Synthesis of ceramides using *N*-hydroxysuccinimide esters

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Summary Fatty acyl esters of *N*-hydroxysuccinimide have been used to directly *N*-acylate sphingenine or sphinganine, forming the corresponding ceramides. The reaction proceeds in excellent yield (84–96%) from small amounts of starting material (10–20 mg). The product ceramides are pure after one recrystallization.

Supplementary key words sphingenine · sphinganine

CERAMIDES have been implicated as intermediates in the biosynthesis of sphingomyelin and cerebrosides and are important constituents of all sphingolipids. ^{14}C-labeled ceramides are useful as substrates for enzyme studies and studies of sphingolipid metabolism in the whole animal.

Some previously reported syntheses of ceramides have used appropriate fatty acid chlorides to acylate benzoyl sphingenine or psychosine (1, 2). The intermediate compounds formed are then hydrolyzed to produce the desired ceramide. Direct acylation of sphingenine or sphinganine by an acid chloride can lead to formation of the di-*O*-acyl,*N*-acyl compound, which provides the ceramide after mild alkaline hydrolysis to cleave the more labile *O*-acyl linkages (3, 4). Choosing a solvent system of *N*,*N*-dimethylformamide and pyridine, Weiss and Raizman (5) were able to use acid chlorides to *N*-acylate sphingenine and sphinganine with no apparent formation of *O*-acyl derivatives. However, yields were low (26–62%).

Hammarström (6) has described a method to prepare ceramides by direct coupling of the long-chain bases and fatty acids in the presence of a mixed carbodiimide. No *O*-acyl derivatives are formed. Yields of ceramide were 60–75% based on long-chain base and 30–37.5% based on fatty acid.

N-Hydroxysuccinimide esters of fatty acids are stable crystalline compounds which are easily prepared in high yield (7). They have been used for the synthesis of *N*-acyl amino acids (7) and fatty acyl CoA (8). As described in this report, they can also be used for the direct *N*-acylation of sphingenine or sphinganine. The reaction proceeds in high yield from small amounts of starting material. The product ceramides are pure after one recrystallization.

Materials. *trans*-2-Hexadecenoic acid was synthesized by the method of Shapiro, Segal, and Flowers (9). Palmitic, stearic, and lignoceric acids were purchased from Sigma Chemical Co., St. Louis, Mo. [1-^{14}C]-Palmitic and [1-^{14}C]stearic acids were purchased from New England Nuclear, Boston, Mass. *N*-Hydroxysuccinimide (Aldrich Chemical Co., Milwaukee, Wis.) was recrystallized from ethyl acetate before use. Dicyclohexylcarbodiimide was obtained from Pierce Chemical Co., Rockford, Ill. DL-Sphinganine (DL-*erythro*-1,3-dihydroxy-2-aminooctadecane) was from Nutritional Biochemical Corp., Cleveland, Ohio. DL-Sphingenine (DL-*erythro-trans*-1,3-dihydroxy-2-amino-4-octadecene) and *N*-(stearoyl) DL-sphinganine were from Miles Laboratories, Elkhart, Ind.

N-Hydroxysuccinimide esters. The esters of *trans*-2-hexadecenoic acid, palmitic acid, stearic acid, and lignoceric acid were synthesized by the method of Lapidot, Rappoport, and Wolman (7). After recrystallization from ethanol, all esters exhibited single spots after thin-layer chromatography on silica gel G with chloroform as the developing solvent. The plates were sprayed with bromothymol blue to visualize the esters (10). Yields were 87–91%. The melting points determined for

* Recipient of USPHS Postdoctoral Fellowship NS-50881.

the fatty acid esters were: *trans*-2-hexadecenoyl ester, 83–84°C; palmitoyl ester, 88°C; stearoyl ester, 93–94°C; and lignoceroyl ester, 103–104°C.

N-(Palmitoyl)-DL-sphingenine. The method described below was used for the preparation of all ceramides containing sphingenine.

A solution of DL-sphingenine (18.0 mg, 60 μmoles) in 3.0 ml of tetrahydrofuran (freshly distilled from $LiAlH_4$) was added to a solution of the *N*-hydroxysuccinimide ester of palmitic acid (21.2 mg, 60 μmoles) in 3.0 ml of tetrahydrofuran. The reaction mixture was left at room temperature in a stoppered test tube for 18 hr. The volume was reduced under a stream of N_2 to approximately 3 ml, and sufficient water was added to induce crystallization of the ceramide; *N*-hydroxysuccinimide remained in solution. White crystalline ceramide (28.0 mg, 87% yield) was collected by filtration. After recrystallization from methanol–water, the product (25.5 mg, 79% final yield) showed a single spot after thin-layer chromatography on silica gel G–$Na_4B_2O_7 \cdot 10H_2O$ 20:1 using the following three solvent systems: chloroform–acetic acid 9:1, chloroform–methanol 9:1, and ethyl acetate–methanol 96:4. Spots were visualized with bromothymol blue spray. The ceramide gave negative reaction with ninhydrin. Spraying with hydroxylamine solution and then with a solution of $FeCl_3$ (which detects, by producing a red color, both *N*-hydroxysuccinimide and its ester) gave no spots (11). The melting point of the recrystallized ceramide was 97–98°C.

