Chemistry and Physics of Lipids, 52 (1990) 57—67
Elsevier Scientific Publishers Ireland Ltd.

Structure determination of phosphonosphingolipids by fast atom bombardment and tandem mass spectrometry

Akira Hayashi[a], Toshiko Matsubara[a], Takemichi Nakamura[b] and Takeshi Kinoshita[b]

[a]*Department of Chemistry, Faculty of Science and Technology, Kinki University, Higashiosaka, Osaka 577 and* [b]*Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd., Shinagawa-ku, Tokyo 140 (Japan)*

(Received March 14th, 1989; revised and accepted May 9th, 1989)

Tandem mass spectrometry (MS/MS) and the fast atom bombardment (FAB) ionization method were used to determine the structures of phosphonosphingolipids. Water-soluble components, aminoethylphosphonic acid and *N*-methylaminoethylphosphonic acid, were distinguished easily by the ions at *m/z* 126 and 140, respectively, in positive ion mode FAB mass spectra and the ions at *m/z* 124 and 138, respectively, in the negative ion mode. The molecular species of the ceramide moiety could also be determined from collisionally activated decomposition (CAD) spectra of each ceramide ion. Long-chain base ions could be clearly detected in positive ion mode FAB/MS.

Keywords: aminoethylphosphonic acid; *N*-methylaminoethylphosphonic acid; phosphonosphingolipids; molecular species; fast atom bombardment mass spectrometry; tandem mass spectrometry.

Introduction

Fast atom bombardment (FAB) mass spectrometry has been widely used to obtain structural information about complex lipids. With glycosphingolipids, this method has been very useful for elucidating the sequence of sugar chains [1—5]. In glycerophospholipids, FAB/MS allowed the determination of molecular weight and the identification of the fatty acid and the polar group bound to the phosphoric acid [6—9]. Moreover, the double bond position in carboxylate moiety in glycerophospholipids could be readily determined by selecting the $[RCOO]^-$ ion formed by negative FAB/MS then using the collisional activated decomposition (CAD) technique [10—12]. Thus, MS/MS combined with FAB ionization (FAB/MS/MS) seems to be a useful method for determining the structure of complex lipids.

We tried using this FAB/MS/MS method to elucidate the structure of phosphonosphingolipids (PnSL), which are unique lipids having the characteristic C—P bond, but not usually a C—O—P bond. They are widely distributed in lower animals, mainly in shellfish. The structural determination of PnSL involves several problems. First, in analyzing the ceramide moiety, as the long-chain base (LCB) and fatty acid (FA) are analyzed separately, the actual combination of LCB and FA cannot be obtained. In the analysis of LCBs this can sometimes lead to incorrect results, because LCBs produce many artifacts during hydrolysis depending upon the conditions [13]. Another problem is that the water-soluble moiety of PnSL is composed of aminoethylphosphonic acid (AEPn) and *N*-methylaminoethylphosphonic acid (MAEPn). To distinguish between AEPn and MAEPn requires the use of tedious hydrolysis of PnSL followed by paper chromatography [14] or gas chromatography [15] of the C—P components. A third problem is that the ion pairs at *m/z* 126 and 140 in positive ion mode FAB and the ions at *m/z* 124 and 138 in negative ion mode FAB seem to indicate the structures of AEPn and MAEPn ions, respectively, but no evidence has yet been

0009-3084/90/$03.50
Published and Printed in Ireland

obtained for this. And finally there is the problem of PnSL containing many molecular species because of the existence of various ceramide molecular species and two different C—P components. These complex combinations cannot be completely separated by usual methods.

To overcome these problems, we used FAB/MS and FAB/MS/MS and were able to prove that they are excellent for the structural analysis of phosphonolipids, especially for analyses of the C—P component and the molecular species of the ceramide moiety.

Materials and methods

Preparation of phosphonosphingolipids

The muscle part (2.2 kg) of abalone, *Haliotis japonica,* was homogenized in acetone, and extracted three times with acetone to remove water and neutral lipids. The residue was then extracted three times with chloroform/methanol (C-M) (2 : 1, v/v) and twice with C-M (1 : 3, v/v). The combined C-M extracts (27.3 g) were partitioned by Folch's method. The lipids partitioned into the lower chloroform phase (10.9 g) were separated by silicic acid column chromatography. They were applied to a column (4.0 × 65 cm) and eluted with 3 l of C-M (98 : 2), 6.5 l of acetone/methanol (A-M) (9 : 1), 4.5 l of A-M (4 : 1), 3 l of C-M (3 : 2), 2.5 l of C-M (1 : 1), 3.5 l of C-M (2 : 3) and finally 1 l of methanol. The PnSL fraction eluted with C-M (3 : 2) was subjected to mild alkaline methanolysis and then partitioned again into the lower chloroform layer. Chloroform was removed and the residual alkali stable lipids were purified by Iatrobeads column chromatography. The purified PnSL fraction (485 mg) was used as a mixture of several kinds of molecular species including AEPn and MAEPn without further separation.

