Squid nerve sphingomyelin containing an unusual sphingoid base

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Abstract **A new methodology has been developed to determine sphingolipid structures by positive-ion fast atom bombardment tandem mass spectrometry (FAB-MS/MS). The method was verified by application to a structurally known glycosphingolipid, and then used in the structural study of an unusual sphingomyelin isolated from squid (*Loligo pealei*) nerve. Our previous study of this squid sphingomyelin indicated that the major base had a branched C_{19} alkyl chain with three double bonds, two of which were conjugated. The positions of the branching as well as the double bonds of this base were unambiguously determined by directly comparing the product ion spectra of the long-chain base ion (LCB^+) of two ceramides, one derived from squid nerve sphingomyelin and another, glucosylceramide, obtained from starfish spermatozoa. The latter served as the standard because the structure had already been determined by nuclear magnetic resonance (NMR). The precursor ion here was LCB^+, that is, $[CH_2 - C(NH_2) = CHR]^+$, rather than $[M + H]^+$, where R represents the backbone hydrocarbon chain counting from C-4. The results clearly showed that the squid nerve base is identical to the base derived from starfish (*Asterias amurensis*), that is, 2-amino-9-methyl-4,8,10-octadecatriene-1,3-diol. This is the first report in which the detailed structure of a branched polyunsaturated sphingoid base was studied by tandem mass spectrometry without derivatization or the aid of NMR. The occurrence of such an unusual sphingoid base in various phyla and tissues suggests the conjugated polyunsaturated branched sphingoid base plays a significant role in animals.**—Ohashi, Y., T. Tanaka, S. Akashi, S. Morimoto, Y. Kishimoto, and Y. Nagai. **Squid nerve sphingomyelin containing an unusual sphingoid base.** ***J. Lipid Res.*** **2000.** 41: **1118–1124.**

Supplementary key words branched, conjugated d19:3 sphingoid base • sphingomyelin • glucosylceramide • squid nerve • starfish spermatozoa • fast atom bombardment tandem mass spectrometry (FAB-MS/MS) • LCB^+ • precursor ion • product ion spectrum

Sphingolipids, such as sphingomyelin and glycosphingolipids, have a common moiety named ceramide. The ceramide is the amide of a long-chain amino-alcohol called sphingoid base. Previously, we reported a simple method by which to obtain information about the sizes of the sphingoid base and fatty acid separately (1).

In the meantime, we encountered a problem with determining the structure of an unusual sphingoid base from the sphingomyelin of the squid nervous system. The above-stated methodology was successfully applied to determine the degree of unsaturation in the base as d19:3, but it was not easy to determine the locations of these double bonds as well as the presence or absence of branchings. At about the same time, sphingoid base of glucosylceramide of starfish spermatozoa was also reported (2) as d19:3, and the structure was unambiguously established by nuclear magnetic resonance (NMR). We were interested in whether these two d19:3 sphingoid bases were identical or structural isomers.

In the present study, we have further developed our methodology to examine the detailed structures of the sphingoid moiety by choosing the long-chain base ions (LCB^+) as the precursor ions for fast atom bombardment tandem mass spectrometry (FAB-MS/MS). The product ion spectrum of the known d19:3 glucosylceramide exhibited features we would predict from the structure. The similar product ion spectrum of the LCB^+ of sphingomyelin was then measured and the two compared. The results indicate that these two d19:3 sphingoid bases were mass spectrometrically identical in spite of their remote relation to each other.

Abbreviations: LCB, long-chain base (which is the same as sphingoid base); positive-ion FAB-MS/MS, positive-ion fast atom bombardment tandem mass spectrometry.

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MATERIALS AND METHODS

The ceramide d18:1/*N*-(C16:0) was purchased from Sigma (St. Louis, MO) and used without further purification. *N*-Acetyl glucosylceramides with d18:3 and d19:3 sphingoid bases from starfish spermatozoa were prepared by A. Irie and M. Hoshi (Tokyo Institute of Technology, Tokyo, Japan), and kindly given to us.

