notes on methodology

A convenient procedure for the synthesis of ceramides

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SUMMARY A procedure for the preparation of ceramides by direct coupling of long-chain bases and fatty acids in the presence of a mixed carbodiimide is described. This method has been used to prepare ceramides containing sphing-4-enine or sphinganine and various saturated and unsaturated fatty acids as well as saturated 2-hydroxy acids. Ceramides containing 4-hydroxy sphinganine and saturated nonhydroxy acids have also been prepared. The yields were 60–75%. The characterization of these compounds by gas–liquid chromatography–mass spectrometry as trimethylsilyl derivatives has been previously reported. Some of the ceramides are further characterized in this report by infrared spectroscopy and one compound, in addition, by elementary analysis. Use of racemic constituents for 2-hydroxy acid ceramide syntheses leads to the formation of diastereoisomers which separate by thin-layer chromatography. These were characterized by gas–liquid chromatography–mass spectrometry as the trimethylsilyl derivatives and by infrared spectroscopy. Their configurations were established by syntheses with optically active constituents.

SUPPLEMENTARY KEY WORDS sphing-4-enine · sphinganine · 4-hydroxy sphinganine · saturated and unsaturated fatty acids · 2-hydroxy acids · infrared spectroscopy · diastereoisomerism · thin-layer chromatography

CERAMIDES have earlier been synthesized from acyl chlorides and sphingosines or sphingosine derivatives (1–4). Generally, the di-*O*-acyl, *N*-acyl sphingosines are formed under these conditions, but the *O*-acyl groups may be removed by mild alkaline hydrolysis without affecting the amide bond. Selective *N*-acylation of long-chain bases has been achieved with acyl chlorides in a mixture of *N*,*N*-dimethylformamide–pyridine (3). Long-chain bases may also be selectively *N*-acetylated with acetic anhydride in methanol (5). Carbodiimides are widely used for peptide syntheses (6). They are simply added to a mixture of the two reactants and cause activation of the carboxylic acid to a mixed carboxylic–ammonocarboxylic anhydride which then reacts with the amine. The procedure gives selective *N*-acylation when both amino and hydroxy groups are present. This report describes an application of the carbodiimide method for the synthesis of ceramides. It has been used in our laboratories to prepare ceramides for mass spectrometric and other investigations (7–15) and for the preparation of cerebrosides from psychosine (16).

