International Journal of Mass Spectrometry 312 (2012) 179–184

Contents lists available at SciVerse ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Studies on (−)ESI-MS/MS of a glycosaminoglycan disaccharide *N*-acetyllactosamine-6,6′-disulfate disodium salt—Charge-localization isomers

Yoko Ohashi[a,*], Masayuki Kubota[b], Hiroshi Hatase[a], Takashi Hirano[a], Shojiro Maki[a], Haruki Niwa[a]

[a] *Department of Engineering and Science, Graduate School of Informatics and Engineering, The University of Electro-Communications, 1-5-1 Chofugaoka, Chofu, Tokyo 182-8585, Japan*

[b] *ThermoFisher Scientific Co., 3-9 Moriya-cho, Kanagawa-ku, Yokohama, Kanagawa 221-1293, Japan*

ARTICLE INFO

Article history:
Received 1 April 2011
Received in revised form 23 August 2011
Accepted 25 August 2011
Available online 1 September 2011

This article is dedicated to Professor Catherine Fenselau with respect and love.

Keywords:
Charge-localization isomers
Dibasic acid
Disaccharide
(−)ESI-MS/MS
GAG
sulfate

ABSTRACT

We previously reported the ESI-MS/MS studies on distinguishing Galβ1-4GlcNAc-6,6′-disulfate disodium salt from 2′-epimer,[1] namely Galβ1-4ManNAc-6,6′-disulfate [1]. Here, we must emphasize first that distinction of the epimeric pair was successful using either FAB or MALDI, but most effectively done by ESI mass spectrometry. However, though totally independent of our original aim of distinguishing epimers, we later realized that there were some inaccuracies in our assignment of the fragmentation path of negative product ions. Through our intensive studies to establish the right fragmentation path, we now propose a new concept of isomers caused by the difference in the localization of a negative charge in apparently the same MS^1 ion of a disaccharide dibasic acid.

1. Introduction

Isomer distinction is naturally performed in organisms, and thus very interesting and important for scientists in order to understand life as well as to design drugs. We have focused our interest on distinguishing isomers of conjugated lipids and sugars on mass spectrometry since the advent of FAB (SIMS). Our study first began with the distinction of positional isomers of phospholipids [2] and proceeded to microheterogeneity analysis of ceramide [3], particularly the long-chain bases [4], to a- and b-series of gangliosides [5], linkage isomers of Le^a and Le^x [6–8], distinction of epimeric GAG isomers [1], and finally the anomeric isomers of a lysoglycero-type ganglioside [9].

It happened in the course of the GAG steroisomer analysis that we made a misassignment in a minor fragmentation path shown in Scheme 6, p. 275 of Ref. [1].

In short, epimer distinction was attributed to a stereochemistry of *N*-acetylhexosamines: in L4, a hydrogen bond may be formed between the 2′-α-equatorial amide-carbonyl oxygen and 4′-hydroxyl hydrogen, resulting in the easier cleavage of 2–3 and 0–1 bonds ($^{0,2}A_2$, *m/z* 463), while the β-axial amide group in *N*-acetylmannosamine in M4 cannot come close to the 4′-OH. Thus, the elimination of 101 Da ($^{0,2}A_2$) in L4 is relatively more abundant than the dehydration ion at *m/z* 546. To the contrary, the dehydration ion is relatively more intense in the epimer M4. We drew the route as follows: $[M''-Na]^-$ (MS^1) at *m/z* 564 > $^{0,2}A_2$ (MS^2) at *m/z* 463.[2]

Up until this point, our rationalization was unequivocally right. Then, routinely we regarded the next loss of 102 u as a replacement of $-SO_3Na$ with H, leading to the MS^3 ion at *m/z* 361, and the following elimination of $O{=}CHCH_2OH$ moiety resulting in the formation of an MS^4 ion at *m/z* 301.