Squids (*Loligo pealei*) were obtained during the summer of 1986 at the Marine Biological Laboratories (Woods Hole, MA). Sphingomyelin was isolated and purified from optic and cerebral lobes as described previously (3).

Positive-ion FAB mass spectrometry

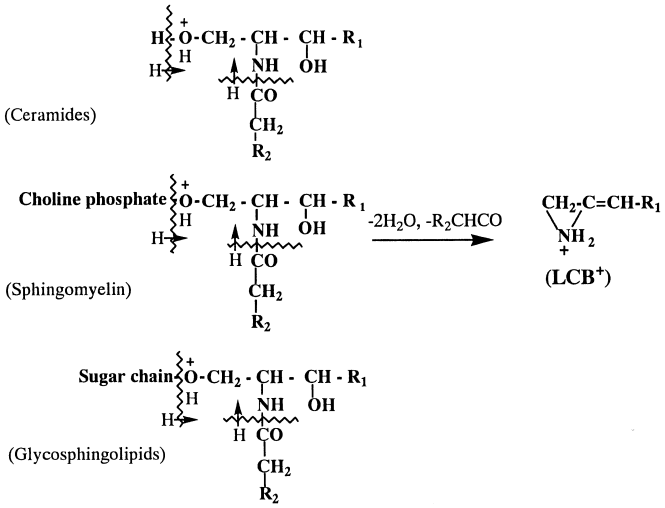
A JMS-HX110/HX110 (EBEB geometry), four-sector tandem mass spectrometer equipped with an array detector (JEOL, Tokyo, Japan) was used for FAB mass spectrometry. The primary beam was xenon gas used at 6 keV and the ion acceleration voltage applied was 10 kV at both M1 and M2. 3-Nitrobenzyl alcohol was used as the matrix. Mass spectra were acquired as positive ions, using either the full scan mode or the collision-induced dissociation (CID) mode. In the latter case, an 8 keV potentially floated helium gas cell inducing 2 keV of collision energy was used.

Because a ceramide is a complex mixture of various fatty acid amides of sphingoid base (long-chain base), it had not been possible to determine each component separately from the *m/z* value of the whole ceramide by FAB mass spectrometry, unless information concerning one of the two variables was known. We (1) have previously reported and Domon and Costello (4) also showed that the sphingoid base structure is represented by a positive ion, LCB^+.[5] This ion is $[CH_2 - C(NH_2) = CHR]^+$, where R represents the backbone hydrocarbon chain, counting from C-4, C-5, and so on until the terminal CH_3. Thus the size of R, and consequently the number of carbons contained in the sphingoid base, is determined according to the above-stated positive-ion structure as a whole mass number. As the size of a sphingoid base increases, it may become difficult to distinguish between isobaric ions $\{-CH = C(OH)-\}$ and a normal $-(CH_2)_3-$, although the former may be distinguished by infrared (IR) or other spectrometry. As illustrated in **Scheme I**, LCB^+ is common among all sphingolipids and does not contain any part of the fatty acid, the polar head groups, such as sugar chains or choline phosphate, or even the primary alcohol itself (ceramide). Thus, the LCB^+ ions of different types of sphingolipids can be directly compared with each other. This is the basis of our present study. Incidentally, the size of the fatty acid amide can also be calculated from the compensation between the $[M + H]^+$ and the size of the sphingoid base thus determined, if M represents the mass of the whole ceramide. For instance, the size of the fatty acyl moiety of a ceramide R′CO−, where R′ represents the fatty alkyl chain, is simply calculated as $\{[M + H]^+ - LCB^+ - 35\}$.

Gross (6) and Adams (7) and many others have extensively studied charge-remote fragmentation (CRF) on the high-energy collision-induced dissociation in the FAB mode. Charge-remote fragmentation shows a product ion spectrum in which the position of a double bond is exhibited as a specific pattern both in the mass interval and in the ion intensities. In addition to the obvious mass interval difference, a double bond, of which the bond energy is larger than a single bond, causes a product ion spectrum with an intensity gap at the double bond and higher ion intensities at the allylic positions.

Thus, we applied this charge-remote fragmentation to a structural study of polyunsaturated sphingoid base structures.