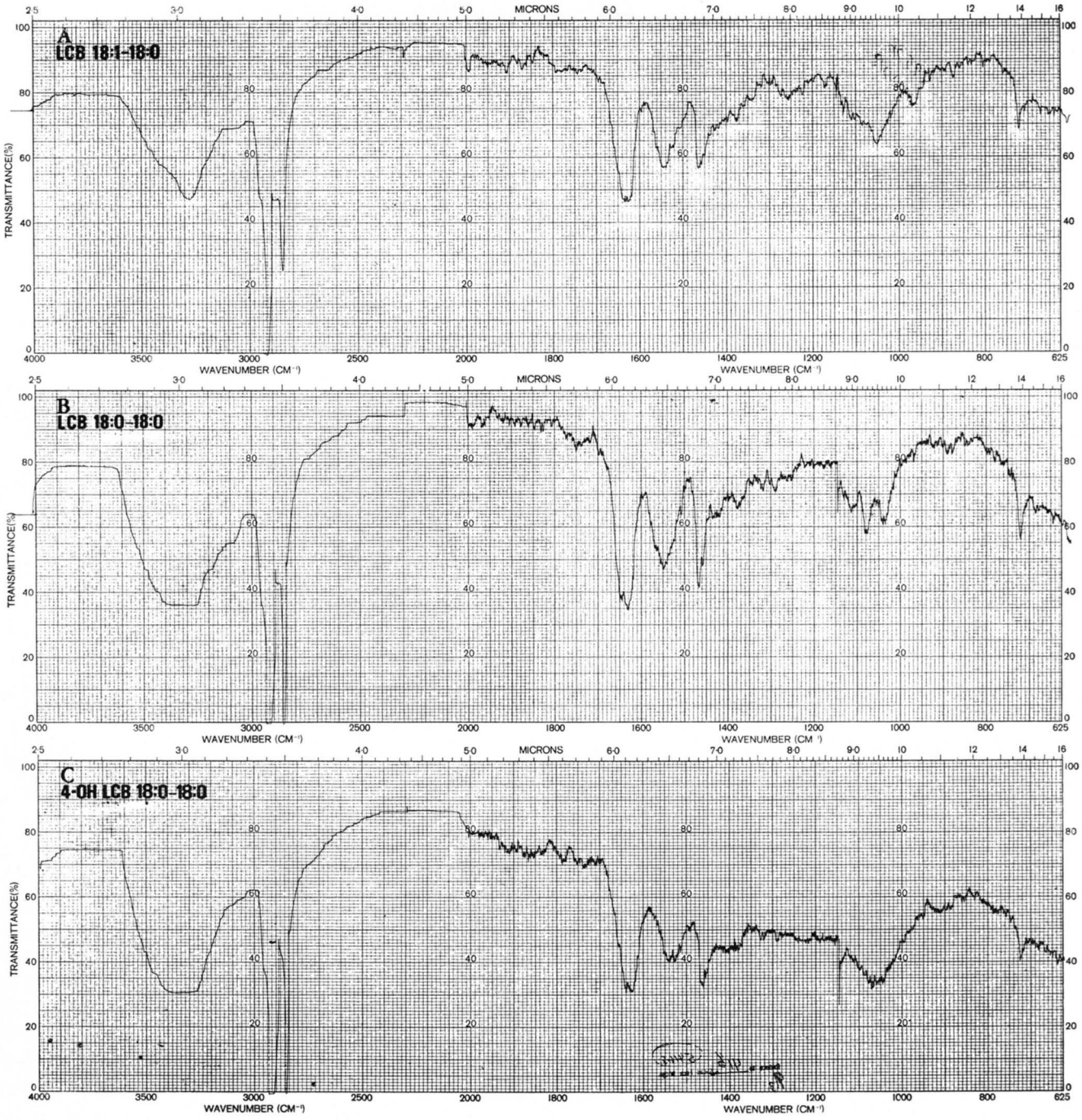
Materials. DL-Sphingenine (DL-*erythro-trans*-1,3-dihydroxy-2-amino-4-octadecene) and DL-sphinganine (DL-*erythro*-1,3-dihydroxy-2-aminooctadecane) were obtained from Miles Laboratories, Inc., Elkhart, Ind. D-Sphingenine was isolated from beef lung lipids (Vio-Bin Corp., Monticello, Ill.) according to Tipton (17). Crystallized from ethyl acetate, it melted between 70 and 80°C (reported 70–82°C [17]). Analysis by GLC–MS of the *N*-acetyl, di-*O*-TMS derivative (5) showed that it was a mixture of sphingosines (LCB 18:1, 85%; LCB 17:1, 5%; LCB 18:0, 2%; *threo*-LCB 18:1, 2%; LCB 16:1, 1%; LCB 19:1, 1%; and LCB 20:1, 1%). Natural phytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-aminooctadecane) was prepared from "$Ba(OH)_2$-insoluble material from corn phytoglycolipid" (kindly provided by Dr. A. Kisic, University of Illinois, Urbana, Ill.) according to a procedure by Dr. Kisic.[1] It was further purified by column chromatography (18). Analysis by GLC–MS of the *N*-acetyl, tri-*O*-TMS derivative[2] confirmed the structure (C value,[3] 24.8; major ions at m/e 560, 401, 383, 349, 299, 276, 246, 218, 204, 187, 174, 157, and 132 [see Ref. 19]). Racemic 2-hydroxy acids were obtained from the sources listed in Ref. 10. Optically active 2-hydroxy acids were prepared from methyl hydrogen 2D-acetoxy succinate and methyl hydrogen 2L-acetoxy succinate by crossed electrolytic couplings with palmitic acid (20). The crude products were hydrolyzed (8% KOH in water, reflux for 30 min), extracted with ether, acidified, and reextracted with ether. The latter extracts were evaporated to dryness and purified by silicic acid column chromatography

