Determination of the Structures of Sphingolipid Bases by Combined Gas Chromatography–Mass Spectrometry*

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ABSTRACT: Analysis of the *N*-acetyl-*O*-trimethylsilyl derivatives of sphingolipid bases by combined gas chromatography–mass spectrometry gives detailed information about the chemical structures of these substances. In addition to defining the chain length and degree of unsaturation of an unknown base, mass spectral data provide information to classify the base as a sphinganine or 4-hydroxysphinganine type. The positions of double bonds in the aliphatic chains of these bases can be determined by mass spectrometry of the osmium tetroxide oxidation products of *N*-acetyl derivatives after conversion of the products into trimethylsilyl derivatives. Characteristic mass spectral fragmentations at positions of vicinal trimethylsilyloxy groups give conclusive evidence for the location of double bonds in the parent base.

A relatively large number of long-chain aliphatic amines of the general type $RCH(OH)CH(NH_2)CH_2OH$ have been found in sphingolipids isolated from animals, plants, and lower organisms. Many of these long-chain sphingolipid bases can be identified by gas chromatography of volatile trimethylsilyl derivatives (Gaver and Sweeley, 1965; Carter and Gaver, 1967b) or the aldehydes liberated by periodate oxidation (Sweeley and Moscatelli, 1959). The absolute assignment of chemical structures from gas chromatographic retention behavior has been made less certain, however, by recent evidence for branching of the aliphatic chains in some long-chain sphingolipid bases (Carter and Gaver, 1967a; Carter and Hirschberg, 1968) and for more than one olefinic group in others (Karlsson, 1967; Polito *et al.*, 1968).

Detailed structural information can be obtained by combined gas chromatography–mass spectrometry of the trimethylsilyl ethers of free long-chain sphingolipid bases (Karlsson, 1965) or their N-acetylated forms (Gaver and Sweeley, 1966), and this technique has been used recently for direct analyses of the trimethylsilyl derivatives of naturally occurring (Samuelsson and Samuelsson, 1969a) and synthetic ceramides (Samuelsson and Samuelsson, 1968, 1969). The positions of double bonds in the aliphatic chains of long-chain sphingolipid bases cannot be determined directly from mass spectra of simple derivatives, however, and we have therefore undertaken an investigation of an approach for the complete structural characterization of unsaturated long-chain sphingolipid bases from animals and plants.

Mass spectra of the trimethylsilyl ethers of vicinal diols have shown strong fragmentation ions for cleavage between the carbon atoms to which these functional groups are attached (Capella and Zorsut, 1968; Eglinton and Hunneman, 1968; Thorpe and Sweeley, 1967). We have accordingly used this derivative for combined gas chromatography–mass spectrometry of the products of osmium tetroxide oxidation of unsaturated *N*-acetyl long-chain sphingolipid bases. Mass spectra of the products from sphingosine, 4-hydroxysphing-8-enine, and sphinga-4,14-dienine have been examined, and characteristic modes of fragmentation that allow definite assignments of double-bond positions are summarized in the present report.

Materials and Methods

Synthetic sphingosine was obtained from Miles Laboratories, Inc., Elkhart, Ind. Sphinga-4,14-dienine was isolated from human plasma sphingomyelin as previously reported (Polito *et al.*, 1968). A mixture of 4-hydroxysphinganine and 4-hydroxysphing-8-enine was isolated from soybean phosphatides obtained from the Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Ill. After hydrolysis of the phosphatides in aqueous methanolic HCl reagent (Gaver and Sweeley, 1965), free long-chain sphingolipid bases were purified by silicic acid chromatography. Selective N acetylation of long-chain sphingolipid bases was carried out according to a previously described procedure (Gaver and Sweeley, 1966).

