Partial synthesis and properties of a series of N-acyl sphingomyelins[1]

P. K. Sripada, P. R. Maulik, J. A. Hamilton, and G. G. Shipley

Biophysics Institute, Departments of Biochemistry and Medicine, Housman Medical Research Center, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118

Abstract A series of sphingomyelins (SM) with different chain length fatty acids (C14:0, C16:0, C18:0, C20:0, C22:0, and C24:0) N-linked to the primary amino group of sphingosine have been synthesized starting with bovine brain SM. Two different acid hydrolysis procedures, *(1a)* butanolic HCl (H. Kaller, 1961. *Biochem. Z.* **334:** 451–456) and *(1b)* methanolic HCl (R. C. Gaver and C. C. Sweeley. 1965. *J. Am. Oil Chem. Soc.* **42:** 294–298), were used and the resultant sphingosylphosphocholine (SPC) was converted to SM using two acylation methods: *(2)* using fatty acid imidazolide to yield the O-acyl, N-acyl SPC, followed by mild alkaline hydrolysis for selective deacylation at the O-acyl linkage, and *(3)* selective acylation at the amino group of SPC using the free fatty acid in the presence of dicyclohexylcarbodiimide. Following chromatographic purification, N-acyl SM were obtained in high yield (80–90%), and were characterized by a combination of thin-layer chromatography, high performance liquid chromatography, chemical analysis, optical rotation, circular dichroism, infrared spectroscopy, ^{13}C NMR, and sphingosine base analysis. The N-acyl SM were chemically homogeneous with respect to fatty acid composition and the sphingosine base composition resembled that of the starting bovine brain SM. However, as a consequence of the epimerization at C-3 of SPC in both acid hydrolysis procedures, the resulting N-acyl SM consisted of mixtures of D-*erythro* and L-*threo* sphingomyelins. By differential scanning calorimetry hydrated C14:0 to C24:0 SM exhibited gel–liquid crystal transitions in the range 30–50°C but the chain length dependence was complex. X-ray diffraction of hydrated C18:0 SM showed that its transition at 45°C was between bilayer gel and bilayer liquid crystalline states.—**Sripada, P. K., P. R. Maulik, J. A. Hamilton, and G. G. Shipley.** Partial synthesis and properties of a series of N-acyl sphingomyelins. *J. Lipid Res.* 1987. **28:** 710–718.

Supplementary key words ^{13}C NMR • circular dichroism • X-ray diffraction • differential scanning calorimetry

Sphingomyelins (SM) are major phospholipid components of many animal cell plasma membranes, as well as circulating plasma lipoproteins. They occur together with the more prevalent glycerol-based phospholipids such as phosphatidylcholine (PC). For example, these two phospholipids (SM and PC) with identical phosphocholine polar groups represent the major phospholipid components of the outer monolayer of red cell membranes (1). Interestingly, in red cell membranes from different species, although the combined contribution of SM and PC remains constant, the SM/PC ratio varies markedly from species to species (2). We have been interested in exploring the structure, properties, and interactions of these two choline-containing lipids with a view to understanding their behavior at biological surfaces. As part of these studies we have made use of synthetic phosphatidylcholines with specific fatty acyl chains attached at the *sn*-1 and *sn*-2 positions of glycerol (3, 4). As a result, a detailed understanding of the behavior of PC bilayers is available (for examples of our own studies, see refs. 5–9).

In contrast, similar detailed studies of SM have been hampered by the lack of, or difficulties with, protocols for SM synthesis. Our own studies of sphingomyelins focused initially on the structure and properties of bovine brain SM (10) and its interaction with egg yolk PC (11) and cholesterol (L. S. Avecilla and G. G. Shipley, unpublished observations). More recently, we developed a protocol for the synthesis of N-palmitoyl SM and defined its structure, properties, and interactions with dimyristoyl PC and cholesterol (12). Excellent reviews of this field are given in references 13 and 14.

Recently, partial-synthetic protocols for SM have been reported by two groups (15, 16). In this study, we report two protocols for the deacylation of bovine brain SM and two protocols for the acylation of sphingosyl phosphocholine (SPC). Using this approach the synthesis of a series of N-acyl SM in which the N-acyl chain is incremented by two methylene groups from C14:0 to C24:0 has been achieved. However, it should be noted that both deacyla-

Abbreviations: SM, sphingomyelin; D-*erythro* SM, (2S, 3R)-N-acyl sphingosyl-1-phosphocholine; L-*threo* SM, (2S,3S)-N-acyl sphingosyl-1-phosphocholine; SPC, sphingosylphosphocholine; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; DSC, differential scanning calorimetry; CDI, carbonyl diimidazole; CD, circular dichroism; DCC, dicyclohexyl carbodiimide; NMR, nuclear magnetic resonance.

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tion procedures result in some epimerization at C-3 of sphingosine and the resulting N-acyl SM consist of mixtures of D-*erythro* and L-*threo* SM. In addition, we include preliminary scanning calorimetry and X-ray diffraction data illustrating the thermotropic behavior of the SM series.