However, we soon realized that such replacement, which involves an extraneous hydrogen, was unfeasible in the MS/MS

Abbreviations: Gal, galactose; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; GAG, glycosaminoglycan; Le^a, Lewis Blood Group (type) a; Le^x, Lewis Blood Group (type) x.

* Corresponding author. Present address: Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako Saitama 351-0198, Japan. Tel.: +81 48 467 6372; fax: +81 48 467 6372.
E-mail address: ohashi@brain.riken.jp (Y. Ohashi).

[1] Carbons in the non-reducing end sugar (galactose, in this case) are numbered without a prime (′), whereas those in the reducing end sugar (*N*-acetylhexosamine, here) are numbered with a prime.

[2] M″ stands for the disodium salt.

1387-3806/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2011.08.024

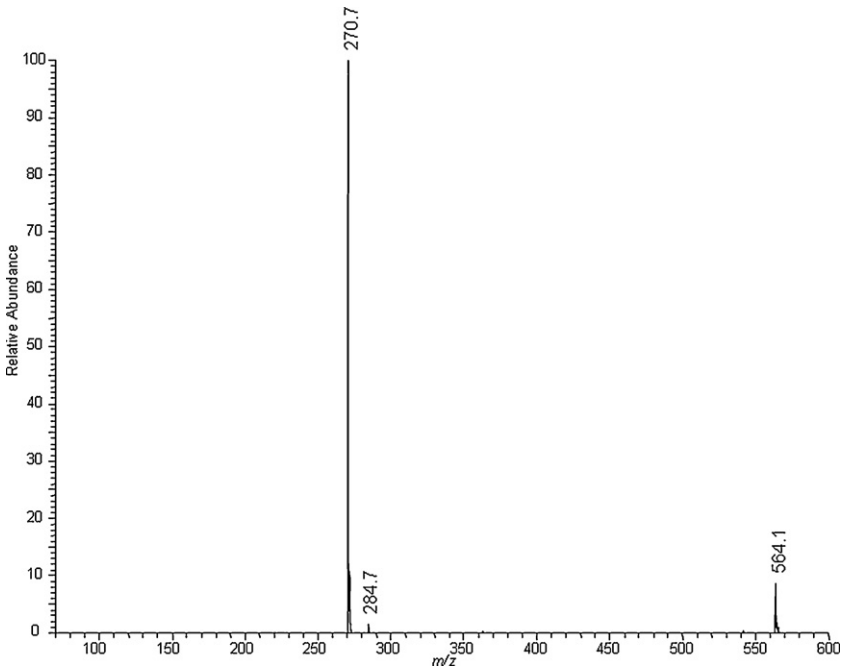


Fig. 1. (−)ESI-MS full scan spectrum of Galβ1-4GlcNAc-6,6′-disulfate disodium salt (L4, M″W 587).

unimolecular dissociation process. Upon this realization we spent some time accounting for the production of all the ions shown in Figs. 1–5 consistently. The point was the fact that each epimer of this dibasic glycosaminoglycan disaccharide showed the doubly negative ion $[M''-2Na]^{2-}$ at *m/z* 270.5 as the MS^1 ion even more intensely than the $[M''-Na]^-$ as shown in Fig. 1. This meant that both sodium sulfate groups in the disaccharide were able to dissociate, producing the sulfate anion individually. Thus, we have come to the conclusion that there are two distinct ion structures for $[M''-Na]^-$ in the gas phase for such a strong dibasic acid, and these two precursors lead to two individual fragmentation paths. As stated in the following Section 2, one series proceeds as *m/z* 564, 463, 361, to 241 while the other as *m/z* 564, 463, 301, to 241. Further detailed studies have led us to propose a new concept of charge-localization isomers.

