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SPHINGOLIPID BASE METABOLISM. CHEMICAL SYNTHESES AND PROPERTIES OF *N*-ACETYL DERIVATIVES OF 4R-, 4S-, 5R-, AND 5S-HYDROXYSPHINGANINE

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The following compounds were prepared by chemical synthesis from tribenzoylsphingosine: (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane, (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane, (2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane, and (2S,3S,5R)-2-acetamido-1,3,5-trihydroxyoctadecane. These compounds were characterized by melting point determination, low and high resolution mass spectra, infrared, optical rotation, chromatographic, and chemical degradation studies. In addition, each of the compounds was converted to the corresponding free base and *N*-benzoyl derivative. (2S,3S,4R)-2-Benzylamino-1,3,4-trihydroxyoctadecane was prepared from the *N*-benzoyl derivative of authentic phytosphingosine.

I. Introduction

Phytosphingosine ((2S,3S,4R)-2-amino-1,3,4-trihydroxyoctadecane or D-*ribo*-4-hydroxysphinganine) is one of the major sphingolipid bases of eukaryotic organisms. For studies of the biosynthesis and metabolism of this compound we required authentic samples of the both the 4R- and 4S-hydroxy isomers of this compound. A simple approach to the chemical synthesis of phytosphingosine from sphingosine ((2S,3R)-2-amino-4-*trans*-1,3-dihydroxyoctadecene or D-erythrosphing-4-enine) was introduced by Weiss [1] and Prostenik et al. [2] and is outlined in Fig. 1. This approach involves epoxidation of the double bond of the tribenzoyl derivative of naturally-occurring sphingosine, reduction of the resulting product with lithium aluminum hydride, and subsequent hydrogenolysis of the resulting *N*-benzyl derivative (Fig. 1). Using this general method, Weiss [1] reported the preparation of (2S,3S,4F)-2-amino-1,3,4-trihydroxyoctadecane and (2S,3S,4S)-2-amino-1,3,4-trihydroxyoctadecane while Prostenik et al. [2] reported the preparation of the former isomer. The epoxidation of the tribenzoylsphingosine should theoretically yield two distinct *trans*-epoxides as shown in Fig. 2. Assuming back-side attack by the hydride, the (4R,5S)-*trans*-epoxide

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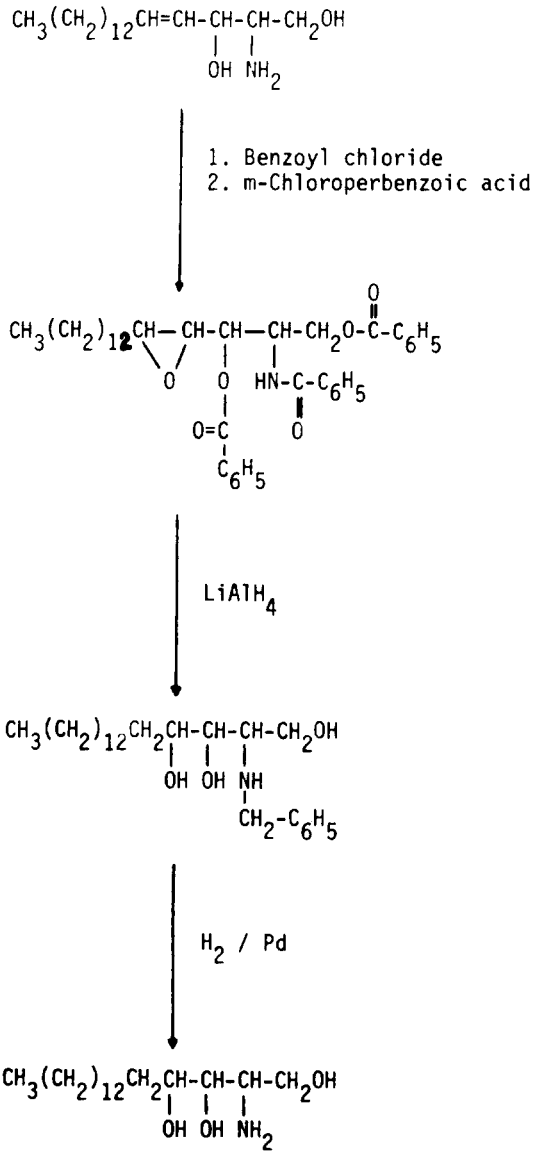


Fig. 1. Outline of chemical synthesis of phytosphingosine from sphingosine described by Weiss [1] and Prostenik et al. [2].

should yield a mixture of the (4R)-hydroxy and the (5S)-hydroxy isomers of phytosphingosine. Similarly, the (4S,5R)-*trans*-epoxide should yield a mixture of the (4S)-hydroxy and (5R)-hydroxy isomers of phytosphingosine. The purpose of this paper is to report that the use of this general approach yields all 4 of the isomers of phyto-

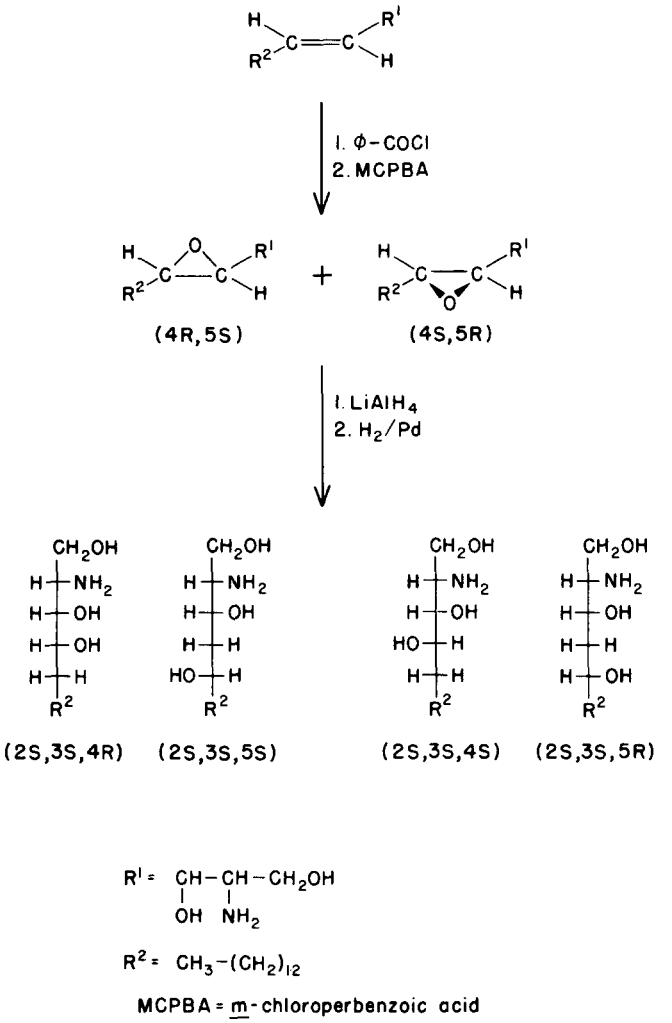


Fig. 2. Structures of the two epoxides and the 4 trihydroxy bases which might arise in the chemical synthesis of phytosphingosine from D-*erythro*-sphingosine described by Weiss [1] and Prostenik et al. [2].

sphingosine. These isomers could be separated from each other, in the form of their *N*-acetyl-derivatives, by medium pressure chromatography on silicic acid columns. The characterization of these compounds by a combination of chemical and physical techniques is described herein. A preliminary account of a portion of this work has been published [3].

II. Experimental procedures and results

A. General methods

Procedures for the recording of melting points (m.p.) [4], optical rotations [5], infrared spectra [4], and low resolution mass spectrometry (MS) [6,7] have been described previously. High resolution MS analyses were performed in the laboratory of Professor J.A. McCloskey (University of Utah) using a Varian MAT 731 mass spectrometer. Gas-liquid chromatography (GLC), combined gas-liquid chromatography-mass spectrometry (GLC-MS), and thin-layer chromatography (TLC) were performed as described previously [4]. Routine preparation of trimethylsilyl (TMS) derivatives was carried out by a minor modification of the procedure of Carter and Gaver [8]. For the larger scale preparation of the TMS derivatives for examination by high resolution MS, the following modification was used. The dried sample (~ 5 mg) was mixed with the silylation reagent (1 ml). After warming at 80°C for 10 min, the solvent was removed with a stream of dry nitrogen. Carbon disulfide (3.0 ml) was added to extract the TMS derivative from the residue and the resulting extract was filtered through glass wool and evaporated to dryness under nitrogen. For the preparation of deuterium-labeled TMS derivatives, the dried sample (~ 0.5 mg) was dissolved in a mixture of dry pyridine (0.020 ml) and d_9-trimethylchlorosilane (0.10 ml) was added. The resulting mixture was warmed at 80°C for 5–10 min prior to analysis. Medium pressure liquid chromatography (MPLC) was carried out using a single-piston pump (Fluid Metering, Inc.; Model RPSY-CSC) which delivered the elution solvent at 103–517 mm Hg through Teflon tubing to glass columns filled with silicic acid. The glass columns, tubing, valves, and fittings were purchased from Altex, Inc. The samples were loaded directly on to the column using a four-way valve. The columns were packed dry with silicic acid (0.032–0.063 mm; ICN Pharmaceuticals, Inc.) and the air displaced with chloroform before the column was equilibrated with the solvent to be used for chromatography. Fatty acid methyl esters were prepared using diazomethane by a modification of the procedure described by Roper and Ma [9]. Periodate oxidation of sphingolipid base derivatives was carried out by a modification of the method of Sweeley and Moscatelli [10]. For combined permanganate-periodate oxidation of the sphingolipid base derivatives a modification of the general procedure described by Scheuerbrandt and Bloch [11] was used.

