Gas–liquid chromatography–mass spectrometry of synthetic ceramides containing phytosphingosine

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ABSTRACT Ceramides containing phytosphingosine as base and one of the fatty acids 16:0, 18:0, 20:0, 22:0, 23:0, and 24:0, were prepared by direct coupling in the presence of a mixed carbodiimide. The ceramides were analyzed as the 1,3,4-tri-*O*-trimethylsilyl ether derivatives by gas–liquid chromatography–mass spectrometry. Gas chromatographic data is presented, and structures of mass spectral ions are suggested. The structures are supported by mass spectra of the homologous ceramides, by deuterium-labeling experiments, and by high resolution mass spectrometry. Some ions, formed by cleavage between C-3 and C-4 in the long-chain base, indicate the phytosphingosine nature of the ceramide.

SUPPLEMENTARY KEY WORDS fatty acids · deuterium labeling · trimethylsilyl ethers · silicic acid chromatography

Gas–liquid chromatography–mass spectrometry (GLC–MS) has been used for the elucidation of the structure of various sphingosines and sphinganines (1, 2), of phytosphingosine (analyzed as the tetraacetyl- and the *N*-acetyl, 1,3,4-tri-*O*-TMS-derivatives [3]), as well as of 2-hydroxy acids derived from sphingolipids (4). A method for determination of double bond positions in LCB by GLC–MS has been described (5). Recently, GLC–MS analysis has been applied to ceramides which contain nonhydroxy fatty acids (6) and 2-hydroxy acids (7). The methods for nonhydroxy fatty acid ceramides have been used to analyze ceramides derived from human plasma sphingomyelins (8), and free ceramides from plasma (9). Furthermore, ceramides of bovine origin have been analyzed by GLC–MS (10). Phytosphingosine, which has been demonstrated to be the most abundant LCB in plant sphingolipids (11), is also present in sphingolipids of animal origin (12, 13). The present report describes gas–liquid chromatographic separation and mass spectrometric analysis of synthetic ceramides containing phytosphingosine as LCB.

Abbreviations: LCB, long-chain base; GLC, gas–liquid chromatography; TMS, trimethylsilyl; TLC, thin-layer chromatography; TGCU, triglyceride carbon units; m.u., mass unit; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; d_9-TMCS, perdeuterated TMCS; d_{35}-octadecanoic acid, perdeuterated stearic acid; GLC–MS, gas–liquid chromatography–mass spectrometry; LCB 18:0-18:0, *N*-(stearoyl) sphinganine; 4-OH-LCB 18:0-18:0, *N*-(stearoyl) phytosphingosine.

MATERIALS AND METHODS

Chemicals

Natural phytosphingosine (D-ribo-1,3,4-trihydroxy-2-aminooctadecane) was a generous gift from Doctors H. E. Carter and A. Kisic of the University of Illinois, Urbana, Ill. Palmitic, eicosanoic, docosanoic, and tricosanoic acids were obtained from Fluka A.G. (Buchs, Switzerland); octadecanoic acid was from E. Merck A.G. (Darmstadt, West Germany); tetracosanoic acid was from Applied Science Laboratories Inc. (State College, Pa.). 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide hyrochloride was purchased from the Ott Chemical Company, Muskegon, Michigan. d_9-TMCS (99 atom %) was synthesized as a special order by Merck, Sharp, & Dohme, (Montreal, Canada). d_{35}-Octadecanoic acid was kindly provided by Prof. E. Stenhagen of the University of Gothenburg, Sweden.

Preparation of Ceramides

Ceramides were prepared according to a procedure which will be described in detail.[1] The fatty acid was

[1] Hammarström, S. To be published.

coupled with the LCB using a carbodiimide to activate the carboxyl group (40°C, 16 hr). The ceramide was isolated by ether extraction and purified by silicic acid column chromatography. It was eluted with ethyl acetate–benzene 40:60 (v/v), and its purity was monitored by TLC.

