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Structural determination of cerebrosides isolated from *Asterias amurensis* starfish eggs using high-energy collision-induced dissociation of sodium-adducted molecules

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Six cerebrosides were isolated from the eggs of the starfish *Asterias amurensis* using solvent extraction, silica gel column chromatography, and reversed-phase high-performance liquid chromatography. This study demonstrated that the structures of cerebrosides could be completely characterized, based on their sodium-adducted molecules, using fast atom bombardment (FAB) tandem mass spectrometry. The high-energy collision-induced dissociation of the sodium-adducted molecule, $[M + Na]^+$, of each cerebroside molecular species generated abundant ions, providing information on the compositions of the 2-hydroxy fatty acids and long-chain sphingoid bases, as well as the sugar moiety polar head group. Each homologous ion series along the fatty acid and aliphatic chain of the sphingoid base was useful for locating the double-bond positions of both chains and the methyl branching position of the long-chain base. The *N*-fatty acyl portions were primarily long-chain saturated or monoenoic acids (C16 to C24) with an α-hydroxy group. The sphingoid long-chain base portions were aliphatic chains (C18 or C22) with two or three degrees of unsaturation and with or without methyl branching. Copyright © 2011 John Wiley & Sons, Ltd.

Cerebrosides are glycosphingolipids consisting of a ceramide and a single sugar residue (glucose or galactose) at C-1. The hydrophobic ceramide portion contains a sphingoid base and an amide-linked fatty acyl chain, while the hydrophilic portion is a sugar ring. These amphipathic molecules have been isolated from diverse natural sources, including plants,[1] marine sources,[2] microorganisms,[3] and humans.[4] In particular, mammalian sphingolipids have various cellular functions, and play important roles in membrane structure and cell signaling.[5]

Several analytical methods have been used to study glycosphingolipids extracted from biological samples.[6,7] Mass spectrometry is an important procedure for structural studies, and the structures of sphingolipids have been characterized using various ionization methods, including electron ionization (EI),[8,9] electrospray ionization (ESI),[10,11] and fast atom bombardment (FAB).[12–14] Recently, ESI tandem mass spectrometry (MS/MS) connected to liquid chromatography has been used to isolate and identify mixtures of sphingolipids, and low-energy collision-induced dissociation (CID) MS/MS has also been used in structural analyses.[15–17] Over the past decades, FAB-MS/MS has been used to characterize various sphingolipids because it offers many advantages for structural elucidation and has high-energy CID capabilities.[13,18–20] In our previous work, FAB-MS/MS with a four-sector instrument was shown to be a useful technique for determining the complete structures of glycerolipids, including glycoglycerolipids,[21] phospholipids,[22] and triacylglycerols.[23] It was used to identify the polar head group and fatty acid compositions, as well as the double-bond position in the fatty acyl chains. Ann and Adams contributed significantly to the characterization of ceramides and neutral glycosphingolipid structures using CID of lithium-adducted molecules.[13,19] The fragmentation mechanisms of product ions observed in the low-energy CID of $[M + Li]^+$ ions of glucosylceramides have been studied extensively.[15] Recently, five glucosylceramides isolated from marine sponges were characterized using FAB-linked scanning at constant B/E.[24]

In the present study, the structures of six cerebrosides (AA-1 to AA-6; Fig. 1) isolated from starfish egg extracts were determined using high-energy CID tandem mass analyses with a four-sector tandem mass spectrometer. Additionally, double-bond configurations and sugar structures were determined using ^1H, ^{13}C NMR and chemical degradation. In this paper, we report on the detailed structural elucidation of cerebrosides isolated from starfish eggs.

EXPERIMENTAL

Extraction and isolation

Raw starfish eggs (4.34 kg) were extracted with methanol at room temperature. The crude extract was partitioned between butanol and water, and the butanol layer was eluted using silica gel column chromatography based on solvent polarity. Neutral lipid fractions were eluted with acetone,