MATERIALS AND METHODS

Materials

Bovine brain SM was obtained from Calbiochem Behring Diagnostics (San Diego, CA) and Sigma Chemical Co. (St.Louis, MO). All solvents were of HPLC grade from EM Science (Gibbstown, NJ). Benzene was distilled over CaH_2 before use and chloroform was distilled over P_2O_5. Fatty acids were obtained from Nu Chek Prep Inc. (Elysian, MN). Carbonyl diimidazole, dicyclohexyl carbodiimide, dimethylaminopyridine, and 4-diphenyl carbonyl chloride were from Aldrich Chemical Co. (Fairlawn, NJ). Unisil activated silicic acid (100–200 mesh) was from Clarkson Chemical Co. (Williamsport, PA). Rexyn I-300 was from Fisher Chemical Co. (Fairlawn, NJ). Thin-layer chromatography (TLC) plates (silica gel G, 5 × 20 cm, 250 μm) were from Analtech (Newark, DW). Sphingomyelinase was obtained from Calbiochem.

Methods

TLC of SM was performed using silica gel G plates developed using two solvent systems: (A) chloroform–methanol–ammonia 25:17:3 (v/v), and (B) chloroform–methanol–water–acetic acid 65:25:4:1 (v/v). The SM were visualized following exposure to iodine. For characterization of SPC, the solvent system of Fujino and Negishi (17), (C) chloroform–methanol–water 60:35:8 (v/v) was also used in addition to solvents A and B.

High performance liquid chromatography (HPLC) analysis of N-acyl SM was performed using a Varian 5000 liquid chromatograph equipped with a Varian UV50 variable wavelength detector and a Hewlett Packard 3390 Å integrator. The conditions used were those described by Jungalwala et al. (18).

For physical analysis, a number of methods were used. Infrared spectra were recorded using a Perkin-Elmer (Norwalk, CT) 1310 infrared spectrophotometer. Optical rotations were measured in an Autopol II polarimeter (Rudolph Research, Flanders, NJ). Circular dichroism spectra of SM in trifluoroethanol were recorded over the range 190–250 nm on a Cary 61 spectropolarimeter (Monrovia, CA). Natural abundance proton-decoupled Fourier transform ^{13}C NMR spectra were obtained at 47 kG (50.3 MHz for ^{13}C) with a Bruker (Billerica, MA) WP-200 spectrometer equipped with an Aspect 2000A data system.

For differential scanning calorimetry (DSC), hydrated (69–81 wt %) samples of SM were sealed in stainless-steel pans. DSC heating and cooling curves were recorded over the temperature range 0–80°C using a Perkin Elmer DSC-2 scanning calorimeter. Heating and cooling rates were 5°C/min. Transition enthalpies were determined from the areas under the peaks using gallium as standard.

For X-ray diffraction, C18:0 SM was weighed into a quartz capillary tube (i.d. 1 mm) and 30 wt % double-distilled water was added. The tube was flame-sealed immediately and centrifuged repeatedly at 60°C to form a multilamellar dispersion. X-ray diffraction patterns were recorded using CuKα radiation from a Jarrell-Ash (Waltham, MA) microfocus generator. The X-rays were line-focused using a single mirror and collimated using a slit optical system. X-ray diffraction data were recorded using a Tennelec (Oak Ridge, TN) linear position sensitive detector and associated electronics (Tracor Northern, Middleton, WI). The capillary tube was placed in a variable temperature sample holder, temperature precision ± 1°C.

RESULTS AND DISCUSSION

(1) Isolation of sphingosylphosphocholine (SPC)

(a) Butanolic HCl (Kaller method, ref. 19). SPC was prepared by acid hydrolysis of bovine brain SM according to the method originally described by Kaller (19, see also ref. 12). The specific rotation of bovine brain SM in $CHCl_3$–CH_3OH 1:1 (v/v) was $[\alpha]_D^{25} = +6.6°$ (C = 3.0). Bovine brain SM (2 g) was dispersed in 10 ml of butanol–6 N HCl 1:1 (v/v) and heated at 95°C for 90 min. Following solvent removal in a rotary evaporator, benzene (15 ml) was added to the residue and then removed by evaporation. This drying procedure was repeated. The dry residue was dissolved in chloroform (10 ml) containing a few drops of methanol, applied to a silicic acid column (40 × 2 cm) and eluted with a chloroform–methanol gradient. The eluted fractions were monitored by TLC. SPC as the hydrochloride salt was eluted in chloroform containing 40–45% methanol. The pooled fractions containing SPC showed a single, ammonium molybdate-positive spot by TLC using solvent system A **(Fig. 1).** Yields in the range 30–35% were obtained. SPC hydrochloride was converted to the free base SPC by passage over Rexyn I-300. However, the specific rotation of SPC in $CHCl_3$–CH_3OH 1:1 (v/v) was found to be $[\alpha]_D^{25} = -2.0°$ (C = 3.5), suggesting that some change in stereochemistry at one of the two optical centers (C-2 and C-3 of sphingosine) had occurred during acid hydrolysis. Further, examination of SPC using TLC solvent system C revealed two spots, suggesting the presence of D-*erythro* and L-*threo* SPC (see ref. 17).