Preparation of Trimethylsilyl Ether Derivatives for GLC and Mass Spectrometry

To 100 μg of ceramide, dissolved in 100 μl of dry pyridine, 20 μl of HMDS and 10 μl of TMCS were added. The mixture was left at room temperature for 30 min, evaporated to dryness using an oil pump, and dissolved in 100 μl of carbon disulfide. To prepare deuterated TMS ethers, 20 μl of d_9-TMCS (14) were added instead of HMDS and TMCS.

GLC

The 1,3,4-tri-*O*-TMS derivatives of ceramides were analyzed in an F & M Biomedical gas chromatograph, model 400, with a hydrogen flame ionization detector. The column contained 1% OV-1, a nonpolar silicone phase (Applied Science Laboratories Inc.) on 60–80 mesh Gas-Chrom Q in a U-shaped 1.2 m glass column 3.5 mm I.D.). This was conditioned at 350°C for 24 hr. The column temperature was kept at 280°C. The detector and flash heater temperatures were kept 25°C above the column temperature. Helium was used as carrier gas with an inlet pressure of 3.0 kg/cm².

Mass Spectrometry

An LKB gas chromatograph–mass spectrometer, model 9000, was used. The electron energy was 22.5 ev, the trap current was 60 μA, the accelerator voltage was 3.5 kv, and the multiplier voltage was 2.9 kv. The separator temperature was 280°C, the ion source temperature 290°C, the scan speed 6, and the scan limits m/e 4–1000. Conditions for GLC were identical with those described above, except that a 1.2 m coiled glass column (3 mm I.D.) was used with the same packing and operating conditions as before.

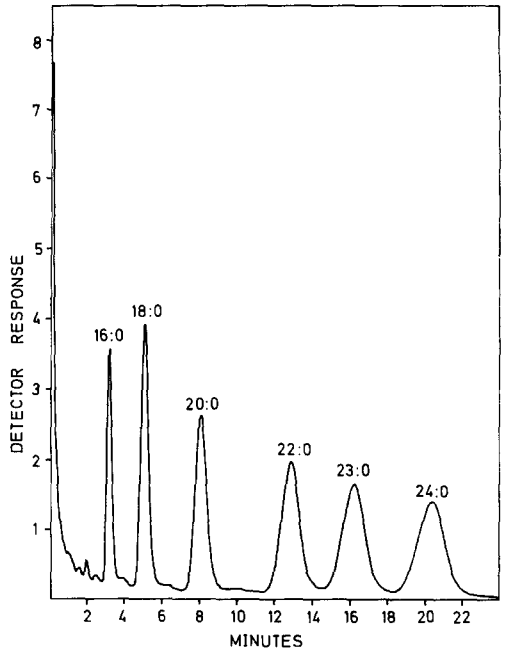


FIG. 1. Gas–liquid chromatogram of 1,3,4-tri-*O*-trimethylsilyl phytosphingosine ceramides with the fatty acids: 16:0, 18:0, 20:0, 22:0, 23:0, and 24:0 on 1% OV-1 at 270°C. The carrier gas was helium, with an inlet pressure of 3.0 kg/cm².

RESULTS AND DISCUSSION

Fig. 1 shows a gas chromatogram of a mixture of homologous phytosphingosine ceramides containing the fatty acids: 16:0, 18:0, 20:0, 22:0, 23:0, and 24:0, respectively. Retention times have been expressed as TGCU (Table 1) obtained by making the linear plot of the logarithm of the retention times for trilaurin, trimyristin, and tripalmitin against their total numbers of carbon atoms and interpolating the logarithm of the retention time for the ceramide. Retention times for other cer-