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; LCB 18:1, *erythro-trans*-1,3-dihydroxy-2-amino-4-octadecene; *threo*-LCB 18:1, *threo-trans*-1,3-dihydroxy-2-amino-4-octadecene; LCB 18:0, *erythro*-1,3-dihydroxy-2-aminooctadecane; 4-OH LCB 18:0, *ribo*-1,3,4-trihydroxy-2-aminooctadecane; LCB 16:1, *erythro-trans*-1,3-dihydroxy-2-amino-4-hexadecene; LCB 17:1, *erythro-trans*-1,3-dihydroxy-2-amino-4-heptadecene, etc.; TLC, thin-layer chromatography; TMS, trimethylsilyl; TGCU, triglyceride carbon units.

[1] Kisic, A. Personal communication.

[2] TMS derivatives were prepared by adding 20 μl of hexamethyldisilazane and 10 μl of trimethylchlorosilane to a solution of 100 μg of ceramide in 100 μl of dry pyridine. After 15 min at room temperature, the solvents were evaporated and the residue redissolved in CS_2.

[3] This was obtained by making the linear plot of the logarithm of the retention times of straight-chain fatty acid methyl esters vs. their numbers of carbon atoms in the fatty acid and interpolating the logarithm of the retention time of the compound in question. The stationary phase was SE-30.



and by TLC after conversion to methyl esters. The free acids (obtained by alkaline hydrolysis) were acetylated with acetic anhydride in pyridine. The 2-acetoxy acids crystallized readily from hexane (mp for both isomers, 63.0–64.0°C; optical rotation measured with a Perkin-Elmer model 141 polarimeter, $[\alpha]_D^{25}$ +9.4 and −9.5° for the D and L isomers, respectively; c, 1.80 in $CHCl_3$). GLC–MS of the *O*-TMS, methyl ester derivatives of the hydroxy stearates confirmed their structures (C value, 20.15 (99% pure); major ions above m/e 300: m/e 327, 343, 371, and 386). The GLC–MS data was the same for both enantiomers. Ethyl (3-dimethyl-aminopropyl) carbodiimide hydrochloride was obtained from the Ott Chemical Co., Muskegon, Mich. All solvents were of reagent grade and were used as supplied by the company (E. Merck A. G., Darmstadt, West Germany) with the exceptions mentioned below. Ethyl acetate, benzene, and acetic anhydride were redistilled; pyridine was refluxed with pellets of KOH, distilled, and stored over pellets of KOH.