2. Experimental

2.1. Samples

Galβ1-4GlcNAc-6,6′-disulfate disodium salt (L4, M″W 587) was obtained from shark fin keratan sulfate by the keratanase II

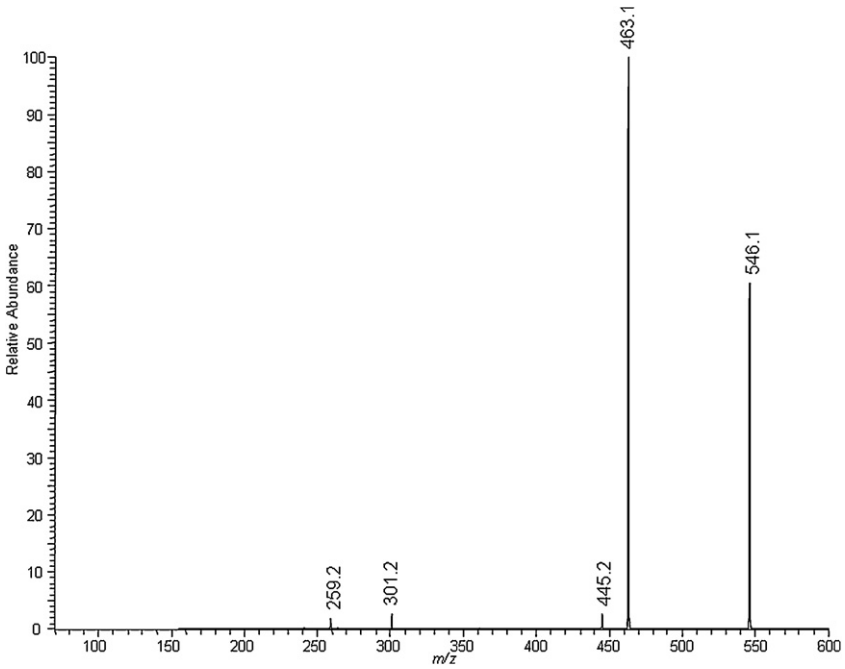


Fig. 2. (−)ESI-MS^2 product ion spectrum of Galβ1-4GlcNAc-6,6′-disulfate disodium salt (L4, M″W 587) having $[M''-Na]^-$ at *m/z* 564 as the precursor ion.

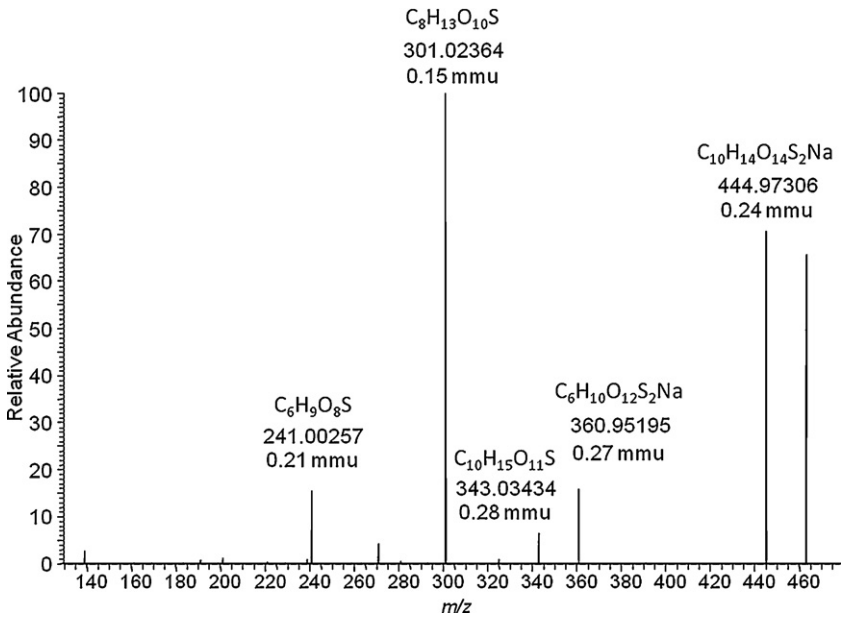


Fig. 3. (−)ESI-MS^3 product ion spectrum of Galβ1-4GlcNAc-6,6′-disulfate disodium salt (L4) in accurate masses having [M″−Na]$^-$ at *m/z* 564 as the precursor and ^{0,2}A ion at *m/z* 463 as the MS^2 ion.