Analytical methods

The phosphorus content was determined by the method of King [16] for both P—O—C and P—C type phosphorus and by Bartlett's method [17] for P—O—C type phosphorus. PnSL was methanolyzed with 1 N aqueous methanolic-HCl [13]. Fatty acid methyl ester was extracted with light petroleum (b.p. 30°—60°C) and the ester bond of the fatty acid was estimated by the slightly modified method of Snyder and Stephens [18]. Methanol layer of hydrolyzate of PnSL was subjected to determine LCB by the method of Lauter and Trams [19]. The thin-layer chromatogram was developed with chloroform/methanol/water (65 : 25 : 4, by vol.) and the spots made visible by spraying with Dittmer reagent [20] for phosphorus and ninhydrin reagent for amino groups. The fatty acid composition was examined by GLC of fatty acid methyl esters on a 2-m column packed with 10% SP-2300 on 100—120 mesh Supelcoport at 220°C. LCBs were analyzed by GLC as trimethylsilyl derivatives on a 2-m column packed with 3% SE-30 on 100—120 mesh Chromosorb W AW at 210°C. Fatty acid methyl esters and trimethylsilyl LCB were identified by GLC-MS using a JEOL HX-100 gas chromatograph-mass spectrometer as reported previously [21].

Analytical conditions of FAB/MS and FAB/MS/MS

All mass spectra and CAD spectra in this paper were obtained with a JEOL JMS-HX100 tandem mass spectrometer, which consisted of a high resolution MS-I of EB configuration followed by an electrostatic analyzer used as MS-II. The instrument was equipped with an FAB gun which was operated at 6 kV, and xenon atoms were used to ionize the sample in a matrix of a mixture of glycerol, dithiothreitol and diethioerythritol in a ratio of approximately 2 : 1 : 1. The mass spectrometer was operated at 5 kV accelerating voltage and a post-acceleration type detector was used for the detection of positive and negative ions. The conversion dinode was given a potential of −15 kV for positive FAB and +15 kV for negative FAB. The FAB-desorbed ions of interest were mass-selected by using MS-I at a resolution of approximately 3000—10,000. Selected ions were then activated by collision with sufficient argon to obtain approximately 80—90% beam suppresion in a collision cell located between MS-I and MS-II. Daughter ions formed in this process were detected by scanning MS-II, with the signals

being averaged for 10—40 scans for each spectrum. Signal averaging and processing of the spectra were done with a JEOL DA5000 data system. As the mass resolving power of MS-II is ca. 50—200, mass assignments were made from the centroids of the broadened peaks.

Results and discussion

Chemical analysis of phosphonosphingolipids

Thin-layer chromatograms of PnSLs are shown in Fig. 1. PnSLs showed positive reactions to ninhydrin and molybdenum blue reagents. The spot positive to molybdenum blue reagent was slightly larger than that positive to ninhydrin reagent. Phosphorus content was 4.25% by King's method and 0.61% by Bartlett's method. The fatty acid was 1.59 μmol/mg and LCB 1.13 μmol/mg. Therefore, the molar ratio of P/ester/LCB was 1.00 : 1.11 : 0.85. These values show that PnSLs obtained from abalone are typical phosphonosphingolipids. LCBs were composed of hexadecasphingenine [$d_{16:1}$; 57.7%], heptadecasphingenine [$d_{17:1}$; 19.2%] and octadecasphingenine [$d_{18:1}$; 23.0%]. The main fatty acid was palmitic acid [$C_{16:0}$; 84.0%], with margaric acid [$C_{17:0}$; 8.3%] and stearic acid [$C_{18:0}$; 7.1%] as minor components. The C-P component was mainly AEPn judging from the paper chromatogram of the acid hydrolyzate of PnSL.

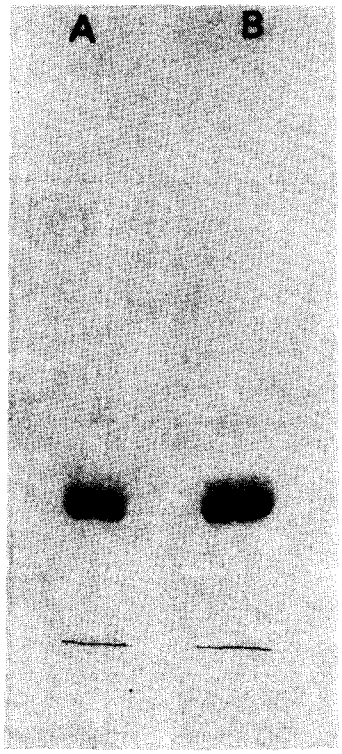


Fig. 1. Thin-layer chromatograms of the PnSLs isolated from abalone muscle. Solvent system was chloroform/methanol/water (65 : 25 : 4, by vol.). Molybdenum blue (A) and ninhydrin (B) reagents were used for detection.

Positive and negative ion FAB/MS of phosphonosphingolipids

Positive and negative ion mode FAB mass spectra of PnSL are shown in Fig. 2. There were a few structurally related fragment ions, and abundant $[M + H]^+$ and $[M - H]^-$ ions were observed. In the molecular ion region, four peaks each appeared: ions at *m/z* 659, 645, 631 and 617 in the positive ion mode and ions at *m/z* 657, 643, 629 and 615 in the negative ion mode. Therefore, PnSLs of abalone are composed of four main molecular species with molecular weights of 658, 644, 630 and 616. Furthermore, in the positive ion mode, three peaks of ceramide indicating ions at *m/z* 520, 506 and 492, three peaks originating from LCB at *m/z* 264, 250 and 236 and two peaks caused by the phosphonic acid moiety at *m/z* 140 and 126 were detected. On the other hand, the negative ion mode provided information about the phosphonic acid component at *m/z* 138 and 124 in addition to the molecular weight.

CAD spectra of phosphonosphingolipids

(1) CAD Spectra of $[M + H]^+$ and $[M - H]^-$. Spectra of the daughter ions produced by collisional activation of $[M + H]^+$ ions at *m/z* 659, 645, 631 and 617 in the positive ion mode (Fig. 2A) are shown in Figs. 3a—d. The ions at *m/z* 534 and 520 in Fig. 3a were ceramide ions which were formed by elimination of the phosphonic acid moiety from the ion at *m/z* 659.