B. Materials

All solvents were of reagent-grade quality and were purchased from Mallinckrodt, Inc. The chloroform contained ethanol (0.75%) as a preservative. Dry pyridine was prepared by distillation from barium oxide and stored over molecular sieves (size 3A, Matheson, Coleman and Bell). Hexamethyldisilazane and trimethylchlorosilane were purchased from Applied Science Laboratories, Inc. d_9-Trimethylchlorosilane was obtained from Merck, Sharp and Dohme of Canada, Ltd. A culture of *Hansenula*

ciferrii (NRRLY-1031; mating type F-60-10) was a gift from Dr. C.P. Kurtzman (U.S. Department of Agriculture, Agricultural Research Service, Peoria, IL). (3RS)-3-Hydroxyhexadecanoic acid was a generous gift from Professor C.C. Sweeley. (2S)-2-Phenyl-propionyl chloride was prepared by the method of Hammarstrom and Hamberg [12]. To racemic 2-phenyl-propionic acid (3.0 g; 20 mmol; Aldrich Chemical Company) in acetone, (60 ml) was added (+) —methylbenzylamine (2.4 g; 20 mmol; Aldrich Chemical Company) in acetone (20 ml). The resulting solution was heated at 70°C for 5 min and then allowed to stand at —20°C overnight. The resulting crystals (4.12 g) were collected and recrystallized 3 times from acetone at 4°C to give 430 mg of the desired salt. The salt was dissolved in water (5 ml) and, after adjustment of the pH to 3 by the addition of 2N HCl, the resulting acid was extracted with ether. Evaporation of the solvent with a stream of nitrogen yielded (2S)-2-phenylpropionic acid as an oil, $[\alpha]_D^{26}$ + 91.2° (*c* 1.01 in benzene; literature: + 88.0° [12] and + 92.5° [13]). The acid was mixed with freshly distilled thionyl chloride at 0°C and heated at 70°C for 30 min. Sulfur dioxide, HCl, and excess thionyl chloride were removed by repeated addition of benzene and subsequent evaporation of the solvent with a stream of nitrogen. The resulting crude acid chloride was dissolved in benzene (2.4 ml) and stored at 4°C until needed. The preparation of (2S)-2-phenylpropionate esters of hydroxy fatty acid methyl esters was carried out by the following procedure. The methyl ester of the hydroxy fatty acid (~ 0.1 mg) was mixed with the solution of the (2S)-2-phenylpropionyl chloride (80 μl) and dry pyridine (20 μl). After 2 h at room temperature, the solvent was evaporated with a stream of nitrogen. The residue was dissolved in chloroform and applied to a silica gel GF plate which was then developed using chloroform as the solvent. After visualization of the components on the plate under ultraviolet light, the component of R_F 0.6 was scraped from the plate and eluted from the adsorbant with ethyl acetate. The resulting (2S)-2-phenylpripionate ester derivative was analyzed by GLC on a 3% QF-1 column.

C. N-Acetylphytosphingosine (authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane) from Hansenula ciferrii

An authentic sample of *N*-acetylphytosphingosine (m.p. 132–133°C) was prepared by a modification [14] of the method of Weiss and Stiller [15]. The compound showed a single component on TLC (chloroform/methanol/15 M ammonia; 100:25:2.5). The specific rotation, $[\alpha]_D^{30}$, was + 5.0° (*c* 1.09 in methanol). The infrared spectrum (Fig. 3) was fully compatible with the assigned structure. Analysis by GLC of the Tris–TMS derivative (3% OV-1; 230°C) showed one major component (~ 98%) with a retention time of 20 min and a minor component (~ 2%) with a retention time of 3 min. Combined GLC (3% OV-17)-MS spectral analysis of the same derivative gave the mass spectrum shown in Fig. 4. The results of detailed studies of the electron-induced fragmentation of this compound are presented in a subsequent section. Phytosphingosine from this source has been shown to have the 2S,3S,4R-configuration [16].

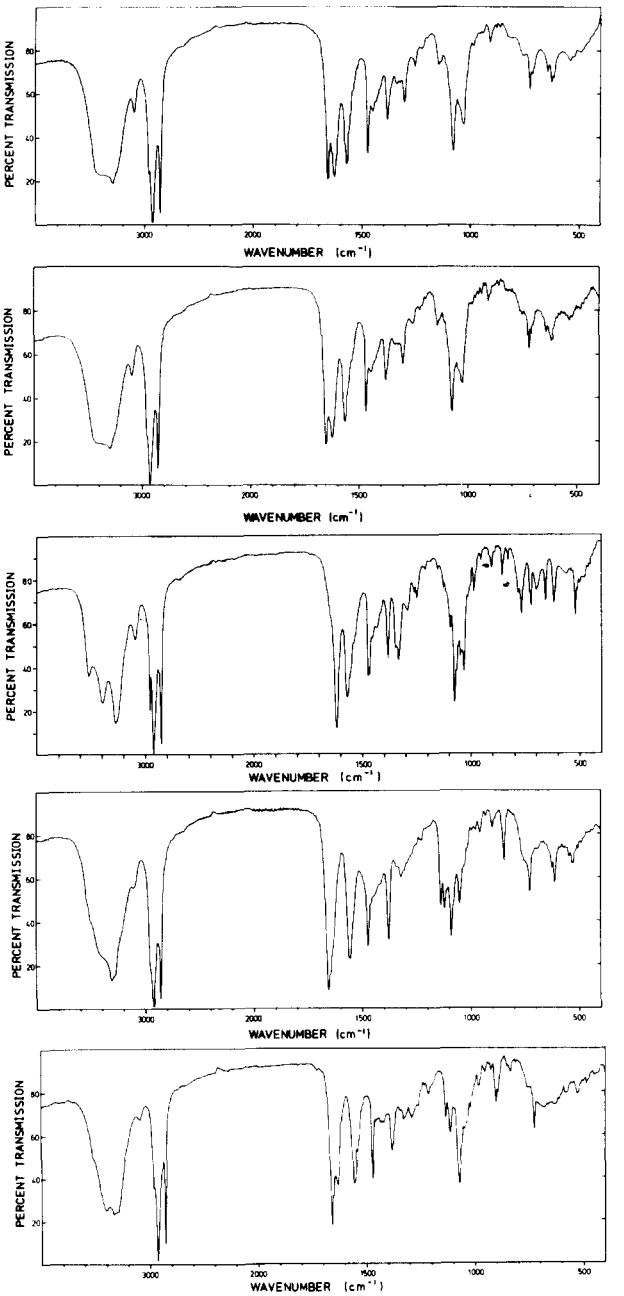


Fig. 3. Infrared spectra of (from top to bottom): authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane (from *H. ciferrii*), synthetic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane, synthetic (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane, synthetic (2S,3S,5R)-2-acetamido-1,3,5-trihydroxyoctadecane, and synthetic (2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane.

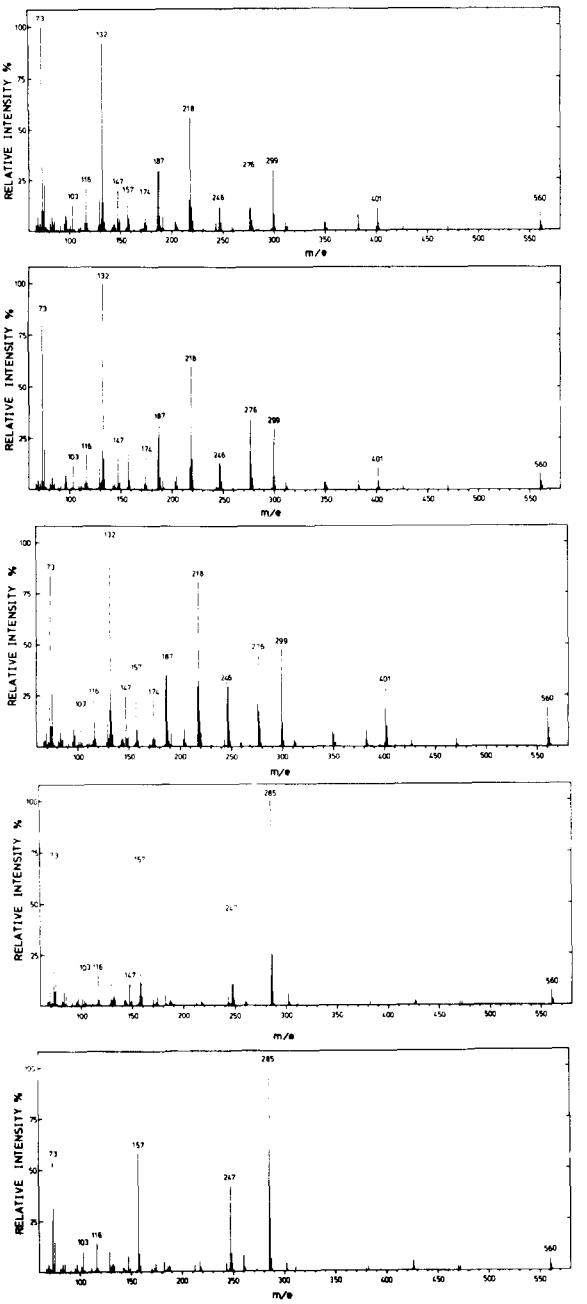


Fig. 4. Low resolution mass spectra of the trimethylsilyl ether derivatives of (from top to bottom): authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane (from *H. ciferrii*), synthetic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane, synthetic (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane, synthetic (2S,3S,5R)-2-acetamido-1,3,5-trihydroxyoctadecane, and synthetic (2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane.