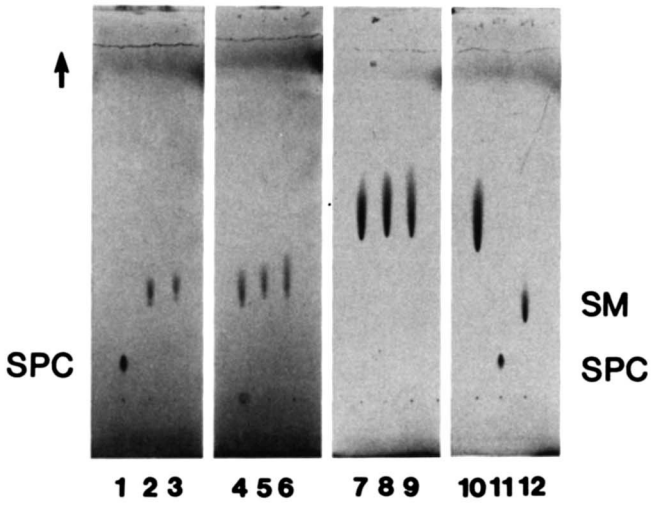

Fig. 1. Thin-layer chromatography of sphingomyelin derivatives on silica gel G plates using chloroform–methanol–ammonia 25:17:3 (v/v). Lane 1, SPC; 2, C16:0 SM; 3, C18:0 SM; 4, C20:0 SM; 5, C22:0 SM; 6, C24:0 SM; 7, O-C18:0, N-C18:0 SPC; 8, O-C20:0, N-C20:0 SPC; 9, O-C22:0, N-C22:0 SPC; 10, O-C24:0, N-C24:0 SPC; 11, SPC; 12, bovine brain SM.

(b) Methanolic HCl (Gaver and Sweeley method, ref. 20). Aqueous methanolic HCl (1 N) was prepared by diluting 8.6 ml of concentrated HCl and 9.4 ml water with methanol to 100 ml. Bovine brain SM (1.8 g) ($[\alpha]_D^{25} = +6.6°$) was hydrolyzed at 70°C with 1 N aqueous methanolic HCl for 20 hr. After evaporation, the dry residue was dissolved in chloroform and purified by silicic acid column chromatography as described above to produce SPC hydrochloride. Rexyn I-300 was used to convert SPC • HCl to the free base SPC. Using solvent system A, SPC was shown to be chromatographically pure as judged by TLC. The yield of SPC was 30–35%. However, SPC again showed a negative specific rotation $[\alpha]_D^{25} = -2.6°$ and TLC analysis using solvent system C showed two spots corresponding to the D-*erythro* and L-*threo* forms of SPC (see above and ref. 17).

(2) Synthesis of sphingomyelin using fatty acyl imidazolide (CDI method)

In step 1, the desired fatty acid (C14:0–C24:0) (1 g) was activated by carbonyl diimidazole (CDI) in the presence of benzene (5 ml). Carbon dioxide was produced to yield, after benzene removal, the activated fatty acyl imidazole. In the second step, dry SPC • HCl (480 mg) was heated in the presence of a ~3 molar excess of fatty acyl imidazole to 70°C and maintained under vacuum for 7–8 hr. After cooling, the reaction mixture was dissolved in chloroform, applied to a silicic acid column (40 × 2 cm) and eluted with a chloroform–methanol gradient. The product, O-acyl, N-acyl SPC, was eluted in chloroform containing 25–30% methanol. The product was further purified by passing over Rexyn I-300 which removed traces of imidazole. The Rexyn treatment can also precede the silicic acid column purification step. The product (yield ~84%) based on SPC was shown to be chromatographically pure using TLC solvent system A (see Fig. 1), or solvent B. In the final step, the O-acyl, N-acyl SM was subjected to alkaline (8 ml of 0.4 N NaOH) hydrolysis for 2 hr at room temperature in order to hydrolyze the O-acyl ester linkage. After neutralization with dilute HCl, the N-acyl SPC or SM was purified by silicic acid column chromatography (elution with a chloroform–methanol gradient). SM was eluted in chloroform containing 45–50% methanol. The purity of the SM product was checked by TLC (solvents A and B) and HPLC. The yield based on SPC is ~66%, giving 250–280 mg of each of the individual SM. TLC using solvent system A showed a single spot in each case with an R_f value identical to that of bovine brain SM (see Fig. 1). HPLC showed a single major peak in each case, the retention time of the major peak increasing with increasing chain length **(Fig. 2).** Minor peaks were probably associated with deacylation and reacylation of SM with other sphingosine compositions (i.e., 18:0, 20:1*t*, or 20:0) rather than the dominant 18:1*t* sphingosine (see below for sphingosine base analysis and ref. 18).

(3) Synthesis of sphingomyelin using O-acyl isourea (DCC method)

In the first step, SPC • HCl was converted to the free base SPC by treatment with Rexyn I-300. SPC was thoroughly dried by repeated (4 times) evaporation in the presence of anhydrous benzene. In the second step, SPC (180 mg) was dissolved in freshly distilled, dry chloroform (10 ml). Fatty acid (160 mg) in a slight molar excess with respect to SPC (1.6:1.0, mol/mol) and dicyclohexyl carbodiimide (DCC) equimolar with respect to fatty acid were added. The reaction mixture was maintained under dry argon and stirred at room temperature. After 1 hr the temperature was raised to 35°C and stirring was continued for 45 min. These reaction conditions optimized the disappearance of SPC and the formation of N-acyl SPC as monitored by TLC. The reaction mixture was filtered and the chloroform was removed on a rotary evaporator. The dry residue was dissolved in 25 ml of methanol–chloroform–water 5:4:1 (v/v) and passed over Rexyn I-300. The solvent was removed by evaporation and the residue was redissolved in 4–5 ml of chloroform–methanol 1:1 (v/v). Following silicic acid chromatography, the N-acyl SPC or SM was eluted in chloroform containing 45–50% methanol. The isolated SM were shown to be pure by TLC using two solvent systems (A and B) and HPLC (see Figs. 1 and 2). The yields based on SPC were in the range 85–90% for the different SM.