digestion. The artifact Galβ1-4ManNAc-6,6′-disulfate disodium salt (M4, M″W 587), generated through the mild alkaline purification process [10], was isolated as the minor component from L4 on an amine-bound silica gel high-performance liquid chromatography. Purity was examined on capillary electrophoresis, of which the result was shown in Fig. 1 of Ref. [1]. Structures of L4 and M4 were confirmed using NMR as shown in Scheme 1. We received these epimeric isomers in the purified form. The samples were dissolved in H_2O/CH_3OH (1:1) in concentration of 10 ng/mL for the mass spectrometric studies.

2.2. Mass spectrometry

2.2.1. Full scanning and product ion scannings

A ThermoFisher Scientific mass spectrometer LTQ FT-ICR was used for (−)ESI-MS/MS experiments. The spray voltage was 1 kV and the resolving power was 100,000. The CID energy parameter for MS^2 and MS^3 was 30%. The full scan spectrum in Fig. 1 showed two MS^1 ions, [M″−Na]$^-$ at *m/z* 564.1 and the doubly charged negative ion [M″−2Na]$^{2-}$ at *m/z* 270.7, the latter in much more abundance. Product ion spectra were obtained by changing energy parameters

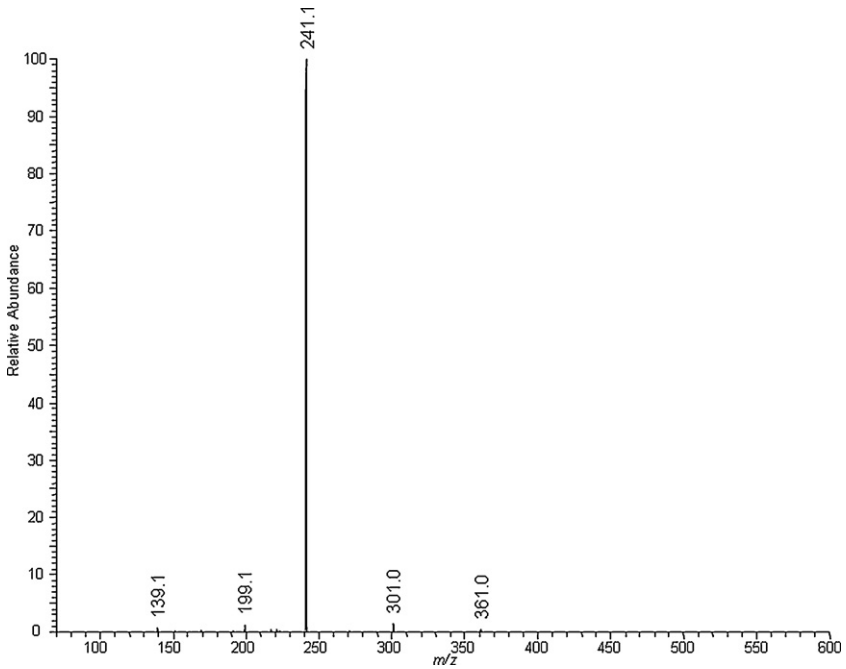


Fig. 4. (−)ESI-MS^4 product ion spectrum of Galβ1-4GlcNAc-6,6′-disulfate disodium salt (L4) having [M″−Na]$^-$ at *m/z* 564 as MS^1, ^{0,2}A ion at *m/z* 463 as MS^2 ion, and the ion in question at *m/z* 361 as the MS^3 ion. The nature of the ion at *m/z* 301 is thought to be a ^{2,4}A ring cleavage product, coincidentally having the same nominal mass as ion d.

