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Fluorine-, pyrene-, and nitroxide-labeled sphingomyelin: semi-synthesis and thermotropic properties

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Abstract A rapid, high-yield method has been developed for the N-acylation of sphingosine-1-phosphocholine (SPC) to obtain a series of sphingomyelin (SM) derivatives bearing different reporter groups in the N-acyl chain. The procedure utilizes a fatty acid activated as the N-hydroxysuccinimide ester. A 1:1 molar mixture of the activated fatty acid and SPC is refluxed in 5% aqueous $NaHCO_3$–ethanol 9:1 (v/v) for 2–3 hr. After acidification, the precipitated SM is purified by column chromatography over silica gel. This procedure offers significant advantages over those reported for the synthesis of well-defined SM: *i*) only the amino (not the hydroxyl) group is acylated; *ii*) only one equivalent of fatty acid is required; and *iii*) the time necessary for the reaction to go to completion is short. The transition temperature and enthalpy of each SM derivative has been measured by differential scanning calorimetry and compared to its unlabeled analog. —**Ahmad, T. Y., J. T. Sparrow, and J. D. Morrisett.** Fluorine-, pyrene-, and nitroxide-labeled sphingomyelin: semi-synthesis and thermotropic properties. *J. Lipid Res.* 1985. **26:** 1160–1165.

Supplementary key words differential scanning calorimetry • N-hydroxysuccinimide esters of fatty acids • sphingosine-1-phosphocholine • ^{13}C nuclear magnetic resonance

Sphingomyelin (SM) is an important component of most biological membranes and the most abundant of extraneural sphingolipids. The variety of fatty acids in the naturally occurring sphingomyelins gives rise to molecular heterogeneity that depends on the source of isolation. Several previous studies on the interaction of sphingomyelin with other phospholipids or proteins have involved the use of naturally occurring SM. However, for studies requiring a high degree of acyl chain cooperativity, chemically homogenous SM is desirable or obligatory. Homogeneous sphingomyelin has been totally synthesized by Shapiro (1) in D,L form. Partially synthetic procedures have involved deacylation of naturally occurring SM (2), then reacylation using activated fatty acids. This latter approach ensures the presence of only the D optical isomer, a structural feature which can have a profound effect on the thermal transition temperature of a bilayer membrane formed from the lipid (3). Generally, acylation can be brought about by fatty acid in a variety of forms: acyl chloride (1), acylimidazole (4) alone or in the presence of dimethylsulfoxide sodium salt (5); *p*-nitrophenyl ester (6); fatty acid in the presence of mixed carbodiimide (7); or fatty acid anhydride (8). The procedures involving these acylating agents have several disadvantages including the requirement for a large molar excess of fatty acid, low yield, long reaction time, and undesirable side reactions such as O-acylation. In this report we describe a rapid, high yield, selective, base-catalyzed reacylation of SPC using the N-hydroxysuccinimide ester of the active fatty acid to obtain the defined SM. With equimolar ratios of the fatty acid and deacylated SM, the reacylation proceeds to completion within 2–3 hr. We have used this procedure to prepare saturated, unsaturated, fluorinated, spin-labeled, and fluorescent-labeled sphingomyelins in greater than 90% yield. The thermotropic properties of these semi-synthetic SM analogs have been determined by differential scanning calorimetry.

Abbreviations: GLC, gas–liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; SM, egg sphingomyelin; doxyl, 3,3-dimethyloxazolidinyl; SPC, sphingosine-1-phosphocholine.

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MATERIALS AND METHODS

Myristic, palmitic, stearic, oleic, and linoleic acids, N-hydroxysuccinimide, and N,N-dicyclohexylcarbodiimide were obtained from the Aldrich Chemical Co. (Milwaukee, WI). 12-Doxyl stearic acid and 12[1-pyrene]-dodecanoic acid were purchased from Molecular Probes (Junction City, OR). Sphingomyelin (egg yolk) was obtained from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 was from Merck (Darmstadt, Germany). The 8,8-difluoro myristic, palmitic, and stearic acids were synthesized by fluorination of the corresponding 8-keto fatty acids with MoF_6 as described previously (9, 10). All solvents were HPLC grade and were purchased from Burdick and Jackson (Muskegon, MI).