[5] In ref. 1 we named this ion Z^+, whereas Domon and Costello (4) named it a w′′-ion and Ann and Adams (5) called it an O′′-ion. To be fair, we wish to call it LCB^+ from now on.



Scheme I. Positive-ion fast atom bombardment tandem mass spectrometry (FAB-MS/MS) analysis of a sphingoid base of various types of sphingolipid sample. Precursor ion is the LCB^+.

RESULTS

Precise information about an unusual sphingoid base was obtained by applying our own method of FAB product ion spectrometry with the in-source fragment ion as the precursor in the positive-ion mode as described previously (1). The method has advanced even further because of the high accuracy of the tandem instrument with an array detector.

As described above, all sphingolipids have LCB^+ regardless of the head group and fatty acid moieties. Thus, direct comparisons of sphingolipids, including ceramide itself, sphingomyelin, and glycosphingolipids, are feasible.

Preliminary study of trimethylsilyl-sphingoid base by GC/EIMS

In one of our previous studies, the sphingoid base from squid nerve sphingomyelin was converted to trimethylsilyl ether and analyzed by GC/MS. The GC/electron ionization mass spectra of these peaks showed fragment ions characteristic of trimethylsilyl derivatives of normal sphingenines, namely *m/z* 73, 75, 93, 117, 132, 145, and 147, in addition to strong $[M - 74]^+$ ions at high intensity (8, 9). Nevertheless, these ions were relatively small compared with another group of peaks. GC/EI spectra of these peaks showed fragment ions characteristic of sphingenine reported previously, but lacked $[M]^{+\bullet}$ or $[M - 74]^{+\bullet}$ ion in the GC/EI mass spectra.

Present study

Step 1: Positive-ion FAB-MS of a commercial, ubiquitous ceramide, d18:1/N-(C16:0), using a full scan mode. To justify our new methodology, we first measured a simple, typical ceramide, d18:1/*N*-(C16:0) (MW 537), using positive-ion FAB mass spectrometry. As shown in **Fig. 1**, there was an $[M + H]^+$ ion at *m/z* 538 and its dehydrated ion at *m/z* 520 in much higher intensity. Besides, LCB^+ was clearly

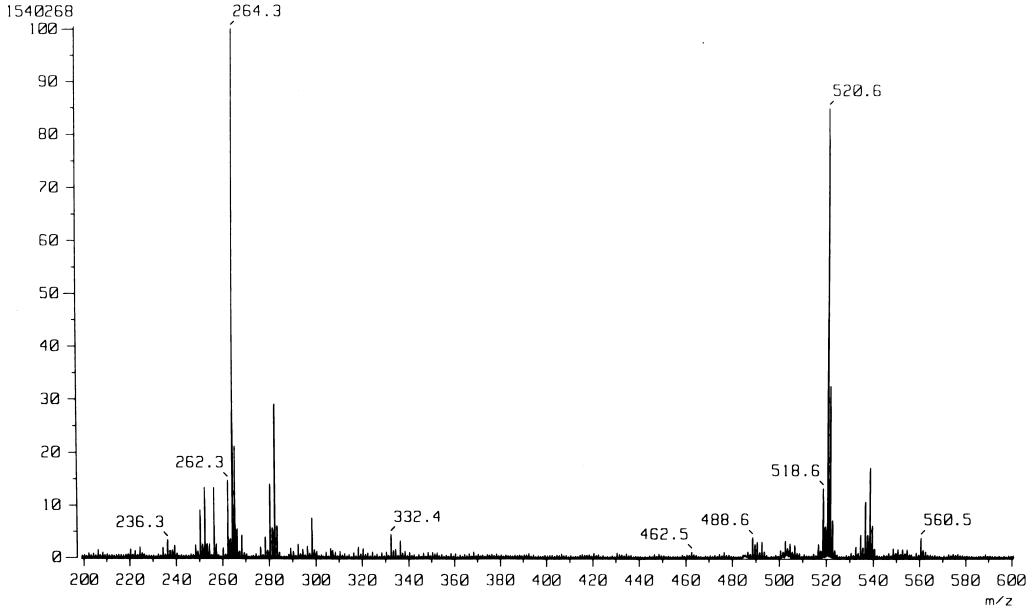


Fig. 1. A positive-ion fast atom bombardment mass spectrum of d18:1/*N*-(C16:0) ceramide in the full scan mode. Matrix: 3-Nitrobenzylalcohol.

observed at *m/z* 264, which is the one we focus on for the MS/MS study of the sphingoid base.

