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# Fast-atom-bombardment chemistry of sulfatide (3-sulfogalactosylceramide)

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ABSTRACT

F.a.b.-m.s. of sulfatide (3-sulfogalactosylceramide, **1**) in the negative-ion mode reveals the molecular weight and the presence of the sulfate ester without ambiguity. The size of the long-chain base is represented as the $(M - H)^-$ ion of the corresponding lyso-form (deacylated molecule) only when the fatty acid is α-hydroxylated. The spectrum in the positive-ion mode indicates the molecular weight by a pair of sodium adduct ions separated by 102 a.m.u. However, the desulfated fragment ion is not shown in the *B*/*E*-constant linked-scanning mode. If the long-chain base ion $[CH_2C(NH_2)=CHR]^+$ is present, *B*/*E*-constant linked scanning confirms that the double bond is located at C-4. *B*/*E*-Constant linked scanning of the $(M - H)^-$ ions in the negative-ion mode indicates the location of a double bond in the fatty acid moiety at C-15 for the most abundant molecular species c24:1.

## INTRODUCTION

The sulfatide from mammalian nervous tissues is 3-sulfogalactosylceramide[1] [β-Gal-3$SO_3$H-(1→1′)-Cer]. Naturally occurring sulfatides show variations in structure, usually in the fatty acid moiety and sometimes in the long-chain base. Sulfatide is one of the two major acidic glycosphingolipids in mammalian tissues and is responsible for some of the negative charge on the cell membranes, accumulating abnormally in the white matter of the brain of patients with metachromatic leukodystrophy. Sulfatide specifically binds to the myelin basic proteins in the brain, is involved in the transport of electrolytes in the kidney, and shows stage-specific appearance in some normal and diseased tissues. The therapeutic effects of protease inhibitors encapsulated with liposomes prepared with sulfatides from different origins were not the same for experimental allergic encephalomyelitis[2]. Attempts to detect structural differences between sulfatides from human brain and other mammalian central nervous tissues led to the f.a.b.-m.s. study now reported.

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## EXPERIMENTAL

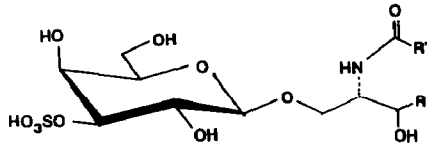
Sulfatide was isolated[3] from human brain, bovine spinal cord, guinea pig brain, and guinea pig spinal cord, and purified. Bovine brain sulfatide (Supelco Co.) was used without further purification. 6-Sulfogalactosylceramide was partially synthesized from human brain galactosylceramide following the method of Taketomi *et al.*[4], and purified by h.p.l.c. T.l.c. was performed on 5 × 20 cm high-performance Kieselgel 60 plates (Merck), using chloroform–methanol–acetone–acetic acid–water (8:2:4:2:1), and detection with $I_2$, orcinol/$H_2SO_4$, or by charring with $H_2SO_4$. Only two spots with $R_F$ 0.35 and 0.42, corresponding to sulfatides of normal and α-hydroxylated fatty acid, respectively, were detected.

For f.a.b.-m.s., typically the sample (1 mg) was dissolved in chloroform–methanol (1:1, 1 mL) and an aliquot (2 μL) was applied onto the f.a.b. target together with 0.5 μL of the matrix (triethanolamine, *m*-nitrobenzyl alcohol, glycerol, or glycerol–thioglycerol). Mass spectra were obtained with a JEOL JMS-HX110 double-focusing mass spectrometer with a JMA-DA5000 data system, Xe atoms at 6 keV (primary beam), and an ion-acceleration energy of 10 keV. Collision-activated dissociation (c.a.d.) was carried out with He gas introduced into a collision chamber placed in the first field-free region of the *EB* geometry. Special care was taken to calibrate the low-mass region in order to detect metastable ions of small *m/z* in the *B/E*-constant linked-scanning mode[5].

## RESULTS AND DISCUSSION

The positive-ion f.a.b.-mass spectrum of sulfatide (**1**) contained peaks* for $(M + Na)^+$, where M represents the Na salt of the sulfate ester **1**, and for $(M' + Na)^+$, where M′ denotes the desulfatated molecule (galactosylceramide, CMH). These ions are separated[6] by 102 a.m.u., and the characteristic ion distributions resulting from the fatty acid diversity are similar, as shown in Fig. 1.