TABLE 1 RETENTION TIMES FOR TMS-DERIVATIVES OF CERAMIDES ON 1% OV-1 AT 280°C EXPRESSED AS TGCU

LCB / Fatty Acid	Phytosphingosine	Sphingosine		Sphinganine	
	Normal	Normal*	2-Hydroxy†	Normal*	2-Hydroxy†
16:0	37.9 ± 0.2‡	37.5 ± 0.1	38.1 ± 0.1	37.5 ± 0.1	38.0 ± 0.1
18:0	40.0 ± 0.1	39.4 ± 0.2	40.0 ± 0.1	39.7 ± 0.1	40.0 ± 0.1
20:0	42.1 ± 0.1	41.4 ± 0.2	42.0 ± 0.1	41.6 ± 0.2	41.9 ± 0.2
22:0	44.1 ± 0.1	43.5 ± 0.1	43.9 ± 0.1	43.7 ± 0.1	43.9 ± 0.1
23:0	45.1 ± 0.1	44.5 ± 0.1	—	44.7 ± 0	—
24:0	46.1 ± 0.1	45.5 ± 0.1	45.8 ± 0.2	45.8 ± 0	45.8 ± 0.2

* These data were obtained from reference 15.
† These data were obtained from reference 7.
‡ SD, five determinations.

amides are included for comparison. Phytosphingosine ceramides and 2′-hydroxy sphinganine ceramides are positional isomers and have similar retention times. The presence of a *trans* double bond in the ceramides (i.e., 2′-hydroxy *sphingosine* ceramides) only slightly alters their gas chromatographic behavior on OV-1, whereas the absence of one trimethylsilyloxy group, (as in sphingosine and sphinganine ceramides containing nonhydroxylated fatty acids), decreases the retention time by approximately 0.5 TGCU.

Fig. 3 shows the mass spectrum of 1,3,4-tri-*O*-TMS-*N*-(stearoyl) phytosphingosine. The designation of fragments is given in the structural formula of this figure. To facilitate the interpretation of fragmentations induced by electron impact, homologous ceramides containing the fatty acids 16:0, 18:0, 20:0, 22:0, 23:0, and 24:0 were prepared. Their mass spectra are shown in a somewhat simplified manner in Fig. 2. Two types of ions can be distinguished, namely, "common ions" which appear at the same m/e value throughout the series, and "homologous ions" which shift towards higher m/e values corresponding to the increase in molecular weight (Table 2). The former ions are formed by elimination of the fatty acyl part of the ceramide whereas the latter ions retain this part. Further experimental evidence for the structures of ions was obtained by recording spectra of 1,3,4-tri-*O*-TMS-*N*-(stearoyl) phytosphingosine containing perdeuterated TMS groups or a perdeuterated stearoyl residue. These mass spectra are shown in Figs. 4 and 5. The mass shifts of the ions (Table 2) provide information on the number of deuterium atoms left in each ion. Table 2 also shows that all common ions but two eliminate the deuterium atoms of the fatty acyl chain. The latter ions retain three of the deuterium atoms and are probably formed by β-cleavage of the acyl chain with a McLafferty type of rearrangement. The homologous ions retain all deuterium atoms of the acyl chain. The ions can be divided into "molecular weight fragments," "LCB-fragments," "fatty acid fragments," and "ceramide fragments" (see Table 3 and cf. references 6, 7).

In the mass spectrum of tri-*O*-TMS-*N*-(stearoyl) phytosphingosine (Fig. 3), several ions of structures analogous to those observed for other ceramides (as suggested by the calculated m/e values and the data in Table 2) are seen. Thus the molecular weight fragments (M–15), (M–90), (M–103), (M–2 × 90), and (M–103–90) are formed respectively by elimination of $\cdot CH_3$ (from a TMS group), $(CH_3)_3Si—OH$, $CH_2{=}\dot{O}—Si(CH_3)_3$ (5), two $(CH_3)_3Si—OH$ and finally $(CH_3)_3Si—OH$ plus $CH_2{=}\dot{O}—Si(CH_3)_3$. The LCB fragments [M–(b + 1 +