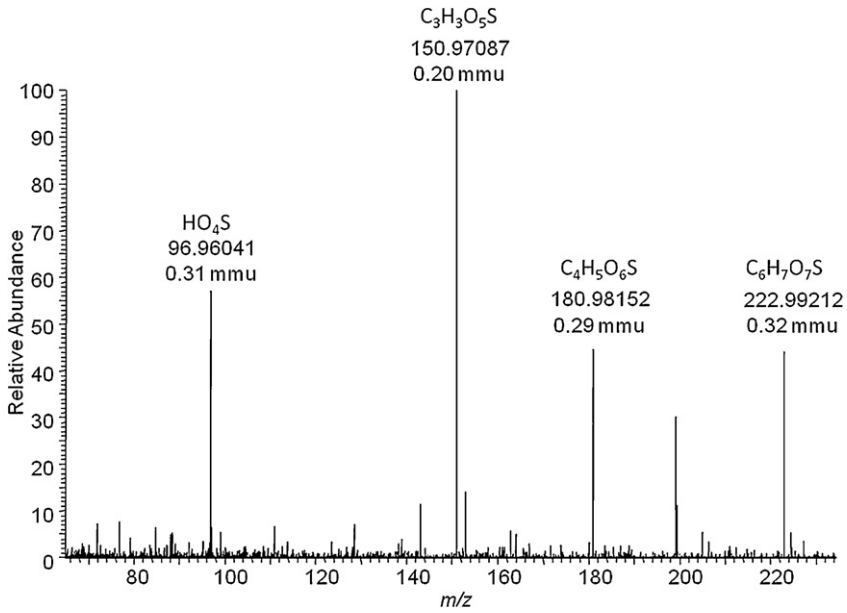


Fig. 5. (−)ESI-MS^5 product ion spectrum of Galβ1-4GlcNAc-6,6′-disulfate disodium salt (L4) in accurate masses having $[M''-Na]^-$ at *m/z* 564 as MS^1, $^{0,2}A$ ion at *m/z* 463 as MS^2, the ion at *m/z* 301 as the MS^3, and the ion at *m/z* 241 as the MS^4 ion. A peak near *m/z* 199 may possibly be a noise.

for these two precursor ions individually; one having $[M''-Na]^-$ as the MS^1 ion to obtain MS^2–MS^5 product ions as shown in Figs. 2–5, while the other having $[M''-2Na]^{2-}$ as the MS^1 ion obtaining MS^2 ions (data not shown).

2.2.2. Determination of product ion compositions

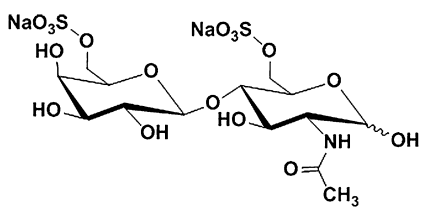
Accurate mass measurements were taken for the product ions at *m/z* 463, 445, 361, 343, 301, 241, 223, 181, 151 and 97 to determine their elemental compositions using the aforementioned FT-ICR instrument. Results are shown in the next section.