The $(M' + Na)^+$ ions could be due to the $(CMH + Na)^+$ ions of contaminants, because the c.a.d.-mass spectrum with $(M + Na)^+$ of sulfatide as the parent failed to show the corresponding $(M' + Na)^+$ ion as its daughter (Fig. 2). However, the following data confirm that $(M' + Na)^+$ ions are true fragment ions of $(M + Na)^+$ ions. (*a*) T.l.c. revealed the sulfatides of normal and α-hydroxylated fatty acids, with only traces of galactosylceramide and even less of gangliosides. (*b*) The f.a.b.-mass spectra of sulfa-

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* The masses are reported as nominal masses except for the c.a.d.-mass spectra.

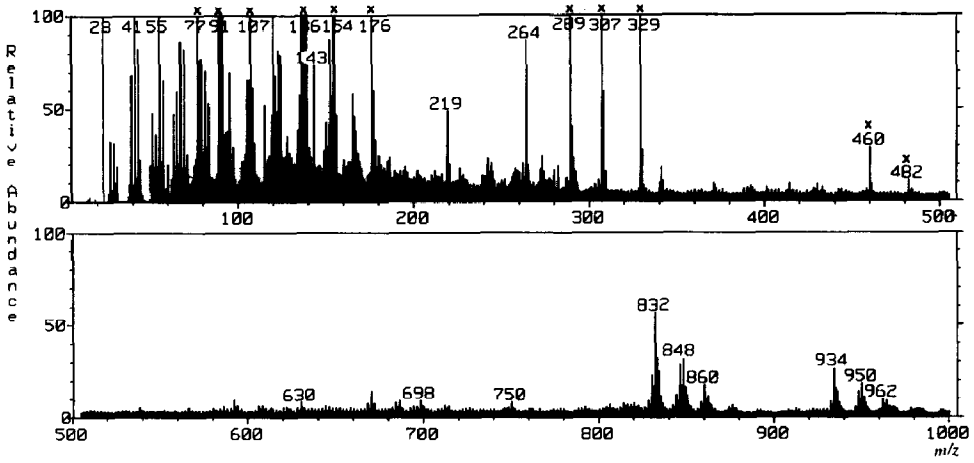



Fig. 1. Positive-ion f.a.b.-mass spectrum of human brain sulfatide (matrix: *m*-nitrobenzyl alcohol): $(M + Na)^+$ ions are at *m/z* 934, 950, and 962, *etc.*, while $(M' + Na)^+$ ions are at *m/z* 832, 848, and 860, *etc.*, The $LCB^+$ ion (*m/z* 264) indicates that the long-chain base is d18:1. Matrix ions are marked ×.