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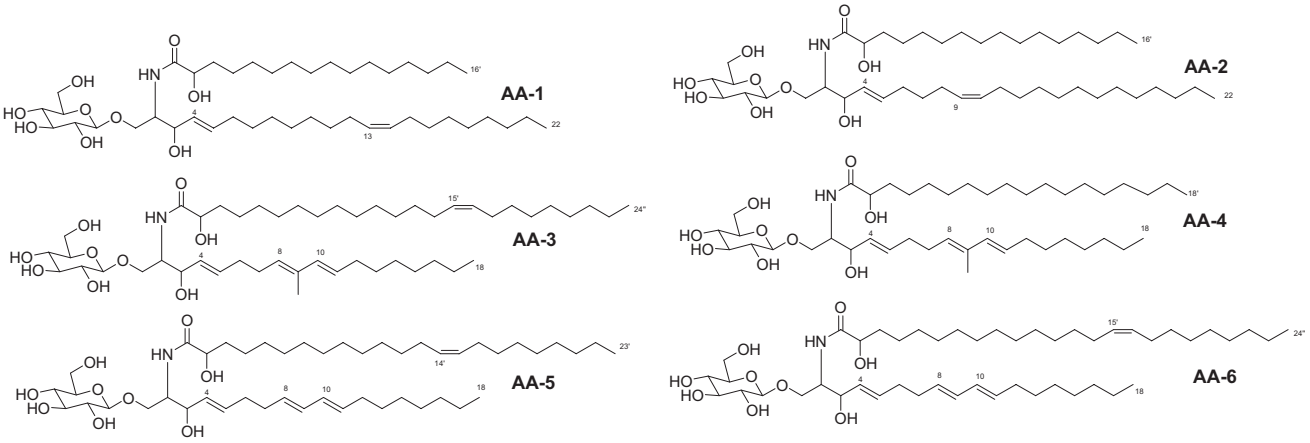

Figure 1. Structures of six cerebrosides isolated from *A. amurensis* starfish eggs.

and the major component was identified as cerebrosides. The acetone fraction (500 mg) was applied to reversed-phase high-performance liquid chromatography (HPLC) on a YMC-ODS column (250 × 10 mm i.d.; YMC Co., Kyoto, Japan), eluting with 100% methanol to yield six cerebrosides: AA-1 (16 mg), AA-2 (12 mg), AA-3 (10 mg), AA-4 (20 mg), AA-5 (10 mg), and AA-6 (15 mg).

Acidic methanolysis

Each cerebroside (3 mg) was heated with 3 mL of 1 N HCl in 80% methanol at 70°C overnight in a small sealed vial. They were methanolized to yield a mixture of fatty acid methyl esters (FAME), free sphingoid long-chain bases (LCB), and methyl-D-glucopyranoside. The reaction mixture was evaporated and partitioned with *n*-hexane and methanol. The hexane and methanol layers were concentrated to produce the FAME and free sphingoid LCB, respectively.

Mass spectrometry

All mass spectrometric analyses were performed using a four-sector tandem mass spectrometer (JMS-HX110/110A, JEOL). The ion source was operated at an accelerating voltage of 10 kV in positive-ion mode. Ions were produced by FAB using a cesium ion beam generated from the ion gun and accelerated to 22 kV. Samples were dissolved in chloroform/methanol (2:1, v/v). An aliquot of 1 μL of the solution was mixed with 1 μL of 3-nitrobenzyl alcohol (3-NBA) saturated with NaI on the FAB probe tip. CID of the precursor ions selected by first mass spectrometer (E_1B_1) occurred in the collision cell located between B_1 and E_2 and floated at 3.0 kV. The product ions were analyzed using a B/E scanning method with a second mass spectrometer (E_2B_2). Helium was introduced into the collision chamber as a collision gas at a pressure sufficient to reduce the precursor ion signal by 70%.

NMR

^{1}H and ^{13}C NMR spectra were recorded on a Varian UNITY 500 NMR spectrometer working at 500 MHz for proton and 125 MHz for carbon. The ^{1}H and ^{13}C NMR chemical shifts were attributed to CD_3OD at 3.30 and 49.0 ppm.

