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Gas Chromatography and Mass Spectrometry of Sphingolipid Bases. Characterization of Sphinga-4,14-dienine from Plasma Sphingomyelin*

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ABSTRACT: A partially purified sample of sphinga-4,14-dienine was obtained by chromatographic procedures from acid-catalyzed methanolysates of human plasma sphingomyelin. The structure of this sphingolipid base was deduced from mass spectrometric data before and after osmium tetroxide oxidation, and by mass spectrometric identification of sebacic acid after permanganate–periodate oxidation of the base. Hexadecasphing-4-enine and heptadecasphing-4-enine were also identified conclusively in sphingomyelin from the mass spectra of *N*-acetyl-*O*-trimethylsilyl derivatives. Gas chromatography of the *N*-acetyl-*O*-trimethylsilyl derivatives on selective liquid phases separates sphingadienines from sphingenines and relative retention data are given for routine gas chromatographic identification of a variety of sphingolipid bases.

Sphingomyelin from human plasma contains several long-chain bases in addition to sphingosine (sphing-4-enine)[1] and sphinganine. They were originally detected by gas–liquid partition chromatography of an aldehyde fraction obtained by periodate oxidation of the bases (Sweeley and Moscatelli, 1959). On the basis of relative retention behavior before and after catalytic hydrogenation, one of the aldehydes was assumed to be derived from a doubly unsaturated base, a sphingadienine, closely related to sphingosine. Later, this new base was partially purified by silicic acid chromatography of a mixture of DNP derivatives and permanganate oxidation led Karlsson (1964) to conclude that it was a mixture of sphinga-4,14-dienine and sphinga-4,12-dienine.

Several lines of evidence have indicated the presence of lower homologs of sphingosine in sphingomyelin from human plasma and other sources. Hexadecasphing-4-enine and heptadecasphing-4-enine have been detected by gas–liquid partition chromatography of aldehydes from periodate oxidation (Sweeley and Moscatelli, 1959), gas–liquid partition chromatography of fatty acids from various types of oxidations at the olefinic center (Proštenik and Majhofer-Oreščanin, 1960; Karlsson, 1964; Popović, 1966), and gas–liquid partition chromatography of the intact bases as trimethylsilyl derivatives (Gaver and Sweeley, 1965; Karlsson, 1965).

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[1] The nomenclature for sphingolipid bases is based on recent recommendations of the Commission on Biochemical Nomenclature of IUPAC and IUB (see The Nomenclature of Lipids, *J. Am. Oil Chemists' Soc. 44*, 548A (1967)). Where new names challenge the imagination at first sight, they are used together with common names for the sake of clarity.

The structures of these homologous bases were firmly established[2] by mass spectrometry of the trimethylsilyl derivatives (Karlsson, 1965). The family of homologous sphingolipid bases also includes nonadecasphing-4-enine (Karlsson and Holm, 1965) and eicosasphing-4-enine (Majhofer-Oreščanin and Proštenik, 1961; Klenk and Gielen, 1961; Stanacev and Chargaff, 1962).

We have confirmed the structure of sphinga-4,14-dienine and the two lower homologs of sphingosine by mass spectrometry of the *N*-acetyl-*O*-trimethylsilyl derivatives of these bases. The position of the second olefinic group in sphingadienine was established by mass spectrometric identification of sebacic acid following permanganate oxidation and by characterization of the product of osmium tetroxide oxidation as 4,5,-14,15-tetrahydroxysphinganine (Polito *et al.*, 1968). The *N*-acetyl-*O*-trimethylsilyl derivative of sphinga-4,-14-dienine can be separated from derivatives of other sphingolipid bases by gas–liquid partition chromatography on columns containing selective silicones such as XE-60[3] or OV-17.