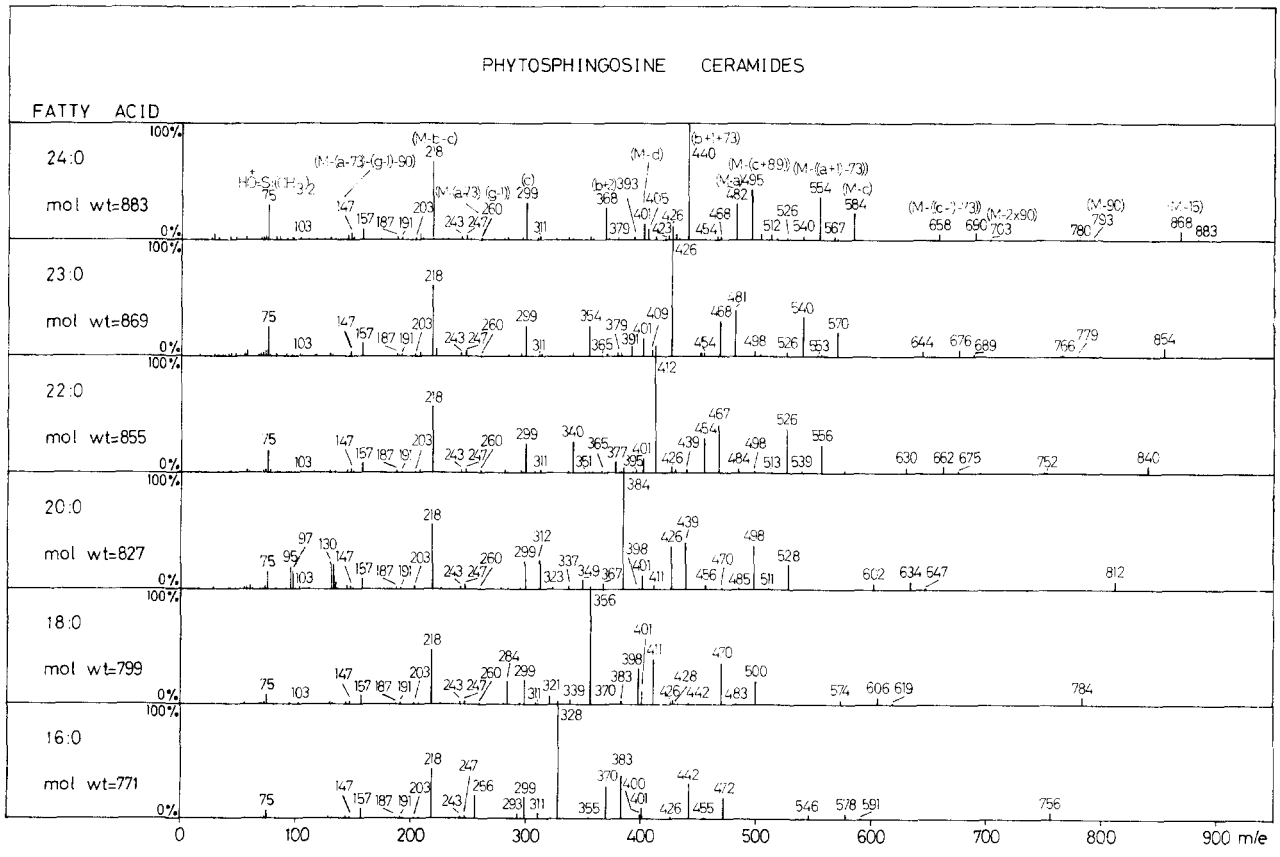


FIG. 2. Mass spectrometric data for TMS derivatives of phytosphingosine ceramides.