RESULTS AND DISCUSSION

FAB of cerebrosides AA-1 to AA-6 isolated from starfish eggs using 3-nitrobenzyl alcohol (3-NBA) saturated with sodium iodide (NaI) yielded prominent sodium-adducted molecules ($[M + Na]^+$) with peaks at *m/z* 792.6, 792.6, 858.6, 776.6, 830.6, and 844.6 in each mass spectrum. The FAB mass spectrum and results for AA-1 are shown in Fig. 2(a) and listed in Table 1, respectively. The molecular formula of AA-1 was determined as $C_{44}H_{83}O_9NNa$ using high-resolution mass measurements at *m/z* 792.5968 (Δ +0.3 mmu). This result is summarized in Table 1 with the results of other cerebrosides. In the FAB mass spectrum of AA-1, abundant fragment ions corresponding to T and O ions identified by Ann and Adams[19] were observed at *m/z* 320 and 538, respectively. High-energy CID of the sodium- adducted molecules fragmented extensively and provided information about the polar head group, the composition of sphingoid bases and the fatty acid portion from the presence of diagnostic ions (Fig. 2(b)). The product ions (^{1,5}X and ^{0,2}X), generated by cross-ring cleavage of the sugar ring, were observed at *m/z* 658 and 672, and B, C, and Y ions, from glycosidic bond cleavage between the sugar ring and the sphingoid long-chain base, were observed at *m/z* 185, 203, and 630, respectively. Our notation follows the common nomenclature for carbohydrate fragmentations proposed by Domon and Costello.[18] These structural features indicated that the sugar residue was the polar head group. The concomitant cleavage of the glycosidic O–C bond and the C–C bond between the 3-hydroxy carbon of LCB and the adjacent carbon formed prominent T ions at *m/z* 320, providing information on the composition (h16:0) of the fatty acyl group. The predominant O ion at *m/z* 538 arose from cleavage of the amide N–CO bond with hydrogen transfer of an α-hydroxy group on the *N*-acyl chain, providing information about the composition (d22:2) of the sphingoid LCB. Moreover, there were two abundant series of high-mass product ions, formed by charge-remote cleavage of the fatty acyl chain and the long chain of the sphingoid base (Fig. 3(a)). A series of homologous ions generated from C_nH_{2n+2} losses along the saturated fatty acyl chain via charge-remote elimination reactions[25] appeared from *m/z* 776 to 594 with a 14 Da interval, with the exception of a 16 Da mass difference, due to CH_4 loss from

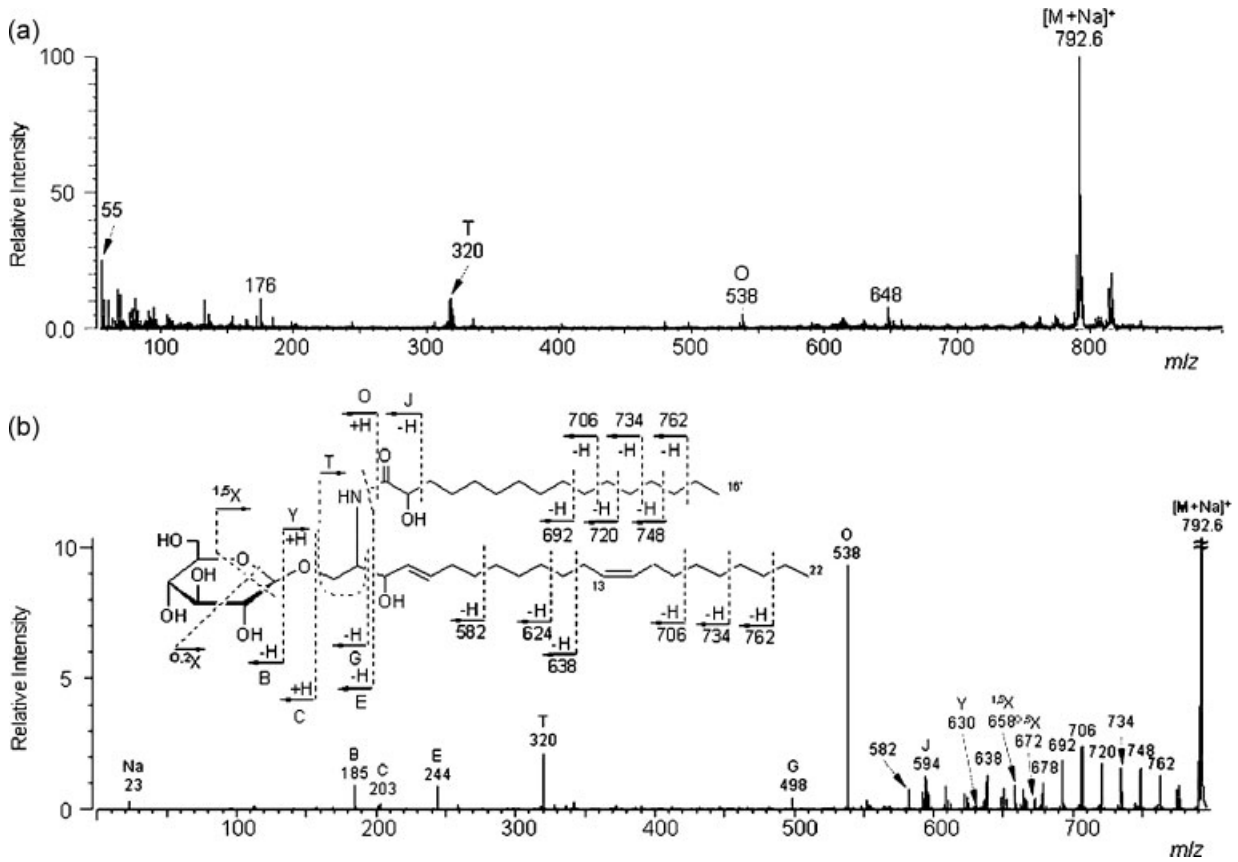

Figure 2. (a) Mass spectrum of AA-1 ionized by FAB after mixing with a 3-NBA matrix saturated with NaI and (b) high-energy CID tandem mass spectrum of the sodium-adducted molecule, $[M + Na]^+$, of AA-1 observed at *m/z* 792.6. Fragmentation pathways observed in the CID of $[M + Na]^+$ are also shown. All product ions contained sodium.