3. Results and discussion

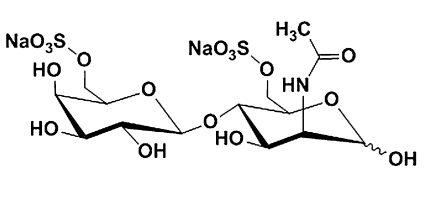
The MS^2-product ion spectrum of the precursor ion $[M''-Na]^-$ at *m/z* 564 is shown in Fig. 2, which mainly provides the dehydration ion at *m/z* 546 and the $^{0,2}A_2$ ion at *m/z* 463. Next, the MS^3 spectrum started from $[M''-Na]^-$ via the $^{0,2}A_2$ is shown in Fig. 3. The fact that Fig. 4 does show an ion at *m/z* 301 may seem to be contradictory to our hypothesis that the ion at *m/z* 301 cannot be produced by the ion at *m/z* 361. However, if the $^{2,4}A$ (or $^{1,3}A$) ring cleavage (loss of HOCH=CHOH) can be granted [11], this ion, though isobaric to the ion d, must have the ion composition of $C_4H_6O_{10}S_2Na$, which is different from the determined composition of d ($C_8H_{13}O_{10}S$) shown in Fig. 3. Thus, the same nominal mass of 301 is decided as pure coincidence. Fig. 5 shows another product ion study in the route of 564 > 463 > 301 > 241>.

Thus, there are two distinct pathways recognized here starting from apparently the same MS^1 and MS^2 ions. One is a series originating from $[M''-Na]^-$ at *m/z* 564 fragmenting to *m/z* 463, 361, 241 and so on, and the other also starting from $[M''-Na]^-$ at *m/z* 564 via *m/z* 463, but then proceeding to 301, 241 and so on. We assume that the ion structure at *m/z* 241 in the two routes are isomeric in the conventional definition, namely, e has the 5-ene-1-sulfated galactose structure while e′ has the 1-deoxy-1-ene-6-sulfated galactose structure. However, the ion intensity of c at *m/z* 361 was only sufficient to show the MS^4 ions, but not to obtain MS^5 ions for the route 564 > 463 > 361 > 241>.

Now, we summarize the fragmentation route as represented in Scheme 2. Ions whose compositions were confirmed with accurate masses in Figs. 3 and 5 are circled. Note that an unusual migration of a sulfate group from the glucosamine-6′ to the C-1 position of galactose (c) is involved in this scheme. Definite pieces of evidence for the galactose disulfate structure for the ion at *m/z* 361 were given by accurate mass measurements for MS^2 and MS^3 ions at *m/z* 463 and 361. The MS^2 ion was found to be 462.9836 ($C_{10}H_{16}O_{15}S_2Na$) (data not shown) while the MS^3 ion was 360.95195 ($C_6H_{10}O_{12}S_2Na$) with the errors of 0.19 mmu and 0.27 mmu, respectively, from the theoretical exact masses. Thus, the loss from the MS^2 ion at *m/z* 463 to the MS^3 ion at *m/z* 361 corresponds to the loss of $C_4H_6O_3$. An ion, though in low intensity, at *m/z* 199 as a next generation of the ion at *m/z* 361 in Fig. 4 suggests a pyrosulfate structure $NaOSO_3SO_3^-$, which supports the presence of two sulfate groups in c. Depending

