methods

Methanolysis of sphingomyelin: toward an epimerization-free methodology for the preparation of D-*erythro*-sphingosylphosphocholine

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Abstract **It is well known that acid hydrolysis of natural sphingomyelin in aqueous methanol or 1-butanol at refluxing temperature is accompanied by epimerization at the C-3 position of the long-chain base. An improved procedure for the hydrolysis of commercially available, naturally occurring sphingomyelin is described. Prolonged exposure (3–4 days) of sphingomyelin to freshly prepared 0.5 M anhydrous methanolic hydrogen chloride (generated by trapping the gas evolved from the reaction of concentrated sulfuric acid with solid sodium chloride in anhydrous methanol) at 50°C resulted in cleavage of the amide side chain. The extent of epimerization of the allylic alcohol stereocenter was quantified by integration of the C-5 signal of the ^{13}C nuclear magnetic resonance spectrum of lysosphingomyelin.** The method described here is superior to the traditional acid hydrolysis methods because it provides the product as a ~10:1 ratio of D-*erythro*/L-*threo* epimers; in contrast, a ratio of ~1.3:1 was obtained by the previous methods. We also report that use of dichloromethane as a cosolvent with *N*,*N*-dimethylformamide in the reaction of lysosphingomyelin with an activated fatty acid reduced the time required for completion of the *N*-acylation reaction.—Bittman, R., and C. A. Verbicky. **Methanolysis of sphingomyelin: toward an epimerization-free methodology for the preparation of D-*erythro*-sphingosylphosphocholine.** ***J. Lipid Res.*** **2000.** 41: **2089–2093.**

Supplementary key words lysosphingomyelin • acid hydrolysis • acylation of lysosphingomyelin

The realization that sphingomyelin is not only a major component of eukaryotic plasma membranes and lipoproteins (1), but also a metabolic source of lipid second messengers that regulate a myriad of physiological events (2, 3), has brought new appreciation of the importance of having a supply of chemically homogeneous sphingomyelin for biochemical studies. The synthesis of chemically defined sphingomyelins has been the topic of many research efforts (4–8). Naturally occurring sphingomyelins have the D-*erythro* configuration (4) but contain a wide degree of variation within the amide side chain (1) and minor variation in the chain length and degrees of unsaturation and hydroxylation of the long-chain base (9). While some early studies of sphingomyelin utilized naturally occurring mixtures, detailed physical studies of the molecular features involved in the interaction of sphingolipids with other membrane components (10, 11) or in domain formation (12) require access to chemically defined sphingomyelins. Some have chosen a total chemical synthetic route to address this issue (4–8); however, the advantages of a degradative route are obvious (i.e., removal of the amide-linked fatty acyl chain to provide the sphingoid base, followed by reacylation). An improved procedure for the preparation of semisynthetic sphingomyelin is presented herein.

The first contribution toward a partial synthesis of homogeneous *N*-acyl sphingomyelins was made in 1961 by Kaller (13), who reported the hydrolysis of commercial sphingomyelin with a 1:1 mixture of 6 M HCl and 1-butanol at 100°C (**Scheme 1**). The impracticality associated with the use (and subsequent removal) of high-boiling solvents prompted Gaver and Sweeley (14) to report a second strategy in which naturally occurring sphingomyelin is heated in 1 M methanolic HCl at reflux. Unfortunately, each of these methods causes a significant loss of configuration at the C-3 (allylic) stereocenter. Although the diastereomeric lysosphingomyelins can be separated on an analytical high performance liquid chromatography (HPLC) column (15, 16), this procedure is not practical on a preparative scale. Indeed, commercially available semisynthetic sphingomyelins that are prepared by *N*-acylation of lysosphingomyelin (obtained by acid hydrolysis of sphingomyelin) are mixtures of D-*erythro* (2*S*,3*R*) and L-*threo* (2*S*,3*S*) diastereomers (17). The need for an efficient route to chemically defined sphingomyelins with a high degree of stereochemical purity as well as a means to evaluate the degree of epimeriza-

Abbreviations: DMF, dimethylformamide; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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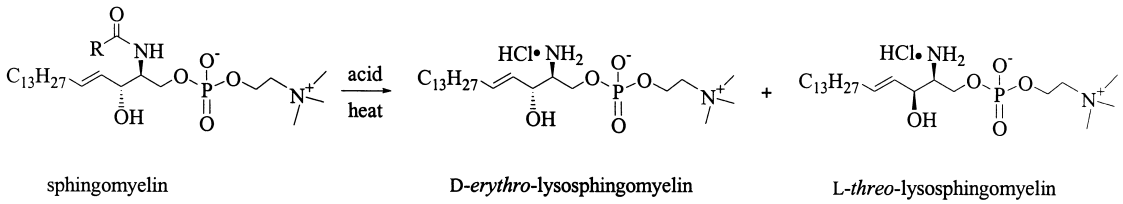

Scheme 1.

tion in the products prompted our investigation, which culminated in the efficient method for the methanolysis of sphingomyelin described herein.