the alkyl end. In the fragmentation study of ceramides by Ann and Adams,[13] the abundant peaks at *m/z* 594 and 608 corresponded to J and K ions, respectively, and provided information on the composition of the *N*-acyl chain. The *N*-fatty acyl group of the AA-1 cerebroside was identified as a 2-hydroxy-*N*- palmitoyl group. The spectral pattern of these homologous ions was similar to that of methyl 2-hydroxylhexadecanoate, synthesized by acidic methanolysis of the cerebroside (Fig. 3(b)). The abundance of peaks observed at *m/z* 594 (J ion) and 111 in Figs. 3(a) and 3(b), respectively, was due to the conjugation stability of the product ion, *N*-acyl aldehyde, which formed via the cleavage of the C–C bond adjacent to an α-hydroxyl group and concomitant hydrogen transfer.

The concurrent observation of an even-electron ion at *m/z* 111 and an odd-electron radical ion at *m/z* 112 in the high-energy CID spectrum of FAME demonstrated that the charge-remote fragmentation (CRF) along the long hydrocarbon chain occurred in two steps: homolytic cleavage of a C–C bond, followed by elimination of a radical (usually H•)

Table 1. Summary of cerebroside species isolated from *Asterias amurensis* starfish eggs

Species	$[M + Na]^+$, *m/z* Observed	$[M + Na]^+$, *m/z* Theoretical	Δm/M (ppm)	Molecular formula	T, O ions, *m/z*[a] T	T, O ions, *m/z*[a] O	Sphingoid base/*N*-acyl group[b]
AA-1	792.5968	792.5966	0.3	$C_{44}H_{83}O_9NNa$	320	538	d22:2$^{\Delta4,13}$/h16:0
AA-2	792.5966	792.5966	0	$C_{44}H_{83}O_9NNa$	320	538	d22:2$^{\Delta4,9}$/h16:0
AA-3	858.6433	858.6435	–0.2	$C_{49}H_{89}O_9NNa$	430	494	d19:3 $^{9\text{-methyl-}\Delta4,8,10}$/h24:1$^{\Delta15'}$
AA-4	776.5658	776.5653	0.7	$C_{43}H_{79}O_9NNa$	348	494	d19:3 $^{9\text{-methyl-}\Delta4,8,10}$/h18:0
AA-5	830.6124	830.6122	0.2	$C_{47}H_{85}O_9NNa$	416	480	d18:3 $^{\Delta4,8,10}$/h23:1$^{\Delta14'}$
AA-6	844.6279	844.6279	0.1	$C_{48}H_{87}O_9NNa$	430	480	d18:3 $^{\Delta4,8,10}$/h24:1$^{\Delta15'}$

[a] T and O ions provided information about the compositions of *N*-fatty acyl groups and sphingoid bases, respectively.
[b] The numbers after Δ indicate the position of the double bonds present in sphingoid bases and fatty acyl chains. The superscript 9-methyl indicates that the methyl group was positioned at C-9 of the sphingoid chain.