Osmium Tetroxide Oxidation and Preparation of Trimethylsilyl Derivatives. Osmium tetroxide oxidations were carried out as follows: to 1 mg of *N*-acetyl long-chain sphingolipid bases in a 20-ml, screw-capped vial was added 1.6 ml of dioxane, 0.2 ml of pyridine, and 0.2 ml of osmium tetroxide in redistilled dioxane (50 mg/ml). The mixture was shaken briefly and allowed to stand at room temperature for 1 hr, during which time a precipitate formed. A suspension of Na_2SO_3 (8.5 ml of 16% Na_2SO_3 in water and 2.5 ml of methanol were mixed immediately before use) was added, after which

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the mixture was left at room temperature for another hour. The precipitate was removed by filtration, and 20 ml of methanol was added to the filtrate, yielding another precipitate immediately. After filtration, the aqueous methanolic solution was evaporated *in vacuo* and the residue was partially dissolved in 5 ml of chloroform–methanol (2:1, v/v). This mixture was filtered, and the product was recovered by evaporation of the solvents under a stream of nitrogen. A freshly prepared solution of pyridine, hexamethyldisilazane, and trimethylchlorosilane (10:2:1, v/v) was added to the residue. About 15 min later the mixture was centrifuged, and analyses of the supernatant solution were made by gas chromatography–mass spectrometry.

Mass Spectrometry. The trimethylsilyl ethers of the highly hydroxylated products from OsO_4 oxidation of *N*-acetyl long-chain sphingolipid bases were chromatographed on 3% OV-1 or 3% OV-17[1] in silanized glass columns ($^1/_8$-in. i.d. × 6 ft) maintained isothermally at 220° or 240°. Low-resolution mass spectra were obtained directly on material eluted from the gas chromatograph, using the LKB 9000 combined gas chromatograph–mass spectrometer with the ion source at 270° and the Becker–Ryhage separator (Ryhage, 1964) at 240°. Spectra were recorded at 70 eV in about 10 sec (*m/e* 15–900) with an accelerating voltage of 3500 V and an ionizing current of 60 μA.

High-resolution mass spectra were obtained with an AEI MS 902 by direct probe analysis of the *N*-acetyltrimethylsilyl derivatives. The observed exact masses of ions for which elemental compositions are given were within 1 ppm of the calculated masses for those formulas.

Results and Discussion

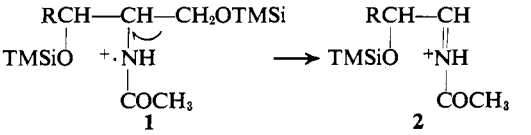
Mass spectra of the trimethylsilyl ethers of *N*-acetylsphinganine, *N*-acetylsphing-4-enine, and *N*-acetylsphinga-4,14-dienine were reported previously (Gaver and Sweeley, 1966; Polito *et al.*, 1968). They are very similar except for the shifts that occur in the locations of many ions according to the degree of unsaturation in the aliphatic chain. Although molecular ions are often too weak to be observed, the molecular weights can be deduced readily from characteristic ions for the loss of $\cdot CH_3$ from one of the trimethylsilyl groups and further elimination of a molecule of trimethylsilanol (TMSiOH). This sequence is illustrated for the trimethylsilyl derivative of *N*-acetylsphinganine

$$M\cdot^+ (m/e\ 487) \longrightarrow \cdot CH_3 + m/e\ 472 \longrightarrow m/e\ 382 + TMSiOH$$

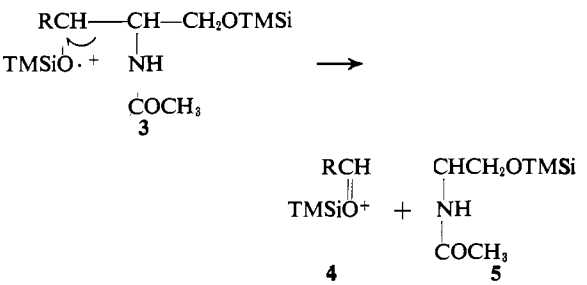
Loss of a methyl group and trimethylsilanol from the sphing-4-enine derivative gives analogous ions at *m/e* 470 ($C_{25}H_{52}NO_3Si_2$) and *m/e* 380 ($C_{22}H_{42}NO_2Si$) while those from the sphinga-4,14-dienine are at *m/e* 468 and 378.

[1] OV-1 is a methylsilicone polymer and OV-17 is a phenylmethylsilicone polymer; both are commercially available on a variety of supports for gas chromatography.

Another ion characteristic of long-chain sphingolipid bases results from the loss from molecular ion of the terminal CH_2OTMSi group, a process that can be assumed to involve the odd-electron species (**1** and **2**).