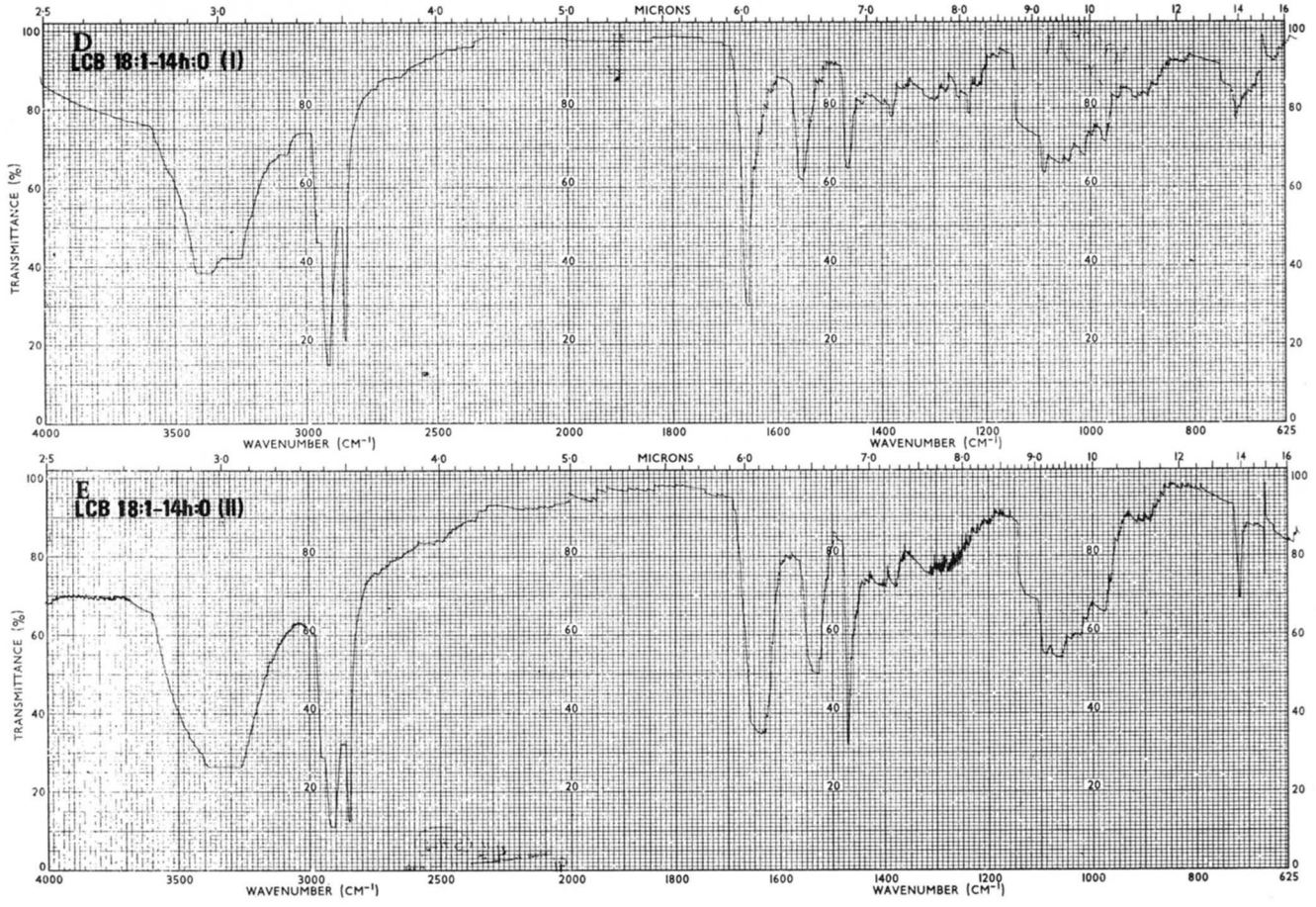


FIG. 1. Infrared spectra of synthetic ceramides, recorded with KBr microdisk techniques (21). (*A*), *N*-(stearoyl) D-sphingenine, (*B*), *N*-(stearoyl) DL-spinganine, (*C*) *N*-(stearoyl) D-phytosphingosine, (*D*), *N*-(2′DL-hydroxymyristoyl) DL-sphingenine (unnatural diastereoisomer), and (*E*), *N*-(2′DL-hydroxymyristoyl) DL-sphingenine (natural diastereoisomer).

Preparation of Nonhydroxy Acid Ceramides. Ceramides containing saturated or unsaturated fatty acids and sphingenine, sphinganine, or 4-hydroxy sphinganine were prepared in the following way. 17 μmoles of long-chain base (about 5 mg) and 35 μmoles of fatty acid were dissolved in 2.5 ml of CH_2Cl_2, 2.5 ml of CH_3CN, and 0.5 ml of CH_3OH. The carbodiimide was dissolved in CH_2Cl_2 (13.5 mg/ml), and 1.25 ml of this solution was added. The flask was stoppered and left in a thermostated chamber at 40–45°C for 16 hr. The solution was then transferred to a separatory funnel with 50 ml of diethyl ether, and the mixture was washed with 3 × 15 ml of 5% $NaHCO_3$, once with 15% NaCl, with 3 × 15 ml of 1 N HCl, and then with 15% NaCl (water solutions) until neutral. After two more washings with distilled water and addition of 5–10 ml of absolute ethanol, the ether was evaporated in vacuo. The residue was dissolved in 2 ml of ethyl acetate–benzene 1:9 (v/v) and chromatographed on a 2-g column of silicic acid (100 mesh, Mallinckrodt; activated at 120°C for more than 24 hr). After washing the column with 40 ml of ethyl acetate–benzene 1:9 (v/v), the ceramide (containing sphingenine or sphinganine) was eluted with 60 ml of ethyl acetate–benzene 3:7 (v/v). Phytosphingosine ceramides were eluted with ethyl acetate–benzene 5:5 (v/v) instead. The yield of ceramide was 60–75% (conversion of LCB to ceramide).