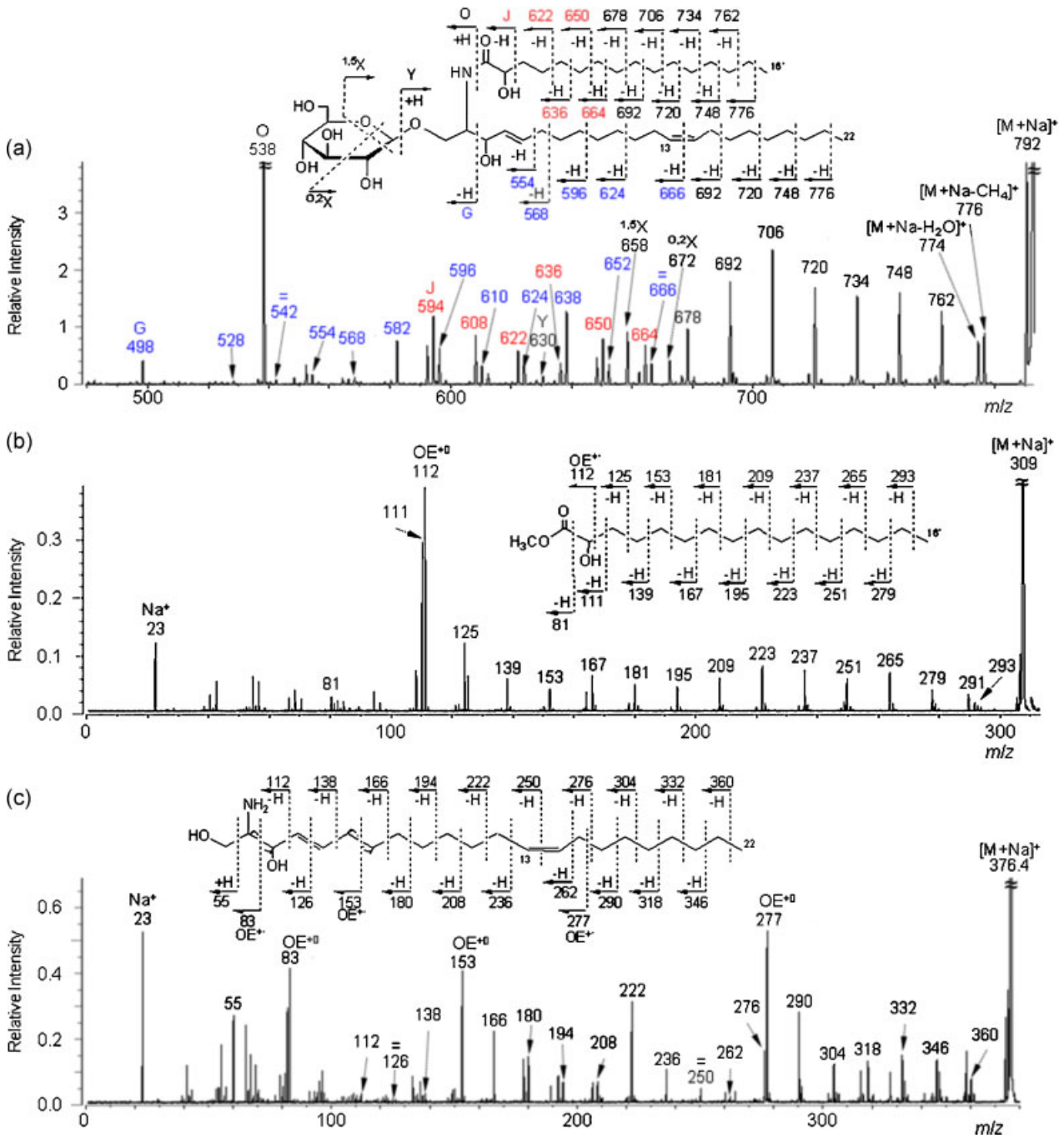

Figure 3. Comparison of the peak patterns from charge-remote fragmentation along the hydrocarbon chains of AA-1 and its fatty acid methyl ester (FAME) and sphingosine derived by acidic methanolysis. (a) High-mass region of the tandem mass spectrum shown in Fig. 2(b) and high-energy CID tandem mass spectra of sodium-adducted molecules, $[M + Na]^+$, of (b) FAME and (c) sphingosine. The pathways of the homologous ions generated by CRFs along the hydrocarbon chains of each $[M + Na]^+$ ion are also shown, along with their structures. In (a), each series of ions from CRFs along the sphingoid chain and the *N*-acyl group are labeled in blue and red, respectively, and the common ions are labeled in black. An equals sign above a peak indicates the double-bond position.

and thermal-like 1,4-eliminations.[26] The CRF along the LCB chain resulted in a series of product ions with the neighboring peaks in the series separated by 14 Da, including common ions of two series from *m/z* 776 to 678. However, the presence of double bonds in the LCB chain reduced the neighboring peak separation to 12 Da, similar to the result from our previous high-energy CID studies of glycoglycerolipids.[21]

The spectral pattern in the LCB series at *m/z* 666(=), (where '=' represents the double-bond position), 652, 638, 624, 610, 596, 582, 568, 554, 542(=), 528, and 498 (G ion, denoted according to Ann and Adams[13]) identified the positions of double bonds and hydroxyl group. Thus, the sphingosine unit of AA-1 was characterized as docosasphinga-(4,13)-dienine. The similarity of the spectral patterns between this