Step 2: Positive-ion FAB-MS/MS spectrum of the standard linear d18:3 sphingoid base. Our new approach was validated as follows: full scan positive-ion FAB mass spectrometry of structurally known glucosylceramide containing straight-chain d18:3/*N*-(C2) (acetamide of straight-chain d18:3) sphingoid base was first carried out as an example of Step 1. The structure of this starfish sphingoid base was previously confirmed as (4*E*,8*E*,10*E*)-2-aminooctadecatriene-1,3-diol by NMR (2). The precursor ion we chose was LCB^+ for d18:3 at *m/z* 260, which was easily detected in the positive-ion FAB full scan spectrum.

As shown in **Fig. 2**, this MS/MS spectrum consisted of a series of prominent charge-remote fragment ions starting from the LCB^+ as well as several charge-mediated fragment ions around the conjugated double bond system (see also **Scheme II**). The charge-remote fragment ions were ob-

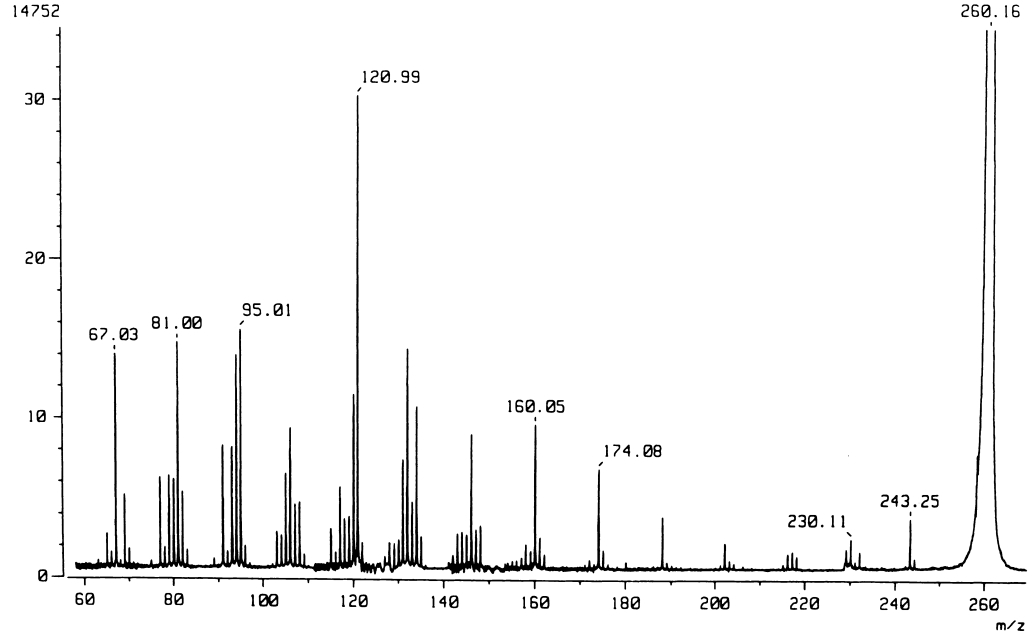
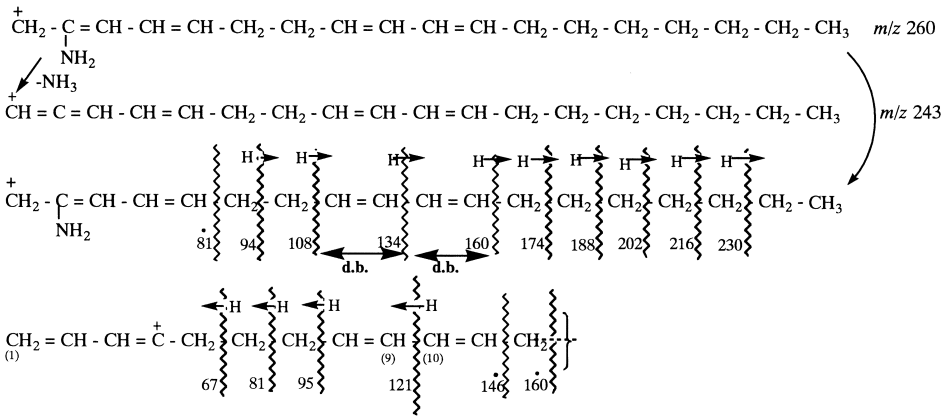