These ions occur at *m/e* 384, 382 ($C_{22}H_{44}NO_2Si$), and 380 in the mass spectra of *N*-acetyltrimethylsilylsphinganine, sphing-4-enine, and sphinga-4,14-dienine. The chain length as well as the degree of unsaturation of a particular base are particularly easy to identify from the location of an intense ion at M − 174; this fragment ion results from cleavage of another odd-electron species of the molecular ion (**3**–**5**). In the mass spectrum of *N*-acetyltrimethylsilylsphinganine this ion is at *m/e* 313; homologous saturated long-chain sphingolipid bases, differing only in chain length, give corresponding ions at *m/e* 285 (C_{16}), *m/e* 299 (C_{17}), *m/e* 327 (C_{19}), etc. The elemental composition ($C_{19}H_{39}OSi$) found for the analogous ion at *m/e* 311 with the sphing-4-enine derivative is in agreement with this structure.



When the molecular ion has its charge on nitrogen as shown in species **1**, cleavage of the bond between C-2 and C-3 results in charge retention on **5** rather than **4** and a strong ion is also observed at *m/e* 174 ($C_7H_{16}NO_2Si$) in the mass spectra of all long-chain sphingolipid base derivatives that have been examined.

Typical pathways of mass spectral fragmentation that have been found with the *N*-acetyltrimethylsilyl ethers also occur with the trimethylsilyl ethers of long-chain *N*-acyl derivatives (ceramides). Samuelsson and Samuelsson (1969b) have made a detailed study of the mass spectra of trimethylsilylceramides prepared synthetically with a variety of fatty acids condensed with sphinganine and sphing-4-enine. It was possible for them to determine the nature of the long-chain sphingolipid bases from a fragment ion $[RCH{=}OTMSi]^+$ of the same type **4** that occurs with *N*-acetyl bases, and the nature of the fatty acid residue was defined by another similar process (**6**–**8**).

An interesting rearrangement also accompanied cleavage at the C-2 to C-3 bond in the mass spectra of ceramides (Samuelsson and Samuelsson, 1969b), leading to ions of a species like **8** but bearing an additional trimethylsilyl group along with related ones at lower *m/e* for the subsequent elimination of trimethylsilanol.

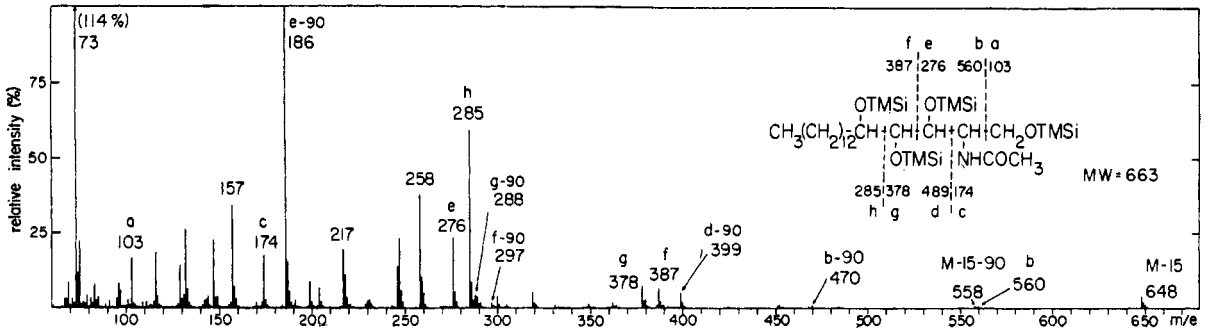
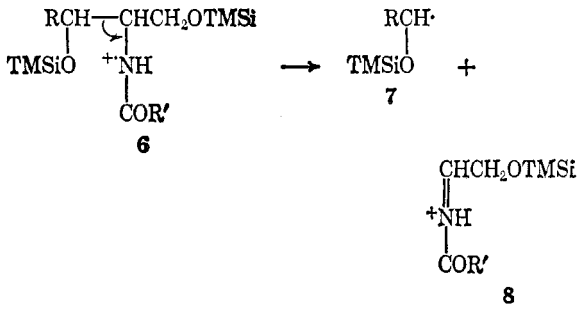
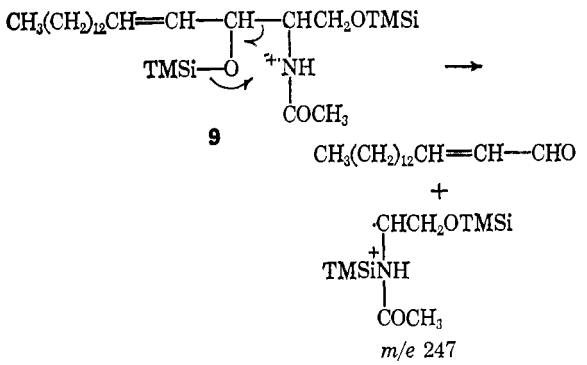


FIGURE 1: Mass spectrum of the *N*-acetyl-*O*-trimethylsilyl derivative of the osmium tetroxide oxidation product from *N*-acetylsphingosine.