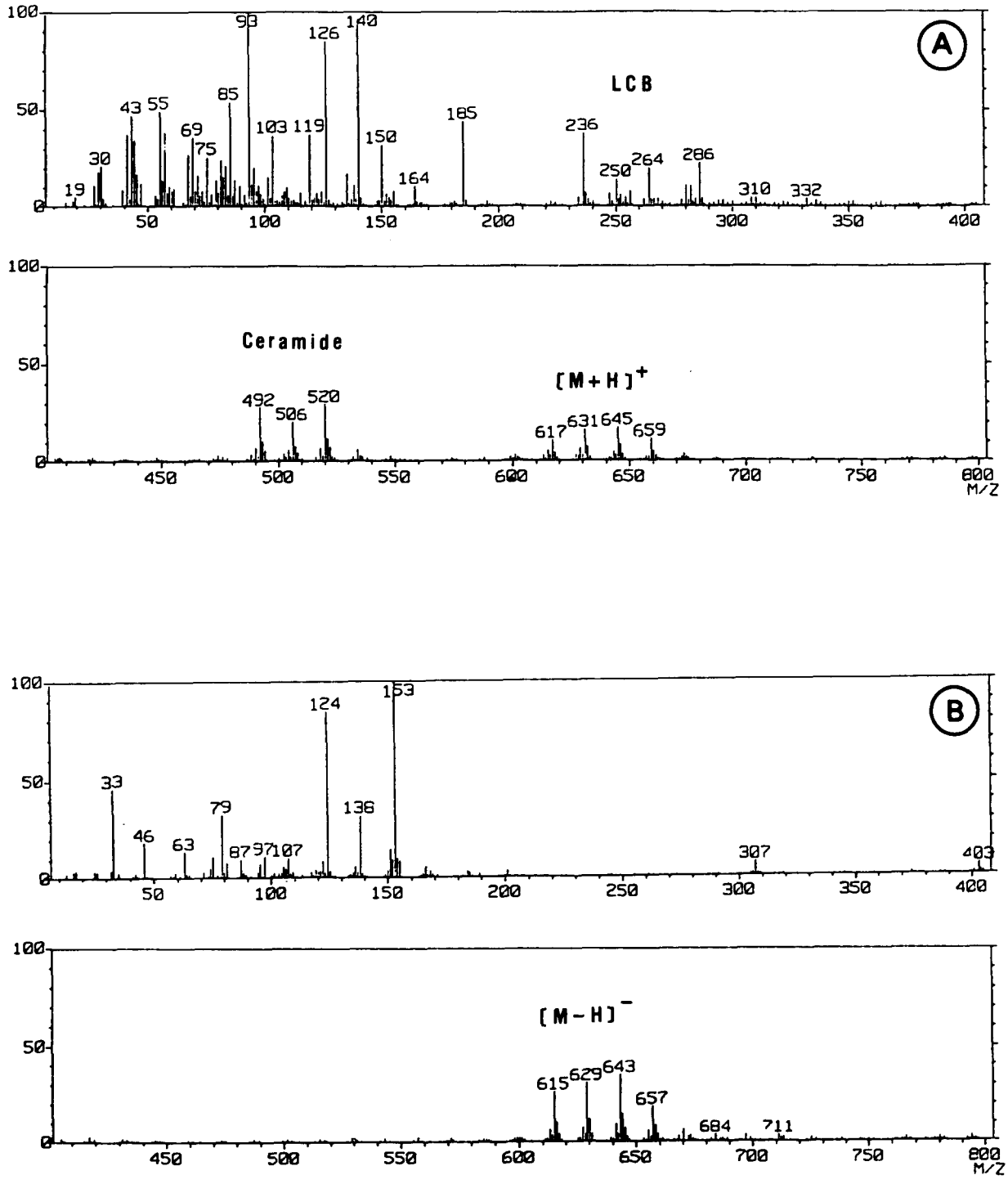


Fig. 2. Positive (A) and negative (B) ion mode FAB mass spectra of PnSL fraction from abalone. A mixture of glycerol, dithiothreitol and dithioerythritol was used as a matrix.