Fig. 2. A positive-ion fast atom bombardment tandem mass spectrum (FAB-MS/MS) of the standard, linear d18:3 sphingoid base. Precursor ion at *m/z* 260. See more detailed descriptions in Scheme II.



Scheme II. Positive-ion FAB fragmentation pattern of LCB^+ ion (*m/z* 260) of standard linear d18:3 [sample: glucosylceramide d18:3/*N*-(C2)]. Reported structure was 2-amino-4,8,10-octadecatriene-1,3-diol.

served at $[M + H - C_2H_6]^+$ at *m/z* 230, $[M + H - C_3H_8]^+$ at *m/z* 216, $[M + H - C_4H_{10}]^+$ at *m/z* 202, $[M + H - C_5H_{12}]^+$ at *m/z* 188, $[M + H - C_6H_{14}]^+$ at *m/z* 174, and $[M + H - C_7H_{16}]^+$ at *m/z* 160. The regularity of the interval of 14 u, corresponding to CH_2, was discontinued at this point. The next two lower-mass fragment ions in this series appeared at *m/z* 134 and 108, with an interval of 26 u. This observation confirmed that two double bonds were located at C-10 and C-8, and thus, conjugated. Furthermore, a lower-mass fragment ion was once more observed at the expected *m/z* value of 94. (The ion at *m/z* 95 was a radical ion and the ion at *m/z* 81 was probably a mixture of a radical ion and a C-1 to C-6 carbohydrate ion after ammonia elimination.) These *m/z* values match the charge-remote fragmentation rule for the reported structure of the sample, and confirmed the validity of our methodology.

The ion observed at *m/z* 243 represented $[M + H - NH_3]^+$. In saturated sphinganine and monoene-sphingenine bases, the elimination of ammonia does not take place because the nitrogen atom is the only positive charge center for their structures. In polyunsaturated sphingoid bases, on the contrary, a conjugated diene system is capable of being another protonation center, and thus, even after elimination of an ammonia molecule, there is still a positive charge center to produce positive fragment ions. A characteristic ion of high intensity appeared at *m/z* 121, representing the vinyl cleavage to split the $[M + H - NH_3]^+$ in half.

Step 3: Positive-ion FAB-MS/MS spectrum of the standard methyl-branched d19:3 sphingoid base. We next examined a structurally known glucosylceramide containing methyl-branched d19:3 sphingoid base. This sample was extracted from the same starfish spermatozoa, and converted to an *N*-acetyl derivative by deacylation/*N*-acetylation as described above. The precursor ion was once again LCB^+, but at *m/z* 274 this time for d19:3.