Preparation of 2-Hydroxy Acid Ceramides. The 2-hydroxy acid (10–50 mg) was dissolved in 1 ml of pyridine and 0.75 ml of acetic anhydride and left at room temperature for 16 hr. 1 ml of ice-cold pyridine–water 1:1 (v/v) was then added. The solution was diluted with 50 ml of diethyl ether and washed with 3 × 15 ml of 2 N HCl, 15% NaCl (water solutions) until neutral, and finally twice with water. Absolute ethanol was added and the solvents were removed in vacuo. The product can be used directly for the following reaction or first purified by silicic acid chromatography and crystallization from hexane. The *N*-acylation reaction was carried out as described above for nonhydroxy acid ceramides, except that a 2-acetoxy

TABLE 1 MELTING POINTS AND APPROXIMATE R_F VALUES* OF SYNTHETIC CERAMIDES

	Observed mp†	Reported mp	R_F
Sphing-4-enine ceramides			
N-(Stearoyl) D-sphingenine	91–93°C	89–91°C (3)	0.6
N-(Nervonoyl) DL-sphingenine	79–80°C		0.6
N-(2′D-Hydroxypalmitoyl) D-sphingenine	100–101°C		0.4
N-(2′D-Hydroxystearoyl) D-sphingenine	103–104°C		0.4
N-(2′L-Hydroxystearoyl) D-sphingenine	98–101°C		0.5
Sphinganine ceramides			
N-(Stearoyl) DL-sphinganine	106–107°C	106–107°C (3)	0.6
N-(2′D-Hydroxypalmitoyl) D-sphinganine	108–114°C		0.4
4-Hydroxy sphinganine ceramide			
N-(Stearoyl) D-4-hydroxy sphinganine	99–100°C		0.5

* TLC on silica gel G plates. The plates were activated at 120°C for 1 hr. The solvent system was $CHCl_3$–CH_3OH 93:7 (v/v).
† Melting points were determined with a Bühler melting point apparatus (E. Bühler, Tübingen, West Germany); they are corrected.

TABLE 2 GLC RETENTION TIMES ON OV-1 FOR TMS DERIVATIVES OF SYNTHETIC CERAMIDES AND REFERENCES TO MASS SPECTRA OF THE SAME DERIVATIVES

LCB	Sphing-4-enine		Sphinganine		4-Hydroxy Sphinganine	
Fatty Acid	Retention Time (TGCU)	MS Reference	Retention Time (TGCU)	MS Reference	Retention Time (TGCU)	MS Reference
16:0	37.5	8	37.5	8	37.9	11
18:0	39.4	8	39.7	8	40.0	11
20:0	41.4	8	41.6	8	42.1	11
22:0	43.5	8	43.7	8	44.1	11
23:0	44.5		44.7		45.1	11
24:0	45.5	8	45.8	8	46.1	11
24:1	45.3	9				
14h:0	36.1	10	36.0	10		
16h:0	38.1	10	38.0	10		
18h:0	40.0	10	40.0	10		
20h:0	42.0	10	41.9	10		
22h:0	43.9	10	43.9	10		
24h:0	45.8	10	45.8	10		
26h:0	47.7	10	47.7	10		