N-Hydroxysuccinimide esters of the desired fatty acids were prepared using the procedure of Lapidot, Rappoport, and Wolman (11). All of these esters were crystalline at room temperature except the derivative of linoleic acid which was liquid. The 12-doxyl stearic acid derivative was used directly without purification. Melting points, infrared carbonyl absorption frequencies, and mass spectral parent ions for selected derivatives are summarized in **Table 1**. SPC was prepared by deacylation of SM as described by Kaller (2) and obtained in about 50% yield after purification by column chromatography on silica gel (12). Although the long chain base composition of this SPC was not determined in the present study, Karlsson (13) has reported the parent base of hen egg yolk SM to be predominantly dihydroxy 18:1 (97%) with traces of dihydroxy 18:0 (3%).

Synthesis of N-palmitoyl sphingomyelin (representative reacylation). SPC (40 mg, 83 μmol) and the N-hydroxysuccinimide ester of palmitic acid (29 mg, 83 μmol) were suspended in about 30 ml of 5% aqueous $NaHCO_3$-ethanol 9:1 (v/v). The reaction mixture was heated to refluxing temperature whereupon it became homogeneous. After refluxing with stirring for 2–3 hr, the mixture was allowed to cool before acidification to pH 3 with 1 M HCl. The precipitate was collected and washed with deionized water, then with acetone. The residue was dissolved in a minimum volume of chloroform–methanol–water 65:25:4 and purified by column chromatography on silica gel (50 g) in the same solvent. The phosphorus-positive fractions were pooled and the solvent was evaporated, resulting in almost quantitative yield of defined sphingomyelin (yield: 57 mg, 92%) that had approximately the same R_f value as authentic egg yolk SM as determined by TLC on silica gel. The IR spectrum showed absorption bands at 1665 and 1645 cm^{-1} for the amide carbonyl and the olefinic double bond, respectively. The ^{13}C-NMR spectrum contained an intense resonance at 173.9 ppm. This resonance was absent in the spectrum of deacylated SPC (**Fig. 1B**), but reappeared in the spectrum of SPC reacylated with the N-hydroxy succinimide ester of myristic acid (Fig. 1C). There were no resonances at 173 ppm or lower that would suggest ester carbonyl. The spectrum was not altered by treating the acylated material with 1.0 N methanolic KOH for 2 hr at room temperature, a condition known to hydrolyze O-acylated sphingomyelin (14, 15).

For DSC experiments, multilamellar sphingomyelin vesicles were prepared by bath sonication of the desired compound (about 100 mg/ml) in deionized water for about 15 min at 30–50°C. From the above mixture, 70 μl was transferred to a 75-μl stainless steel pan and hermetically sealed. DSC was performed with a Perkin-Elmer DSC-2 instrument equipped with a subambient cooling unit. The calorimeter was calibrated with an indium standard to an accuracy of ± 0.3°C. Scans were corrected for thermal lag by extrapolating to zero the apparent transition temperature (T_c) obtained at heating rates of 2.5 and 5.0°C/min. Scans were performed at least in triplicate. After the last scan of a sample, the pan was opened and the contents were transferred to a known volume of methanol–chloroform 1:1. The actual amount of lipid in each pan was determined by phosphorus analysis (16) and

TABLE 1. Physical and spectroscopic properties of N-hydroxysuccinimide esters of selected fatty acids

Fatty Acid	Observed Melting Point[a]	Reported Melting Point (11)	Infrared Carbonyl Absorption (cm^{-1})[b]	Calc'd Mol Wt	Parent Ions[c] m/e
C14:0	88–89		1820; 1790; 1745, 1725(sh) Nujol	325.45	326,327
C16:0	90–91	91	1825; 1790; 1745(sh), 1725 KBr	353.50	354,355
C18:0	93–94	94	1825; 1790; 1745, 1725(sh) Nujol	381.56	382,383
C18:1	43–44		1820; 1790; 1750, 1730(sh) Nujol	379.54	380,381
8,8-F_2-C14:0	66–67		1815; 1785; 1740(sh), 1725 Nujol	341.42 (– HF)	342,343
8,8-F_2-C16:0	79–80				
8,8-F_2-C18:0	82–83		1825; 1790; 1745(sh), 1725 Nujol	397.53 (– HF)	398,399
12[1-Pyrene]C12:0	91–92		1810; 1775; 1730 (broad) KBr	497.65	497,498,499

[a] Determined in capillaries on a Hoover melting point apparatus.
[b] Determined on a Beckman Acculab 4 spectrophotometer.
[c] Determined on a Ribermag R10-10C mass spectrometer using desorption chemical ionization conditions with isobutane.