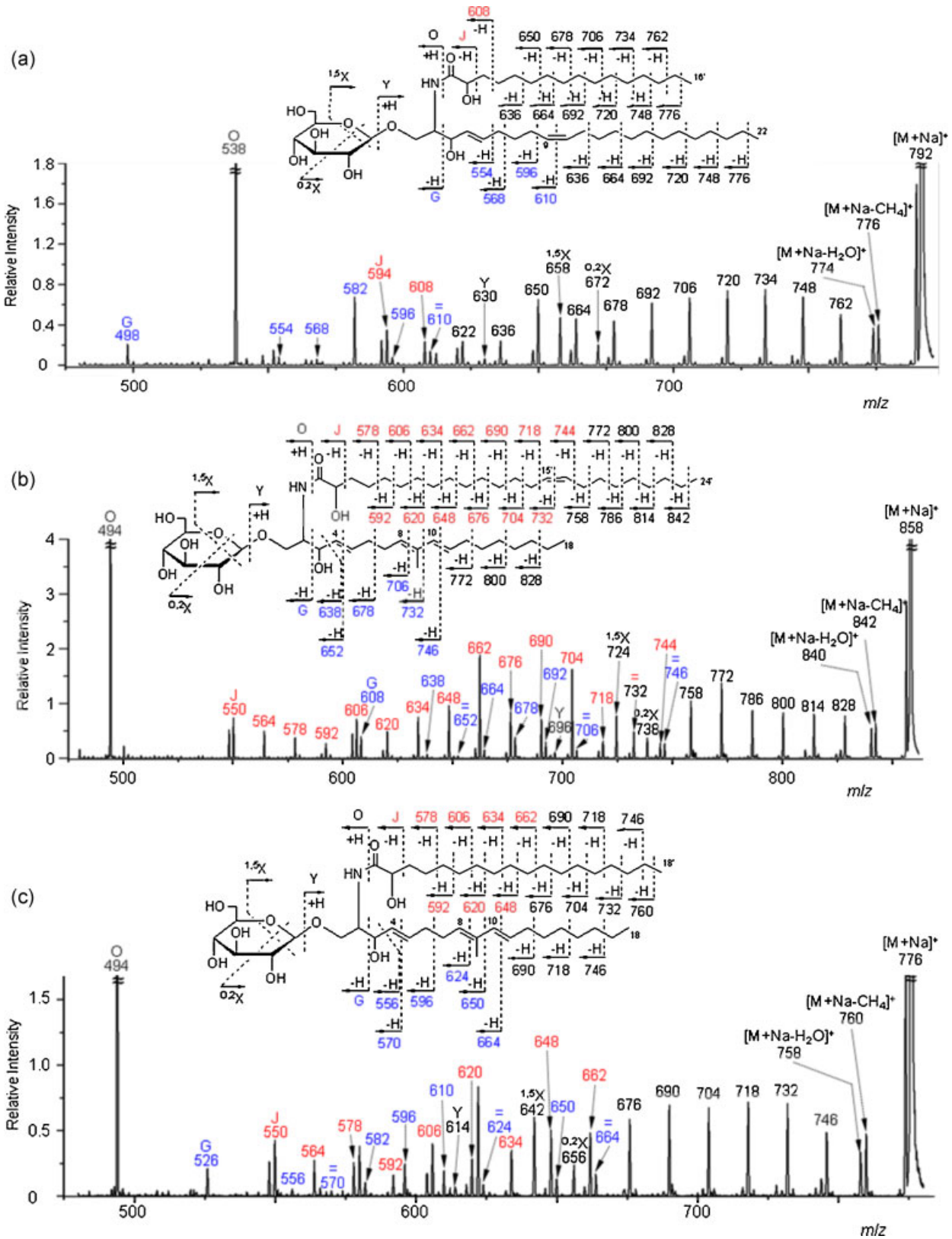

Figure 4. High-mass region of tandem mass spectra for the $[M+Na]^+$ ions of (a) AA-2, (b) AA-3, and (c) AA-4. Each series of ions from CRFs along the sphingoid chain and the *N*-acyl group are labeled in blue and red, respectively, and the common ions are labeled in black. An equal sign above a peak indicates the double-bond position.

series of ions and the corresponding ions of sphingosine obtained by methanolysis of AA-1 confirmed the structure of the sphingosine unit (with the exception of odd-electron ions at *m/z* 83, 153, and 277). In particular, the abundance of the peaks at *m/z* 498, 582, 638, and 706 and the weakness of the peaks at *m/z* 528, 542(=), 554, and 568 (Fig. 3(a)) corresponded to those of the ions at *m/z* 82, 166, 222, and 290 and the ions at *m/z* 112, 126(=), 138, and 152 (Fig. 3(c)), respectively. The odd-electron ions in Fig. 3(c), formed via homolytic cleavage of C–C bonds, indicated abundant peaks because the allylic radical was stabilized by a double bond. Thus, in addition to the mass difference between neighboring ions, relative peak intensities were also helpful in the determination of the double-bond positions in the *N*-acyl chain and the sphingoid LCB.