N-Benzoylphytosphingosine (authentic (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane) from yeast N-acetylphytosphingosine

To yeast *N*-acetylphytosphingosine (159 mg; 0.44 mmol) in methanol (13 ml) was added 2N KOH (2.5 ml) and the resulting mixture was heated under reflux for 11 h. After cooling to room temperature, the mixture was extracted 3 times with ether (70 ml portions). The combined extracts were washed twice with water (10 ml portions), dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. The resulting free base was suspended in ether (5 ml) and 1 N NaOH (1.9 ml) and benzoyl chloride (0.30 ml) was added. After 1.5 h at room temperature, water was added and the resulting mixture was extracted 3 times with ether (100, 50, and 50 ml portions). The combined extracts were washed twice with water (15 ml portions), dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. The resulting crude product (245 mg) was recrystallized from hexane/methanol to give (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane (116 mg; 0.28 mmol; 63% yield) melting at 137–138°C (literature: 135–136°C [17], 137.8–138.8°C [18] and 135–136°C [1]). Analyses by TLC. showed a single component in two solvent systems (chloroform/methanol, 10 : 1 and benzene/methanol, 9 : 1). The infrared spectrum was compatible with the assigned structure. Analysis of the Tris–TMS derivative by GLC on a 3% OV-1 column (260°C) showed a single component. Combined GLC (3% OV–17)-MS analysis showed the following major ions: 622 (6%; M–CH_3), 444 (7%), 401 (8%; C-2–C-3 cleavage with charge retention by the C-3 fragment), 338 (23%; C-3–C-4 cleavage with charge retention on the C-3 fragment), 309 (14%; $(CH_3)_3Si-N(C_6H_5-C=O)-CH-CH_2-O-Si(CH_3)_3$, 308 (14%), 299 (19%; C-3–C-4 cleavage with charge retention on the C-4 fragment), 249 (41%, 236 (31%; C-3–C3 cleavage with charge retention by the C-3 fragment), 218 (50%), 194 (92%; 236-ketene), 147 (35%; $(CH_3)_3Si-O=Si(CH_3)_2$), 105 (96%; C_6H_5CO), and 73 (100%; $(CH_3)_3Si$).

E. Authentic (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyoctadecane

N-Benzoylphytosphingosine (56 mg; 0.13 mmol) from above was heated under reflux for 5 h with lithium aluminum hydride (100 mg; 2.63 mmol) in ether (25 ml). The excess hydride was cautiously decomposed by the successive addition of ethyl acetate and water. The resulting mixture was extracted 3 times with ether (75 ml portions) and the combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The resulting crude product (27 mg) was subjected to preparative TLC on silica gel GF plates (1 mm thick) using a mixture of chloroform, methanol, and 15 M ammonia (100 : 25 : 2.5). Two bands were observed; one with the same mobility as the starting material and a more intense band of higher R_F. The latter material was scraped from the plate and chloroform/methanol (2 : 1) and methanol were used to elute the product from the adsorbant. Evaporation of the solvent under reduced pressure gave (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyocta-

decane (8.7 mg) which melted at 90.0–91.5°C (literature: 91–92°C [1] and 55–58°C [2]). The compound showed a single component on TLC analyses in 3 solvent systems (chloroform/methanol, 10 : 1; benzene/methanol, 9 : 1; and chloroform/methanol/15 M ammonia, 100 : 25 : 2.5). The infrared spectrum was compatible with the assigned structure. Analysis of the Tris-TMS derivative by GLC (3% OV-1) showed one major component (> 98%). Combined GLC (3% OV-17)-MS analysis of the same derivative showed the following major ions: 608 (2%; M–CH_3), 520 (5%; C-1–C-2 cleavage with charge retention on the C-1 fragment), 430 (14%), 401 (5%; C-2–C-3 cleavage with charge retention on the C-3 fragment), 299 (16%; C-3–C-4 cleavage with charge retention on the C-4 fragment), 222 (100%; C-2–C-3 cleavage with charge retention on the C-2 fragment), 147 (12%; $(CH_3)_3Si\text{-}O{=}Si(CH_3)_2$), 131 (16%), 103 (9%; C-1–C-2 cleavage with charge retention by the C-1 fragment), 91 (47%; C_7H_7) and 73 (50%; $(CH_3)_3Si$).

F. D-erythro-Sphingosine ((2S,3R)-2-amino-1,3-dihydroxyoctadecane) from bovine brain sphingolipids

The procedure employed was a modification of that described by Gaver and Sweeley [19]. In a typical run 50 g of bovine brain sphingolipid, prepared by the procedure of Carter et al. [20], was heated under reflux with a mixture of 5 N HCl and methanol (1 : 4; 1000 ml) for 18 h. After cooling to room temperature, the resulting mixture was extracted 3 times with petroleum ether (500 ml portions; b.p. 35–60°C) to remove fatty acids. The pH of the residual aqueous methanol solution was, after chilling in an ice bath, adjusted to 12 by the addition of 15 N NaOH. Chloroform (1600 ml) and water (350 ml) were added, the mixture thoroughly shaken, and the phases allowed to separate. The lower layer was removed and washed twice with an equal volume of a mixture of chloroform, methanol, and water (6 : 94 : 96) and the solvent was evaporated to dryness under reduced pressure. The resulting crude base (10 g) was dissolved in a mixture of chloroform and methanol (9 : 1; 40 ml) and was subjected to MPLC on a silicic acid column (2.5 cm × 100 cm) using the same solvent mixture as the eluting solvent at a flow rate of 4 ml/min. After 200 ml of solvent had been eluted from the column, fractions 16 ml in volume were collected and the eluate was monitored by TLC. Under these conditions the elution of sphingosine started at ~ fraction 68. The contents of fractions 75 through 110 were pooled, evaporated to dryness under reduced pressure, decolorized with charcoal, and recrystallized from ethyl acetate/methanol to give pure sphingosine (3.0 g) which melted at 84–86°C (literature: 79–81°C [21]); $[\alpha]_D^{26}$ + 10.9° (*c* 1.50 in methanol). The infrared spectrum was compatible with the assigned structure. Analysis by TLC (chloroform/methanol/15 M ammonia; 100 : 25 : 2.5) showed one major band with an R_F 0.6 and a very faint band with a slightly lower R_F. Examination of the TMS derivative by gas-liquid chromatography on a 3% OV-1 column at 220°C indicated a purity of ~ 97% with a minor component (~ 3%) with the same retention time as that of the TMS derivative of authentic dihydrosphingosine. Examination of the TMS derivative of the *N*-acetylated compound by GLC (3% OV-17) showed a single sharp

peak which was easily distinguished from that produced by the corresponding derivative of *threo-N*-acetylsphingosine. Combined GLC-MS analysis of the TMS derivative of the free base showed the following major ions: 428 (1%; $M-CH_3$), 340 (6%; $CH_3-(CH_2)_{12}-CH=CH-\underset{\underset{O-Si(CH_3)_3}{|}}{CH}-CH-NH_2$), 311 (8%; $CH_3-(CH_2)_{12}-CH=CH-CH-$ $O-Si-(CH_3)_3$), 250 (12%), 155 (36%), 132 (97%; $NH_2-CH-CH_2-O-Si-(CH_3)_3$), 116 (55%), 75 (100%; $(CH_3)_2-Si-OH$), and 73 (80%; $(CH_3)_3-Si$).

G. D-erythro-Tribenzoylsphingosine

D-*erythro*-Tribenzoylsphingosine was prepared by a modification of the method of Carter et al. [21]. To D-*erythro*-sphingosine (3.61 g; 12.1 mmol) in pyridine (60 ml) was added benzoyl chloride (10.2 ml; 87 mmol) with stirring. After continued stirring for 2.5 h at room temperature, water (30 ml) was added. After 5 min, additional water (600 ml) was added and the resulting precipitate was collected by filtration, washed with water, and dried in a vacuum desiccator over P_2O_5 to give crude-*D-erythro*-tribenzoylsphingosine (7.22 g). The crude product was dissolved in chloroform (40 ml) and subjected to MPLC on a silicic acid column (2.5 cm × 100 cm) using chloroform as the eluting solvent at a flow rate of 2.25 ml/min. After a total of 200 ml had been eluted from the column, fractions 9 ml in volume were collected. The contents of fractions 7 through 16 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from hexane/methanol to give pure D-*erythro*-tribenzoylsphingosine (6.06 g; 9.92 mmol; 82% yeild) which melted at 122–123°C (literature: 121.5–123.5°C [21] and 120–122°C [2]); $[\alpha]_D^{29} - 5.5°$ (*c* 1.66 in chloroform) (literature: −11.2° [21] and −10.7° [2]). The infrared spectrum was compatible with the assigned structure. Analysis by TLC (chloroform) showed a single component of R_F 0.18. The mass spectrum showed the following major ions: 489 (6%; M-benzoic acid), 384 (7%; M-benzoic acid–C_6H_5CO), 367 (100%; M–benzoic acid–benzoic acid), 354 (52%), 268 (14%; $C_6H_5CO-NH-CH-CH_2-O-OC-C_6H_5$), 251 (22%), 198 (43%), 172 (33%), 159 (20%), 146 (268-benzoic acid), 130 (67%), 122 (64%, benzoic acid), 105 (90%; C_6H_5CO), and 77 (69%; C_6H_5).