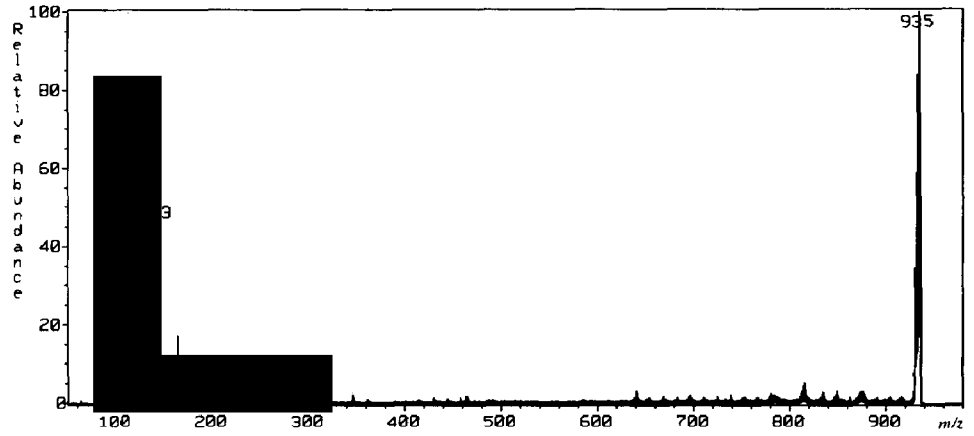
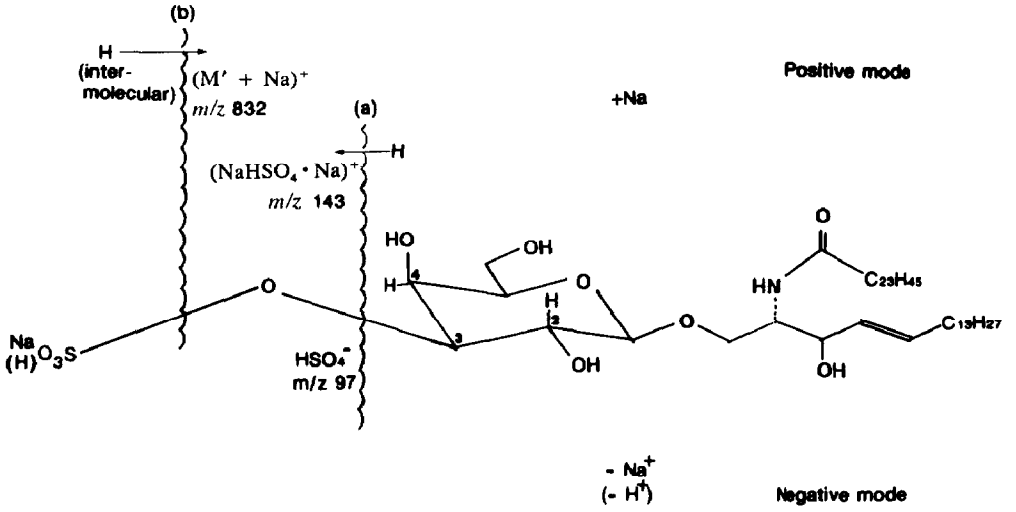



Fig. 2. Positive-ion f.a.b.-c.a.d.-mass spectrum. Daughters of $(M + Na)^+$ ion of bovine spinal cord sulfatide (d18:1/c24:1, *m/z* 935). Matrix: triethanolamine plus a trace of aqueous NaCl in order to enhance the intensity of the $(M + Na)^+$ ions.

tides in the negative-ion mode did not show the $(M - H)^-$ ions of the corresponding CMHs. (*c*) A FRIT*-f.a.b.-mass chromatogram in the positive-ion mode, using reverse-phase h.p.l.c. (ODS column) with a constant mobile phase (acetonitrile–water–acetic acid 97:3:0.2 plus 0.5% of *m*-nitrobenzyl alcohol), showed exactly the same retention times for $(M + Na)^+$ ions and the $(M + Na - 102)^+$ ions.

A failure to show the $(M + Na - 102)^+$ ions as daughters of the $(M + Na)^+$ ions (Fig. 2) is due to the fact that desulfatation is effected only with a hydrogen supply from

* Continuous flow-type l.c.-interface.



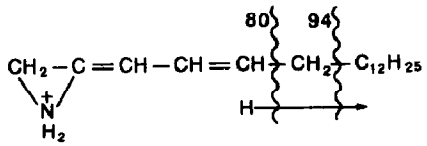

Scheme 1. Illustration of C-3—O (a) and O—S (b) cleavages of sulfatide. Cleavage (a) involves intramolecular hydrogen transfer and produces the sulfate-side fragment ions both in the positive- and negative-ion modes. Cleavage (b) involves intermolecular hydrogen transfer and gives rise to the $(M' + Na)^+$ ion, corresponding to the mass of $(CMH + Na)^+$ in the positive-ion mode. An NFA-type sulfatide (d18:1/c24:1) was chosen as representative.

other molecules such as the matrix, moisture, or a neighbouring molecule of the same species (Scheme 1), *i.e.*, desulfatation with O–S bond breaking is not a gas-phase unimolecular dissociation.

For most neutral sphingolipids[7–9], the long-chain base is shown as $LCB^+$* $[CH_2C(NH_2)=CHR]^+$. However, $LCB^+$ is not always formed from acidic glycolipids, such as $G_{Q1b}[IV^3(NeuAc)_2,II^3(NeuAc)_2–GgOse_4Cer]$. When a stable $LCB^+$ ion is formed, the size of the long chain-base can be determined. The $LCB^+$ ion was detected at *m/z* 264 for all the sulfatides examined, at least for the major long-chain base (Fig. 1).