Experimental Procedures

Materials. Synthetic sphingosine and sphinganine were obtained from Miles Laboratories, Inc., Elkhart, Ind. Natural 4-hydroxysphinganine and 4-hydroxy-sphing-8-enine were gifts from H. E. Carter. A small sample of 3-deoxysphinganine (sphingine) was prepared by catalytic hydrogenation of triacetylsphingosine (Carter and Humiston, 1951). A mixture of *erythro* and *threo* isomers of *N*-acetylsphingosine was obtained by sodium borohydride reduction of the 3-oxo-*N*-acetyl derivative as previously described (Gaver and Sweeley, 1966). The *O*-methyl ethers of sphingosine were prepared by acid-catalyzed methanolysis of sphingomyelin and beef lung ceramide in dry reagent (Gaver and Sweeley, 1966); *N*-acetyl-*O*-trimethylsilyl derivatives of 3-*O*-methylsphingosine and 5-methoxy-3-deoxy-sphing-3-enine (Weiss, 1964) were differentiated by mass spectrometry–gas chromatography.[4]

Isolation of Sphingolipid Bases. Sphingomyelin was obtained from outdated ACD (acid–citrate–dextrose) human plasma by a method previously described (Sweeley, 1963) and from freshly drawn, heparinized human plasma by a modification of the procedure used to isolate glycosyl ceramides (Vance and Sweeley, 1967). In the latter method, sphingomyelin was separated from crude polar lipids by mild alkali-catalyzed methanolysis (Vance and Sweeley, 1967) and preparative thin-layer chromatography of the alkali-stable lipid on plates, coated with silica gel G (Brinkman Instruments Inc., Great Neck, Long Island, N. Y.) and developed in chloroform–methanol–water (100:42:6). Approximately 20 mg of purified sphingomyelin was recovered from 100 ml of plasma.

Long-chain bases were liberated from sphingomyelin by acid-catalyzed methanolysis with a modified aqueous methanolic HCl (Gaver and Sweeley, 1965). Approximately 3 mg of a purified fraction of bases was obtained from 100 ml of plasma.

An unresolved mixture of sphingosine and sphingadienine was separated from sphinganine and *O*-methyl ethers by preparative thin-layer chromatography in chloroform–methanol–water (100:42:6). Appropriate zones on the plates were detected by spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol; the bases were recovered from these zones by eluting them from the silica gel with methanol.

Preparation of Volatile Derivatives. Selective N acetylation of the free bases and conversion of the *N*-acetyl derivatives into 1,3-di-*O*-trimethylsilyl ethers were accomplished by procedures outlined previously (Gaver and Sweeley, 1966), but with conditions for N acetylation and trimethylsilylation that were given by Carter and Gaver (1967). The more highly hydroxylated products of osmium tetroxide oxidation were converted into trimethylsilyl derivatives by the same method with a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1).

Gas–Liquid Partition Chromatographic Purification of Sphingosine and Sphingadienine. Samples containing only *N*-acetylsphingosine and *N*-acetylsphingadienine, required for oxidations to establish the positions of olefinic groups, were prepared on a small scale from the mixture of free bases recovered from thin-layer chromatographic plates. After conversion into the *N*-acetyl-*O*-trimethylsilyl derivatives, repetitive injections were made of aliquots onto a gas–liquid partition chromatography column packed with 3% XE-60 (100–120 mesh acid-washed, silanized Gas Chrom S from Applied Science Laboratories, State College, Pa.). The column was maintained isothermally at 220° and the injection port was at 220–250° (higher temperatures can result in partial decomposition of these derivatives). Samples containing both bases, but relatively enriched with the sphingadienine, were collected in 30-in. lengths of Teflon tubing attached to the exit tube from the gas–liquid partition chromatographic instrument (F & M Instruments, Avondale, Pa., Model 400, with postcolumn stream-splitting accessory).