Using a similar protocol for selective SPC acylation, Cohen et al. (16), reacted SPC with the appropriate fatty acid in the presence of a large excess of DCC (10 mol of

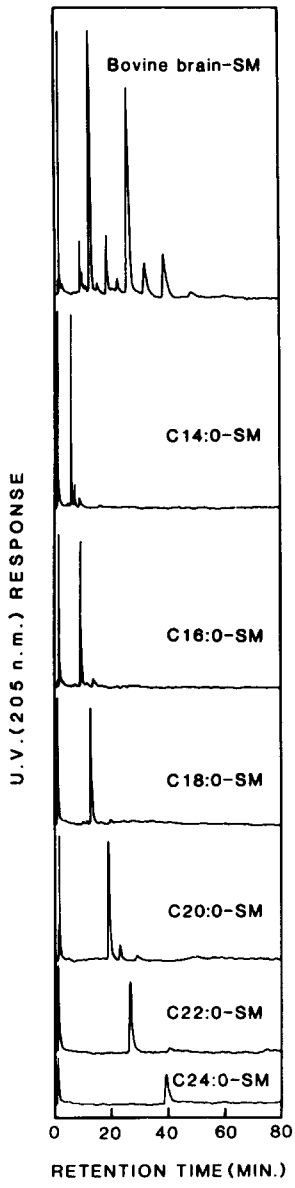

Fig. 2. High performance liquid chromatography of (a) bovine brain SM; (b) C14:0 SM; (c) C16:0 SM; (d) C18:0 SM; (e) C20:0 SM; (f) C22:0 SM; (g) C24:0 SM on an Altex C18 column. The solvent was methanol–5 mM phosphate buffer (pH 7.4), 98:2 (v/v) with a flow rate of 2 ml/min. All samples (300 μg for (a); 40 μg (b)-(g) were dissolved in dichloromethane–methanol 1:1 (v/v) and 20-μl samples were injected.

DCC per mol of fatty acid) in a mixture of solvents (methylene chloride, methanol, and triethylamine). The tertiary base was added to insure the complete conversion of SPC hydrochloride to SPC. In the small-scale synthesis described (16), <25 mg quantities of SPC were used. If 300 mg of SPC were used as in our protocol, the procedure of Cohen et al. (16) would require 1.1 g of DCC, which would complicate the purification steps. Thus, our protocol requires *1*) a much smaller amount of DCC, and *2*) a simpler solvent system, anhydrous chloroform.

(4) Sphingosine base analysis

A modification of the method described by Jungalwala et al. (18) was used. Bovine brain SM or C16:0 SM were incubated with sphingomyelinase to remove the phosphocholine yielding ceramide. The amide bond in the resulting ceramide was hydrolyzed with methanolic KOH (24 hr; 70°C) and the sphingosine bases, after derivatization with 4-biphenyl carbonyl chloride, were analyzed by HPLC. The sphingosine bases were identified according to reference 18. For bovine brain SM, the predominant sphingosine base was C18:1*t* (90.2%) with minor amounts of C20:1*t* (7.3%) and C18:0 (2.6%). C16:0 SM prepared by procedures (*1b*) and (*3*) retained C18:1*t* (85.7%) as the predominant sphingosine base, C20:1 (1.0%) decreased, and some enrichment in C18:0 (14.3%) occurred. It is not clear at what stage in the deacylation, reacylation, and purification procedures these minor changes in base composition occur.

(5) Analysis of C16:0 SM

C16:0 SM prepared by procedures *(1b)* and *(3)* was analyzed for nitrogen and phosphorus (N calculated 3.8%, N found 3.72%; P calculated 4.2%, P found 3.9%). Infrared spectra of C16:0 SM in KBr pellets showed absorptions at 960, 1040, 1090, 1240, 1470, 1645, 2840, and 2190 cm^{-1} and were essentially identical with those of bovine brain SM (data not shown; see also ref. 16). Circular dichroism spectra were recorded for C16:0 SM *(1b, 3)*, C20:0 SM *(1a, 2)*, and C24:0 SM *(1a, 2)* over the range 190–250 nm. A trough at 195 nm (molar ellipticity = – 69,000, for C16:0 SM) was observed in each case and the spectra were superimposable. Bovine brain SM exhibited a similar CD spectrum with the trough at 197 nm and a similar molar ellipticity to C16:0 SM. Following acylation (method *3*) of SPC prepared by either *(1a)* or *(1b)*, C16:0 SM showed specific rotations in the range $[\alpha]_D^{25} = -6.7°$ to $-10.0.°$ These values, compared with that of bovine brain SM ($[\alpha]_D^{25} = +6.6°$), provide clear evidence that the epimerization occurring during the deacylation step (with either method *1a* or *1b*) is present in the reacylated SM, although this change in stereochemistry is not evident in the 190–250 nm CD spectra. It should be noted that Shapiro (21) synthesized C16:0 SM de novo, isolated D-*erythro* and L-*erythro* isomers, and showed that they had essential ly identical but opposite sign specific rotations.