Moreover, c.a.d.-m.s. having the $LCB^+$ ion as the parent showed a series of ions as a result of charge-remote fragmentation[10] (c.r.f.). C.r.f. is observed primarily as a result of high-energy collisions using a double-focusing instrument. One such ion of highest intensity at *m/z* 94 corresponds to $C_6H_8N^+$ and indicates that there are four double-bond equivalents within C-1/6 of the sphingenine moiety. Double bonds or their equivalents are produced between C-2 and C-3 by dehydration of the allylic alcohol (leaving one hydrogen at C-3), between C-1 and C-2 by the formation of the $LCB^+$ ion (aziridinium ion), and by ring formation within C-6 and C-1. Therefore, the remaining double bond must have been present in the original sphingenine structure either at C-4 or C-5. However, if another ion at *m/z* 80 is also taken into consideration, the only possible location is at C-4 (Scheme 2). Details about the c.r.f. of sphingolipids will be reported elsewhere. The ion at *m/z* 143 corresponds to $(NaHSO_4 + Na)^+$ (Scheme 1),

* Originally, this ion was designated[8] as $Z^+$, but the more characteristic designation $LCB^+$ is now used.




Scheme 2. Possible scheme for the formation of the ion at $m/z$ 94 as a daughter of the LCB$^+$ ion ($m/z$ 264) for $\Delta^4$-sphingenyl compounds.

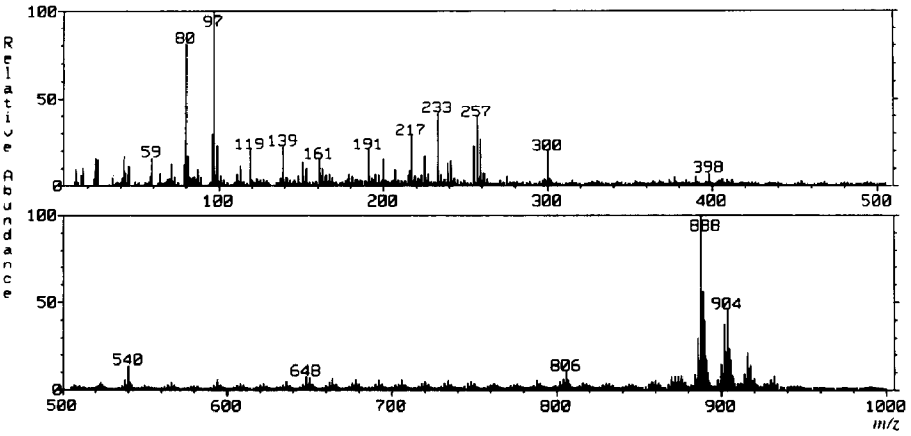



Fig. 3. Negative-ion f.a.b.-mass spectrum of human brain sulfatide (matrix:triethanolamine). A peak at $m/z$ 888 is for the (M − H)$^-$ ion of c24:1, since the long-chain base is already known as d18:1 from the ion at $m/z$ 264 produced in the positive-ion mode (Fig. 1). The ion at $m/z$ 904 corresponds to the molecular species of α-hydroxylated fatty acid of the same chain-length (c24h:1). The fragment ion at $m/z$ 540 is a daughter of such sulfatides with α-hydroxylated fatty acids, corresponding to the (M − H)$^-$ ion of the lyso-form (sulfohexosylsphingenine; d18:1). The ions at $m/z$ 97 and 80 for $HSO_4^-$ and $SO_3^{-\cdot}$, respectively, confirm the presence of a sulfate group.