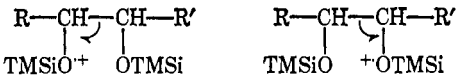
We have found the same kind of ions in the mass spectra of *N*-acetyltrimethylsilyl bases, and postulate the process shown with sphing-4-enine



The first-formed ion at *m/e* 247 ($C_{10}H_{25}NO_2Si_2$) and the product formed from it by loss of trimethylsilanol, at *m/e* 157 ($C_7H_{15}NOSi$), have formulas which are in agreement with the process shown above.

It is difficult to deduce the location of double bonds from the locations of these ions in the mass spectra of unsaturated compounds, although we previously noted (Gaver and Sweeley, 1966) that the intensity of *m/e* 174 was high (60%) in the mass spectrum of the sphing-4-enine compound and low (7.5%) in that of sphinganine. This observation has been extended to include sphinga-4,14-dienine with a strong (65%) *m/e* 174 (Polito *et al.*, 1968) and 4-hydroxysphinganine with a weak (15.7%) *m/e* 174 (Thorpe and Sweeley, 1967). The assignment of a double bond at Δ^4 is probably justified when an *N*-acetyltrimethylsilyl base has an intensity of 50% or more for this ion. Other locations for double bonds cannot be determined from these mass spectra, however.

The positions of vicinal diol groups that are produced by OsO_4 oxidation of unsaturated long-chain sphingolipid bases can be determined readily from mass spectra of the poly-*O*-trimethylsilyl ethers. A mass spectrum of the product obtained from *N*-acetylsphing-4-enine is shown in Figure 1. All of the assignments made in the figure have the support of elemental compositions calculated from exact mass measurements. Ions at *m/e* 157, *m/e* 174, and *m/e* 247 are indicative of a sphingolipid base of the classical type, as discussed before. The molecular weight of 663 is derived from ions at *m/e* 648 ($C_{31}H_{70}NO_5Si_4$) for loss of $\cdot CH_3$, *m/e* 560 ($C_{28}H_{62}NO_4Si_3$) for loss of the CH_2OTMSi group, and *m/e* 558 ($C_{28}H_{60}NO_4Si_3$) for loss of $\cdot CH_3$ and trimethylsilanol; the values for these *m/e* correspond to those predicted for an *N*-acetyl C_{18} base with four OTMSi groups. The OTMSi groups that are on adjacent carbon atoms, derived from the olefinic group in the parent base, are assigned to C-4 and C-5 by the presence of ions for simple cleavage between the vicinal trimethylsilyl ethers, located at *m/e* 285 ($C_{17}H_{37}OSi$) or *m/e* 378 ($C_{15}H_{36}NO_4Si_3$) depending upon which oxygen bears the charge in the molecular ion.



In the particular case of bases with a double bond at Δ^4, OsO_4 oxidation produces another vicinal pair of OTMSi groups on C-3 and C-4, and additional fragmentation of the same type as above leads to formation of ions at *m/e* 276 ($C_{11}H_{26}NO_3Si_2$) and M − 276, which is at *m/e* 387 ($C_{21}H_{47}O_2Si_2$) for the sphing-4-enine product (Figure 1). Ions that contain several OTMSi groups can undergo further rearrangement with the loss of trimethylsilanol, giving ions with 90 less mass units. In Figure 1, several such ions are at *m/e* 186 ($C_8H_{16}NO_2Si$), *m/e* 288 ($C_{12}H_{26}NO_3Si_2$), and *m/e* 297 ($C_{18}H_{37}OSi$).