The product ion spectrum, shown in the upper part of **Fig. 3**, indicated charge-mediated fragment ions at *m/z* 257 (elimination of NH_3), *m/z* 243 (elimination of CH_3NH_2), and *m/z* 231 (elimination of an enamine, $CH_2 = CHNH_2$). The MS/MS spectrum of LCB^+ also showed a series of charge-remote fragmentations, starting with the elimination of C_4H_{10} (*m/z* 216) from the far end of the protonated site of the sphingoid base, at *m/z* 274. A series of charge-remote fragment ions were detected at *m/z* 202, 188, and 174, as expected. A characteristic ion was observed at *m/z* 108, which is 66 u lower than *m/z* 174, corresponding to C_5H_6 instead of C_5H_{10} (70 u). Thus, it is obvious that two unsaturations are located between the ions of *m/z* 174 (C-8) and *m/z* 108 (C-11). After this interval, the normal charge-remote fragmentation reappears at *m/z* 94, while the next lower-mass ion at *m/z* 81 is a radical caion. Thus, in this fragmentation series no unexpected ion due to branching of the sphingoid chain was observed. However, the ion at *m/z* 257, that is, the deammoniated hydrocarbon ion, showed unusual fragment ions at *m/z* 91 and *m/z* 131, indicating branching at C-9. The ion at *m/z* 91 may appear even for a linear sphingoid base as the conjugated carbocation of the C-1 to C-7 chain, as actually observed in the MS/MS spectrum of a linear d18:3 sphingoid base measured under the same conditions. This can be explained by assuming the rearrangement of the C-6 methylenic hydrogen to the unsaturated C-8, causing cleavage between C-7 and C-8. On the other hand, the ion at *m/z* 131 was uniquely observed only in the case of branched d19:3 sphingoid base. Although a switchover of the array detector in the vicinity may have produced a suspected product ion also for the linear d18:3 sphingoid base, detailed examination showed the mass to be *m/z* 132 rather than *m/z* 131. Our hypothesis is that for methyl-branched sphingoid base, the methylenic hydrogen at the C-6 position may also attack the double bond at C-10, producing an unusual ion at *m/z* 131 as shown in **Scheme III**.

Thus, the difference between linear, conjugated, and polyunsaturated sphingoid base and the conjugated polyunsaturated base bearing an extra methyl group at C-9 can be shown by fast atom bombardment tandem mass spectrometry (FAB-MS/MS) with LCB^+ as the precursor.

Step 4: MS/MS spectrum of the d19:3 sphingoid base isolated from sphingomyelin of squid nerve. On the basis of the above-described studies of sphingolipids from *Asterias amurensis*, we examined ceramide obtained from squid nerve sphingomyelin. The ceramide had molecular diversity both in the