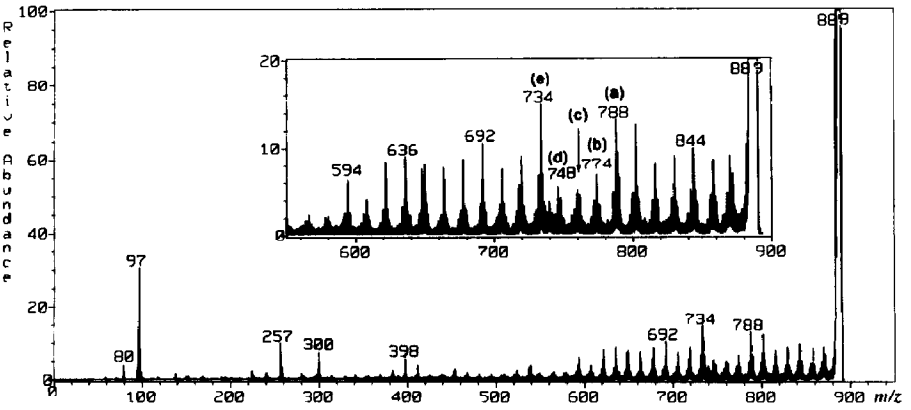


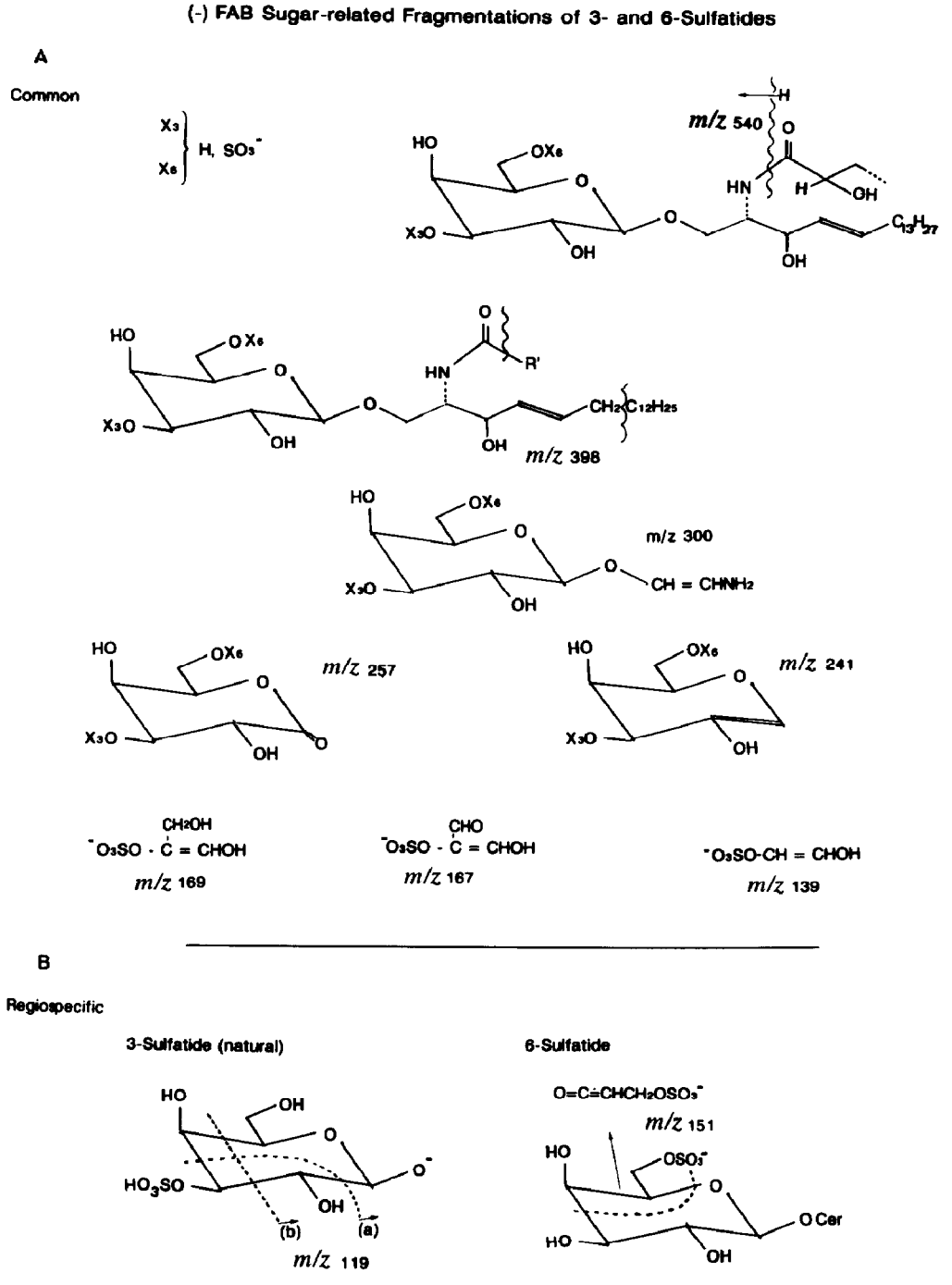

Fig. 4. Negative-ion f.a.b.-c.a.d.-mass spectrum: daughters of the (M − H)$^-$ ion of bovine spinal cord sulfatide (d18:1/c24:1; molecular weight, 889). Matrix: triethanolamine.