N-[1-^{14}C](Stearoyl)-DL-sphinganine. The method described below was used for the preparation of ceramides containing sphinganine.

A solution of DL-sphinganine (10.0 mg, 33 μmoles) in 2.0 ml of tetrahydrofuran was added to a solution of the *N*-hydroxysuccinimide ester of [1-^{14}C]stearic acid (11.4 mg, 30 μmoles) in 2.0 ml of tetrahydrofuran. White crystals of ceramide began to form 5 min after mixing. The reaction mixture was left at room temperature in a stoppered test tube for 18 hr. After cooling the reaction mixture in ice to promote further crystallization, the product ceramide was collected by filtration (15.2 mg, 90% yield). The ceramide was recrystallized from methanol (13.8 mg, 82% final yield), and its melting point was determined to be 107–108°C. The specific activity of the ceramide was identical with that of the *N*-hydroxysuccinimide ester used (1.0 μCi/μmole). Thin-layer chromatography with the three systems described above showed single spots after visualization with bromothymol blue spray; these spots corresponded to those found after autoradiography. The correspondence of the synthesized ceramide to standard *N*-(stearoyl) DL-sphinganine is shown in Fig. 1. The purchased standard showed two minor impurities, but the major

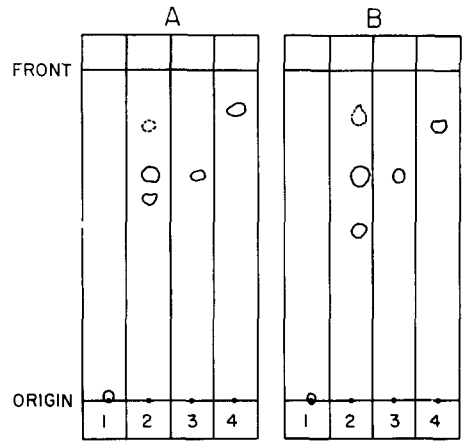

FIG. 1. Thin-layer chromatograms on plates coated with silica gel G–$Na_4B_2O_7 \cdot 10H_2O$ 20:1 (0.5 mm thick). Solvent systems: *A*, chloroform–acetic acid 9:1; *B*, chloroform–methanol 9:1. Compounds were detected with bromothymol blue spray (10). *1*, DL-sphinganine; *2*, standard *N*-(stearoyl) DL-sphinganine; *3*, *N*-(stearoyl) DL-sphinganine; *4*, *N*-hydroxysuccinimide ester of stearic acid.

component corresponded to our synthesized ceramide. The ceramide lanes on the thin-layer plates were divided into 1-cm bands and the silica gel was scraped off and counted. More than 99% of the radioactivity present corresponded with the ceramide position on the plate.

Infrared spectra. Infrared spectra were taken on a Perkin-Elmer 257 grating infrared spectrophotometer, using KBr pellets for each ceramide synthesized (1.0 mg of ceramide/240 mg of KBr). Representative spectra are shown in Fig. 2. All spectra were essentially similar and resemble those of natural (12) and synthetic ceramides (6) published previously. All spectra showed

TABLE 1. Yields and melting points of synthetic ceramides

Ceramide	Yield	Melting Point	
		Observed	Reported
	%	°C	
N-(*trans*-2-Hexadecenoyl) DL-sphinganine	88	110–111	
N-(*trans*-2-Hexadecenoyl) DL-sphingenine	92	89–90	
N-([1-^{14}C]Palmitoyl) DL-sphinganine	87	104–105	103–103.5[a] (3)
N-(Palmitoyl) DL-sphingenine	87	97–98	90.5–91.5[a] (3)
N-([1-^{14}C]Stearoyl) DL-sphinganine	90	107–108	106–107 (6)
N-(Stearoyl) DL-sphingenine	84	101–102	91–93[b] (6)
N-(Lignoceroyl) DL-sphinganine	96	104–105	
N-(Lignoceroyl) DL-sphingenine	89	95–96	

[a] Optical isomer of long-chain base not reported.
[b] Long-chain base was a mixture of sphingosines, 85% D-sphingenine.