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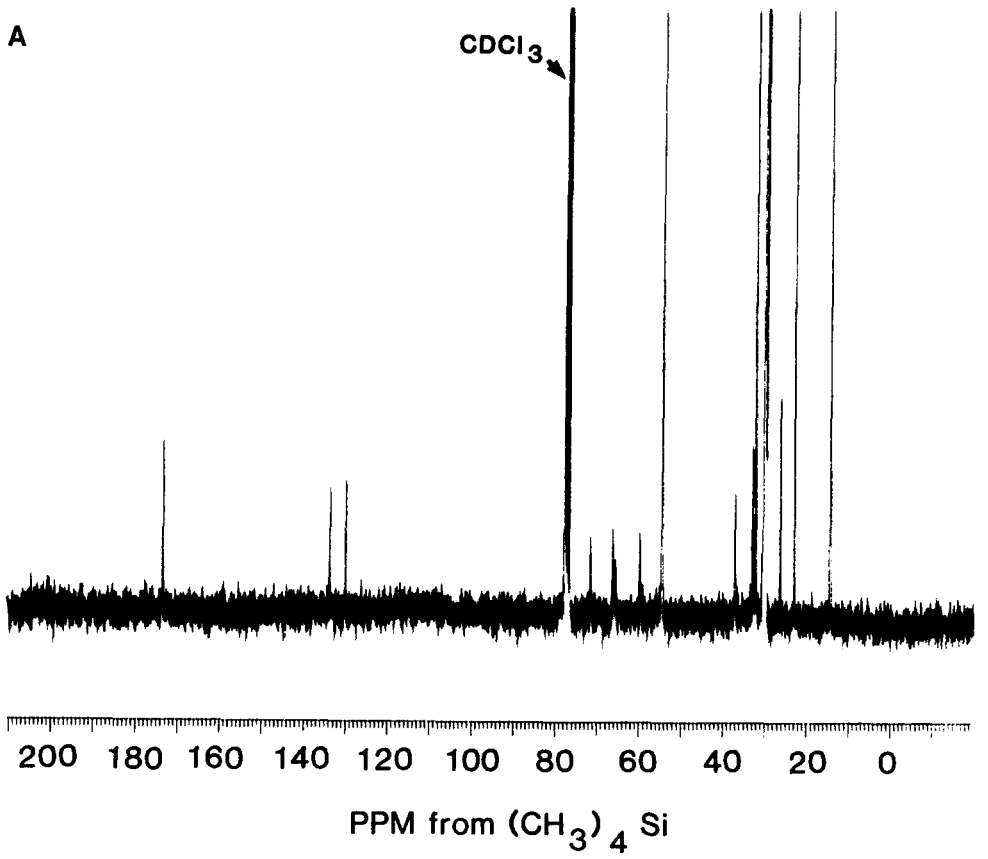