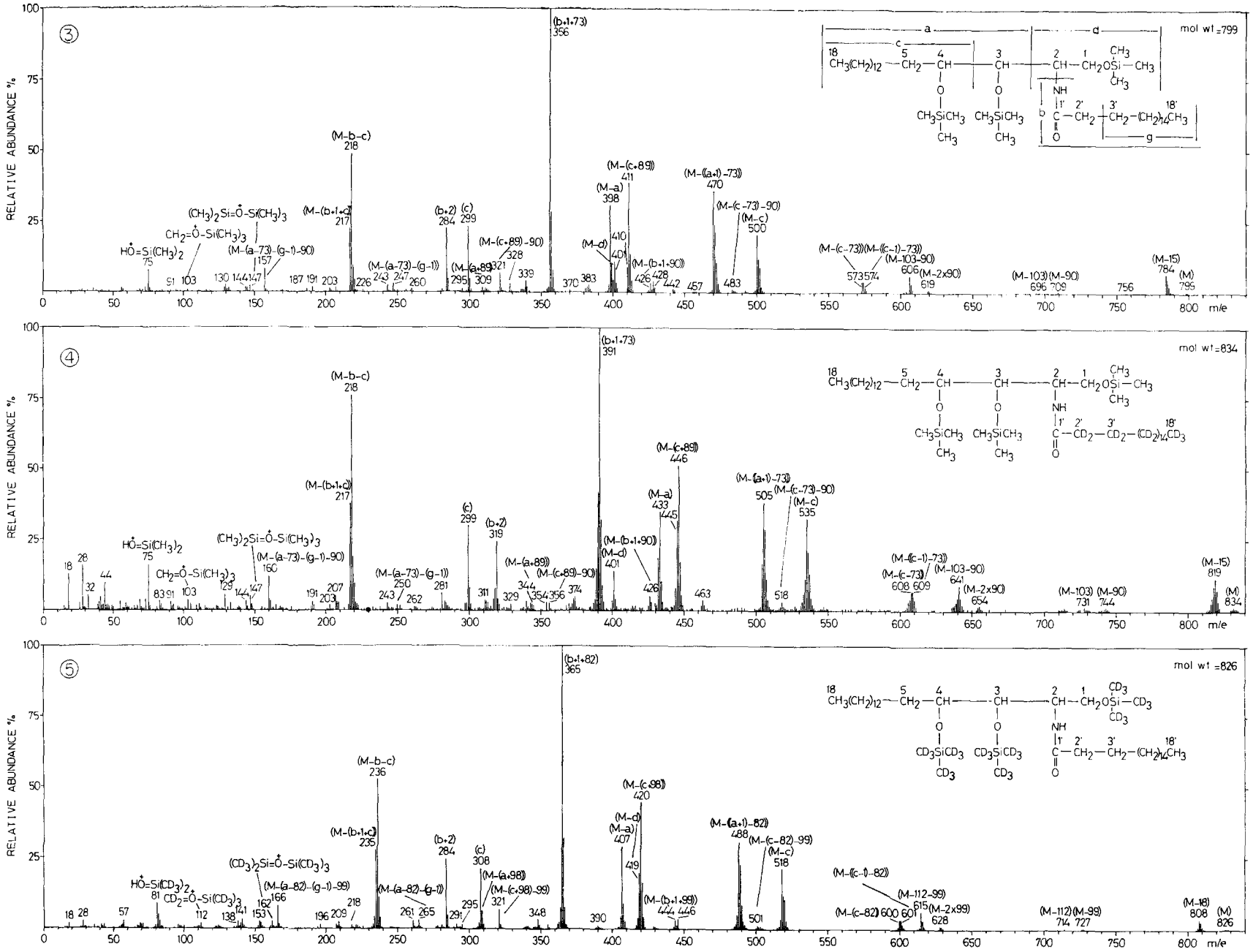


FIG. 3. Mass spectrum of 1,3,4-tri-*O*-trimethylsilyl-*N*-(stearoyl) phytosphingosine.
FIG. 4. Mass spectrum of 1,3,4-tri-*O*-trimethylsilyl-*N*-(d_{35}-stearoyl) phytosphingosine.
FIG. 5. Mass spectrum of 1,3,4-d_{27}-tri-*O*-trimethylsilyl-*N*-(stearoyl) phytosphingosine.