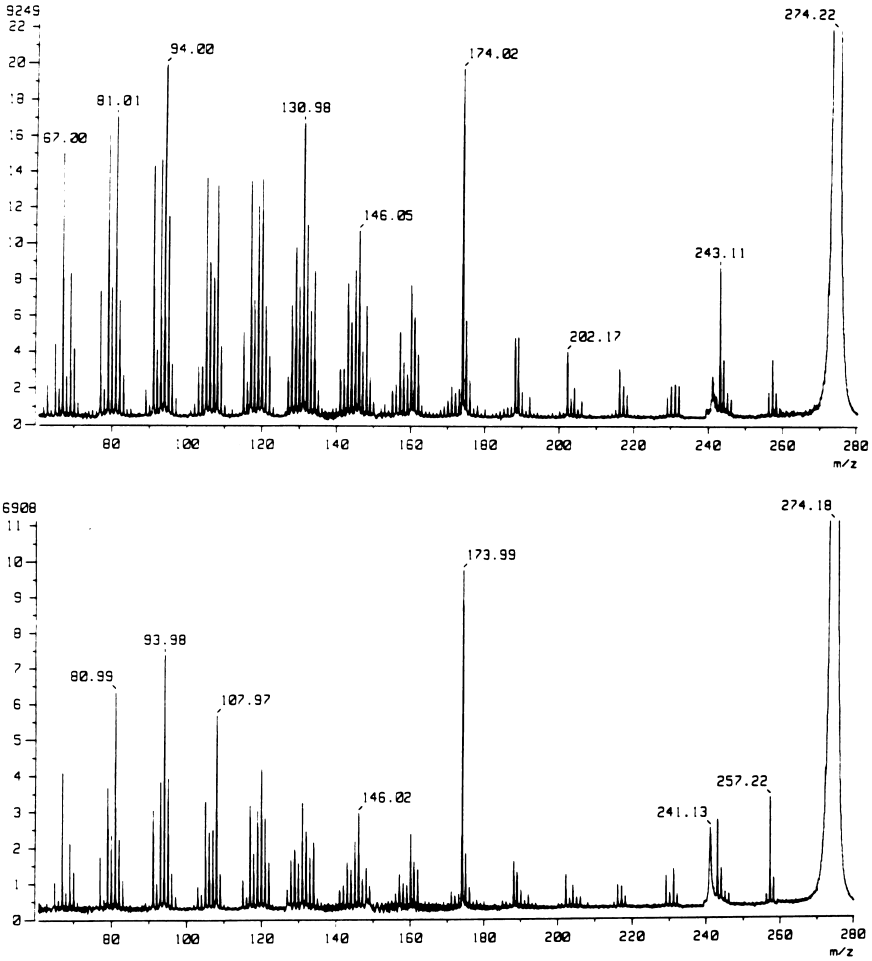


Fig. 3. Top: A positive-ion fast atom bombardment tandem mass spectrum (FAB-MS/MS) of the standard, branched d19:3 glucosylceramide. Precursor ion at *m/z* 274. See more detailed descriptions in Scheme III. Bottom: A positive-ion fast atom bombardment tandem mass spectrum (FAB-MS/MS) of the d19:3 ceramide sample in question, derived from squid nerve sphingomyelin. Precursor ion at *m/z* 274. See Scheme III, which applies not only to the standard, branched sphingoid but also to the unknown d19:3 sphingoid base of ceramide from the squid nerve system.

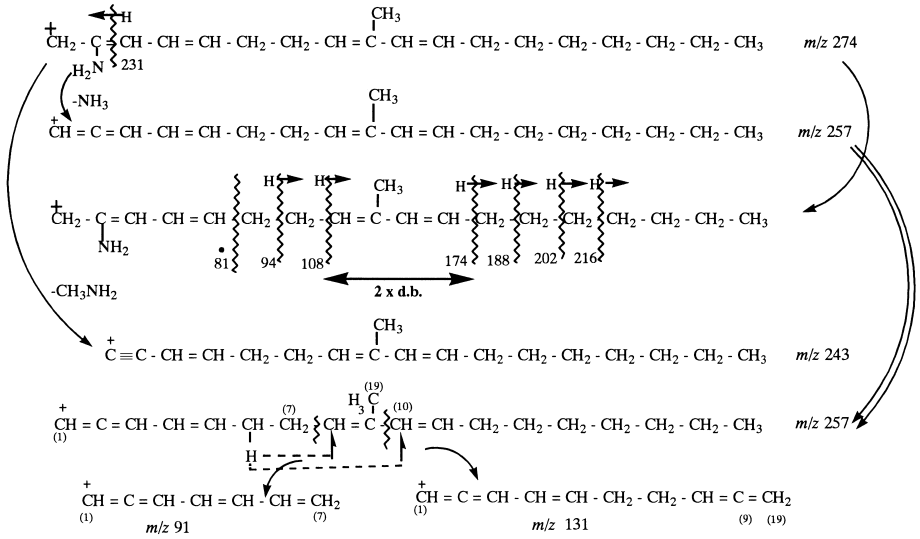
fatty acid (mostly *N*-C16:0 and *N*-C18:0) and sphingoid bases, including the d19:3 species in addition to the more common d18:1 and several other species. Accordingly, the normal scan positive-ion FAB spectrum showed many $[M + H]^+$ ions and LCB^+ ions. Ignoring such diversities of molecule-related ions, we focused, however, on a particular LCB^+ ion of medium intensity at *m/z* 274, which corresponded to the LCB^+ of d19:3. The locations of the three double bonds in this triunsaturated sphingoid chain were determined without much difficulty in a similar manner as for straight-chain d18:3. Then we attempted to determine whether the d19:3 polyunsaturated sphingoid base from the squid nerve was straight chained or branched, and if branched, where the branching was located.

The MS/MS spectrum of the sample was measured under conditions as described for the starfish glucosylceramide, which contained methyl-branched d19:3 sphingoid base as stated above.