which is a daughter of the $(M + Na)^+$ ion of sulfatide in the c.a.d.-mass spectrum (Fig. 2). This ion, together with a negative ion at *m/z* 97 (Figs. 3 and 4), means that the cleavage between C-3 and O of the 3-sulfogalactopyranose ring occurs not only inside the f.a.b. ion source but also in the first field-free region as a result of a unimolecular dissociation of the parent in the gas phase. Studies using space-filling molecular models (CPK) show that both H-2*αx* and H-4*eq* are available for abstraction by $^-O_3SO$– *via* 6-membered transition states. Desulfatation by cleavage of the C–O bond through an intramolecular H transfer contrasts with the above-mentioned cleavage of the O–S bond which involves intermolecular H transfer (Scheme 1).

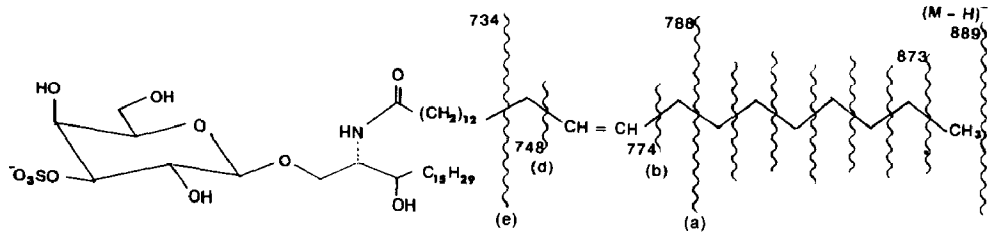
Negative-ion f.a.b.-m.s. of sulfatide (Fig. 3) gave $(M - H)^-$ ions. For HFA(α-hydroxylated fatty acid)-type sulfatides, the size of the long-chain base is shown as $(M - H)^-$ of the corresponding deacylated molecule (lyso-form). The ion at *m/z* 540 (Fig. 3) appears to be related to the $LCB^+$ ion at *m/z* 264 for d18:1 in the positive-ion mode (Fig. 1). It is clear that this ion is not the $(M - H)^-$ of a contaminant lyso-sulfatide, because it appears as a daughter of the $(M - H)^-$ ion of an HFA-type sulfatide such as c24h:1. However, the ion at *m/z* 540 was not detected as a daughter of *m/z* 889 (nominally 888), the $(M - H)^-$ ion of the c24:1 species, when the low-energy tandem m.s. (m.s./m.s.) study was performed using a triple-stage quadrupole mass spectrometer[3]. The presence of a hydroxyl group next to the carbonyl group facilitates the elimination of ketene from the amide *via* an enediol structure, which is not feasible for a non-hydroxylated amide. Thus, the fragment ion with the lyso-form structure is derived from the HFA-type sulfatide only, at least for low-energy c.a.d.-m.s.

The presence of the fragment ion at *m/z* 97 in the negative-ion c.a.d.-spectrum (Figs. 3 and 4) is not sufficient to confirm a sulfate derivative, because a phosphate derivative also produces an ion with *m/z* 97 $(H_2PO_4)^-$, but ions with *m/z* 79 $(PO_3)^-$ and 63 $(PO_2)^-$ discriminate phosphate from sulfate, the second characteristic ion of which has *m/z* 80 $(SO_3)^{-\cdot}$. These diagnostic fragment ions for sulfate and phosphate are daughters of the respective $(M - H)^-$ ions regardless of the type of fatty acid. Two other negative daughter ions related to the sulfate ester of hexosylceramide appear at *m/z* 398 and 300, and are assignable to the partial structure shown in Scheme 3.