Gas–Liquid Partition Chromatographic Analysis of Sphingolipid Bases. The relative retention behavior of *N*-acetyl-*O*-trimethylsilyl derivatives of the sphingolipid bases was studied on packed columns (6 ft × 3 mm i.d.) containing nonpolar phases (3% SE-30 or 3% OV-1) and polar phases (3% XE-60 or 3% OV-17) on the support described above. Instruments used in these analyses were Hewlett-Packard's Model F & M 402 and Victoreen's Model 4000, both of which were equipped with flame ionization detectors. The columns were condi-

[2] The stereochemistry of their asymmetric carbon atoms has not been assigned.

[3] XE-60, a commercially available nitrile silicone polymer; OV-1 and SE-30, commercially available methyl silicone polymers; OV-17, commercially available methyl phenyl silicone polymer.

[4] We previously erred in the assignments of retention times and yields of these *O*-methyl ethers (Gaver and Sweeley, 1965). Unknown A in the previous study is 3-*O*-methylsphingosine, while the compound that was called 3-*O*-methylsphingosine is the 5-methoxy-3-deoxysphing-3-enine. The latter substance is the predominant product from anhydrous methanolysis (Weiss, 1964).

TABLE I: Relative Retention Behavior of *N*-Acetyl-*O*-trimethylsilyl Derivatives of Sphingolipid Bases.

Compound	Rel Retention Times[a] and mμ Values[b]		
	SE-30	XE-60	OV-17
Hexadecasphing-4-enine	0.55 (24.90)	0.56	0.52 (25.55)
Heptadecasphing-4-enine	0.74 (25.98)	0.75	0.72 (26.61)
3-Deoxysphinganine (sphingine)	0.76 (26.08)	1.10	0.92 (27.38)
3-*O*-Methylsphingosine	0.84 (26.40)		1.09 (27.93)
trans-D-*threo*-Sphing-4-enine	0.92 (26.70)	0.85	
threo-Sphinganine	0.95 (26.81)	0.83	0.91 (27.35)
5-Methoxy-3-deoxysphing-3-enine	0.96 (26.84)	1.68	1.42 (28.88)
Sphingosine	1.00 (26.97)	1.00	1.00 (27.66)
Sphinga-4,14-dienine	1.00 (26.97)	1.09	1.07 (27.87)
5-Methoxy-3-deoxysphinganine	1.05 (27.13)	1.74	1.52 (30.00)
Sphinganine	1.11 (27.29)	1.10	1.07 (27.87)
4-Hydroxysphing-8-enine	1.36 (27.92)	1.01	1.20 (28.28)
4-Hydroxysphinganine	1.46 (28.14)	1.05	1.20 (28.28)

[a] The retention time of *N*-acetyl-1,3-di-*O*-trimethylsilylsphingosine was 18 ± 2 min on the SE-30, XE-60, and OV-17 columns, each containing 3% liquid phase and held isothermally at 220°. [b] The mμ values (VandenHeuvel *et al.*, 1964) are given in parentheses; they are equivalent to *I*/100, where *I* is the Kovats (1958) index.

tioned in the usual way before use, and precautions were taken to assure that the injection port was always below 250°. Mixtures of the *N*-acetyl-*O*-trimethylsilyl derivatives with small amounts of tetracosane, octacosane, and dotriacontane were injected together to establish Kovats indices (Kovats, 1958).

Oxidation of N-Acetyl Derivatives. Samples (about 2 mg) of the *N*-acetyl derivatives of sphingosine and sphingadienine, isolated as a mixture by thin-layer chromatography and gas–liquid partition chromatography procedures, were oxidized with von Rudloff's reagent by a procedure previously developed for the oxidation of polyenoic acids (Chang and Sweeley, 1962). A solution of the sample in 3 ml of *t*-butyl alcohol was mixed with 4 ml of the reagent (0.018 M $NaIO_4$ and 0.0025 M $KMnO_4$) and 2 ml of 0.1 M sodium carbonate. After stirring for 6 hr at room temperature, the reaction was stopped with the addition of sodium metabisulfite and potassium hydroxide, and the acids were isolated as described earlier (Chang and Sweeley, 1962). Methyl esters were prepared with diazomethane by the procedure of Schlenk and Gellerman (1960), and gas–liquid partition chromatographic analyses were made on 3% SE-30 at 142°, using reference samples of authentic methyl esters for comparison. The results obtained with the mixture of unsaturated bases were compared with those from a pure synthetic sample of sphingosine. Dimethyl sebacate was finally characterized by combined gas chromatography–mass spectrometry.