As shown in the lower part of Fig. 3, the product ion spectrum of the sample having *m/z* 274 as the precursor was indistinguishable from that of the standard, branched d19:3 sphingoid base from glucosylceramide of the starfish spermatozoa (Fig. 3, top). Only the ion intensities were different, because of the smaller quantity of the sample. The product ion spectrum of the sample also showed an ion at *m/z* 131, characteristic of the C-9 methyl-branched structure, as described above. It was thus concluded that the d19:3 sphingoid base, the major base of the sphingomyelin ceramide from the squid nerve system, has a branched structure identical to that of the d19:3 sphingoid base from the starfish spermatozoa glucosylceramide.

DISCUSSION

We previously found that the sphingomyelin in the axoplasm of squid giant fibers is metabolically more active than that in other parts of the fiber. We now show that a sphingoid base of sphingomyelin in squid nerve has a



Scheme III. Positive-ion FAB fragmentation pattern of LCB^+ ion (*m/z* 274) of standard, branched d19:3 [sample: glucosylceramide d19:3/*N*-(C2)]. Reported structure was 2-amino-9-methyl-4,8,10-octadecatriene-1,3-diol. However, the fragmentation scheme was also applicable to the unknown sphingoid base from the squid nervous sphingomyelin.

unique structure, that is, the same unique branched, conjugated triunsaturated structure as that found in glucosylceramide of starfish (*Asterias amurensis*) spermatozoa (2).

Sphingolipids are most enriched in the mammalian nervous system. They are mostly composed of straight carbon chains with one or no double bond. The presence of a branched-chain sphingoid base in sphingolipids has been reported in tissues of shellfish (10), which are phylogenetically closely related to squid. More recently, another starfish (*Ophidiaster ophidiamus*) was found to have a glucosylceramide similar to that of *Asterias amurensis,* containing an unusual d19:3 sphingoid base (11). This glucosylceramide showed cytotoxicity. One more example is the sphingoid of phosphocholine-galactosylceramide from the clam worm (*Marphysa sanguinea*), which contains the same branched triunsaturated sphingoid base (12). In lipids, the occurrence of methyl branches in alkyl chains was attributed to the facilitation of membrane function at high salt concentrations (13). A branched base with two isolated double bonds has been found in cerebrosides from the sea anemone *Metridium senile* (14) and mycelia of the fungus *Schizophyllum commune* (15). Interestingly, this cerebroside stimulates formation of fruiting bodies in the same fungus (15). The discovery of a branched, conjugated triunsaturated sphingoid base in the squid nerve system, glucosylceramide of starfish spermatozoa (2), and even in other animal species may indicate an essential role for such unusual sphingoid bases in the cell membranes of primitive animals.

If the unusual squid sphingoid base is synthesized and degraded by identical pathways in squid nerves, the biosynthetic precursor of the product nonadecasphingatriene should have a branched C_{17} alkyl chain with two conjugated double bonds. Details of the synthetic pathway of this interesting squid sphingatriene remain to be elucidated.

CONCLUSION

The methodology described in this article depends on the fact that any type of sphingolipid has the same LCB^+ ion provided the sphingoid moiety is the same, and also on the fact that the LCB^+ ion may be used as the precursor ion for product ion spectra to directly compare sphingoid structures of different types of sphingolipids. The method enables us to obtain structural information, including the positions of unsaturation, conjugation, and methyl branching in the sphingoid base, with a much smaller amount of samples than is required by NMR.

The two standard glucosylceramide samples, which played an important role in this study, were kindly provided by Professor Motonori Hoshi and Dr. Atsushi Irie of the Tokyo Institute of Technology. The assistance of Drs. Michael Stoskopf and Robert Gould, especially at the beginning of the study, is greatly appreciated. Professor Robert Cotter of Johns Hopkins University measured the molecular weight of sphingomyelin by plasma desorption mass spectroscopy. Dr. Koji Takio and Mr. Yasuaki Esumi kindly let us use the HX110/HX110 instrument. Our special thanks go to Dr. Ron Orlando of the Suntory Institute of Bioorganic Research (present address: CCRC/UGA), who made a special effort to obtain the MS/MS spectra for us.

Manuscript received 27 July 1999 and in revised form 20 March 2000.

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