TABLE 2 EXPERIMENTAL EVIDENCE FOR STRUCTURES OF MASS SPECTRAL IONS OF PHYTOSPHINGOSINE CERAMIDES

m/e	Common/ Homologous Ion (cf. Fig. 3)	Mass Shift with Perdeuterated TMS Groups (cf. Fig. 4)	Number of Methyl Groups Originating in TMS Groups	Mass Shift with Perdeuterated Stearoyl Residue (cf. Fig. 5)	Proposed Structure
799		27	9	35	M
784	Homologous	24	8	35	M–15
709		18	6	35	M–90
696		18	6	35	M–103
619	Homologous	9	3	35	M–2 × 90
606	Homologous	9	3	35	M–103-90
574	Homologous	27	9	35	M–[(*c* − 1)-73]
573	Homologous	27	9	35	M–(c − 73)
500	Homologous	18	6	35	M–c
483	Homologous	18	6	35	M–(c − 73)-90
471	Homologous	18	6	35	M–(a − 73)
470	Homologous	18	6	35	M–[(a + 1)-73]
457	Homologous				
442	Homologous				
428	Homologous	18	6	35	
426	Common	18	6	0	M–(b + 1 + 90)
411	Homologous	9	3	35	M–(c + 89)
410	Homologous	9	3	35	M–(c + 90)
401	Common	18	6	0	M–d
398	Homologous	9	3	35	M–a
383	Homologous				
370	Homologous				
356	Homologous	9	3	35	b + 1 + 73
339	Homologous	9	3	35	
328	Homologous	27	9		
321	Homologous	0	0	35	M–(c + 89)-90
311	Common			0	
309	Homologous	0	0	35	M–(a + 89)
299	Common	9	3	0	c
295	Homologous	0	0		
284	Homologous	0	0	35	b + 2
260	Common	18	6		
247	Common	18	6	3	M–(a − 73)–(g − 1)
243	Common	18	6	0	
218	Common	18	6	0	M–b–c
217	Common	18	6	0	M–(b + 1 + c)
203	Common			0	
191	Common	18	6	0	
187	Common	9	3		
157	Common	9	3	3	M–(a − 73)–(g − 1)-90
147	Common	15	5	0	$(CH_3)_2Si{=}\overset{+}{O}{-}Si(CH_3)_3$*
144	Common	9	3	0	
132		9	3		
103	Common	9	3	0	$CH_2{=}\overset{+}{O}{-}Si(CH_3)_3$*
75	Common	6	2	0	$H\overset{+}{O}{=}Si(CH_3)_2$*
73	Common	9	3	0	$+Si(CH_3)_3$*

m/e values are given for tri-*O*-TMS-*N*-(stearoyl) phytosphingosine.
* cf. Reference 14.

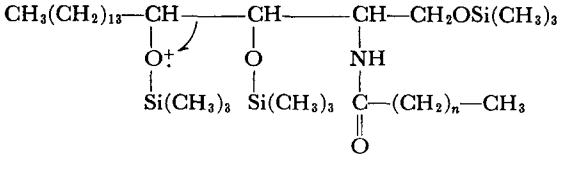
90)] and (M–d) appear at m/e 426 and m/e 401. The fragments [M–(b + 1)] and [M–(g − 1)] (7) are not seen. The fatty acid fragments m/e 398 (M − a), m/e 309 (M–a-89), and m/e 284 (b + 2) have been described in an earlier report (7). The base peak of phytosphingosine ceramides is considered to be due to (b + 1 + 73) which has also been previously observed (6, 7). It is a homologous ion which contains one TMS group and

TABLE 3 CLASSES OF MASS SPECTRAL IONS IN PHYTOSPHINGOSINE CERAMIDES

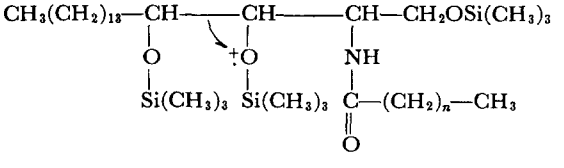
Molecular Weight Fagments	LCB Fragments	Fatty Acid Fragments	Ceramide Fragments
M–15	M–(b + 1)-90	M–a	M–(a − 73)-(g − 1)
M–90	M–d	M–a-89	M–(a − 73)-(g − 1)-90
M–103	c	b + 2	M–(b + 1 + c)
M–2 × 90		b + 1 + 73	M–b-c
M–103-90		M–[(c − 1)-73]	
		M–(c − 73)	
		M–c	
		M–(c − 73)-90	
		M–[(a + 1)-73]	
		M–c-89	
		M–c-89-90	

retains all fatty acyl hydrogen atoms (Table 2). A mechanism for its formation which is compatible with these data is discussed below.