The trimethylsilyl derivative prepared from the OsO_4 oxidation product of 4-hydroxysphing-8-enine was sufficiently volatile for analysis by combined gas chromatography–mass spectrometry. The mass spectrum (Figure 2) showed the usual ions at M − 15 (*m/e* 736), M − 103 (*m/e* 648), and M − 15 − 90 (*m/e* 646), from which the proper molecular weight of 751 was deduced. The position of the double bond was readily apparent

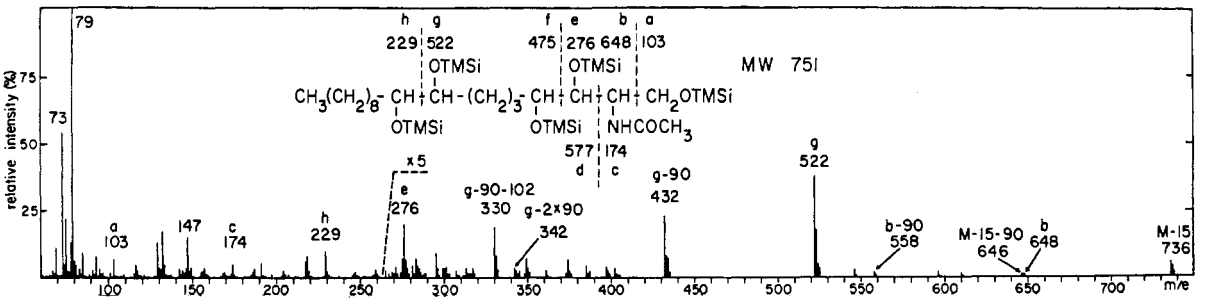


FIGURE 2: Mass spectrum of the *N*-acetyl-*O*-trimethylsilyl derivative of the osmium tetroxide oxidation product from *N*-acetyl-4-hydroxyshing-8-enine.