H. Epoxidation of D-erythro-tribenzoylsphingosine

The following modification of the procedure described by Weiss [1] was utilized. D-*erythro*-Tribenzoylsphingosine (5.94 g; 9.73 mmol) was dissolved in chloroform (20 ml) and mixed with a solution of *m*-chloroperbenzoic acid (5.9 g; 29 mmol) in benzene (200 ml). After standing for 24 h in the dark at room temperature, the reaction mixture was washed with a 5% solution of $NaHCO_3$ and repeatedly with water until neutral, and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure at ~ 40°C to give the crude epoxide (8.4 g). The product was dissolved in a mixture of hexane and chloroform (1 : 1; 45 ml) and subjected to MPLC

on an activated silicic acid (heated at 120°C for 2 h prior to use) column (2.5 cm × 100 cm) using the same solvent mixture as the eluting solvent at a flow rate of 4 ml per min. After a total of 1105 ml had been eluted from the column, the eluting solvent was changed to chloroform at a flow rate of 8 ml per min. Fractions 16 ml in volume were collected and the eluate was monitored by TLC. The contents of fractions 62 through 110 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from hexane/methanol to give the tribenzoyl epoxide (4.08 g; 6.51 mmol; 67% yield) which melted at 136.5–137.5°C (literature: 135–137°C [1] and 134–135°C [2]); $[\alpha]_D^{28} - 5.9°$ (*c* 1.21 in chloroform). Analyses by TLC in 3 solvent systems (chloroform; benzene/ether, 9 : 1; and hexane/ether, 6 : 4) showed a single band. The infrared spectrum showed the presence of complex absorption at ~ 900 cm^{-1} due to the oxirane moiety and absence of the absorption due to the *trans*-olefin at 965 cm^{-1}. The mass spectrum showed the following major ions: 522 (1%; M–C_6H_5CO), 492 (2%; C-1–C-2 cleavage with charge retention by the C-2 fragment), 487 (8%), 400 (7%), 383 (29%; M-benzoic acid–benzoic acid), 370 (12%), 359 (6%; C-2–C-3 cleavage with charge retention by the C-3 fragment), 354 (7%), 292 (17%), 268 (19%; C-2–C-3 cleavage with charge retention by the C-2 fragment), 188 (37%), 171 (57%), 159 (66%), 146 (94%; 268-benzoic acid), 122 (47%; benzoic acid), 105 (100%; C_6H_5CO) and 77 (71%; C_6H_5). It should be noted that while the epoxide appeared homogeneous in three different solvent systems and had a narrow melting range, the optical rotations of the products derived from 4 synthetic runs had values of −5.9°, −8.4°, −8.8°, and −11.2°. Epoxidation of the *trans*-olefinic bond of D-*erythro*-tribenzoylsphingosine should theoretically give both the (2S,3S, 4R,5S)- and (2S,3S,4S,5R)-tribenzoylsphingosine *trans*-epoxides. The variation in the ratios of the two isomers probably accounts for the observed variation in the optical rotation values. This interpretation is consistent with the fact that 4 isomers of the trihydroxy sphingolipid base are obtained upon reduction of the epoxide with lithium aluminum hydride (see below).

I. Lithium aluminum hydride reduction of D-erythro-tribenzoylsphingosine trans-epoxides

The mixture of tribenzoylsphingosine *trans*-epoxides (3.61 g; 5.76 mmol; $[\alpha]_D^{28} -5.9°$) from above was slurried in ether (350 ml) containing molecular sieves (15 g; size 3A; Matheson, Coleman and Bell) and allowed to stand for 15 min. Lithium aluminum hydride (1.08 g; 28.5 mmol) was added and the mixture was heated under reflux for 5.5 h. The mixture was cooled in an ice bath and methanol (350 ml) and 2.5 N NaOH (350 ml) were added. After transfer to a separatory funnel, the upper phase was removed and the lower phase was extracted 3 times with a 1 : 1 mixture of ethyl acetate and ether (480 ml portions). The extracts were combined with the initial upper phase, washed 4 times with water (100 ml portions), dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. The crude product (3.0 g), presumably a mixture of the (2S,3S,4R) and (2S,3S,4S) isomers of 2-benzylamino-

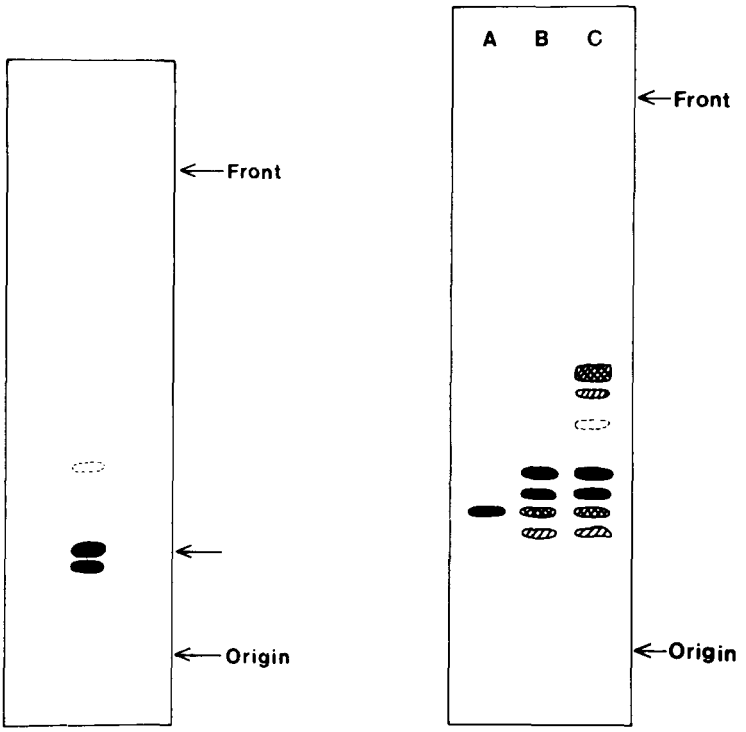


Fig. 5. (*left*) Thin-layer chromatogram of crude mixture of *N*-benzylated trihydroxy bases (solvent: $CHCl_3/CH_3OH$, 10 : 1). The middle arrow indicates the mobility of authentic (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyoctadecane.

Fig. 6. (*right*) Thin-layer chromatogram (solvent: $CHCl_3/CH_3OH$/15 M NH_4OH, 100 : 25 : 2.5) of: (A) authentic phytosphingosine; (B) Free base Mixture A; (C) Free Base Mixture B.

1,3,4-trihydroxyoctadecane and the (2S,3S,5R) and (2S,3S,5S) isomers of 2-benzylamino-1,3,5-trihydroxyoctadecane, was analyzed by TLC (chloroform/methanol, 1 : 1) and showed two major components, the least polar of which had the same mobility as authentic (2S.3S.4R)-2-benzylamino-1,3,4-trihydroxyoctadecane (Fig. 5).

J. Hydrogenation of mixture of 2-benzylaminotrihydroxyoctadecanes

The mixture of 2-benzylaminotrihydroxyoctadecanes (3.0 g) from above was dissolved in ethanol (175 ml) and hydrogenated for 5.5 h at 260 mm Hg using a 5% palladium on charcoal catalyst (9.1 g). The catalyst was removed by filtration and washed with methanol (50 ml) and hot methanol (200 ml). The initial filtrate and the washes were combined to give Free Base Mixture A. Further washing of the catalyst with a 2 : 1 mixture of chloroform and methanol (300 ml) gave Free Base Mixture B. Analyses by TLC (chloroform/methanol/15 M ammonia; 100 : 25 : 2.5) showed, for

both mixtures, two abnormally broad major bands and several less polar minor components (Fig. 6).

J. N-Acetylation of free base mixtures A and B

The material designated as free base mixture A from above was dissolved in pyridine (5 ml) and acetic anhydride (5 ml) was added. After 2 h at room temperature the mixture was evaporated to dryness under a stream of nitrogen and the resulting residue was dried overnight in a vacuum desiccator over P_2O_5. The crude product was subjected to mild alkaline hydrolysis using 0.1 N KOH in 90% methanol (100 ml) for 5 h at room temperature. The methanol was evaporated under reduced pressure and water (25 ml) was added. The resulting mixture was extracted 3 times with ether (250 ml portions) and the combined extracts were washed 3 times with water (50 ml portions), dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure to give N-acetylated base misture A (920 mg). In a similar fashion, free base mixture B was N-acetylated to give N-acetylated base mixture B (427 mg). The two mixtures of N-acetylated bases appeared similar upon TLC (chloroform/methanol; 10 : 1). Each mixture showed 4 major polar components (Fig. 7). In addition, mixture B showed several less polar components. Mixtures A and B were combined.

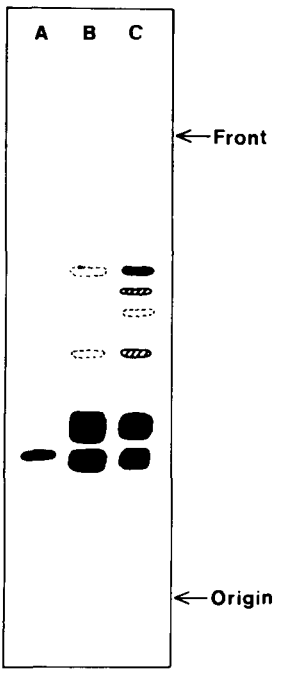


Fig. 7. Thin-layer chromatogram (solvent: $CHCl_3/CH_3OH$, 10 : 1) of: (A) authentic *N*-acetylphytosphingosine; (B) N-Acetylated Base Mixture A; (C) N-Acetylated Base Mixture B.

Table 1

Medium pressure liquid chromatography of N-acetylated bases

Fractions	Weight (mg)
1–18	256
19–23	371
24–29	283
30–40	211
41–65	121

K. Medium pressure silicic acid column chromatography of N-acetylated bases

The N-acetylated base mixture from above was dissolved in a mixture of chloroform and methanol (93 : 7; 20 ml) and subjected to MPLC on a silicic acid column (1.5 cm × 100 cm) using the same solvent mixture as the eluting solvent at a flow rate of 1.5 ml/min. After a total of 100 ml had been eluted from the column, fractions 12 ml in volume were collected. After 58 fractions had been collected in this manner, the fraction size was increased to 24 ml. Based upon the results of TLC analyses of the eluate, fractions were combined as indicated in Table 1 and the solvent was evaporated under reduced pressure.