(6) ^{13}C NMR

High resolution ^{13}C NMR spectra of the starting bovine brain SM, SPC prepared by both hydrolysis methods *(1a* and *1b)*, various O-acyl, N-acyl SPC synthesized using method *(2)*, and various N-acyl SPC (SM) synthesized using both methods *(2)* and *(3)* were recorded at 50.32 MHz. Two issues were focused on: first, the

stereochemical changes occurring during deacylation of SM, and second, the acylation site of SM.

Possible inversion at C-3 during hydrolysis using either hydrolytic procedure was monitored at three different sites; *i*) C-3; *ii*) C4–C5 of sphingosine in both SPC and reacylated (C16:0) SM; and *iii*) C-1' of the fatty acid (C16:0) in reacylated SM. Previous ^{13}C NMR studies have shown that these resonances are sensitive to the molecular configuration at C-3. For example, Sarmientos, Schwarzmann, and Sandhoff (22) in a comprehensive ^{13}C NMR study of sphingosines, ceramides, and glucosyl ceramides have shown that the C4–C5 sphingosine resonances provide excellent markers for the D-*erythro* and L-*threo* configurations. In addition, Hara and Taketomi (23) pointed out that the sphingosine C-3 resonance is useful for the determination of D-*erythro* (downfield shift) and L-*threo* (upfield shift) isomers. The SPC prepared by *(1a)* or *(1b)* showed pairs of resonances corresponding to C-3 at 69.91 and 69.81 ppm *(1a,* see **Fig. 3D)** and 70.07 and 69.93 ppm *(1b,* see Fig. 3C). Hara and Taketomi (23) reported a value of 69.8 ppm for the C-3 of their SPC containing mainly the L-*threo* isomer. Thus, SPC prepared from bovine brain SM using the hydrolytic conditions of both Kaller (19) and Gaver and Sweeley (20) is a mixture of D-*erythro* (C-3, ~70.0 ppm) and L-*threo* (C-3, ~69.85 ppm) isomers. The peak intensities show that the Kaller hydrolysis (19) results in greater inversion at C-3 than does the Gaver and Sweeley method (20). SPC prepared by *(1a)* and *(1b)* showed two resonances at 137.13 and 137.0 ppm *(1a,* see Fig. 3D) and 137.18 and 137.0 ppm *(1b,* see Fig. 3C), corresponding to C-4 of sphingosine; similarly the resonances at 127.93 and 127.06 ppm *(1a,* see Fig. 3D), and 128.15 and 127.25 ppm *(1b,* see Fig. 3C) correspond to the C-5 of sphingosine. The pairs of resonances correspond to the D-*erythro* (~137.0 and ~127.0 ppm) and L-*threo* (~137.2 and ~128.0 ppm) isomers of SPC. Again, consideration of the ^{13}C resonances from C4–C5 suggests that the D-*erythro* to L-*threo* inversion is more pronounced in the Kaller deacylation procedure (19) than the Gaver and Sweeley method (20), but both procedures lead to epimerization at C-3 of sphingosine (c.f. Figs. 3C and D).

The ^{13}C NMR spectra of C16:0 SM prepared by *(1a)* and *(3)* showed resonances at 134.77 and 133.16 ppm (C-4), and at 129.70 and 129.28 ppm (C-5), the region