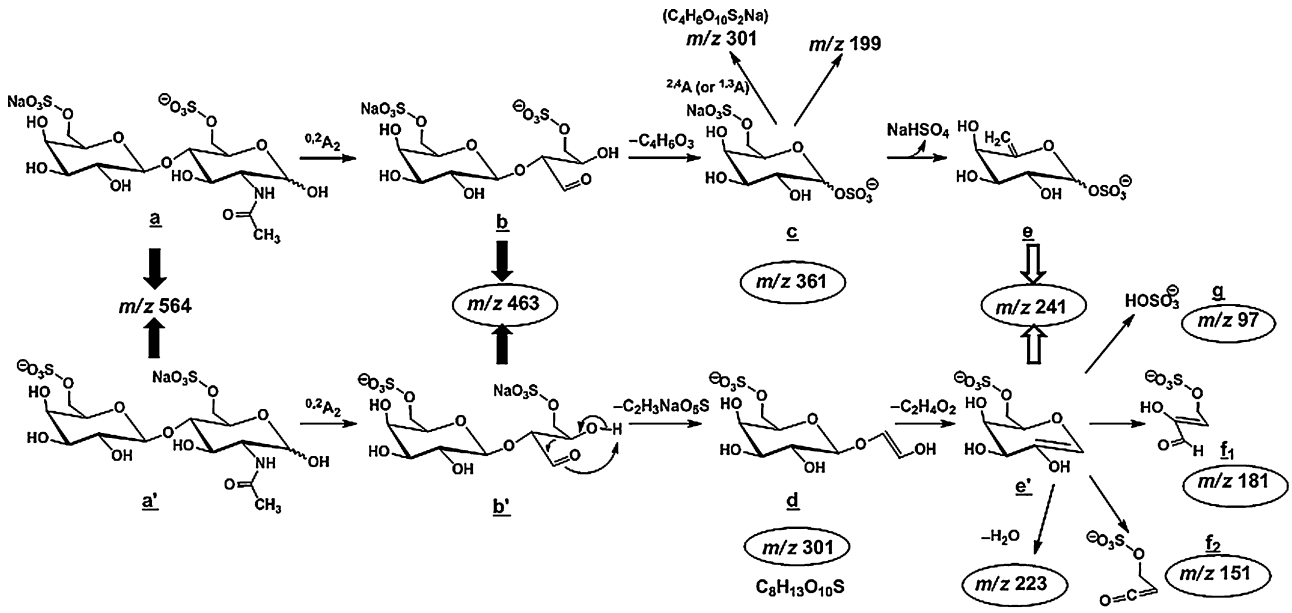


L4 (Galβ1-4GlcNAc-6, 6'-disulfate disodium salt): M''W 587



M4 (Galβ1-4ManNAc-6, 6'-disulfate disodium salt): M''W 587

Scheme 1.



Scheme 2.

on the direction of the intramolecular S_N2 of the GlcNAc-6-sulfate to the C1 position of Gal-6-sulfate, resulting anomeric sulfate may take α or β-position.

To elucidate all these data without contradiction, we propose a new phase of isomers, in which the effective negative charge localizes at either one of the two sulfate groups in the molecular-weight-related negative precursor ion, while a sodium cation resides in the immediate vicinity of the other sulfate group so as to mask the negative charge as represented as (a) and (a′) in Scheme 2. We hereby name this phenomenon "charge-localization isomers". Not only the precursor ion (a) and (a′) but even the MS^2 ion (b) and (b′) have the relationship of "charge-localization isomers", which are different from the conventional structural isomeric relations between (e) and (e′) in Scheme 2.

We also considered another possibility in which both sulfate groups are dissociated, forming a doubly negative entity to which a sodium cation is loosely associated so as to compensate one negative charge and showing an apparently singly charged negative ion $([M''-2Na]^{2-}+Na^+)^-$ at *m/z* 564 in the ion source. However, if this had been the case, charge-localization could not have existed [12,13]. Although the negative-ion ESI full scanning showed the doubly charged negative ion $[M''-2Na]^{2-}$ at *m/z* 270.7 as the base peak as shown in Fig. 1, this doubly negative ion was not a product ion of the precursor at *m/z* 564 as evidenced in Fig. 2. Thus, the possibility of "doubly negative ion plus Na''^+ being the precursor structure may safely be eliminated.

4. Conclusions

It is well known that the same one molecular-weight-related ion may give rise to more than one product ions according to the charge-localizations in the product ions. In such cases, the structure of the precursor ion is the same. In our hypothesis, however, the precursor ion consists of two chemical species. This phenomenon is likely to occur in strongly acidic dibasic acids in the negative-ion ESI-MS.

Since two anionic structures are possible for the precursor ion, each of them may take a different course in the fragmentation processes. This phenomenon is entirely different from the well-known production of plural numbers of product ions originating from the same precursor. Since negatively charged sugars are important in relation to basic protein, this concept may give some suggestions regarding the mechanism of certain kinds of interaction in living organisms

Acknowledgments

The precious samples were the gift from Mr. Keiichi Yoshida formerly of Seikagaku-Kogyo. Dr. Yoshiyuki Ito, then a graduate student of the University of Electro-Communications, diligently carried out the initial part of the work. Dr. Mamoru Ohashi, Professor Emeritus of the University of Electro-Communications, has been always willing to discuss with us. Had it not been for their help, we would have been unable to perform this work.