L. (2S,3S,4S)-2-Acetamido-1,3,4-trihydroxyoctadecane, (2S,3S,4S)-2-Amino-1,3,4-trihydroxyoctadecane, and (2S,3S,4S)-2-Benzoylamido-1,3,4-trihydroxyoctadecane

The material corresponding to fractions 19 through 23 from the MPLC (Table 1) was recrystallized from ethyl acetate to give pure (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane (335 mg; 0.93 mmol; 16% yield, calculated from the epoxide) which melted at 118–119°C, $[\alpha]_D^{28}$ −0.5° (*c* 1.06 in methanol). Analysis by TLC (chloroform/methanol, 10 : 1) showed a single component which was clearly separated from authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane (Fig. 8). Analysis by GLC (3% OV-1) of the Tris–TMS derivative showed a single component which had a retention time identical with that of the corresponding derivative of authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane. Periodate oxidation proceeded to completion (as indicated by TLC) to yield a product which, upon GLC analysis (3% OV-1), showed one component with a retention time identical to that of authentic pentadecanal. Reduction of the product of the periodate oxidation with sodium borohydride gave pentadecanol which was characterized, as its TMS ether derivative, by GLC (3% OV-17). A single component was observed with a retention time identical to that of the TMS derivative of authentic pentadecanol. The infrared spectrum of the N-acetylated base was fully compatible with the assigned structure but distinguishable, in the fingerprint region, from that of authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane (Fig. 3). Combined GLC (3% OV-17)-MS analysis of the Tris–TMS

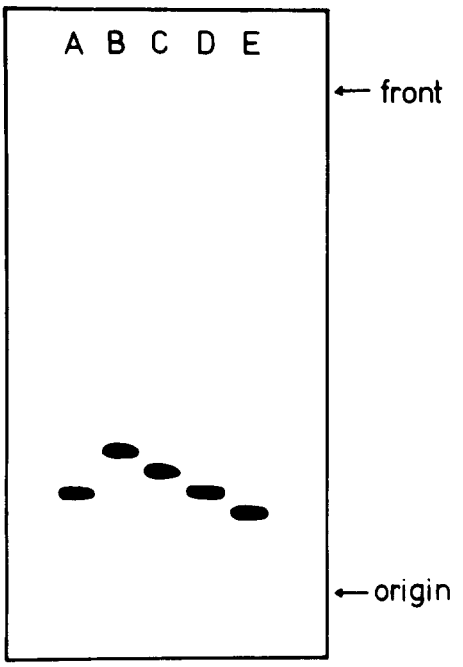


Fig. 8. Thin-layer chromatogram (solvent: $CHCl_3/CH_3OH$, 10 : 1) of: (A) authentic *N*-acetylphytosphingosine; (B) (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane; (C) (2S,3S,5R)-2-acetamido-1,3,5-trihydroxyoctadecane; (D) (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane; (E) (2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane.

derivative gave the mass spectrum shown in Fig. 4. The results of analysis of this spectrum, studies of the corresponding d_9-TMS derivative, and high resolution mass spectral analyses were fully compatible with the assigned structure (Table 2). The ions of *m/e* 401, 299, and 276 were of particular importance in establishing the location of the hydroxyl function at carbon atom 4.

The results of the periodate oxidation studies and the mass spectral studies clearly indicate the overall structure to be that of a 2-acetamido-1,3,4-trihydroxyoctadecane. Since the absolute configurations at carbon atoms 2 and 3 of the starting material for the synthesis, sphingosine, are well established and since the reactions employed should not alter the stereochemistry at carbon atoms 2 and 3 of the product, the absolute configurations at C-2 and C-3 are 2S,3S. Only the absolute configuration of the hydroxyl group at C-4 remained to be specified. The configuration of C-4 must be 4S since the compound differed from its isomer, authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane, in its m.p., optical rotation, infrared spectrum, and mobility on silicic acid columns and on TLC on silica gel.

The compound was further characterized by conversion to the free base and to the corresponding *N*-benzoyl derivative.

Table 2

Major ions of diagnostic significance in the mass spectrum of the Tris-trimethylsilyl derivative of (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane

Nominal mass	Relative intensity (%)	Exact mass		Shift with d_9-TMS		Proposed structure
		Observed	Calculated	Observed	Predicted	
560	19%	560.3963	560.3987	24	24	M-CH_3
401	29%	401.3274	401.3271	18	18	CH_3-$(CH_2)_{13}$-CH-CH-O-Si(CH_3) O-Si$(CH_3)_3$
299	48%	299.2751	299.2770	9	9	CH_3-$(CH_2)_{13}$-CH-O-Si$(CH_3)_3$
276	46%	276.1451	276.1451	18	18	$(CH_3)_3$Si-O-CH-CH-CH_2-O-Si$(CH_3)_3$ NH-$COCH_3$
247	30%	247.1400	247.1425	18	18	CH-CH_2-O-Si$(CH_3)_3$ $(CH_3)_3$Si-NH-$COCH_3$
174	23%	174.0945	174.0950	9	9	CH-CH_2-O-Si$(CH_3)_3$ NH-$COCH_3$
157	35%	157.0907	157.0924	9	9	CH=CH_2 $(CH_3)_3$Si-N-$COCH_3$
147	25%	147.0656	147.0662	15	15	$(CH_3)_2$Si=O-Si$(CH_3)_3$
132	100%	132.0844	132.0845	9	9	CH-CH_2-OSi$(CH_3)_3$ (ion 174-ketene) NH_2

The N-acetylated base (26 mg) in methanol (13 ml) was heated under reflux with 2 N KOH (2.5 ml) for 8 h. Water (10 ml) was added and the resulting mixture was extracted 3 times with ether (50 ml portions). The combined extracts were washed 3 times with water (20 ml portions), dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure to give (2S,3S,4S)-2-amino-1,3,4-trihydroxyoctadecane. The product showed a single component on TLC (chloroform/methanol/15 M ammonia, 100 : 25 : 2.5) with R_F 0.58. Analysis of the Tris–TMS derivative by GLC (3% OV-17) showed one component with a retention time identical to that of the derivative of authentic phytosphingosine. Combined GLC (3% OV-17)-MS analysis of the TMS derivative showed the following major ions: 518 (2%; M–CH_3), 430 (2%; C-1–C-2 cleavage with charge retention by the C-2 fragment), 340 (4%), 312 (17%), 299 (31%; C-3–C-4 cleavage with charge retention by the C-4 fragment), 204 (62%), 132 (100%; C-2–C-3 cleavage with charge retention by the C-2 fragment), 129 (48%), 116 (31%), 75 (44%; $(CH_3)_2$–Si–OH), and 73 (82%; $(CH_3)_3$Si).

The free base (3 mg) was suspended in ether (1 ml). 1 N NaOH (0.4 ml) and benzoyl chloride (0.025 ml) were added. After 1 h at room temperature, water (1 ml) was added and the mixture was extracted with ether (4 ml). The ether extract was washed 3 times with water (1 ml portions) and the solvent was evaporated under a stream of nitrogen. The residue was dissolved in methanol (0.9 ml), 1 N KOH (0.1 ml) was added, and the mixture was left at room temperature for 1 h. After removal of the methanol with a stream of nitrogen, water (1 ml) was added, and the mixture was extracted with ether (4 ml). The extract was washed 3 times with water (0.5 ml) and the solvent was evaporated under nitrogen to give (2S,3S,4S)-2-benzoylamido-1,3,4-trihydroxyoctadecane as a white residue. Analysis by TLC (chloroform/methanol, 10 : 1) showed a major band with R_F 0.48 and a minor band with R_F 0.77. Analysis by GLC (3% OV-17) of the Tris–TMS derivative showed a major component (~ 90%) with a retention time of 1.04 (relative to that of Tris–TMS *N*-benzoyl derivative of authentic phytosphingosine) and a minor component (~ 10%) with a relative retention time of 0.87. Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative of the major component showed the following major ions: 622 (9%; M–CH_3), 401 (19%; C-2–C-3 cleavage with charge retention by the C-3 fragment), 338 (25%; C-3–C-4 cleavage with charge retention by the C-3 fragment), 309 (31%; $(CH_3)_3$–Si–N(–C_6H_5–C=O)–CH–CH_2–O–$Si(CH_3)_3$), 299 (26%; C-3–C-4 cleavage with charge retention by the C-4 fragment), 249 (43%), 236 (35%; C-2–C-3 cleavage with charge retention by the C-2 fragment), 218 (45%), 194 (73%), 147 (25%; $(CH_3)_3$Si–O=Si$(CH_3)_2$), 105 (62%; C_6H_5CO), and 73 (100%; $(CH_3)_3$–Si). The mass spectrum was essentially the same as that of the Tris–TMS derivative of authentic (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane.