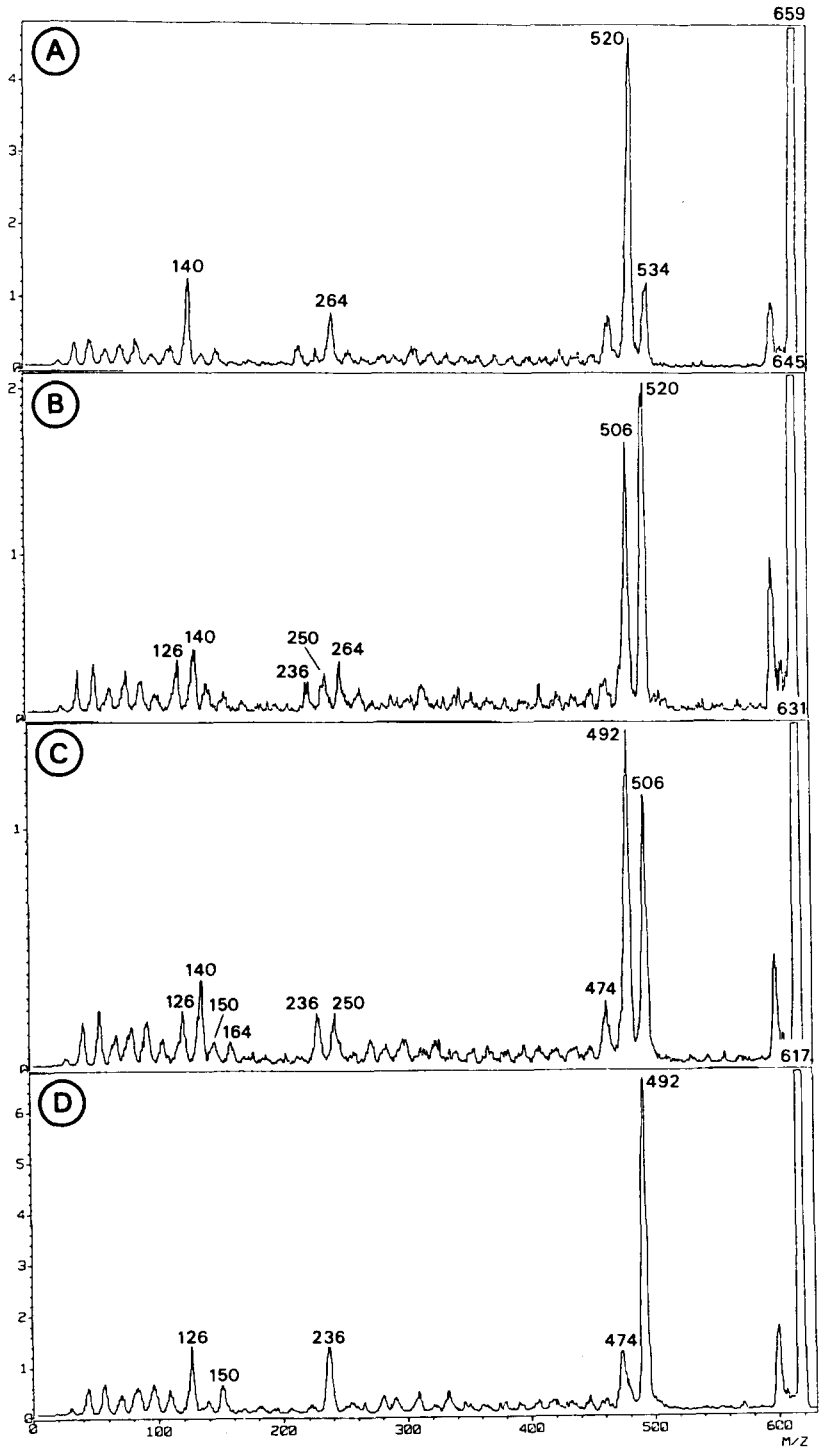


Fig. 3. CAD spectra of the ions at *m/z* 659, 645, 631 and 617 in the positive ion FAB mass spectra of PnSLs from abalone.

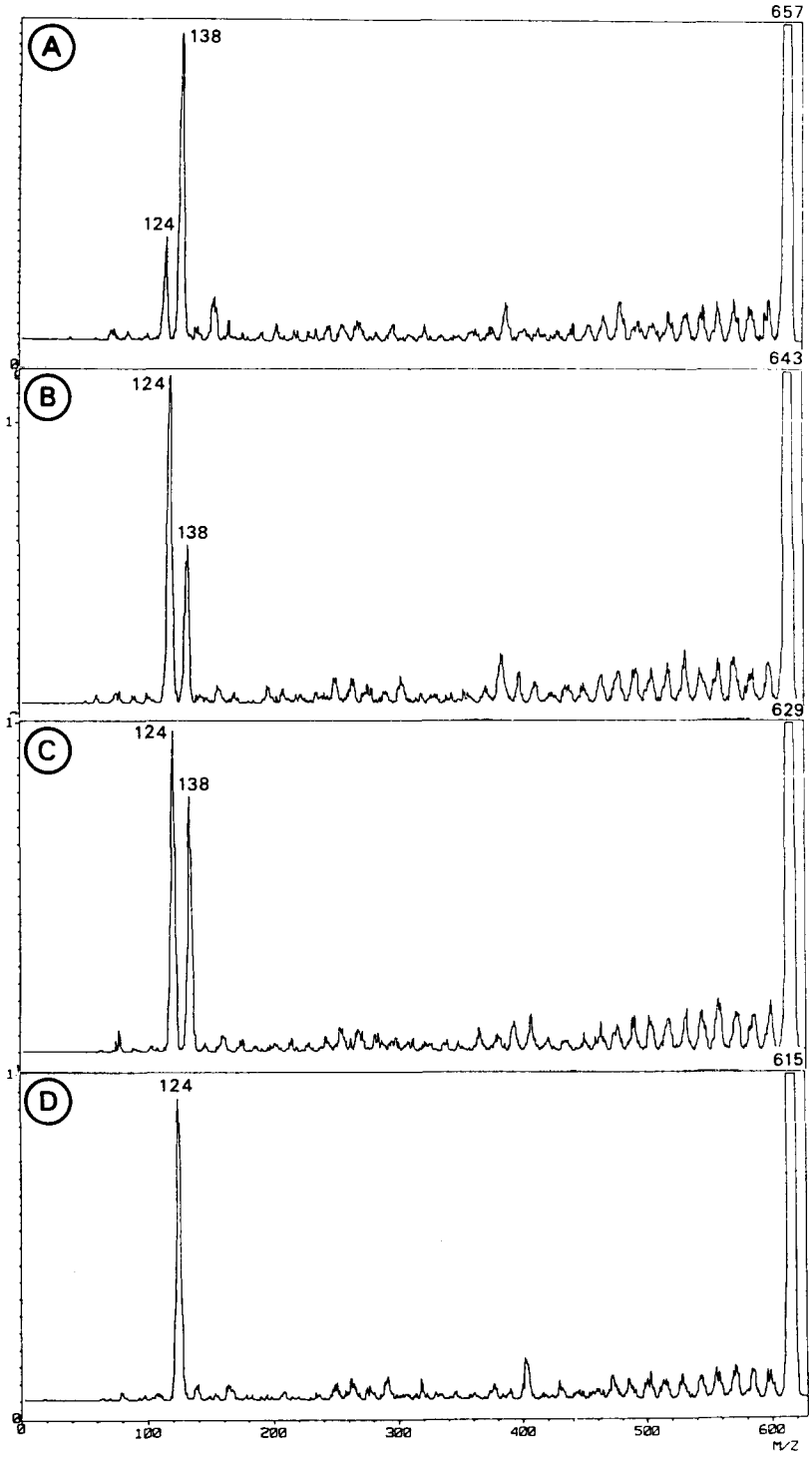
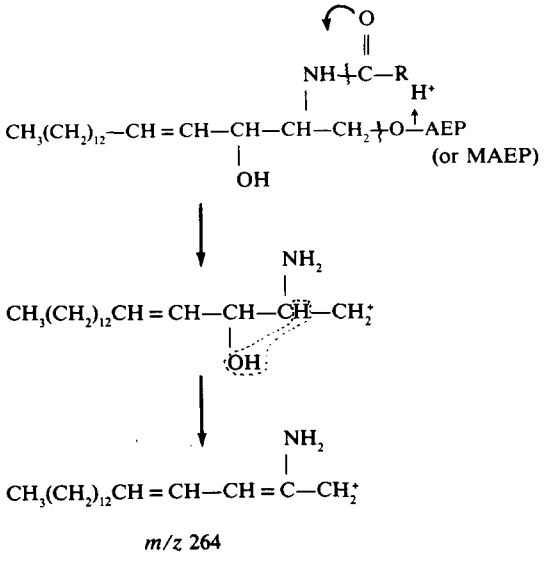