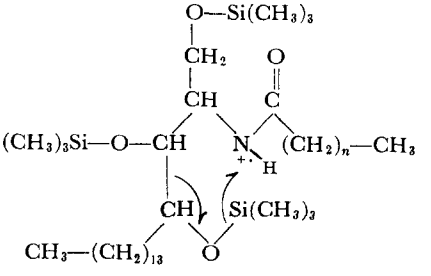
The presence of a trimethylsilyloxy group at C-4 in the LCB chain of phytosphingosine ceramides, in addition to the one at C-3, causes cleavage of the C—C bond between C-3 and C-4. (The same kind of fragmentation has been observed in mass spectra of *N*-acetyl, *O*-TMS derivatives of unsaturated LCB which had been oxidized by OsO_4 (5). These derivatives also contain vicinal *O*-TMS groups.) Thus, the ion at m/e 299 (c) is probably formed by α-cleavage after charge localization on the oxygen at C-4:



Several fatty acid fragments are also formed by cleavage of the same bond. m/e 500 (M-c) is probably formed by α-cleavage after charge localization on the oxygen at C-3 instead of the oxygen at C-4:



The intramolecular transfer of a trimethylsilyl radical in the formation of [M–(a-73)] has been demonstrated before (6, 7, see also 5). This reaction is also operating in the fragmentation of phytosphingosine ceramides. Thus, m/e 573 [M–(c-73)], is an ion retaining three TMS groups and the whole acyl chain. It is formed by transfer of the TMS radical from the oxygen at C-4 and cleavage of the bond between C-3 and C-4. The acceptor site for the TMS group could be any of the three heteroatoms on the main fragment with a pair of lone electrons. However, a transfer to the nitrogen seems most likely as this reaction proceeds through a six-membered transition state:



Elimination of a molecule of trimethylsilanol from this ion gives rise to the ion, m/e 483, (M–(c-73)-90). The presence of a metastable ion at the calculated m/e 262.39 indicates that this ion is the precursor of the base peak (b + 1 + 73). A tentative mechanism for the transformation is shown in Fig. 6. m/e 411 (M–c-89) is probably formed by elimination of a trimethylsilyloxy radical from (M–c); m/e 321 (M–c-89-90) is probably formed by further elimination of a trimethylsilanol. There are two fatty acid fragments at m/e 574 and m/e

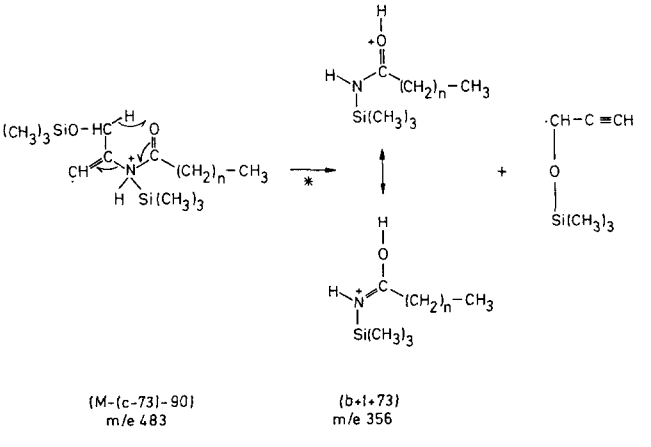


FIG. 6. Proposed formation of the ion (b + 1 + 73).