M. (2S,3S,4R)-2-Acetamido-1,3,4-trihydroxyoctadecane, (2S,3S,4R)-2-amino-1,3,4-trihydroxyoctadecane, and (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane

The material corresponding to fractions 30 through 40 from the MPLC (Table 1)

was dissolved in a mixture of chloroform and methanol (92.5 : 7.5; 10 ml) and subjected to MPLC on a silicic acid column (1.5 cm × 100 cm) using the same solvent mixture as the eluting solvent at a flow rate of 1.5 ml/min. Fractions 12 ml in volume were collected. The contents of fractions 48 through 65 were pooled and, after evaporation of the solvent under reduced pressure, the residue was recrystallized from ethyl acetate/methanol to give pure (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane (88 mg; 0.25 mmol; 4% yield, calculated from the epoxide) which melted at 133–134°C; $[\alpha]_D^{30}$ + 4.7° (*c* 0.772 in methanol). The product showed a single component on TLC (chloroform/methanol, 10 : 1) with a R_F of 0.20 which was identical with that of authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane (Fig. 8). The Tris–TMS derivative showed a single component on GLC (3% OV-1) which had the same retention time as that of the TMS derivative of the authentic compound. Periodate oxidation proceeded to completion (as indicated by TLC) to yield a product which, upon GLC analysis (3% OV-1), showed one peak with a retention time identical to that of authentic pentadecanal. Sodium borohydride reduction of the product of the periodate oxidation gave pentadecanol which was characterized, as its TMS derivative, by GLC (3% OV-17). A single peak was observed with a retention time identical to that of the TMS derivative of authentic pentadecanol. The infrared spectrum of the N-acetylated base was fully compatible with the assigned structure and essentially identical with that of an authentic sample of *N*-acetylphytosphingosine (Fig. 3). Combined GLC (3% OV-17)-MS analysis gave the mass spectrum shown in Fig. 4. The results of the analysis of this spectrum, studies of the corresponding d_9-TMS derivative, and high resolution mass spectral studies were fully compatible with the assigned structure (Table 3). The ions at *m/e* 401, 299 and 276 were of particular importance in establishing the location of the hydroxyl function at carbon atom 4.

The results of the periodate oxidation studies and the mass spectral studies clearly establish the overall structure to be that of a 2-acetamido-1,3,4-trihydroxyoctadecane. Since the absolute configurations of the substituents at carbon atoms 2 and 3 of the starting material for the synthesis, sphingosine, are well established and since the reactions employed should not affect the stereochemistry at carbon atoms 2 and 3 of the product, the absolute configurations at C-2 and C-3 are 2S,3S. The configuration at C-4 must be 4R since all the physical properties (melting point, infrared, optical rotation, mass spectral, and chromatographic) were essentially the same as that for authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane. The compound differed from the chemically synthesized (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane in its m.p., optical rotation, infrared spectrum, and chromatographic mobility on TLC on silica gel.

The compound was further characterized by conversion to the free base and to the corresponding *N*-benzoyl derivative.

The N-acetylated base (22 mg) in methanol (5 ml) was heated under reflux with 2 N KOH (0.95 ml) for 7 h. Water (5 ml) was added and the resulting mixture was extracted 3 times with ether (20 ml portions). The combined extracts were washed 3 times with water (5 ml portions), dried over anhydrous $MgSO_4$, and evaporated to

Table 3

Major ions of diagnostic significance in the mass spectrum of the Tris-trimethylsilyl derivative of (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane

Nominal mass	Relative intensity	Exact mass		Shift with d_9-TMS		Proposed structure
		Observed	Calculated	Observed	Predicted	
560	8%	560.3983	560.2987	24	24	M-CH_3
401	11%	401.3251	401.3271	18	18	CH_3-$(CH_2)_{13}$-CH(O-Si$(CH_3)_3$)-CH-O-Si$(CH_3)_3$
299	30%	299.2768	299.2770	9	9	CH_3-$(CH_2)_{13}$-CH-O-Si$(CH_3)_3$
276	34%	276.1451	276.1451	18	18	$(CH_3)_3$Si-O-CH(NH-$COCH_3$)-CH-CH_2-O-Si$(CH_3)_3$
247	12%	247.1398	247.1425	18	18	CH(CH_2-O-Si$(CH_3)_3$)-NH-$COCH_3$
						$(CH_3)_3$Si-
174	16%	174.0949	174.0950	9	9	CH(CH_2-O-Si$(CH_3)_3$)-NH-$COCH_3$
157	19%	157.0935	157.0924	9	9	CH(CH_2)-N($COCH_3$)-Si$(CH_3)_3$
147	20%	147.0655	147.0662	15	15	$(CH_3)_2$Si=O-Si$(CH_3)_3$
132	100%	132.0844	132.0845	9	9	CH(CH_2-O-Si$(CH_3)_3$)-NH_2 (ion 174-ketene)

dryness under reduced pressure to give (2S,3S,4R)-2-amino-1,3,4-trihydroxyoctadecane. The product showed one major component with R_F 0.53 upon TLC (chloroform/methanol/15 M ammonia, 100 : 25 : 2.5) with a trace component of R_F 0.75. The TMS derivative showed a single (>99%) component of GLC (3% OV-17) which had the same retention time as the same derivative of authentic phytosphingosine. Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative showed the same major ions as described above in the case of the corresponding derivative of the (2S,3S,4S)-isomer.

The free base (5 mg) was benzoylated as described previously for the case of the (2S,3S,4S)-isomer to give (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane. Analysis by TLC (chloroform/methanol, 10 : 1) showed a single component with an R_F of 0.43. Analysis of GLC (3% OV-17) of the Tris–TMS derivative showed one major component (>98%) which had the same retention time as did the corresponding derivative of authentic (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane. Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative gave a spectrum which was essentially identical to that of the same derivative of authentic (2S,3S,4R)-2-benzoylamido-1,3,4-trihydroxyoctadecane.

N. (2S,3S,5R)-2-Acetamido-1,3,5-trihydroxyoctadecane, (2S,3S,5R)-2-amino-1,3,5-trihydroxyoctadecane, and (2S,3S,5R)-2-benzoylamido-1,3,5-trihydroxyoctadecane

The material corresponding to fractions 24 through 29 from the MPLC (Table 1) was dissolved in a mixture of chloroform and methanol (94 : 6; 15 ml) and subjected to MPLC on a silicic acid column (1.5 cm × 100 cm) using the same solvent mixture as the eluting solvent at a flow rate of 2 ml/min. After elution of 70 ml from the column, fractions 8 ml in volume were collected. The contents of fractions 70 through 90 were combined and, after evaporation of the solvent under reduced pressure, recrystallized from ethyl acetate/methanol to give (2S,3S,5R)-2-acetamido-1,3,5-trihydroxyoctadecane (178 mg; 0.50 mmol; 9% yield, calculated from the epoxide) which melted at 119–120°C; $[\alpha]_D^{28} - 4.5°$ (*c* 1.51 in methanol). The compound showed a single component on TLC (chlorform/methanol, 10 : 1) with an R_F of 0.24 (Fig. 8). Analysis by GLC (3% OV-1) of the Tris–TMS derivative showed a single component with a retention time of 0.98, relative to that of the same derivative of authentic (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane. The infrared spectrum (Fig. 3) was compatible with the assigned structure and clearly distinguishable from that of (2S,3S,4S) and (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane. No reaction was observed upon periodate oxidation as indicated by recovery of only starting material upon TLC. Combined GLC (3% OV-17)-MS analysis of the Tris-TMS derivative gave the mass spectrum shown in Fig. 4. The results of the analysis of this spectrum, studies of the corresponding d_9-TMS derivative, and high resolution mass spectral analyses were fully compatible with the assigned structure (Table 4).

The *N*-acetylated base (27 mg) was subjected to alkaline hydrolysis as described previously for the case of (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane to give

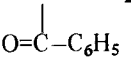
Table 4

Major ions of diagnostic significance in the mass spectrum of the Tris-trimethylsilyl derivative of (2S,3S,5R)-2-acetamido-1,3,5-trihydroxyoctadecane

Nominal mass	Relative intensity	Exact mass		Shift with d_9-TMS		Proposed structure
		Observed	Calculated	Observed	Predicted	
560	7%	560.3992	560.3987	24	24	$M\text{-}CH_3$
285	100%	285.2611	285.2614	9	9	$CH_3\text{-}(CH_2)_{12}\text{-}CH\text{-}O\text{-}Si(CH_3)_3$
247	44%	247.1422	247.1425	18	18	$CH\text{-}CH_2\text{-}O\text{-}Si(CH_3)_3$ $\vert$ $(CH_3)_3Si\text{-}NH\text{-}COCH_3$
174	5%	174.0951	174.0950	9	9	$CH\text{-}CH_2\text{-}O\text{-}Si(CH_3)_3$ $\vert$ $NH\text{-}COCH_3$
147	11%	147.0665	147.0662	15	15	$(CH_3)_2\text{-}Si{=}O\text{-}Si(CH_3)_3^+$
116	15%	116.0506	116.0532	6	6	$(CH_3)_2Si{=}NH\text{-}COCH_3$
103	15%	103.0559	103.0580	9	9	$CH_2{=}O\text{-}Si(CH_3)_3$

the corresponding free base. Analysis by TLC (chloroform/methanol/15 M ammonia, 100 : 25 : 2.5) showed one major component of R_F 0.60 and a trace component of R_F 0.69. Analysis by GLC (3% OV-17) showed one major component (~ 97%) which had the same retention time as that of the same derivative of authentic phytosphingosine. Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative showed the following major ions: 518 (3%; M–CH_3), 430 (18%; C-1–C-2 cleavage with charge retention by the C-2 fragment), 340 (15%), 285 (47%; C-4–C-5 cleavage with charge retention by the C-5 fragment), 204 (16%), 147 (19%; $(CH_3)_2$–Si=O–$Si(CH_3)_3$, 132 (100%; C-2–C-3 cleavage with charge retention by the C-2 fragment), 116 (28%), 103 (19%; C-1–C-2 cleavage with charge retention on the C-1 fragment), 75 (31%; $(CH_3)_2$–Si–OH), and 73 (70%; $(CH_3)_3Si$).