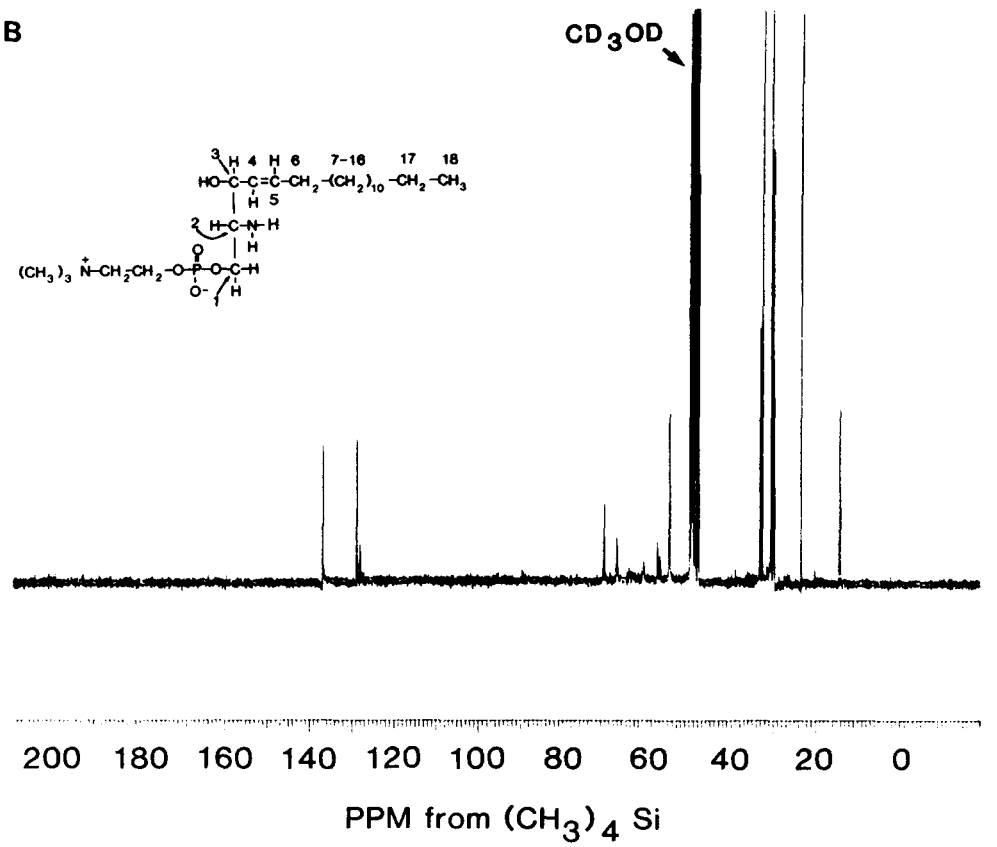

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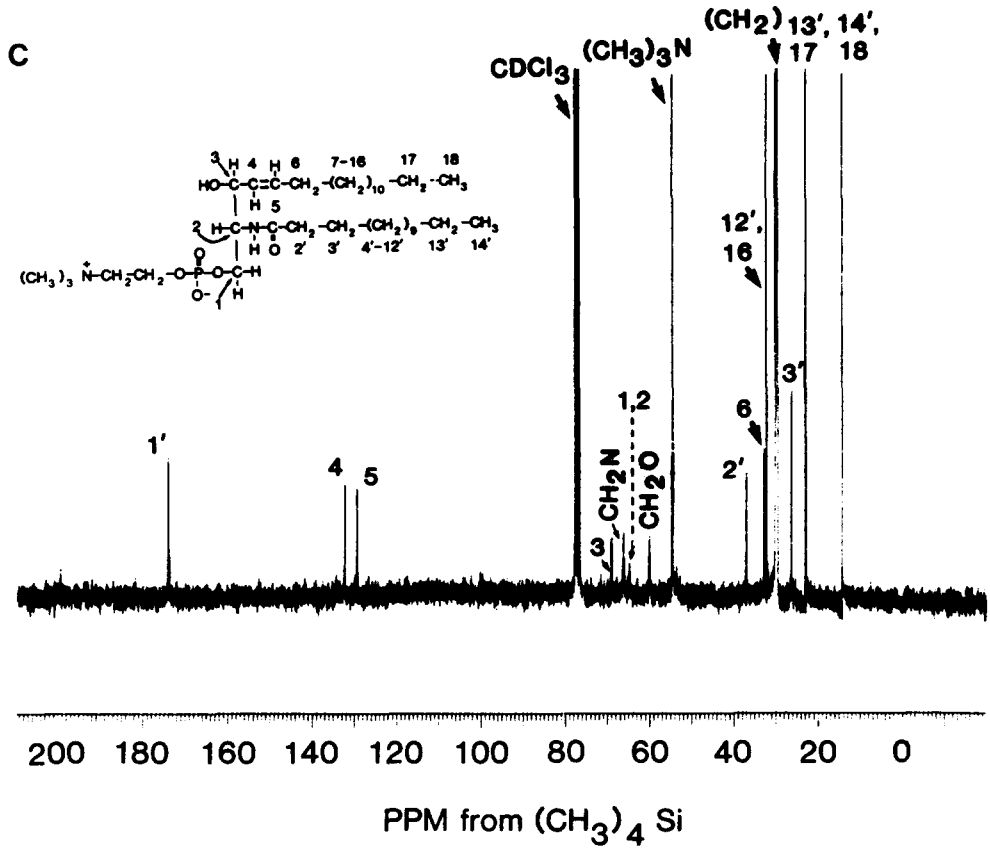

Fig. 1. Natural abundance, proton-decoupled 67 MHz ^{13}C NMR spectrum of (A) egg sphingomyelin, 50 mg/ml in $CDCl_3$; (B) sphingosine-1-phosphocholine, 60 mg/ml in CD_3OD; and (C) N-myristoyl sphingomyelin, 20 mg/ml in $CDCl_3$. The phospholipids were dried in vacuo over P_2O_5 for 4 days before dissolving in freshly opened solvent (Stohler). For all three samples the following conditions were used: 15,050 Hz spectral width, 16,384 real data points (0.92 Hz resolution); 3.0 sec recycle time; 1.0 Hz line broadening; 2,048, 3,500, and 16,780 accumulations for (A), (B), and (C), respectively. Assignments are based on those of Metcalfe et al. (23) and Birdsall et al. (24) for dipalmitoyl phosphatidylcholine and on unpublished work (Hamilton, J. A., E. Williams, and J. D. Morrisett).