On the other hand, the ^{1}H and ^{13}C NMR study of AA-1 revealed that the sugar moiety was a *β*-glucopyranoside, and Δ^{4} and Δ^{13} double bonds in the sphingoid LCB had *E* and *Z* geometries, respectively. Thus, the gross structure of the AA-1 cerebroside was determined to be (4*E*,13*Z*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[2-hydroxyhexadecanoyl]amino-4,13-docosadiene. The high-energy CID spectrum (Fig. 4(a)) of sodium-adducted molecules, *m/z* 792.6 of AA-2, was analogous to that of AA-1, except for a spectral difference due to the double bonds positioned at C-4 and C-9 on the sphingoid base. The gross structure of the AA-2 cerebroside was characterized as (4*E*,9*Z*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[2-hydroxyhexadecanoyl]amino-4,9-docosadiene. The T and O ions in the high-energy CID spectrum of the sodium-adducted molecules, *m/z* 858.6 of AA-3, were observed at *m/z* 430 and 494, respectively (Fig. 4(b)). The compositions of the *N*-acyl chain and the sphingoid LCB from these ions were assigned as h24:1 and d19:3, respectively. The product ion peak at *m/z* 732 from the series of ions generated by the fragments of the *N*-acyl chain, starting from the J ion at *m/z* 550, had a mass difference of 12 Da relative to the neighboring ion at *m/z* 744. The double-bond position at C-15′ was confirmed by the