The *N*-benzoyl derivative was prepared from the free base (5 mg) as outlined previously for the case of the (2S,3S,4S)-2-amino-1,3,4-trihydroxyoctadecane. Analysis by TLC (chloroform/methanol, 10 : 1) showed one major component of R_F 0.45 and a trace component of R_F 0.28. Analysis by GLC (3% OV-17) of the TMS derivative showed a single component which had the same retention time as that of the TMS derivative of authentic *N*-benzoylphytosphingosine. Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative showed the following major ions: 622 (9%; M–CH_3), 426 (9%), 322 (9%), 309(68%; $(CH_3)_3Si$–NH–CH_2–CH_2–O–$Si(CH_3)_3$, 285



(100%; C-4–C-5 cleavage with charge retention by the C-5 fragment), 236 (16%; C-2–C-3 cleavage with charge retention on the C-2 fragment), 219 (67%; $(CH_3)_3Si$–NH-CH–CH_2), 178 (17%), 147 (25%; $(CH_3)_3Si$–O=$Si(CH_3)_2$, 105 (63%; C_6H_5CO), 103 (20%; C-1–C-2 cleavage with charge retention by the C-1 fragment), 75 (24%; $(CH_3)_2$-Si–OH), and 73 (82%; $(CH_3)_3Si$).

The combined results of the periodate oxidation studies and the mass spectral analyses clearly establish the location of the hydroxyl function at carbon atom 5. The ion at *m/e* 285 in the spectrum of the Tris–TMS derivative of the N-acetylated base and the ions at *m/e* 285 in the spectra of the Tris–TMS derivative of the *N*-benzoylated base and the Tris–TMS derivative of the free base were of particular importance in the localization of the hydroxyl function at carbon atom 5.

The absolute configuration of the hydroxyl function at carbon atom 5 was established in the following manner. The free base was subjected to permanganate-periodate oxidation to yield a 3-hydroxyhexadecanoic acic which, after methylation of the carboxyl function with diazomethane, was esterified with (2S)-2-phenylpropionyl chloride. As reported by Hammarstrom and Hamberg [12], the use of this optically active derivative permits the separation of enantiomorphic 3-hydroxyhexadecanoates upon gas-liquid chromatography on QF-1 column. Separation of the (2S)-2-phenylpropionate esters of the (3R)- and the (3S)- isomers of methyl 3-hydroxyhexadecanoate was achieved on a 3% QF-1 column. The structures of the eluting derivatives were confirmed by comined GLC–MS analysis. The (3R)-isomer eluted from the column prior to the (3S)-isomer as indicated by the retention time of the (2S)-2-phenyl-

propionate derivative of the authentic methyl (3R)-3-hydroxyhexadecanoate and by experiments in which the derivative of the (3R)-isomer was co-injected with the corresponding derivative of the (3RS)-mixture. The order of elution of the R and S isomers was, therefore, the same as that reported by Hammarstrom and Hamberg [12] who utilized a 5% QF-1 column. Combined GLC (3% QF-1)-MS analysis gave a mass spectrum which showed the same major ions as reported by Hammarstrom and Hamberg [12], i.e., *m/e* 418 (M), 300, 269, 237, 219, 195, 132, and 105.

The 3-hydroxy fatty acid obtained upon permanganate-periodate oxidation of the 2-amino-1,3,5-trihydroxyoctadecane was esterified with diazomethane and reacted with (2S)-2-phenylpropionyl chloride. Analysis by GLC (3% GF-1) showed a single component with the same retention time as that of the corresponding derivative of authentic methyl (3R)-3-hydroxyhexadecanoate. Combined GLC-MS analysis gave a mass spectrum which was essentially identical to that of the same derivative of authentic methyl (3R)-3-hydroxyhexadecanoate. Thus, combined periodate-permanganate oxidation of the free sphingolipid base gave exclusively (3R)-3-hydroxyhexadecanoic acid, a finding establishing the configuration of the hydroxyl function at carbon atom 5 of the sphingolipid base as 5R.

O. (2S,3S,5S)-Acetamido-1,3,5-trihydroxyoctadecane, (2S,3S,5S)-2-Amino-1,3,5-trihydroxyoctadecane, and (2S,3S,5S)-2-benzoylamido-1,3,5-trihydroxyoctadecane

The material corresponding to fractions 41 through 65 from the MPLC (Table 1) was dissolved in a mixture of chloroform and methanol (92 : 8; 10 ml) and subjected to MPLC on a silicic acid column (1.5 cm × 100 cm) using the same solvent mixture as the eluting solvent at a flow rate of 2.25 ml/min. The contents of fractions 65 through 85 were combined and, after evaporation of the solvent under reduced pressure, recrystallized from ethyl acetate to give (2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane (45 mg; 2% yield, calculated from the epoxide) which melted at 133–134°C; $[\alpha]_D^{28}$ + 2.4° (*c* 0.37 in methanol). The compound showed a single component on TLC (chloroform/methanol, 10 : 1) with an R_F of 0.16 (Fig. 8). Analysis by GLC (3% OV-17) of the Tris–TMS derivative showed one major component (95%) with a retention time (relative to that of the corresponding derivative of authentic *N*-acetylphytosphingosine) of 1.06 and a minor component (5%) with a relative retention time (as defined above) of 1.41. The infrared spectrum (Fig. 3) was compatible with the assigned structure. No reaction was observed upon periodate oxidation as indicated by recovery of only starting material upon TLC. Combined GLC (3% OV-17)-MS analysis of the major component gave the mass spectrum shown in Fig. 4. To obtain sufficient material for further detailed analyses, the entire synthetic procedure from the *trans*-epoxide (1.95 g) was repeated as described above. After two recrystallizations from acetonitrile, pure (2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane (23.2 mg) melting at 138.5–139.5°C was obtained. The compound had the same mobility on TLC and the same infrared spectrum as the sample described above. On GLC (3% OV-1) analysis of the Tris–TMS derivative a single component was observed.

Table 5

Major ions of diagnostic significance in the mass spectrum of the Tris-trimethylsilyl derivative of(2S,3S,5S)-2-acetamido-1,3,5-trihydroxyoctadecane

Nominal mass	Relative intensity	Exact mass		Shift with d_9-TMS		Proposed structure
		Observed	Calculated	Observed	Predicted	
560	6%	560.3992	560.3987	24	24	M-CH_3
285	100%	285.2613	285.2614	9	9	CH_3-$(CH_2)_{12}$-CH-O-Si$(CH_3)_3$
247	42%	247.1423	247.1425	18	18	CH-CH_2-O-Si$(CH_3)_3$ NH $(CH_3)_3$Si-NH-$COCH_3$
174	4%	174.0943	174.0950	9	9	CH-CH_2-O-Si$(CH_3)_3$ NH-$COCH_3$
147	8%	147.0657	147.0662	15	15	$(CH_3)_2$Si-O-Si$(CH_3)_3$
116	14%	116.0499	116.0532	6	6	$(CH_3)_2$Si=NH-$COCH_3$
103	10%	103.0558	103.0580	9	9	CH_2=O-Si$(CH_3)_3$

The results of the analysis of this spectrum, studies of the corresponding d_9-TMS derivative, and high resolution mass spectral analyses were fully compatible with the assigned structure (Table 5).

The N-acetylated base (17 mg) was subjected to alkaline hydrolysis as described previously for the case of (2S,3S,4S)-2-acetamido-1,3,4-trihydroxyoctadecane to give the corresponding free base. Analysis by TLC (chloroform/methanol/15 M ammonia, 100 : 25 : 2.5) showed one major component of R_F 0.53 and a trace component of R_F 0.66. Analysis by GLC (3% OV-17) of the TMS derivative showed one component (> 99%). Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative gave a mass spectrum which was essentially identical to that of the same derivative of the (2S,3S,5R)-isomer.

The *N*-benzoyl derivative was prepared from the free base (3 mg) as outlined previously for the case of the (2S,3S,4S)-2-amino-1,3,4-trihydroxyoctadecane. Analysis by TLC (chloroform/methanol, 10 : 1) showed one major component of R_F 0.40 and a trace component of R_F 0.22. Analysis by GLC (3% OV-17) of the TMS derivative showed a single component with a retention time of 1.10 (relative to that of the same derivative of authentic *N*-benzoylphytosphingosine). Combined GLC (3% OV-17)-MS analysis of the Tris–TMS derivative gave a spectrum which was essentially identical to that of the same derivative of the (2S,3S,5R)-isomer. The configuration of the hydroxyl function at carbon atom 5 was established in the same fashion as described above for the case of the (2S,3S,5R)-isomer. The (2S)-2-phenylpropionate ester of the 3-hydroxyhexadecanoic acid showed major peak with the same retention time as that of the corresponding derivative of (3S)-3-hydroxyhexadecanoate. Combined GLC-MS analysis gave a mass spectrum which was essentially the same as that of the corresponding derivative of authentic methyl (3R)-3-hydroxyhexadecanoate. The combined results of these experiments establish the absolute configuration of the hydroxyl function at carbon atom 5 of the sphingolipid base as 5S.