Fig. 4. CAD spectra of the ions at m/z 657, 643, 629 and 615 in the negative ion FAB mass spectra of PnSLs from abalone

The ion at *m/z* 534 corresponds to [M + H]$^+$ − 125 (AEPn) and *m/z* 520 [M + H]$^+$ − 139 (MAEPn). The ion at *m/z* 264 is the LCB ion with 18 carbon atoms, $CH_3(CH_2)_{12}CH{=}CH - CH{=}C(NH_2){-}CH_2^+$, which also appeared in Fig. 2A. Its fragmentation may occur by the following process as described by Ohashi [22].



m/z 264

The ion at *m/z* 140 was [MAEPn + H]$^+$, HO—P(O)(OH)—$CH_2CH_2NHCH_3$ + H as shown in the following section (2). Therefore, the [M + H]$^+$ ion at *m/z* 659 was composed mainly of $C_{16:0}$-$d_{18:1}$-MAEP*. $C_{17:0}$-$d_{18:1}$-AEPn may be a minor component.

[M + H]$^+$ ion at *m/z* 645 produces two main ceramide ions, at *m/z* 520 and 506 as shown in Fig. 3b. The ion at *m/z* 520 corresponds to [M + H]$^+$ − AEPn and *m/z* 506, [M + H]$^+$ − MAEPn. Ions at *m/z* 264, 250 and 236 show d18 : 1, d17 : 1 and d16 : 1 LCB, respectively. The main fatty acid may be $C_{16:0}$, because the value 256 was obtained for all spectra in Fig. 3 by subtraction of the C—P component and then the LCB ion from the molecular ion. This value corresponds to $C_{15}H_{31}COOH$. Two types of phosphonic acids were present in the [M + H]$^+$ ion at *m/z* 645, one was the ion at *m/z* 140 and the other that at *m/z* 126. The latter ion was [AEPn + H]$^+$, HO—P(O)(OH)—$CH_2CH_2NH_2$ + H from the CAD spectrum as shown later. Therefore, the [M + H]$^+$ ion at *m/z* 645 was composed of two molecular species of PnSLs, i.e. $C_{16:0}$-$d_{18:1}$-AEPn and $C_{16:0}$-$d_{17:1}$-MAEPn. Another possible minor molecular species was $C_{17:0}$-$d_{16:1}$-MAEPn.

Based on similar considerations, the [M + H]$^+$ ion at *m/z* 631 contains $C_{16:0}$-$d_{16:1}$-MAEPn and $C_{16:0}$-$d_{17:1}$-AEPn as shown in Fig. 3c. The [M + H]$^+$ ion at *m/z* 617 only has one species, $C_{16:0}$-$d_{16:1}$-AEPn (Fig. 3d).