acid was used instead of nonhydroxy acid. Ethyl acetate–benzene 3:7 (v/v) eluted 2′-acetoxy ceramides. The eluate was evaporated to dryness in a 50-ml centrifuge tube. The residue was dissolved in 9 ml of 0.6 N NaOH in CH_3OH and 9 ml of $CHCl_3$ and left for 1 hr at room temperature. After adding water (18 ml) and mixing the contents, the tube was centrifuged and the upper phase was discarded. The lower phase was washed once with water and then evaporated to dryness. To obtain the natural diastereoisomer when either the long-chain base, the acid, or both were racemic, the residue was dissolved in CH_2Cl_2–CH_3OH 5:1 (v/v) and applied as a band on a TLC plate coated with 0.5 mm of silica gel G and previously activated at 120°C for 1 hr. The solvent system was $CHCl_3$–CH_3OH 93:7 (v/v). The plate was sprayed with 0.2% (w/v) 2′,7′-dichlorofluorescein in ethanol, and the positions of the ceramides were marked in UV light. The lower zone was scraped off the plate and eluted with three 10-ml portions of 0.6 N NaOH in CH_3OH mixed with $CHCl_3$ (9:1, v/v). To each eluate were added 8 ml of $CHCl_3$ and 18 ml of H_2O. The contents were mixed and centrifuged. The upper phases, containing the dye, were discarded. The lower phases were washed with water, combined, and evaporated to dryness after addition of some absolute ethanol. The yield of natural ceramide diastereoisomers was 30–40% when racemic compounds were used and 60–75% with optically active constituents (% conversion of LCB to ceramide).

Results. The purities of the synthetic compounds after silicic acid chromatography were generally high as judged by TLC (solvent system $CHCl_3$–CH_3OH 93:7, v/v). Synthetic nonhydroxy acid ceramides cochromatographed with ceramides derived from brain sphingomyelin (Sigma Chemical Co., St. Louis, Mo.) (9), and the slower migrating 2-hydroxy acid ceramide diastereoisomer (see below) had the same R_F value as 2-hydroxy acid ceramides derived from brain cerebrosides (12). 4-Hydroxy sphinganine cer-

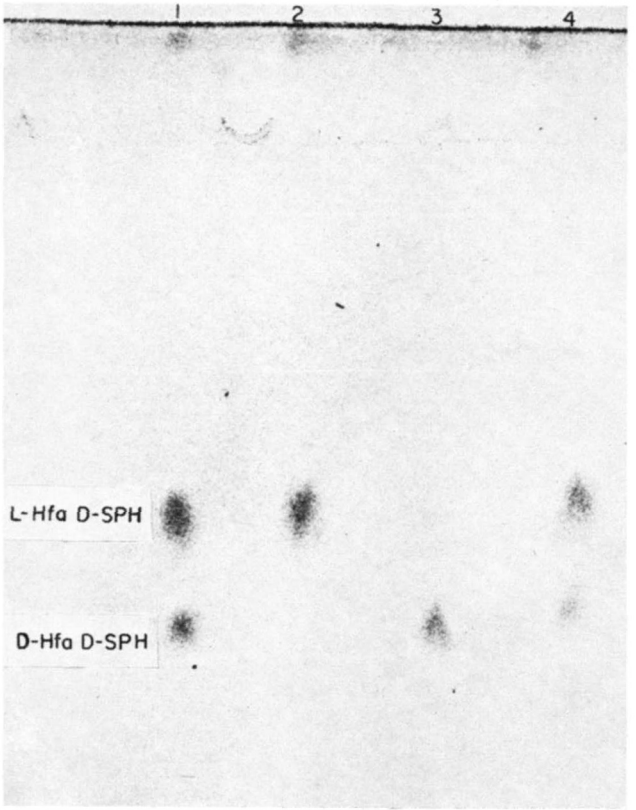


FIG. 2. Thin-layer chromatogram of diastereoisomeric 2′-hydroxy acid ceramides. Lanes *1* and *4*, *N*-(2′DL-hydroxystearoyl) D-sphingenine; lane *2*, *N*-(2′L-hydroxystearoyl) D-sphingenine; and lane *3*, *N*-(2′D-hydroxystearoyl) D-sphingenine. The plate was coated with 0.50 mm of silica gel G and was activated for 1 hr at 120°C before use. The solvent system was $CHCl_3$–CH_3OH 93:7 (v/v), and the compounds were detected by charring with with H_2SO_4.

amides containing nonhydroxy acids had somewhat higher R_F values than 2-hydroxy acid ceramides. Approximate R_F values are given in Table 1.