TABLE 4 MAJOR IONS IN MASS SPECTRA OF CERAMIDES

LCB Fatty Acid	Phytosphingosine	Sphingosine		Sphinganine	
	Normal	Normal	2-Hydroxy†	Normal*	2-Hydroxy†
	%	%	%	%	%
M	1	1	1	1	2
M–15	8	7	4	8	11
M–90	1	5	6	5	5
M–103	1	3	3	13	13
M–2 × 90	1	2	4	1	2
M–103-90	6	4	9	8	1
M–a	31	100	100	16	25
M–a-16	—	—	13	—	8
M–(a − 73)	—	20	19	33	100
M–(a − 73)-16	—	—	3	—	8
M–(a − 73)-90	—	—	3	—	8
M–((a + 1)-73)	36	—	—	—	—
M–a-89	2	—	6	44	86
M–a-89-16	—	—	1	—	5
M–(a − 73)–(g − 1)	4	7	3	15	14
M–(a − 73)–(g − 1)-90	9	23	6	62	17
M–(b + 1)	—	45	22	4	5
M–(b + 1)-90	4	6	10	—	—
M–(b + 1 + e)	—	24	12	—	—
M–(b + 1 + c)	21	—	4	28	30
M–(b + c)	48	—	—	—	—
b + 1 + 73	100	4	14	5	24
b + 2	21	—	5	—	20
M–c	21	—	—	—	—
M–[(c − 1)-73]	4	—	—	—	—
M–(c − 73)	4	—	—	—	—
M–(c − 73)-90	2	—	—	—	—
M–c-89	39	—	—	—	—
M–c-89-90	7	—	—	—	—
c	21	—	—	—	—
M–d	11	60	31	100	58
f	—	—	20	—	30
M–(g − 1)	—	—	—	—	5

Relative abundancies given are for the C_{18}-fatty acid ceramides.
* See reference 6.
† See reference 7.

470, which differ by 1 m.u. from ions previously discussed. Their designations, [M–(c-1)-73] and [M–(a + 1)-73], respectively, indicate a hydrogen atom transfer prior to cleavage of the LCB chain. The mechanism of formation of the ceramide fragments [M–(a-73)–(g-1)] and [M–(a-73)–(g-1)-90] at m/e 247 and m/e 157, respectively, has been discussed (7). An ion at m/e 217, which has earlier been observed in sphinganine ceramides is also present in spectra of phytosphingosine ceramides. A tentative structure for this ion is [M–(b + 1 + c)]. In the spectra of phytosphingosine ceramides, the ion at m/e 218, however, is of greater abundance. Like [M–(b + 1 + c)], this ion contains two TMS groups but lacks the acyl chain.

A comparison between the occurrence and the relative abundancies of ions in the mass spectra of ceramides containing 18 carbon atoms in the fatty acid residue is shown in Table 4. Eight ions are present only in the mass spectra of phytosphingosine ceramides, namely the ceramide fragment [M–(b + c)] and the fatty acid fragments (M–c) [M–(c-1)-73], [M–(c-73)], [M–(c-73)-90], (M–c-89), (M–c-89-90), and c. On the other hand, all molecular weight fragments in addition to the fatty acid fragments (M–a) and (b + 1 + 73), the LCB fragment (M–d), and the ceramide fragment [M–(a-73)-(g-1)], and [M–(a-73)-(g-1)-90] are present in mass spectra of all ceramides listed in Table 4.

The results presented show that TMS derivatives of phytosphingosine ceramides are suitable for GLC–MS analysis and that the structures of the constituent LCB and fatty acids can be determined by this method.

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Note Added in Proof: Additional evidence for structures of mass spectral ions was obtained from a high resolution mass spectrum of 1,3,4-tri-*O*-TMS-*N*-(stearoyl)phytosphingosine. An Atlas SM-1 instrument with direct probe inlet and a comparator from Gaertner Scientific Corp., Chicago, Ill. was used. The observed m/e values for the ions: m/e 606, 574, 573, 500, 471, 470, 411, 401, 398, 356, 321, 299, 284, 247, 218, 217, and 157 differed less than 10 p.p.m. from the exact m/e values calculated on basis of the proposed structures. (There were five ions with the integral m/e value 247. One of these had an exact m/e value which differed less than 10 p.p.m. from the value calculated for [M–(a-73)-(g-1].)

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