Samples (1 mg) of the *N*-acetyl derivatives of sphingosine and sphingadienine were oxidized with osmium tetroxide according to previously described conditions (McCloskey and McClelland, 1965; Niehaus and Ryhage, 1967). The hydroxylated products were isolated and converted into poly-*O*-trimethylsilyl ethers as described in detail in another report (Polito *et al.*, 1968). Aliquots of the trimethylsilylation reaction mixture were analyzed by combined gas chromatography–mass spectrometry with a gas–liquid partition chromatography column of 3% OV-17, maintained isothermally at 240°.

Mass Spectrometry. Mass spectra of the *N*-acetyl-*O*-trimethylsilyl derivatives of the bases and osmium tetroxide oxidation products were obtained from compounds eluted into the ion source from a gas–liquid partition chromatography column (3% OV-1 or 3% OV-17) maintained isothermally at 220–240°. The instrument was an LKB 9000, operated with an ion source temperature of 270°, ionizing current of 60 μA, and accelerating voltage of 70 eV. The scanning time was about 4 sec for a mass range from 15 to 500, and about 10 sec for 15–900. Mass spectra of the methyl esters from permanganate–periodate oxidations of sphingolipid bases were obtained using a 3% OV-1 column at 140°.

Results and Discussion

Although a more highly unsaturated form of sphingolipid base was detected among the aldehydes produced on periodate oxidation of bases from human plasma sphingomyelin (Sweeley and Moscatelli, 1959), gas–liquid partition chromatography of the intact bases in the form of volatile 1,3-di-*O*-trimethylsilyl derivatives failed to resolve this component from sphingosine (Gaver and Sweeley, 1965). The *N*-acetyl-*O*-trimethylsilyl derivatives are more useful for the determination of this sphingadienine by gas–liquid partition chromatography. On either XE-60 or OV-17 columns, it is well separated from *N*-acetyl-*O*-trimethylsilylsphingosine (Table I). The retention time of the sphingadienine derivative is nearly identical with that of the sphinganine

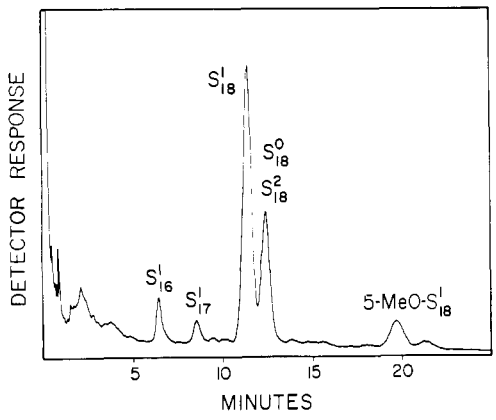

FIGURE 1: Gas–liquid partition chromatography of *N*-acetyl-*O*-trimethylsilyl bases from plasma sphingomyelin; 3% XE-60 at 230°. Superscripts indicate degree of unsaturation in the bases and subscripts indicate their chain lengths.

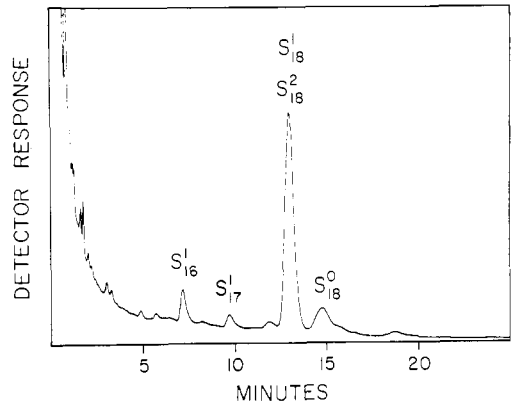

FIGURE 2: Gas–liquid partition chromatography of *N*-acetyl-*O*-trimethylsilyl bases from plasma sphingomyelin; 3% SE-30 at 230°. Superscripts indicate degree of unsaturation in the bases and subscripts indicate their chain lengths.