Discussion

The findings reported herein are in contrast to those reported by two other laboratories who utilized a similar approach from the chemical synthesis of phytosphingosine from sphingosine. In 1965 Weiss [1] reported that treatment of tribenzoylsphingosine with perbenzoic acid gave, after standard work-up and crystallization from ethanol/petroleum ether an epoxide which was characterized by m.p. (135–137°C; cf 136.5–137.5°C reported herein), elemental analysis, and its infrared spectrum. The epoxide, presumably a mixture of the (4S,5R)- and (4R,5S)-isomers, was reduced with lithium aluminum hydride which was reported to give a mixture of (2S,3S,4S)-2-benzylamino-1,3,4-trihydroxyoctadecane and (2S,3S,4R)-1,3,4-trihydroxyoctadecane. The latter compounds were reported to have been separated by fractional crystallization and were characterized only by m.p. (91–92°C for the (2S,3S,4R)-isomer and 48–58°C for the (2S,3S,4S)-isomer), elemental analysis, by their infrared spectra, and by the

formation of pentadecanal (as judged by GLC and by formation of the 2,4-dinitrophenylhydrazone derivative) upon periodate oxidation of the two compounds. The two *N*-benzylamines were subjected to hydrogenolysis over a palladium oxide catalyst to give the corresponding free bases. The (2S,3S,4R)-isomer melted at 97–107°C (cf. with values of 102–104°C [20], 102.0–103.7°C [21], and 106°C [2], for phytosphingosine isolated from natural sources) and gave a positive ninhydrin reaction. The (2S,3S,4S)-isomer was reported to be a wax which showed a positive ninhydrin reaction. The free bases were not characterized further. The corresponding *N*-benzoyl derivatives were prepared. Reported characterization of the *N*-benzoyl derivative of the (2S,3S, 4R)-isomer was based upon m.p. (135–136°C), elemental analysis, and its infrared spectrum. Reported characterization of the (2S,3S,4S)-isomer was based upon m.p. (101–104°C), elemental analysis, and its infrared spectrum (which showed the same major absorptions as its (2S,3S,4R)-isomer). The compounds were studied further. While no comment was made by Weiss regarding the stereochemistry of the isolated 4,5-epoxide, the reported isolation of both the (2S,3S,4R) and (2S,3S,4S)-isomers of 2-benzylamino-1,3,4-trihydroxyoctadecane implies that the isolated epoxide must have been a mixture of the (4S,5R)-epoxide and its (4R,5S)-isomer. Weiss interpreted his results as indicating that the hydride reduction of the tribenzoyl derivative of the sphingosine epoxide proceeded exclusively at carbon atom 5 to give the 4-hydroxy isomers and none of the 5-hydroxy isomers. This finding is in clear variance with the results reported herein.

Prostenik et al. [2] also reported the epoxide of tribenzoylsphingosine (m.p. 134–135°C) by treatment of tribenzoylsphingosine with perbenzoic acid. They noted that two *trans*-tribenzoylsphingosine epoxides might result from the reaction. However, they interpreted their findings that the product purified by crystallization appeared to be homogeneous as indicating that the epoxidation proceeded stereo-specifically to yield only one of the two isomers of the *trans*-epoxide. These workers reported the isolation of (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyoctadecane upon lithium aluminum hydride reduction of epoxide. The reported *N*-benzylamino compound melted at 55–58°C (cf. with value reported herein of 90.0–91.5°C for (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyoctane prepared by hyride reduction of authentic (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyoctadecane and the value of 91–92°C for the (2S,3S,4R)-2-benzylamino-1,3,4-trihydroxyoctadecane reported by Weiss [1]). The *N*-benzylamino compound isolated by Prostenik et al. [2] was further characterized by optical rotation, elemental analysis, and by its infrared spectrum. Hydrogenolysis of the benzylamino compound with a palladium on charcoal catalyst was reported to give (2S,3S,4R)-2-amino-1,3,4-trihydroxyoctadecane which was characterized by m.p. (96–97°C; cf. value of 97–109°C reported by Weiss [1] and values of 102–104°C [20], 102.0–103.7°C [21], and 106°C [2] for phytosphingosine isolated from natural sources), optical rotation, elemental analysis, and its infrared spectrum. Prostenik et al. [2] also reported that catalytic reduction of the tribenzoylsphingosine *trans*-epoxide gave, in high yield, optically active (2S,3S,4R)-2-cyclohexananoylamino-1,3,4-trihydroxyoctadecane which was characterized by its m.p. (94–97°C), optical rotation,

elemental analysis, and its infrared spectrum. Upon treatment of the above compound with periodate, 93% of the quantity of periodate required for the oxidation of 1 mol of the compound was consumed. The products of the periodate oxidation were not characterized. Treatment of the *N*-cyclohexanoyl derivative with methanolic sulfuric acid followed by base treatment was reported to give (2S,3S,4R)-amino-1,3,4-trihydroxyoctadecane which was characterized by its m.p. (95–97°C; cf. with values cited above for authentic phytosphingosine isolated from natural sources), optical rotation, elemental analysis, and its infrared spectrum.

The results presented herein indicate that epoxidation of tribenzoylsphingosine with *m*-chloroperbenzoic acid yields two isomeric 4,5-*trans*-epoxides. Attempts at separation of the two epoxides by chromatography were unsuccessful. However, the results of recent detailed ^{13}C nuclear magnetic resonance studies of the 4,5-*trans*-epoxy-derivative of tribenzoylsphingosine are compatible with the presence of two isomers of the 4,5-*trans*-epoxide (M. Tsuda et al., unpublished results). Lithium aluminum hydride reduction of the epoxide mixture gave a mixture of 2-benzylamino-1,3,4-trihydroxyoctadecanes (presumably 2S,3S,4R and 2S,3S,4S) and 2-benzylamino-1,3,5-trihydroxyoctadecanes (presumably 2S,3S,5R and 2S,3S,5S). Attempts at separation of the individual components by chromatography were unsuccessful. Hydrogenolysis of the *N*-benzyl function followed acetylation of the resulting free bases yielded a mixture comprised of 2-acetamido derivatives of (2S,3S,4S)-1,3,4-trihydroxyoctadecane, (2S,3S,4R)-1,3,4-trihydroxyoctadecane, (2S,3S,5S)-1,3,5-trihydroxyoctadecane, and (2S,3S,5R)-1,3,5-trihydroxyoctadecane. The latter 4 compounds could be separated from each other by TLC on silica gel (Fig. 8) or, on a preparative basis, by MPLC on silicic acid columns. The compounds were characterized by TLC, GLC of their Tris–TMS derivatives, m.p. determination, optical rotation, infrared spectroscopy, low and high resolution MS analysis of their Tris–TMS derivatives, and by periodate oxidation studies. The compounds were further characterized by conversion to the corresponding free bases and *N*-benzoyl derivatives which were studied by chromatography and by MS analysis of their TMS derivatives. The free bases of the 5-hydroxy isomers were further studied by combined permanganate-periodate oxidation studies to yield 3-hydroxyhexadecanoic acids whose absolute configurations were established by GLC analyses of the (2S)-2-phenylpropionate derivatives of the methyl esters of the 3-hydroxy fatty acids. The properties of the various derivatives of the 4- and 5-hydroxy isomers of sphinganine are summarized in Table 6. The two isomers of the *N*-acetyl derivative of 4-hydroxysphinganine were readily distinguishable by their melting points, optical rotation, and chromatographic properties. A similar situation existed with respect to the two isomers of the *N*-acetyl derivative of 5-hydroxysphinganine. The *N*-acetyl derivative of the 4- and 5-hydroxy-sphinganines were readily distinguished from each other by MS analysis in the form of their TMS ether derivatives (Fig. 4). However, the mass spectra of the two isomers of the same derivative of 4-hydroxysphinganine were essentially identical. The same situation also obtained for the case of the two isomers of 5-hydroxysphinganine. In addition to these low resolution studies, high resolution MS analyses and studies of the

Table 6

Properties of derivatives of natural phytosphingosine and of synthetic phytosphingosines

	Natural phytosphingosine (2S,3S,4R)	Synthetic phytosphingsines			
		(2S,3S,4R)	(2S,3S,4S)	(2S,3S,5R)	(2S,3S,5S)
Melting point of N-acetyl derivative	132–133°C	133–134°C	118–119°C	119–120°C	138.5 139.5°C
Optical rotation ($[\alpha]_D$) of N-acetyl derivative in methanol	+5.0	+4.7°	+0.5°	–4.5°	+2.4°
Thin-layer chromatography of N-acetyl derivative, R_F ($CHCl_3$-CH_3OH, 10 : 1)	0.20	0.20	0.28	0.24	0.16
Thin-layer Chromatography of free base, R_F (CH_3Cl_3-CH_3OH-15M NH_3 100:25:2.5)	0.53	0.53	0.58	0.60	0.53
Thin-layer chromatography of *N*-benzoyl derivative, R_F ($CHCl_3$-CH_3OH, 10 : 1)	0.43	0.43	0.48	0.45	0.40

electron ionization induced fragmentation of the corresponding perdeuterated TMS ether derivatives have been completed. These results are summarized in Tables 2–5. The infrared spectra of the *N*-acetyl derivative of the various isomers of phytosphingosine were of special interest (Fig. 3). The infrared spectrum of the synthetic sample of (2S,3S,4R)-2-acetamido-1,3,4-trihydroxyoctadecane was essentially identical with that of the N-acetyl derivative of authentic phytosphingosine prepared from *H. ciferrii*. However, the spectra of each of the 4 synthetic isomers, i.e., the (2S,3S,4R)- and (2S,3S,4S)-isomers of 2-acetamido-1,3,4-trihydroxyoctadecane and the (2S,3S,5S)- and (2S,3S,5R)-isomers of 2-acetamido-1,3,5-trihydroxyoctadecane, differed significantly from each other. Each of the 4 spectra differed from each other in the 3200–3400 cm^{-1} region (O–H stretch and N–H stretch), in the 1600–1700 cm^{-1} region (predominantly amide C=O stretch), and in the 1000–1200 cm^{-1} region (predominantly C–O stretch).

Further studies of the ^{13}C nuclear magnetic resonance spectral properties of the concerned compounds are in progress as well as studies employing these compounds as substrates for sphinganine kinase.

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