set of ions were observed 14 Da lower in mass numbers for the molecular species containing sphingenine. Further complexity of the spectrum is caused by the variety (H, Na or K) of carboxylate forms of, and adduct ions to, these two molecular species.

The positive-ion ESI mass spectra of other gangliosides so far studied also showed molecular distribution similar to that of GQ1b. These results suggest that the positive-ion ESIMS is also capable of giving information on the molecular masses, although ESI spectra of acidic glycolipids in the positive-ion mode show more complexity than in the negative-ion mode.

Positive-ion ESI CIDMS/MS spectra of gangliosides, GD1a and GD1b.—The positive-ion ESI CIDMS/MS of the aforementioned two isomeric gangliosides GD1a and GD1b indicate a possibility to distinguish the structural isomers up to this sugar chain size equally well as with the negative-ion mode. The ESI CIDMS/MS spectra of the disodium salt of GD1a and GD1b having their double-charged ion $[(M_2 - 2H + 2Na) + 2Na]^{2+}$ (m/z 977.5) as the precursor ion are shown in Figs 7a and 7b. One of the isomers, GD1a, showed a pair of complementary ions at m/z 336 and 1619, both being single-charged ions. On the contrary, in a similar spectrum, GD1b showed two diagnostic peaks at m/z 649 and 1306 of the single-charged state. The former corresponds to the Na-adduct ion of NeuAc–NeuAc as one unit, and the latter ion (m/z 1306) represents the rest of the molecule that is associated with two Na^+. The ion at m/z 336 of GD1b is less abundant than that of GD1a, which has two terminal sialic acids. Moreover, the ion at m/z 1619, observed in the spectrum of GD1a and corresponding to the elimination of NeuAc–Na^+, was not detected for GD1b, reflecting the number of terminal sialic acids. These observations clearly indicate that the positive-ion CIDMS/MS is also useful to distinguish two structural isomers. Owing to the acidic nature of gangliosides, soft-ionization mass spectrometry is applied in most cases in the negative-ion mode for structural studies. However, our results indicate that the positive-ion ESIMS also gives molecular weight information with high sensitivity, and that CIDMS/MS is also useful for characterization of native gangliosides. These findings prove that ESIMS is currently the most efficient analytical method for the structural studies of polysialoglycolipids, and our studies are being extended further to the analysis of trace amounts of unknown samples.

Acknowledgements

The authors are most thankful to our colleague Dr Sadamu Kurono, without whose efforts in maintaining the instrument in the best condition, we could not have carried out the present study. This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Area No. 06240247 from the Ministry of Education, Science and Culture of Japan.

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