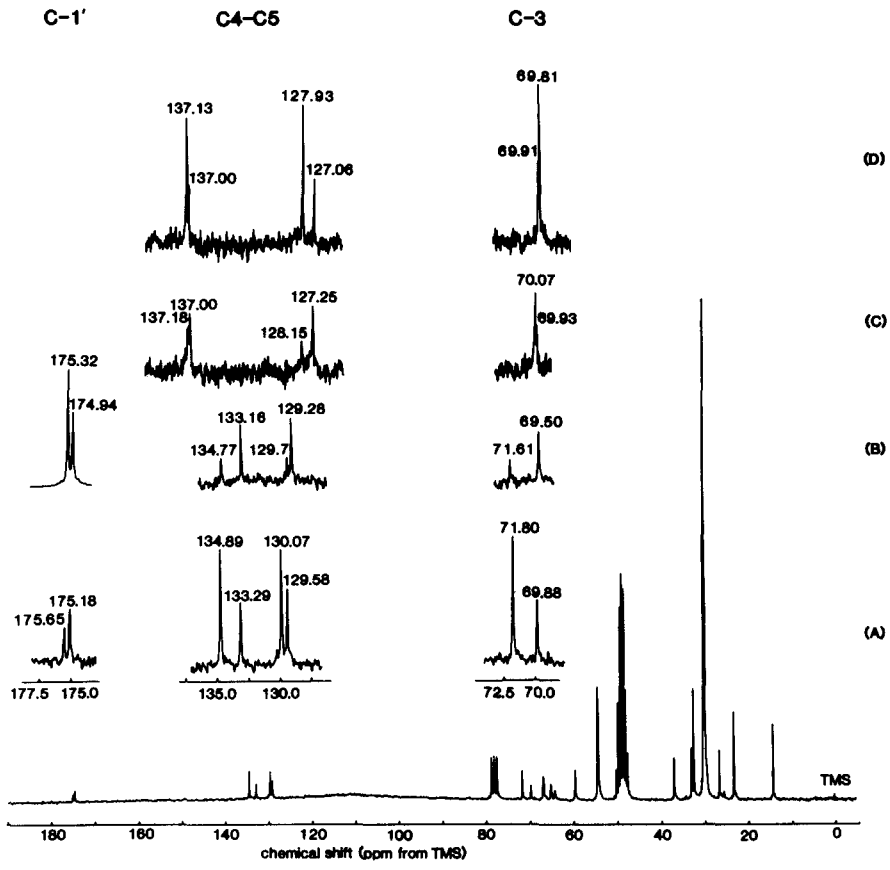

Fig. 3. (A) ^{13}C NMR spectrum of C16:0 SM (method *1b, 3*) in $CDCl_3$–CD_3OD 1:1 (v/v). Scale expansions of the C-3 (sphingosine), C4–C5 (sphingosine) and C-1'(fatty acid) regions are shown above. Corresponding regions of C16:0 SM (method *1a, 3:* the improved signal-to-noise in the C-1' (fatty acid) region is due to ^{13}C-enrichment at the carbonyl carbon), SPC (method *1b*) and SPC (method *1a*) are shown in (B), (C), and (D), respectively.

characteristic of sphingosine C4–C5 (Fig. 3B). Similarly the C16:0 SM prepared by *(1b)* and *(3)* showed resonances at 134.89 and 133.29 ppm (C-4), and 130.07 and 129.58 ppm (C-5) (Fig. 3A). The downfield resonances (~135 and ~130 ppm) correspond to D-*erythro* SM and the upfield pair (~133 and ~129 ppm) to L-*threo* SM. The separation of the C-4 and C-5 resonances was ~5 ppm for the D-*erythro* isomer and ~4 ppm for the L-*threo* isomer (see ref. 22). Synthetic C16:0 SM prepared by *(1a)* and *(3)* showed two C-3 resonances at 71.61 and 69.50 ppm (Fig. 3B), the downfield peak corresponding to D-*erythro* SM and the upfield peak corresponding to the L-*threo* SM (isomer shift, $\Delta = 2.11$ ppm). C16:0 SM prepared by *(1b)* and *(3)* showed the corresponding peaks at 71.80 and 69.88 ppm (Fig. 3A). This confirmed that synthetic C16:0 SM prepared from either hydrolysis procedure *(1a* or *1b)* contained both D-*erythro* and L-*threo* isomers. Finally, the chemical shift in the fatty acyl carbonyl region (~175 ppm) is also diagnostic for D-*erythro* and L-*threo* isomers in other sphingolipids (22). C16:0 SM synthesized from SPC [*(1a)* and *(3)* or *(1b)* and *(3)*] gave two resonances at 175.32 and 174.94 ppm *(1a, 3;* see Fig. 3B) and 175.65 and 175.18 ppm *(1b, 3;* see Fig. 3A). The upfield resonances (~175.0 ppm) in each case correspond to the D-*erythro* isomer and the downfield resonances (~175.5 ppm) to the L-*threo* isomer. Clearly, epimerization was more pronounced (i.e., more L-*threo* SM) using the Kaller hydrolysis procedure (c.f. Figs. 3A and B).

With respect to the acylation state of the C-2 amine and the C-3 hydroxyl groups, a particularly sensitive region to monitor is the α- and β-methylene region (20–40 ppm) of the linked fatty acids. Bovine brain SM in $CDCl_3$–CD_3OD showed an amide-linked α-CH_2 resonance at 36.9 ppm and a β-CH_2 resonance at 26.4 ppm. SPC in D_2O showed no resonances at ~35 and ~25 ppm. The O-C18:0 N-C18:0 SPC in $CDCl_3$ showed two pairs of resonances in this region, one pair at 36.2 and 26.1 ppm attributable to the α- and β-methylenes of the amide-linked fatty acid and an additional pair at 34.5 and 25.1 ppm due to the α- and β-methylenes of the ester-linked fatty acid. Similar spectral characteristics were observed for the O-C16:0, N-C16:0 SPC. Finally, removal of the O-linked fatty acid as in method *(2)* or direct acylation as in method *(3)* resulted in, for C16:0 SM (dissolved in $CDCl_3$–CD_3OD), only one pair of resonances at 36.9 and 26.4 ppm attributable to the α- and β-methylenes of the amide-linked fatty acid (see above). The additional resonances due to ester-linked fatty acid were not present.

(7) Differential scanning calorimetry of hydrated SM

DSC heating curves of the series C14:0- to C24:0-SM at full (69–81 wt. %) hydration are shown in **Fig. 4.** C14:0 SM exhibited two endothermic transitions with peak maxima at 25.9 and 29.8°C which were reversible on cooling (data not shown). C16:0 SM and C18:0 SM showed

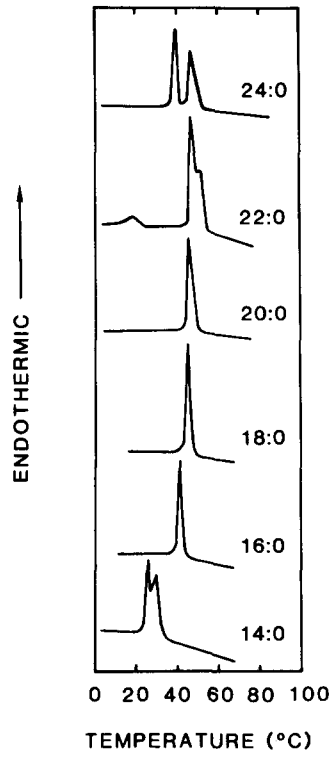

Fig. 4. Differential scanning calorimetry heating curves of hydrated SM. Bottom to top: C14:0 SM, C16:0 SM, C18:0 SM, C20:0 SM, C22:0 SM, and C24:0 SM. Heating rate was 5°C/min.