References

[1] Y. Ohashi, Y. Itoh, M. Kubota, K. Hamada, M. Ohashi, T. Hirano, H. Niwa, Analysis of sugar epimers using mass spectrometry: *N*-acetyllactosamine-6,6′-disulfate and the 2′-epimer, Eur. J. Mass Spectrom. 10 (2004) 269–278.
[2] Y. Ohashi, Structure determination of phospholipids by secondary ion mass spectrometric techniques: differentiation of isomeric esters, Biomed. Mass Spectrom. 11 (1984) 383–385.
[3] Y. Ohashi, M. Iwamori, T. Ogawa, Y. Nagai, Analysis of long-chain bases in sphingolipids by positive ion fast atom bombardment or matrix-assisted secondary ion mass spectrometry, Biochemistry 26 (1987) 3990–3995.
[4] Y. Ohashi, T. Tanaka, S. Akashi, S. Morimoto, Y. Kishimoto, Y. Nagai, Squid nerve sphingomyelin containing an unusual sphingoid base, J. Lipid Res. 41 (2000) 1118–1124.
[5] T. Ii, Y. Ohashi, Y. Nagai, Structural elucidation of underivatized gangliosides by electrospray-ionization tandem mass spectrometry (ESIMS/MS), Carbohydr. Res. 273 (1995) 27–40.
[6] T. Ii, Y. Ohashi, T. Ogawa, Y. Nagai, A New Approach to the characterization of sulfated and sialyl Lewis-type glycosphingolipids using positive-ion FABMS, ESIMS and CID-MS/MS, J. Mass Spectrom. Soc. Jpn. 44 (1996) 183–195.
[7] T. Ii, Y. Ohashi, T. Ogawa, Y. Nagai, Negative-ion fast atom bombardment and electrospray ionization tandem mass spectrometry for characterization of sulfated and sialyl Lewis-type glycosphingolipids, Glycoconjugate J. 13 (1996) 273–283.
[8] Y. Ohashi, T. Ii, M. Kubota, S. Nunomura, H. Niwa, M. Ohashi, T. Ogawa, Y. Nagai, FAB CID-MS/MS of in-source fragment ions as a means to differentiate linkage isomers of branched oligosaccharides: Le^a and Le^x type glycoconjugates, J. Mass Spectrom. Soc. Jpn. 46 (1998) 45–52.
[9] Y. Ohashi, M. Kubota, H. Hatase, M. Nakamura, T. Hirano, H. Niwa, Y. Nagai, Distinction of sialyl anomers on ESI- and FAB-MS/MS: stereo-specific fragmentations, J. Am. Soc. Mass Spectrom. 20 (2009) 394–397.

[10] T. Toida, I.R. Vlahov, A.E. Smith, R.E. Hileman, R.J. Linhardt, C-2 epimerization of *N*-acetylglucosamine in an oligosaccharide derived from heparan sulfate, J. Carbohydr. Chem. 15 (1996) 351–360.
[11] D. Harvey, Fragmentation of negative ions from carbohydrates: part 2. Fragmentation of high-mannose *N*-linked glycans, J. Am. Soc. Mass Spectrom. 16 (2005) 631–646.
[12] H. Budzikiewicz, C. Djerassi, D. Williams, Note to the Reader, in: Interpretation of Mass Spectra of Organic Compounds, Holden-Day, Inc., San Francisco, 1964, pp. xi–xiii.
[13] F. W. McLafferty, F. Turecek, Interpretation of Mass Spectra, fourth ed., University Science Books, Mill Valley, CA, 1993.