derivative on these two columns, however, as shown by the chromatogram in Figure 1. Since the *N*-acetyl-*O*-trimethylsilyl derivatives of sphingosine and the sphingadienine separate from that of sphinganine on nonpolar columns such as SE-30, as shown in Figure 2, all three components can be determined in mixtures by gas–liquid partition chromatography on both polar and nonpolar columns. Other sphingolipid bases have characteristic retention behavior on these columns, and the Kovats indices or methylene unit values (Vanden-Heuvel *et al.*, 1964) can be used for the qualitative identification of a variety of bases from animal and plant lipids.

Positions of olefinic groups in bases such as dehydrophytosphingosine (4-D-hydroxysphing-8-enine) and sphingadienines cannot be determined from relative retention behavior on any gas–liquid partition chromatography columns that have been tested to date. Some stereochemical assignments can be made from the gas–liquid partition chromatography data, however. For example, the *erythro* and *threo* isomers of sphingosine and sphinganine are readily separated as *N*-acetyl-*O*-trimethylsilyl derivatives on either polar or nonpolar columns, as shown in Table I.[5] Although suitable compounds have not been available to study the effects of *cis–trans* differences in unsaturated sphingolipid bases, it is doubtful that separations of these isomers will be possible.

The mass spectrum of *N*-acetyl-1,3-di-*O*-trimethylsilylsphinga-4,14-dienine is shown in Figure 3. A molecular ion is not observed, but the molecular weight is calculated from fragment ions at *m/e* 468 $(M - 15)^+$, *m/e* 424 $(M - 59)^+$, and *m/e* 309 $(M - 174)^+$, and these values are appropriate for a doubly unsaturated C_{18} base. The intensity (66%) of the peak at *m/e* 174 for $[CH(NHCOCH_3)CH_2OSi(CH_3)_3]^+$ is typical for allylic cleavage between C-2 and C-3, as previously observed in the case of sphingosine (59%) (Gaver and Sweeley, 1966). The lower homologs of sphingosine have appropriately intense ions at *m/e* 174 as well, as shown in Table II. The intensity of this ion is much less in the mass spectra of *N*-acetyl-1,3-di-*O*-trimethylsilylsphinganine (7.5%) (Gaver and Sweeley, 1966) and *N*-acetyl-1,3,4-tris-*O*-trimethylsilyl-4-hydroxysphinganine (16%) (Thorpe and Sweeley, 1967), and the intensity of this ion is considered a diagnostic test for the presence or absence of an olefinic group at Δ^4. The position of the second double bond in sphingadienine from plasma sphingomyelin cannot be determined from mass spectral data alone, although the intense fragment ion at *m/e* 309 $(M - 174)^+$, as compared with *m/e* 311 in the sphingosine derivative, is indicative of its presence in the side chain. In most respects the mass spectra of sphingosine and the diene were identical except for the shift of some of the ions by two mass units.

The position of the second double bond in this sphingadienine was determined by two independent methods. A sample containing a mixture of sphingosine and sphingadienine was oxidized with permanganate–periodate and the resulting mono- and dicarboxylic acids were compared by gas–liquid partition chromatography of their methyl esters with reference standards. The major component had a retention time on 3% SE-30 that was identical with that of methyl myristate. Small amounts of methyl laurate (2.5% of the myristate) and methyl tridecanoate (10% of the myristate) were found in the products from a mixture of sphingosine and sphingadienine as well as from pure sphingosine; these compounds were attributed to some over oxidation of the sphingosine. Methyl sebacate and a small amount of methyl azelate were identified in the mixture from sphingosine and sphingadienine by gas–liquid partition chromatography retention times and by mass spectrometry. These compounds were not produced on oxidation of synthetic sphingosine and were therefore attributed to the sphingadienine. Assignment of the position of the second double bond at Δ^{14} was based on the oxidative cleavage to sebacic acid. The small amount of azelaic acid found in this mixture is believed to be entirely from over oxidation of the sphinga-4,14-dienine.