Figures 4a—4d show CAD spectra of the ions at *m/z* 657, 643, 629 and 615 in the negative ion FAB spectra (Fig. 2b). The CAD spectra of [M − H]$^-$ are remarkably simple compared to those of [M + H]$^+$. Only two major ions at *m/z* 138 and 124 were detected from each [M − H]$^-$ ion, and they were identified as [MAEPn − H]$^-$ and [AEPn − H]$^-$, as reported in the following section 2). The [M − H]$^-$ ion at *m/z* 657 produced the ion at *m/z* 138 with fairly strong intensity by the loss of the ceramide moiety. From the ion at *m/z* 643 and 629, ions at *m/z* 138 and 124 were produced. The [M − H]$^-$ ion of *m/z* 615 gave only one major ion at *m/z* 124. These results agreed well qualitatively with the corresponding ions at *m/z* 140 and 126 obtained from the CAD spectra of [M + H]$^+$, although the intensities of these two ions in CAD spectra produced from [M + H]$^+$ or [M − H]$^-$ differ from each other. From the above data, the possible major molecular species of abalone PnSL were summarized in Table I. The PnSLs with moelcular weights 658 and 616 seem to be of only one major molecular species each; the former being $C_{16:0}$-$d_{18:1}$-MAEPn and the latter $C_{16:0}$-$d_{16:1}$-AEPn. The PnSLs with molecular weights 644 and 630 seem to be mixtures, i.e. the former of $C_{16:0}$-$d_{18:1}$-AEPn and $C_{16:0}$-$d_{17:1}$-MAEPn and the latter of $C_{16:0}$-$d_{17:1}$-MAEPn and $C_{16:0}$-$d_{16:1}$-MAEPn.

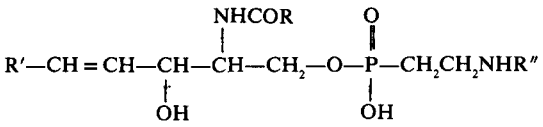
(2) CAD spectra of AEPn and MAEPn

CAD spectra of the ions at *m/z* 140 and 126 in the positive ion mode FAB mass spectra and the ions at *m/z* 138 and 124 in the negative ion mode (Fig. 2) are shown in Figs. 5A and 5B.

*The abbreviation shows the order of the FA—LCB—C-P component.

TABLE I

Molecular species of abalone PnSL obtained from the CAD spectra of $[M+H]^+$ and $[M-H]^-$.



	PnSL Mol. wt.					
	658	644		630		616
		(a)	(b)	(a)	(b)	
R	$C_{15}H_{31}$	$C_{15}H_{31}$	$C_{15}H_{31}$	$C_{15}H_{31}$	$C_{15}H_{31}$	$C_{15}H_{31}$
R′	$C_{13}H_{27}$	$C_{13}H_{27}$	$C_{12}H_{25}$	$C_{12}H_{25}$	$C_{11}H_{23}$	$C_{11}H_{23}$
R″	CH_3	H	CH_3	H	CH_3	H

From the ions at *m/z* 140 and 126, daughter ions such as *m/z* 109, 97, 81 and 44 or 30 were detected and also daughter ions at *m/z* 107, 94, 79 and 63 appeared from the both ions at *m/z* 138 and 124. The mechanisms of the generation of these daughter ions from their percursor ions are explained from the fragmentation pattern shown in the formula in Fig. 5. Abundant ion at *m/z* 79 in the negative ion mode is strong evidence for phosphonic acid. The existence of a daughter ion at *m/z* 44 produced from the ion at *m/z* 140 shows that hydrogen of the amino group of AEPn is substituted by a methyl group, i.e. this ion is characteristic of MAEPn. Therefore, the ions at *m/z* 126 and 140 in the positive ion mode and ions at *m/z* 124 and 138 in the negative ion mode represent AEPn and MAEPn, respectively.

(3) CAD spectra of ceramide ions

CAD spectra of the ceramide ions at *m/z* 520, 506 and 492 in the positive ion mode FAB mass spectra (Fig. 2A) are shown in Fig. 6. These ions were produced by the loss of phosphonic acid from $[M + H]^+$ ion. Therefore, measurement of the CAD spectra of ceramide ions may clarify the molecular species of the ceramide. From the ions at *m/z* at 520, 506 and 492 the ions at *m/z* 264, 250 and 236 appeared as LCB ions, respectively. These ions correspond to $d_{18:1}$, $d_{17:1}$ and $d_{16:1}$, respectively. LCB ions could be clearly detected in the positive ion mode FAB/MS. The intensities of the LCB ions correspond to the composition of LCB in PnSLs which were obtained by chemical analysis. In addition to LCB ions, each spectrum had one more ion at *m/z* 280, which seemed to be due to $C_{16:0}[C_{15}H_{31}CONH{-}CH{=}CH_2 - H]^+$. These results showed that ceramide ions have constituent acyl and LCB groups, as follows: the ion at *m/z* 520 shows the ceramide in a combination of $C_{16:0}$-$d_{18:1}$, *m/z* 506, $C_{16:0}$-$d_{17:1}$, and *m/z* 492, $C_{16:0}$-$d_{16:1}$. These results coincided with those obtained from the CAD sprectrum of $[M + H]^+$.

Abalone PnSL was found to be a mixture of two types of PnSLs, ceramide aminoethylphosphonate (CAEP) and ceramide *N*-methylaminoethylphosphonate (CMAEP), depending on the C—P component. The FAB/MS/MS method was useful for identifying the C—P component, i.e. the ions at *m/z* 126 and *m/z* 124 were shown to have the structures of $[AEPn + H]^+$ and $[AEPn - H]^-$, and the ions at *m/z* 140 and *m/z* 138 to be $[MAEPn + H]^+$ and $[MAEPn - H]^-$, respectively. The intensities of these ions led to the conclusion that PnSL is CAEPn, and/or CMAEPn. In addition to the determination of the C—P component, informa-