A large number of synthetic ceramides have previously been characterized by GLC–MS as the TMS derivatives. The retention times of these compounds, expressed as TGCU (10), are summarized in Table 2. This table also gives information on where to find the published mass spectra of ceramides. The ceramides in Table 2 were generally prepared on a small scale and not characterized by infrared spectroscopy and elementary analyses. However, high resolution mass spectrometry confirmed the elementary compositions of 1,3,2′-tri-*O*-TMS-*N*-(2′-hydroxytetradecanoyl) sphinganine (10) and 1,3,4-tri-*O*-TMS-*N*-(stearoyl)-4-hydroxy sphinganine (11).

For other studies (14, and footnote 4), some ceramides were prepared in larger quantities and crystallized from acetone or acetone–water mixtures. Melting points of some of these compounds are shown in Table 1, and GLC–MS analyses of TMS derivatives confirmed structures and purities. Elementary analyses of *N*-(stearoyl) DL-sphinganine (Lantbrukshögskolan, Uppsala, Sweden) gave 76.0% C, 13.2% H, and 2.5% N (calculated for $C_{36}H_{73}O_3N$: 76.1% C, 13.0% H, and 2.5% N), thus providing evidence of a correct elementary composition of the underivatized ceramide.

Infrared spectra of some of the synthetic ceramides are shown in Fig. 1. The spectra were recorded with KBr microdisks (21). A Perkin-Elmer 257 infrared spectrophotometer equipped with a beam condensor (model C-41, Research and Industrial Instruments Co., London) was used for recording the spectra. O—H and N—H stretching vibration was seen as a broad absorption band between 3700 and 3000 cm^{-1}. The C—H stretching bands were at 2960 cm^{-1} (CH_3) and 2920 plus 2860 cm^{-1} (CH_2). The presence of an amide bond was indicated by bands at 1650–1630 cm^{-1} (amide band I, C=O absorption) and 1550–1540 cm^{-1} (amide band II, C—N stretch and N—H deformation). The absence of bands in the ester carbonyl region (1755–1735 cm^{-1}) showed that the hydroxy groups of the constituent long-chain bases (and 2-hydroxy acids) were not acylated. Evidence for a *trans* double bond in the sphingenine-containing ceramides was provided by the band at 970 cm^{-1}. This band was absent from spectra of sphinganine- and 4-hydroxy sphinganine-containing ceramides. The infrared spectra of Fig. 1 resemble those of natural and synthetic ceramides published before (22, 23).

When 2-hydroxy acid ceramides were prepared from racemic long-chain bases or fatty acids, or both, two spots were seen on TLC (Fig. 2). If optically active constituents were used, only one spot appeared. The upper spot appeared when 2L-hydroxy stearic acid and D-sphingenine were used; the lower spot when both components had the D configuration (Fig. 2). Similar results have been reported before (24, 25). When analyzed by GLC–MS as the 1,3,2′-tri-*O*-TMS derivatives, both components had the same retention time with OV-1 as the stationary phase. Furthermore, their mass spectra were indistinguishable. The syntheses with optically active compounds and the GLC–MS analyses show that the two TLC components of 2-hydroxy acid ceramides are diastereoisomers. The slower migrating one is the naturally occurring isomer, since both long-chain bases and sphingolipid 2-hydroxy acids of mammalian origin have D configurations (26, 27). It was shown that 2-hydroxy acid ceramides obtained by degradation of brain cerebrosides (12) had the same R_F value as this isomer. The TLC separation of diastereoisomeric ceramides can be used on a preparative scale to obtain optically pure ceramides when only one of the components (long-chain base and 2-hydroxy acid) is available in optically active form (13).

[4] Hammarström, S. Unpublished experiments.

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