On oxidation of the *N*-acetyl derivatives of sphingosine and sphingadienine with osmium tetroxide, rapid

[5] See also data on retention times reported by Gaver and Sweeley (1966) and Carter and Gaver (1967).

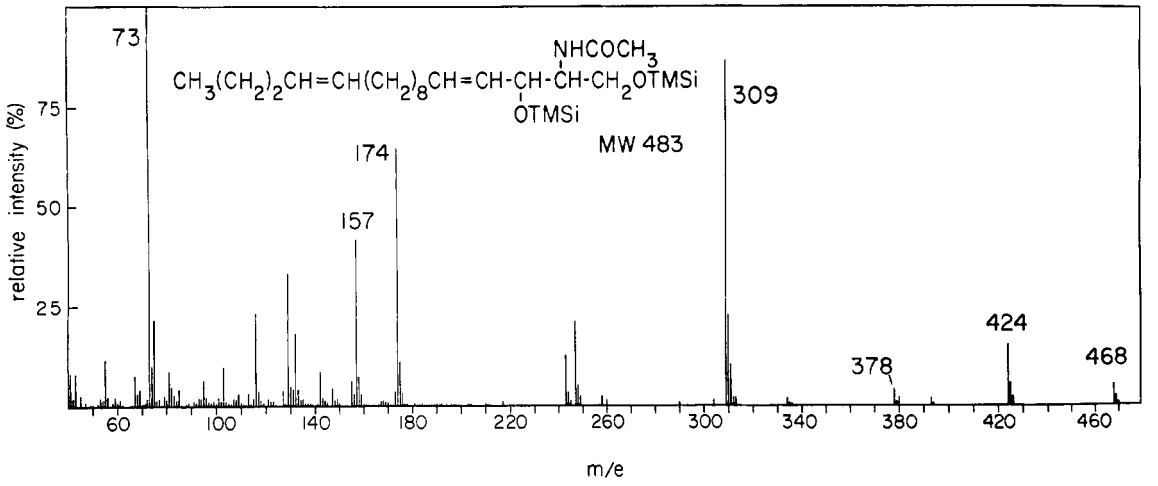

FIGURE 3: Mass spectrum of the *N*-acetyl-*O*-trimethylsilyl derivative of sphinga-4,14-dienine isolated from plasma sphingomyelin.

TABLE II: Mass Spectrometric Identification of Homologous *N*-Acetyl-*O*-trimethylsilylsphingolipid Bases.

Hexadecasphing-4-enine			Heptadecasphing-4-enine			Sphingosine		
m/e	Δ	Intensity (%)	*m/e*	Δ	Intensity (%)	*m/e*	Δ	Intensity (%)
73		100	73		100	73		100
75		24	75		25	75		29
103		11	103		12	103		10
116		28	116		29	116		24
129		44	129		43	129		36
132		18	132		13	132		17
157		42	157		39	157		45
174	M − 283	53	174	M − 297	40	174	M − 311	59
243		11	243		10	243		11
247		13	247		15	247		15
283	M − 174	55	297	M − 174	46	311	M − 174	52
352	M − 105	3	366	M − 105	2	380	M − 105	3
398	M − 59	5	412	M − 59	5	426	M − 59	6
442	M − 15	2	456	M − 15	2	470	M − 15	3
457	Molecular weight		471	Molecular weight		485	Molecular weight	