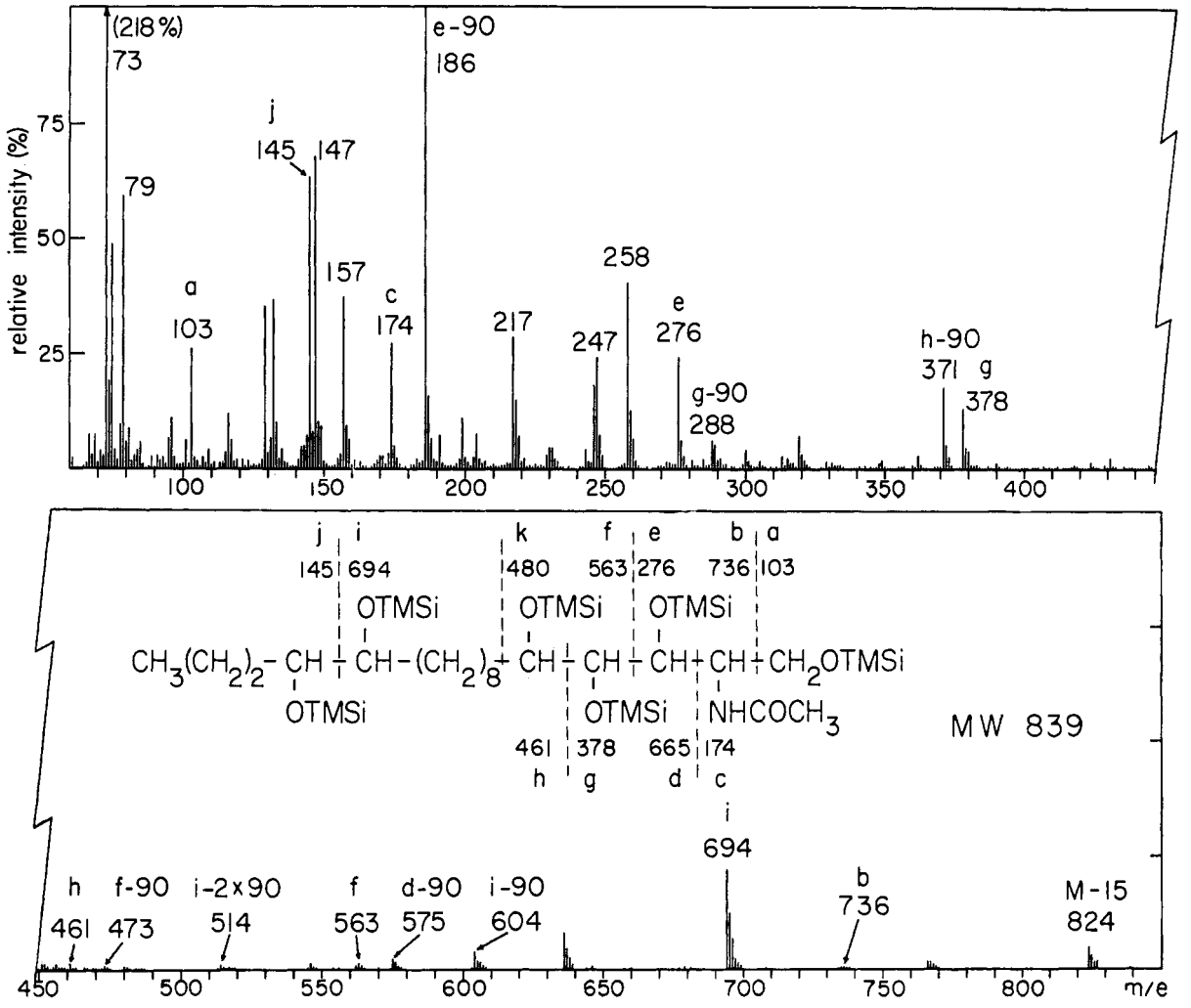


FIGURE 3: Mass spectrum of the *N*-acetyl-*O*-trimethylsilyl derivative of the osmium tetroxide oxidation product from *N*-acetyl-sphinga-4,14-dienine.

from ions at *m/e* 229 (h) and *m/e* 522 (g) for cleavage between C-8 and C-9. Relatively strong ions that resulted from secondary rearrangement losses of trimethylsilanol were also observed at *m/e* 432 (g − 90), *m/e* 342 (g − 2 × 90), and *m/e* 385 (f − 90).

The mass spectrum of the trimethylsilyl derivative of the OsO_4 product from *N*-acetylsphinga-4,14-dienine is shown in Figure 3. Even though the molecular weight of this product is 839, it is suitably volatile for gas chromatography on 3% OV-17 at 240°. Ions were observed at *m/e* 824 $(M - 15)^+$, *m/e* 736 $(M - 103)^+$, and *m/e* 734 $(M - 105)^+$. The assignment of one pair of vicinal TMSiO groups to C-4 and C-5 was based on the fragment ions at *m/e* 461 (h), *m/e* 371 (h − 90), *m/e* 378 (g), and *m/e* 288 (g − 90). Additional fragmentation gave ions at *m/e* 563 (f), *m/e* 473 (f −90), *m/e* 276 (e), and *m/e* 186 (e − 90). These resulted from the adjacent TMSiO groups on C-3 and C-4, and provided addi-

tional evidence for the location of an olefinic group at Δ^4 in the original base.

The position of the isolated double bond in sphinga-4,14-dienine was evident from prominent ions at *m/e* 145 (j) and *m/e* 694 (i), along with secondary ions at *m/e* 604 (i − 90) and *m/e* 514 (i − 2 × 90). These ions could only have arisen from cleavage between TMSiO groups at C-14 and C-15.

It is evident from these examples that the structures of unsaturated long-chain sphingolipid bases might be determined from information in the mass spectra of *N*-acetyltrimethylsilyl ethers after OsO_4 oxidation of the base. Since the derivatives are volatile, mixtures can be analyzed by combined gas chromatography–mass spectrometry without isolation of individual long-chain sphingolipid bases that are sometimes present in minute amounts. Reasonably complete separations can be expected with chain-length isomers and with compounds which differ in the number of double bonds, but products from positional isomers of unsaturated long-chain sphingolipid bases are likely to be difficult to resolve by gas chromatography.

An analysis has been made of the locations of mass spectral ions that would be expected from various chain-length isomers of sphinganine, 4-hydroxysphinganine, and the OsO_4 products of mono- and diunsaturated bases of each of these types. Unique sets of ions will be obtained, from which the following information can be derived: molecular weight of the derivative; the number of TMSiO groups in the molecule; chain length of the parent long-chain sphingolipid base; classification as a sphinganine, a 4-hydroxysphinganine, or a sphinganine with an OH group at some position other than C-4; and the positions of all vicinal TMSiO groups.

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