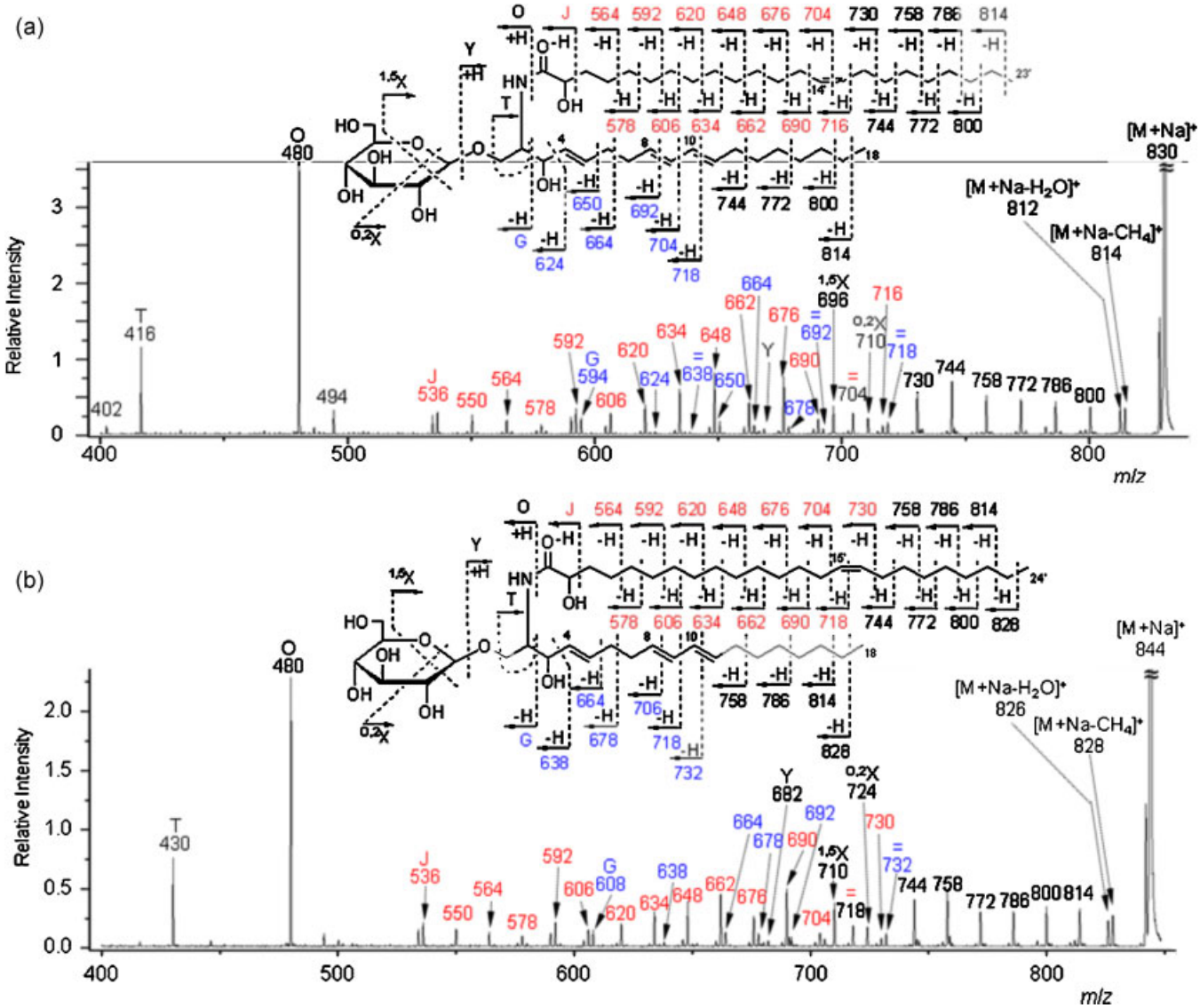

Figure 5. High-mass region of tandem mass spectra for the $[M + Na]^{+}$ ions of (a) AA-5 and (b) AA-6. The peak intensity in each MS/MS spectrum was normalized to that of the most abundant O ion.

abundant peak at *m/z* 704, due to the cleavage of the allylic C–C bond. On the other hand, the positions of double bonds in the LCB chain were at C-4, C-8, and C-10, according to the spectral pattern of the series of ions appearing at *m/z* 608 (G ion), 638, 652(=), 664, 678, 692, 706(=), 732, and 746(=), including common ions of two series from *m/z* 842 to 758. However, the methyl group was expected to be at C-9 or C-10 due to the presence of ions at *m/z* 718 and 732, which commonly occur with CRF ions of the *N*-acyl group. The peak intensity at *m/z* 732 was higher than that at *m/z* 718 because of the peak that occurred with the fragmentation of the *N*-acyl group. The high-energy CID spectrum (Fig. 4(c)) of AA-4, with the same sphingoid composition but the saturated *N*-acyl group (h18:0), showed that the peaks at *m/z* 624(=), 650, and 664(=) were not common to those of the saturated *N*-acyl group. The comparison demonstrated that the methyl group on the sphingoid LCB was positioned at C-9 in the middle of the (4,8,10)-sphingenine residue. Based on the these results and NMR data, AA-3 and AA-4 were characterized as (4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[2-hydroxy-15-tetracosenoyl]amino-9-methyl-4,8,10-octadecatriene and (4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[2-hydroxyoctadecanoyl]amino-9-methyl-4,8,10-octadecatriene, respectively.

Similarly, another species of AA-5, *m/z* 830.6, which was identified by an unsaturated *N*-acyl group (h23:1) and sphingoid base (d18:3) from the T and O ions at *m/z* 416 and 480, respectively, contained 2-hydroxy-14-tricosenoyl and 3-hydroxy-4,8,10-octadecatrienyl groups (Fig. 5(a)). It appears that there are two ions each ±14 mass unit (*m/z* 494 and 402) from the O and T ions identified. This suggests that isomeric species of the type d19:3/h22:1 may be present as isobaric minor component. However, as shown in Fig. 5(a), the peak intensity of the minor O ion at *m/z* 494 relative to that of the major O ion at *m/z* 480 was smaller than 10%. The spectral pattern of AA-6 (Fig. 5(b)) was similar to that of AA-5, but there were differences in their chain lengths and double-bond position, C-15′ of the *N*-acyl group (h24:1). Thus, the structures of AA-5 and AA-6 were assigned as (4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[2-hydroxy-14-tricosenoyl]amino-4,8,10-octadecatriene and (4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[2-hydroxy-15-tetracosenoyl]amino-4,8,10-octadecatriene, respectively.

The structural determination of cerebrosides, which have important biological functions, was completely performed by FAB-CID-MS/MS. The high-energy CID tandem mass spectra of sodium-adducted molecules of cerebrosides provided the information on the composition of the *N*-acyl group and the sphingoid LCB, as well as the sugar moiety. In particular, charge-remote fragmentation that is only possible in high-energy CID occurred along the hydrocarbon chains of fatty acyl groups, and the LCB chains were used to locate the double-bond positions and methyl branching. This direct and rapid method has great potential for determining the complete structure of individual components present in a mixture of sphingolipids isolated from biological sources, including sphingosine, ceramides, and sphingomyelins.

Acknowledgements

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