reversible transitions at 41.0 and 45.0°C, respectively. C20:0 SM showed a reversible transition at 46.5°C, with a poorly resolved shoulder on the high temperature side. C22:0 SM exhibited a high enthalpy transition at 46.9°C with a resolved shoulder at 51.2°C. In addition, a broad low enthalpy transition was present at 18.4°C. Finally, C24:0 SM showed two major transitions at 40°C and 47.5°C, with the latter transition showing evidence of a high temperature shoulder. In all cases, in spite of the complex thermotropic behavior, essentially reversible behavior was observed for all members of the series. **Table 1** summarizes the transition temperature and enthalpy data for the complete SM series. For C14:0, C16:0, and C18:0 SM, good agreement is observed with the transition temperature values reported by Ahmad, Sparrow, and Morrisett (15) for a series of synthetic SM starting with egg yolk SM; however, our values for the transition enthalpy are lower. For C16:0, C18:0, C22:0, and C24:0 SM, reasonable agreement with the transition temperatures reported by Cohen et al. (16) for their series of SM synthesized from bovine brain SM is again demonstrated.

Our data for the complete C14:0 to C24:0 SM series indicate a complex pattern of thermotropic behavior. For intermediate chain lengths (C16:0 and C18:0) quite simple reversible behavior is observed with conversions between bilayer gel and liquid crystalline states demonstrated by

TABLE 1. DSC data of hydrated N-acyl sphingomyelins

N-Acyl SM	Wt % H_2O	Transition 1		Transition 2		Transition 3	
		T_m(°C)	ΔH(a) Kcal/mol	T_m(°C)	ΔH(b) Kcal/mol	T_m(°C)	ΔH(c) Kcal/mol
C14:0	81			25.9	1.63	29.8	2.73
C16:0	69					41	5.78
C18:0	79					45	6.49
C20:0	75					46.5	6.27
C22:0	75	18.4	0.63	46.9	3.83	51.2	2.90
C24:0	75			40	4.35	47.5	6.25

X-ray diffraction (see below, and ref. 24). In contrast, the short (C14:0) and long (C20:0, C22:0, and C24:0) chain SM exhibit multiple transitions. This is indicative of gel state polymorphism presumably due to packing problems of the short and long chain fatty acids, with the fixed length sphingosine chain (P. R. Maulik and G. G. Shipley, unpublished results).

(8) X-ray diffraction of hydrated (30 wt %) C18:0 SM

X-ray diffraction data were recorded for hydrated C18:0 SM over the temperature range 12–68°C **(Fig. 5).** At 12°C the diffraction pattern consisted of a series of lamellar low angle reflections (h = 1–4) with a bilayer periodicity d = 71 Å. In the wide-angle region, a single sharp reflection at 1/4.17 $Å^{-1}$ was indicative of hexagonal hydrocarbon chain packing. On raising the temperature, the diffraction pattern remained essentially the same up to 40°C; the bilayer periodicity decreased slightly and the wide-angle reflection shifted progressively to 1/4.26 $Å^{-1}$. At 44°C the wide-angle reflection began to broaden and shifted to lower angles and at 48°C only the broad diffuse maximum at 1/4.53 $Å^{-1}$ indicative of the melted chain state was present. At 48°C the low-angle reflections showed a bilayer periodicity d = 65 Å. Only minor shifts in the positions of the lamellar and wide-angle reflections occurred over the temperature range 48–68°C. Thus, for C18:0 SM, the transition observed by DSC at 45°C corresponded to a bilayer gel to bilayer liquid crystal ($L\beta \rightarrow L\alpha$) structural transition. Similar structural studies for the complete SM series are currently in progress (P. R. Maulik and G. G. Shipley, unpublished results).

SUMMARY

We have developed several protocols for the partial synthesis of a series of SM in which the N-acyl chain length increases from C14:0 to C24:0. The least efficient step is the production of SPC from bovine brain SM by acid hydrolysis. It appears that without an enzyme capable of hydrolyzing the N-acyl amide linkage in SM, this step will continue to be a problem. The most difficult problem is that associated with inversion at C-3 during hydrolytic deacylation procedures. Using both butanolic HCl (according to Kaller, ref. 19) and methanolic HCl (according to Gaver and Sweeley, ref. 20) significant amounts of the L-*threo* stereoisomer of SPC are formed. As revealed by TLC using solvent system C (see also ref. 17) and ^{13}C NMR, both procedures lead to mixtures of D-*erythro* SPC and L-*threo* SPC. The methanolic HCl protocol appears milder and less L-*threo* SPC is formed. So far we have not been able to isolate the D-*erythro* and L-*threo* isomers of SPC using column chromatography, and separation experiments using preparative TLC are in progress. While this issue of epimerization at C-3 of sphingosine has been carefully addressed for glycosphingolipids (see ref. 22), relatively few studies (see refs. 17, 23, 25, and 26) have focused on this problem during the preparation of SPC and the synthesis of SM.