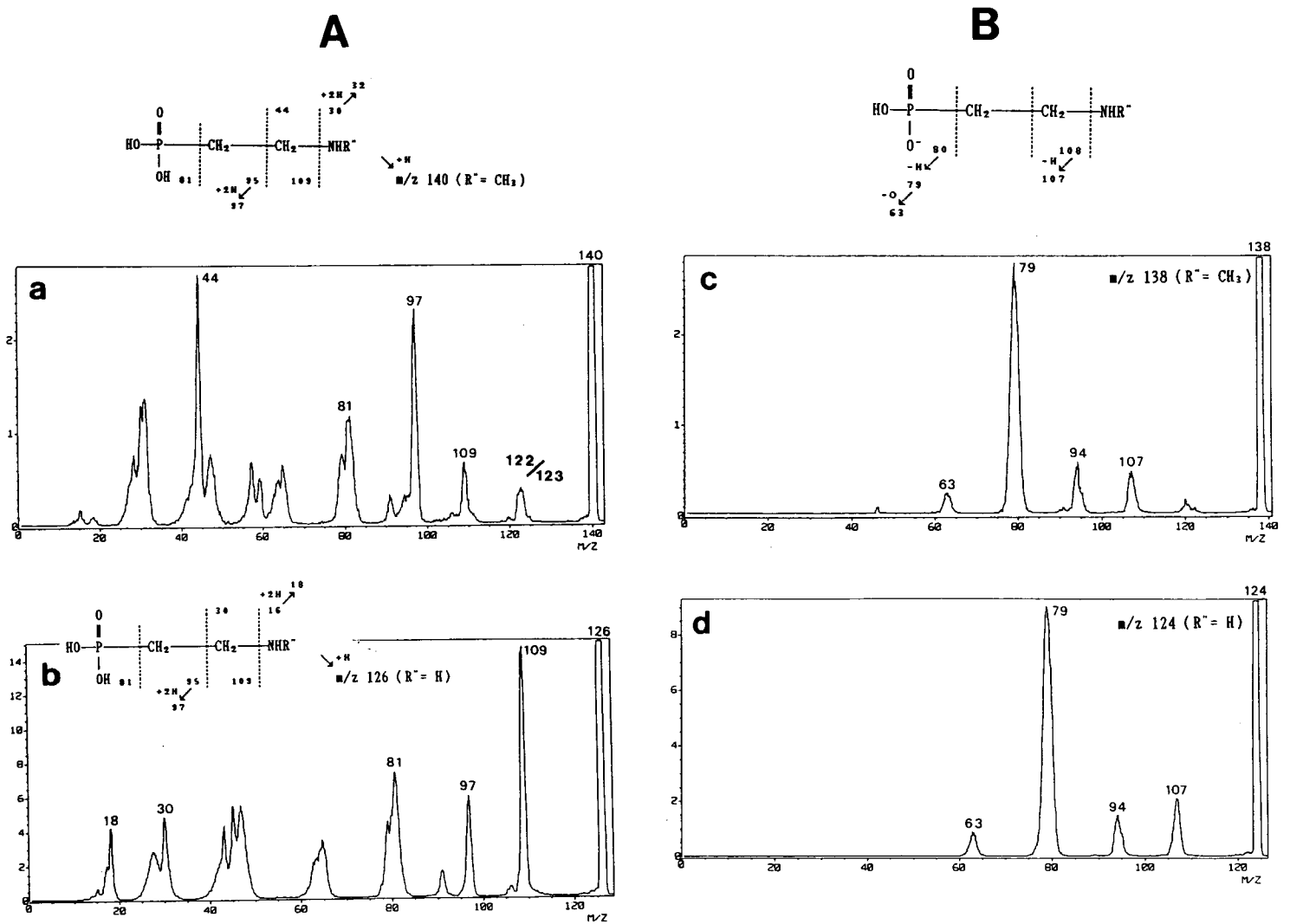


Fig. 5. CAD spectra of the ions at *m/z* 140 and 126 in the positive ion mode (A) and the ions at *m/z* 138 and 124 in the negative ion mode (B) FAB mass spectra of PnSLs from abalone.