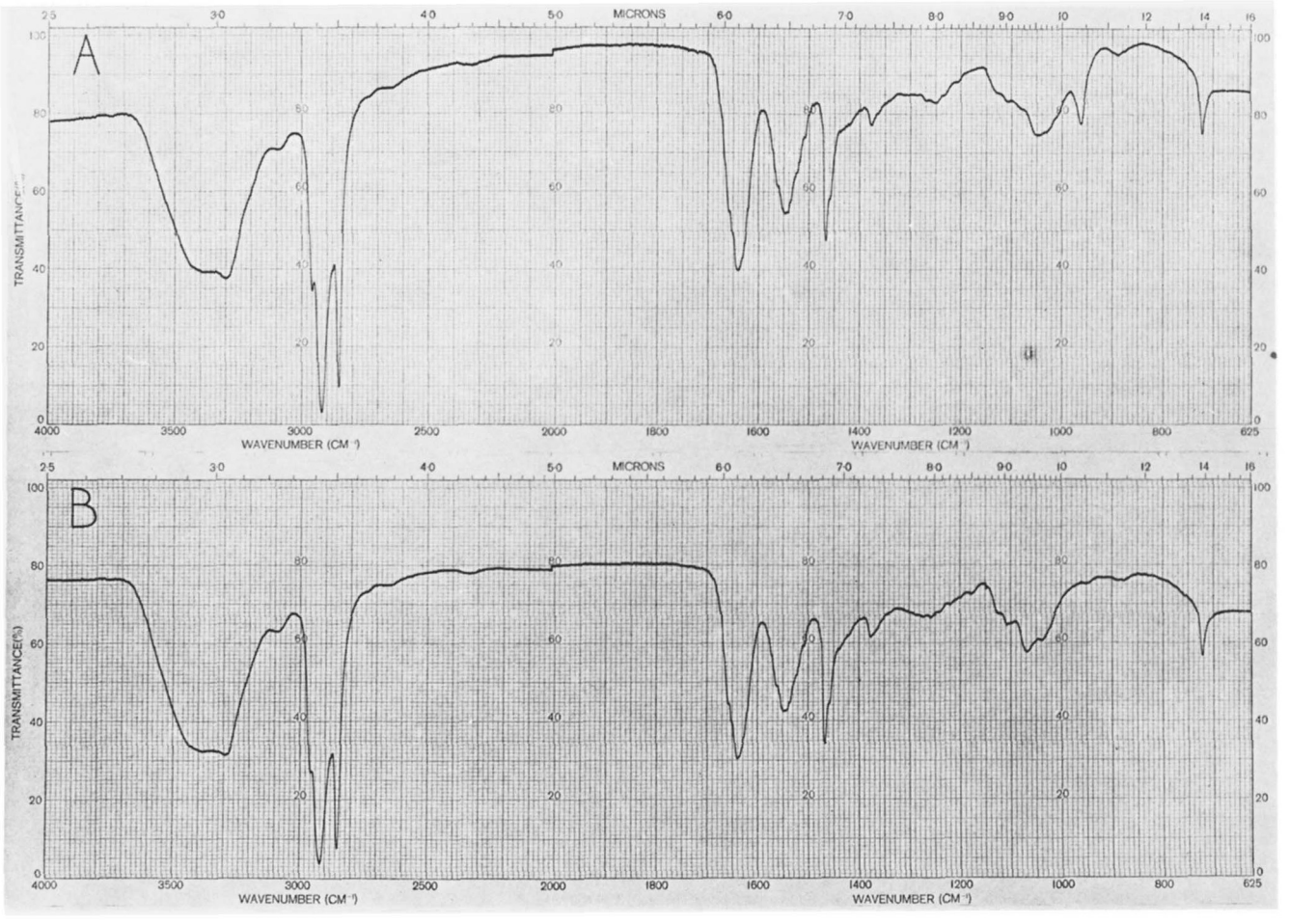

FIG. 2. Infrared spectra of synthetic ceramides, recorded from KBr discs (1.0 mg of ceramide/250 mg of KBr). *A*, *N*-(palmitoyl) DL-sphingenine; *B*, *N*-(stearoyl) DL-sphinganine.

the amide band I at 1645–1630 cm^{-1} and amide band II at 1550–1545 cm^{-1}. There was no absorption in the ester carbonyl region (1755–1735 cm^{-1}), indicating no acylation of the hydroxyl groups of the long-chain bases. Ceramides containing sphingenine or *trans*-2-hexadecenoic acid, or both, showed absorption for a *trans* double bond at 970–960 cm^{-1}; this band was absent in ceramides composed of sphinganine and saturated fatty acids.

Discussion. Table 1 summarizes the yields and melting points of the ceramides that were synthesized. In all syntheses using sphinganine as the acyl acceptor, the product ceramide crystallized spontaneously from the reaction solution as it was formed. Ceramides with sphingenine were more soluble in the tetrahydrofuran and required addition of water to induce crystallization after the reaction was completed. Thin-layer chromatography of the products at this point would occasionally show a trace amount of the *N*-hydroxysuccinimide ester used in the synthesis. No side products were detected. Recrystallization of the *N*-acyl sphinganine from methanol or the *N*-acyl sphingenine from methanol–water removed traces of starting material. After recrystallization, yields based on long-chain base were 75–86% and on free fatty acid, 67–77%.

The ease of preparation and handling of the esters and the high yields and ease of purification of the product ceramides make this synthesis useful when it is desired to use ^{14}C-labeled starting material. This procedure probably could be used for the synthesis of cerebrosides, employing psychosine as the acyl acceptor.

This work was supported in part by grant 430 from the Nutrition Foundation and grant NS-09249 from the National Institutes of Health, U.S. Public Health Service.

Manuscript received 21 April 1972; accepted 10 July 1972.

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