For the SPC acylation step, the time-consuming CDI method acylates both the amino and hydroxyl groups of SPC and extra steps associated with the base hydrolysis used to remove selectively the O-acyl chain are required. Clearly, selective acylation at the amino group of SPC is desirable and we have developed the DCC method for this purpose. As indicated above, our protocol differs somewhat from that described by Cohen et al. (16). Good yields are obtained with both of our acylation procedures, but currently we favor the shorter DCC method. Ahmad et al. (15) used the fatty acid activated as the N-hydroxysuccinimide ester for selective acylation at the amino group of SPC and reported high yields. Again, we have shown that acylation of both D-*erythro* and L-*threo* SPC takes place and the resulting SM are mixtures of the D-*erythro* and L-*threo* stereoisomers. It should be stressed that other protocols for the partial synthesis of sphingomyelins do not address this problem directly. For example, Ahmad et al. (15) used the butanolic HCl method for producing SPC, whereas Cohen et al. (16) favored the "milder" methanolic HCl method and claimed that, using the solvent system of Fujino and Negishi (17), only the D-*erythro* isomer is present. In our hands at least, both protocols lead, to a greater or lesser extent, to inversion at C-3 of SPC and, following acylation, to mixtures of D-*erythro* and L-*threo* SM. In contrast to Cohen et al. (16), we do not find the CD spectrum (at least in the region 190–250 nm) to be a

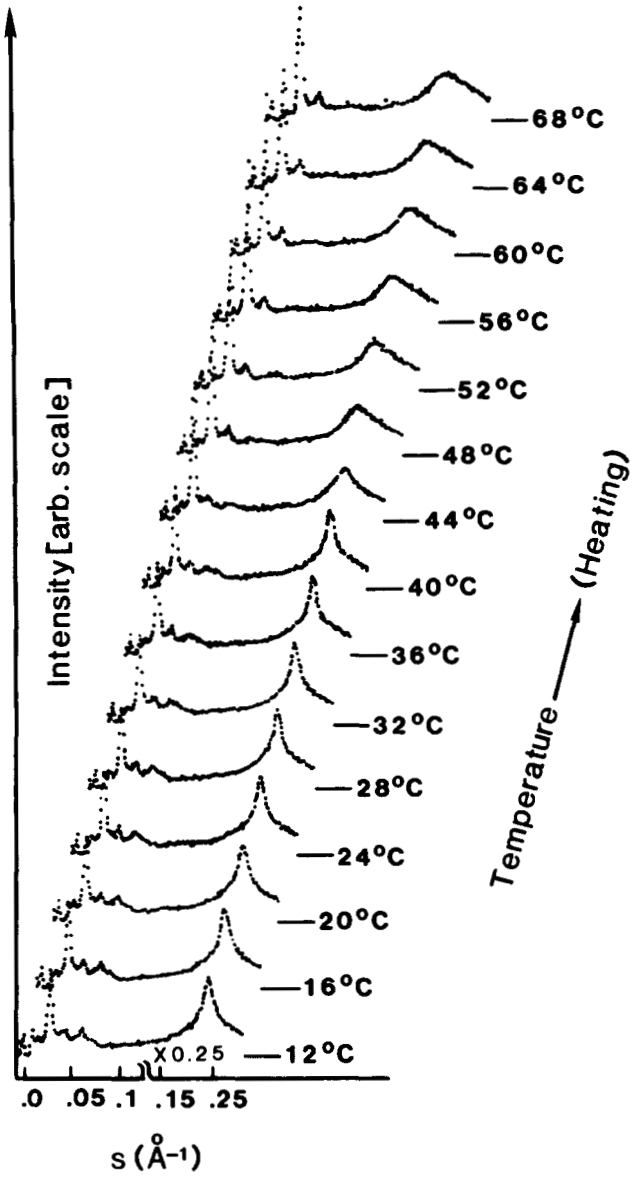

Fig. 5. X-ray diffraction patterns of hydrated (30 weight % water) C18:0 SM as a function of temperature. Intensity data were recorded using a position sensitive detector, sample-to-detector distance = 104.2 mm. The acquisition time for each diffraction pattern was 10 min. $s = 2 \sin \Theta/\lambda$; 2Θ, diffraction angle; $\lambda = 1.5418$ Å.

good monitor of epimerization. In our view, direct measurement of the specific rotation plus ^{13}C NMR provide compelling evidence of epimerization. While the two stereoisomers of SM may not differ significantly in terms of their physical properties, methods for the synthesis (or separation) of "native" D-*erythro* SM must be developed.

We have shown by DSC that the thermotropic behavior of some members of the C14:0- to C24:0-SM series is quite complex, whereas C18:0 SM exhibits a relatively simple, reversible chain melting transition between bilayer gel and liquid crystalline states. Bilayer thickness measurements in single bilayer vesicles of this series of SM show a linear dependence on SM chain length and evidence of significant chain interdigitation across the bilayer center for the longer chain length SM (see ref. 27). Detailed studies of the structure and properties of C16:0-SM and C18:0-SM and their interactions with PC and cholesterol will be reported later (P. R. Maulik, P. K. Sripada, and G. G. Shipley, unpublished observations). Studies of the short (C14:0) and long (C24:0) chain members of the SM series are in progress. Only with the availability of protocols for SM synthesis such as those described here and in refs. 15 and 16, together with methods to produce or separate D-*erythro* and L-*threo* SM, are extensive biophysical studies of this type made possible.

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