and complete conversion into cyclic osmate esters occurred; after liberation of the polyhydroxy compounds with sodium sulfite, *N*-acetyl-*O*-trimethylsilyl derivatives were prepared for analysis by combined gas chromatography–mass spectrometry. On 3% OV-17 at 240°, the retention times of the products from sphingosine and sphingadienine were 19 and 33 min, respectively. The mass spectrum of the product from sphingadienine provided conclusive evidence for two double bonds, and for their locations at Δ^4 and Δ^{14}. A molecular weight of 839 was readily apparent from *m/e* 824 $(M - 15)^+$ and *m/e* 736 $(M - 103)^+$. This molecular weight is exactly that which is predicted for the addition of four trimethylsilyloxy groups (4 × 89) to the molecular weight (483) of an *N*-acetyl-di-*O*-trimethylsilylsphingadienine. The position of one double bond is fixed at Δ^{14} by *m/e* 694 $(M - 145)^+$, for loss of $CH_3CH_2CH_2CHOSi(CH_3)_3$, and a companion ion at high intensity at *m/e* 145. Assignment of the other double bond at Δ^4 is based on the ion at *m/e* 378 for $(CH[OSi(CH_3)_3]CH[OSi(CH_3)_3]CH[NHCOCH_3]CH_2OSi(CH_3)_3)^+$; this fragment ion is also in mass spectra of 4-hydroxysphinganine derivatives, but it is absent from the mass spectrum of sphinganine.

It has not been possible to deduce the stereochemical configuration of the double bond at Δ^{14} in sphingadienine. Nor has the configuration of the double bond at Δ^4 been determined, but it is assumed to be *trans* like those in other naturally occurring sphingenines. Some

information about the configuration of asymmetric carbons 2 and 3 can be inferred from gas–liquid partition chromatography data given in Table I. The retention time of the sphingadienine derivative was identical with that of *erythro*-sphingosine on SE-30, but considerably later than the retention time of the *threo*-sphingosine on SE-30. If the sphingadienine were *threo*, the relationship of retention times for *threo*-Δ^4 and *threo*-$\Delta^{4,14}$ would be exactly opposite from classical behavior according to boiling points (dienes have a *lower* retention time than monoenes). That the retention time of the sphingadienine derivative was nearly the same as that of the *erythro*-sphing-4-enine suggests, in fact, that it has the normal *erythro* configuration.

Two lower homologs of sphingosine, hexadecasphing-4-enine and heptadecasphing-4-enine, are also observed on gas–liquid partition chromatography of the *N*-acetyl-*O*-trimethylsilyl derivatives of the bases from plasma sphingomyelin, as shown in Figures 1 and 2. Their chemical characterization by mass spectrometry is summarized in Table II. These data also illustrate the relatively high degree of reproducibility in the intensities of mass spectral ions that are important for identifications.

Of interest is the virtual absence of hexadecasphingadienine and heptadecasphingadienine in plasma sphingomyelin. Although very small amounts of these homologous dienes were found, the biosynthesis of this type of sphingolipid base must be relatively specific for the C_{18} chain length, at least in the system for plasma sphingomyelin. No evidence was found for positional isomers, as reported by Karlsson (1964) earlier. Although a small amount of azelaic acid was formed on permanganate–periodate oxidation, osmium tetroxide oxidation and mass spectrometry of the trimethylsilyl product failed to reveal evidence for a corresponding $\Delta^{4,12}$ isomer.

If sphingadienine has an endogenous origin, it may result from the condensation of 12-hexadecenoic acid with serine, or it might be formed by a desaturase system similar to the one for biosynthesis of unsaturated fatty acids in mammalian organisms. Since 12-hexadecenoic acid has never been found in living cells, the latter hypothesis is perhaps the more likely one. Of course, this new type of sphingolipid base might equally well originate in some ingested sphingolipid. Long-chain bases with olefinic groups at positions remote from the aminodiol moiety are known in types such as 4-hydroxysphing-8-enine (Carter and Hendrickson, 1963) from a variety of plant seeds. We have obtained preliminary evidence for the existence of a family of related sphingadienines in yeasts.

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