A c.r.f. observed in the negative-ion mode is also of special interest. The inset in Fig. 4 shows the higher-mass region of a negative-ion mode f.a.b.-c.a.d. mass spectrum with the $(M - H)^-$ ion of the (d18:1/c24:1) species as the parent, and Scheme 4 illustrates C–C bond cleavages by the c.r.f. A series of c.r.f. ions with hydrogen transfer appearing at $(M - H - 16)^-$ and $[(M - H - 16) - (CH_2)_n]^-$ (ref. 10) show maxima at *m/z* 788(a) and 734(e). There are ions of low intensity in between, appearing at *m/z* 774(788 − 14) (b), an odd-numbered ion at *m/z* 761(774 − 13) (c), and another at *m/z* 748 (761 − 13 = 734 + 14) (d). These five ions suggest that the double bond in the c24:1 is located between peaks (b) and (d), by showing intensive allylic cleavages (a and e) and moderate vinylic cleavages (b and d) with near-symmetrical intensities of the peaks. Since peak (c) is the tenth peak from the parent (*m/z* 889 or nominally 888), the position of the double bond must be at C-15 counting from the carbonyl carbon in the fatty acid moiety. Indeed, this series of ions was observed clearly down to *m/z* 594 (for C-3).




Scheme 3. Negative-ion f.a.b. fragmentations related to the sugar moiety: A, fragment ions that include hexopyranosyl sulfate; B, ring-opening fragment ions. For simplicity, the structures of the ions for *m/z* 169, 167, and 139 are represented by one typical resonance or isomeric form.




Scheme 4. Charge-remote fragmentation of the $(M - H)^-$ ion of bovine spinal cord sulfatide (d18:1/c24:1) shown in Fig. 4: (a) and (e) indicate allylic cleavages, (b) and (d) indicate vinylic cleavages. The integral mass numbers shown are 1 a.m.u. higher than the corresponding nominal masses in the mass range $m/z > 844$ because of the accumulation of the mass defect of hydrogen.

Also of importance in the negative-ion mode is another family of daughter ions related to the structure of galactose sulfate. In Figs. 3 and 4, as well as the corresponding spectra taken with the low-energy instrument TSQ 70 (data not shown), these daughter ions are detected at $m/z$ 257 or 241 (dehydrogenated or dehydrated hexose sulfate), 169 or 167 (low intensity) [$^-O_3SOC(CH_2OH)=CHOH$ or its dehydrogenated form], and 139 [$^-O_3SOCH=CHOH$] for both naturally occurring 3-sulfogalactosylceramide (3-sulfatide) and a semi-synthetic C-6 isomer (6-sulfatide). However, an ion at $m/z$ 119 was observed in the negative-ion normal-scanning mode f.a.b.-mass spectrum of 3-sulfatide, but not of the 6-sulfatide. By analogy with neutral glycosphingolipids, it is assumed that this ion is a reducing-side fragment produced by rupture of the ring either at C-1–C-2 and C-3–C4(a) or C-2–C-3 and C-4–C-5(b). In either cleavage, the resulting ion at $m/z$ 119 does not contain the strongly negative functional group. As mentioned above, desulfatation to give neutral CMHs occurs only inside the ion source, and it is of interest that the fragment ion at $m/z$ 119 is more prominent in the normal scanning mode but hardly detected in the $B/E$-constant linked-scanning mode. On the other hand, only for the 6-isomer was a fragment ion detected in both the normal scanning and $B/E$-constant linked-scanning modes at $m/z$ 151, which probably corresponds to $OC=CHCH_2O-SO_3^-$ derived from the sulfate ester of a primary alcohol (Scheme 3). These two ions, which are characteristic of individual structures, were also confirmed by the m.s./m.s. study using a triple-stage quadrupole mass spectrometer.

Daughter ions of a molecular ion give more specific information than normal scans in mass spectrometry. Especially is this true where matrix-derived ions interfere in the assignment of fragment ions, such as in f.a.b. or s.i.m.s. Fragment ions are detected as daughters only if the molecular ion decomposes unimolecularly in the gas phase after being accelerated fully but before entering a field. The example is now reported where an ion (hexosylceramide + $Na^+$), possibly suspected as a contaminant because of its absence in the $B/E$-constant linked-scanning mode, is a real fragment ion of the sulfogalactosylceramide. Thus, f.a.b.-m.s. with a double-focusing instrument is even more useful for elucidating the structure of glycolipids if the linked scanning and normal scanning modes are combined.

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