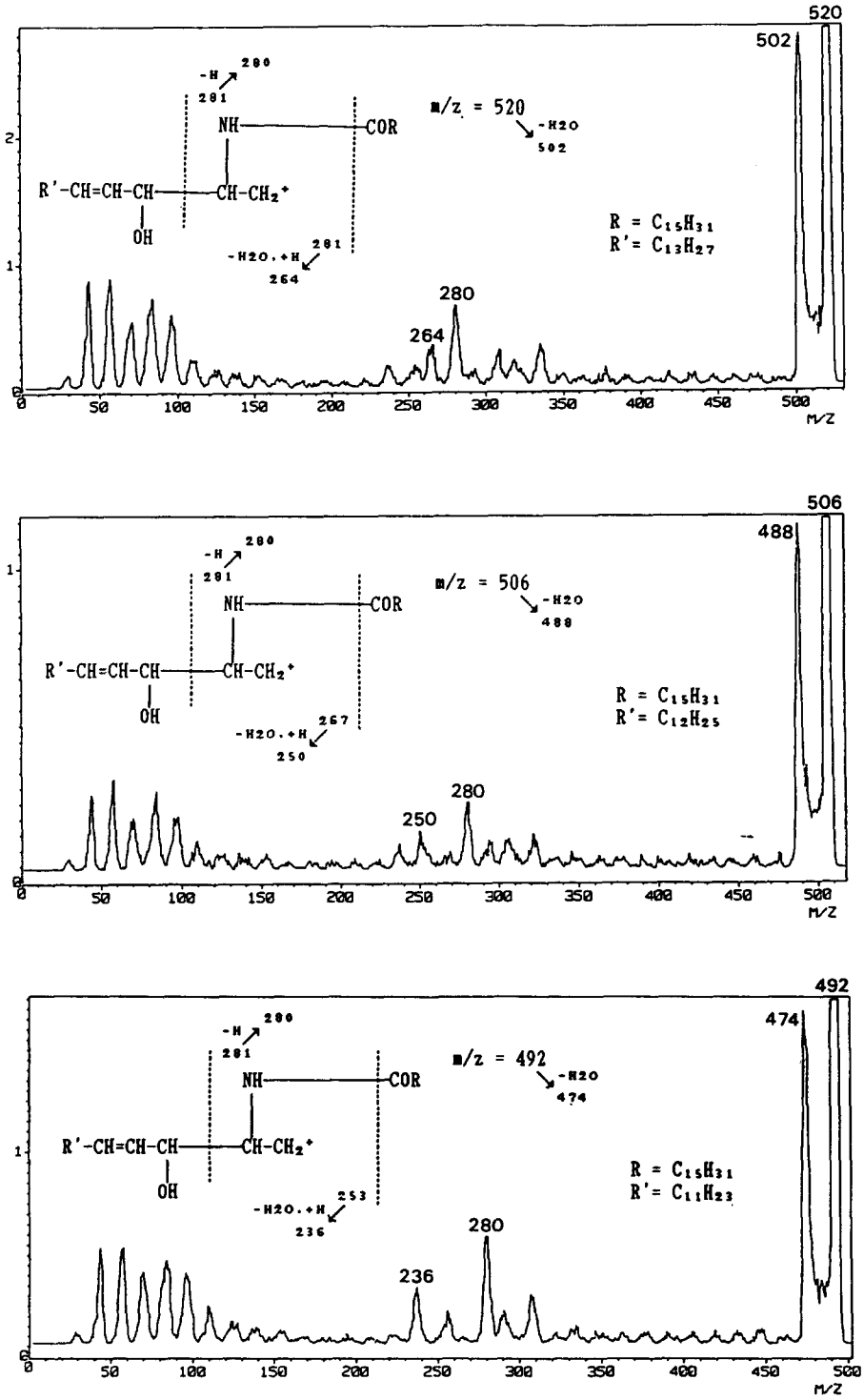


Fig. 6. CAD spectra of the ions at *m/z* 520, 506 and 492 in the positive ion FAB mass spectra of PnSLs from abalone

tion on the molecular species of the ceramide moiety could be easily obtained from the CAD spectra of the $[M + H]^+$ or $[M - H]^-$ ions and the FAB/MS of the intact lipids without degradation or derivatization. Separating such complex PnSL mixtures into the component molecules is extremely difficult by chemical methods, e.g. repeated column chromatography. Consequently, the combination of FAB/MS and MS/MS should be very useful for structural study of PnSLs. We will next apply this method to the analysis of PnSLs obtained from other marine animals.

References

1 M. Arita, M. Iwamori, T. Higuchi and Y. Nagai (1983) J. Biochem. (Tokyo) 93, 319—322.
2 S. Handa, Y. Kushi, H. Kambara and K. Shizukuishi (1983) J. Biochem. (Tokyo) 93, 315—318.
3 M. Arita, M. Iwamori, T. Higuch and Y. Nagai (1984) J. Biochem. (Tokyo) 95, 971—981.
4 Y. Kushi, S. Handa and I. Ishizuka (1985) J. Biochem. (Tokyo) 97, 419—428.
5 H. Egge, J.P.-Katalinic, G. Reuter, R. Schauer, R. Ghidoni, S. Sonino and G. Tettamanti (1985) Chem. Phys. Lipids 37, 127—141.
6 G.R. Fenwick, J. Eagles and R. Self (1983) Biomed. Mass Sepctrom. 10, 382—386.
7 H. Munster, J. Stein and H. Budzikiewitcz (1986) Biomed. Environ. Mass Spectrom. 13, 423—427.
8 A. Hayashi and M. Morita (1985) Kinkidaigaku Rikogakabu Kenkyuhokoku 21, 117—122 (in Japanese).
9 F.T. Chiltin II and R.C. Murphy (1986) Biomed. Environ. Mass Spectrom. 13, 71—76.
10 W.R. Sherman, K.E. Ackerman, R.H. Bateman, B.N. Green and I. Lewis (1985) Biomed. Mass Spectrom. 12, 409—413.
11 N.J. Jensen, K.B. Tomer and M.L. Gross (1986) Lipids 21, 580—588.
12 A. Hayashi, T. Matsubara, M. Morita, T. Kinoshita and T. Nakamura (1989) J. Biochem. (Tokyo) 106, 264—269.
13 R.C. Gaver and C.C. Sweeley (1965) J. Am. Oil Chem. Soc. 42, 294—298.
14 J.S. Kittredge, A.F. Isbell and R.R. Hughes (1967) Biochemistry 6, 289—295.
15 D.J. Harvey and M.G. Horning (1973) J. Chromatogr. 79, 65—74.
16 E.J. King (1932) Biochem. J. 26, 292—297.
17 G.R. Bartlett (1959) J. Biol. Chem. 234, 466—468.
18 F. Snyder and N. Stephens (1959) Biochim. Biophys. Acta 34, 244—245.
19 C.J. Lauter and E.G. Trams (1962) J. Lipid Res. 3, 136—138.
20 J.C. Dittmer and R.L. Lester (1964) J. Lipid Res. 5, 126—127.
21 T. Matsubara, A. Hayashi, Y. Banno, T. Morita and Y. Nozawa (1987) Chem. Phys. Lipids 43, 1—12.
22 Y. Ohashi, M. Iwamori, T. Ogawa and Y. Nagai (1987) Biochemistry 26, 3990—3995.