its chemical integrity was checked by TLC. The transition temperature was determined from the peak of the endotherm. Transition enthalpy was determined from the area under the endotherm by weighing the trace bounded by the peak and the baseline. Values for ΔH were computed by comparison of the experimental area with the area of the indium standard. At least five weighings were used to compute the mean enthalpy of transition. Transition entropy was determined by assuming that at the transition temperature, T_c, free energy $\Delta G = 0$ so that $\Delta H = T\Delta S$.

RESULTS AND DISCUSSION

The capacity of N-hydroxysuccinimide esters of fatty acids to acylate only the amino group of serine, threonine, and hydroxylamine (11) led us to utilize this reagent in the acylation of SPC. The progress of the reaction could be monitored by TLC on silica gel plates; usually more than 95% of the sphingomyelin had formed in 2–3 hr. The product was purified by column chromatography using silica gel and eluted with chloroform–methanol–water 65:25:4, conditions where unreacted N-hydroxysuccinimide ester or fatty acid is eluted first, followed by sphingomyelin. The IR and NMR spectra of the semi-synthetic sphingomyelins clearly indicated that only N-acylation had occurred (Fig. 1). Using the above methods, we have prepared several chemically homogeneous sphingomyelins in greater than 90% yield. The transition temperatures (T_c) of C14:0, C16:0, and C18:0 semi-synthetic hydrated sphingomyelins prepared by our method are in good agreement with the reported values (**Table 2**).

The effect of N-acyl chain structure on the thermal transition temperature of the fully hydrated SM is significant. C14:0 gives an SM that exhibits two DSC transitions (25.0 and 29.0°C). When two fluorine atoms are substituted at C8, only a single transition is observed (20.0°C). C16:0 gives SM with one transition (41.5°C). Difluorination at C8 causes this transition temperature to decrease by 9.5°C (32.0°C). Similarly, C18:0-SM displays a single

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TABLE 2. Thermotropic properties of liposomes formed from egg yolk sphingomyelin (EY-SM) and some of its N-acyl derivatives

Fatty Acyl Chain	T_c, °C		ΔH, kcal/mol		ΔS, cal/mol per deg		Ref.
	Obs	Lit	Obs	Lit	Obs	Lit	
EY-SM[a]	39.0-40.0	40.0	7.3	5.9	23.4	27.7	17
C14:0	25.0, 29.0		6.9		23.2		
C16:0	41.5	41.3	7.1	6.8	24.2	21.6	18
		41.5		5.8		18.4	14
C18:0	45.2	44.5	7.4	6.7	23.3	21.1	3
		45.0					22
C18:1	33.0		6.8		22.4		
C18:2	26.0		6.5		25.1		
8,8-F_2-C14:0	20.0		7.3		25.0		
8,8-F_2-C16:0	32.0		7.4		24.3		
8,8-F_2-C18:0	36.0		7.8		25.2		
12-doxyl-C18:0	30.5		9.1		30.0		
12[1-Pyrene]C12:0	47.0		8.6		26.9		

[a]EY-SM is a heterogeneous mixture of molecules with different acyl chains. However, C16:0-SM is the most abundant species (about 85%).

transition at 45.2°C, and C8 difluorination diminishes this by 9.2°C (36.0°C). Substituting a bulky paramagnetic doxyl group at C12 reduces this by 14.7°C (30.5°C). Hence, the effect of difluorination or doxylation is roughly equivalent to introducing a *cis* double bond (C18:1-SM; 33.0°C). It has been shown that fluorinated phosphatidylcholines generally exhibit transition temperatures about 10°C below that of the unlabeled compound (19, 20). The doxyl group has also been shown to significantly lower the transition temperature of phosphatidylcholines (21). The effect of substituting a pyrene moiety on the N-acyl chain of SM contrasts strongly with the effects of fluorine or nitroxide substitution. Addition of this aromatic moiety to the hydrophobic terminus of C12:0 *raises* the transition temperature of the resulting SM to 47.0°C. Comparison of this T_c to that of C18:0-SM (45.2°C) suggests that the effect of appending the pyrenyl group is roughly equivalent to extending the chain by more than six methylene units. These sphingomyelin derivatives are currently being used in our laboratories to study acyl chain motions in native lipoproteins, lipoprotein recombinants, and biological membranes.

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