It is well known that chromatography of lysosphingomyelin on silica gel requires the use of highly polar solvent systems, such as chloroform–methanol–water 65:35:8 (by volume) and chloroform–methanol–concentrated ammonium hydroxide–water 65:35:2.5:2.5 (by volume) (18, 19). When such polar solvent systems are used on silica gel columns, substantial amounts of silica gel are present as a contaminant of the product. As a result of the solubility properties of lysosphingomyelin, removal of the silica gel impurity can be troublesome. Furthermore, the presence of silica gel in the product lysosphingomyelin causes an inaccurate determination of the yields of both the hydrolysis and reacylation reactions.

The inability to quantify the degree of epimerization of the product has made it difficult to compare the efficiency of the known methods for hydrolysis of sphingomyelin. Thin-layer chromatography (TLC) and optical rotation have been used unsuccessfully in efforts to illustrate the extent of inversion at the C-3 position of sphingolipids. The reliability of these measurements is complicated by the use of chemically undefined systems, and therefore must be considered to be of dubious value. The use of ^{13}C nuclear magnetic resonance (NMR) spectroscopy to evaluate the occurrence of epimerization in semisynthetic lysosphingomyelin represents an accurate means of observing the result of epimerization (20). The spectral region arising from the vinyl carbons does not overlap with the signals of any other carbons in lysosphingomyelin. Previous ^{13}C NMR studies showed that the C-4 and C-5 signals of the long-chain base of various sphingolipids are qualitative markers of the presence of the D-*erythro* and L-*threo* configurations. Hara and Taketomi (21) assigned the signals at 137.2 and 128.0 ppm in lysosphingomyelin to C-4 and C-5, respectively. In other sphingolipids, the C-4 and C-5 signals are also well separated. For example, the signals at 134.3 and 129.3 ppm in D-*erythro*-ceramide (21) and at 135.0 and 129.9 ppm in D-*erythro*-sphingosine (22) and at ~135.0 and ~130.7 ppm in sphingomyelin (17, 23) were assigned to C-4 and C-5, respectively. A large chemical shift difference between the C-4 and C-5 signals [δ(C-4)-δ(C-5)] may be indicative of the presence of the L-*threo* configuration (17, 22). Indeed, Sripada et al. (20) showed that the C-4 and C-5 NMR signals in lysosphingomyelin are indicative of epimerization at C-3. However, no attempt was made to determine the relative amounts of each epimer in the hydrolysis product, that is, only the occurrence of epimerization was noted. We illustrate here the first use of ^{13}C NMR spectroscopy to evaluate the degree of epimerization with the known strategies for acid hydrolysis of sphingomyelin, and we describe a new method for preparing lysosphingomyelin with a low extent of C-3 epimerization.

MATERIALS AND METHODS

Reagents

Sphingomyelin from egg yolk was purchased from Avanti Polar Lipids (Alabaster, AL) and was used without purification. Anhydrous methanolic hydrogen chloride was prepared by dripping concentrated sulfuric acid onto solid sodium chloride and trapping the evolved gas in methanol, which had previously been dried by distillation from sodium methoxide. The concentration of hydrogen chloride was determined by potassium carbonate titration of an aqueous solution of the methanolic hydrogen chloride to an end point marked by phenolphthalein. Anhydrous dimethylformamide (DMF) was prepared by distillation from P_2O_5 under nitrogen. Dichloromethane was stored over calcium hydride. Flash chromatography with silica gel was carried out with Merck (Rahway, NJ) silica gel 60 (230-400 ASTM mesh). TLC plates (200-μm-thick silica gel $60F_{254}$ precoated on aluminum) were from EM Science (Cherry Hill, NJ). Amberlite IRA-400 strongly basic anion-exchange resin and Cameo filters (0.45 μm) were purchased from Fisher Scientific (Pittsburgh, PA). Phosphorus-sensitive molybdic acid spray was prepared by dissolving molybdenum metal (0.4 g) in concentrated sulfuric acid (150 ml), and diluting with a solution consisting of molybdic acid anhydride (8 g) dissolved in water (200 ml).

NMR spectroscopy

^1H NMR spectra were recorded at 400 MHz. Proton-decoupled ^{13}C NMR spectra with a relaxation delay of 2 s were recorded in methanol-d_4 at 100 MHz on a Bruker (Billerica, MA) spectrometer, and were referenced to deuterated methanol at 49.0 ppm. The vinylic region of lysosphingomyelin (see Fig. 1) resulting from the transformed free induction decay was phased and integrated repetitively. The ratios of D-*erythro*- and L-*threo*-lysosphingomyelin reported in Table 2 reflect the average values of the integrals.

Temperature control

With the exception of the reactions carried out at 65°C and at ambient temperature, the reactions were carried out in an Orbit microprocessor shaker water bath (Lab-Line Instruments, Melrose Park, IL), and the temperatures reported are uncorrected.

Preparation of lysosphingomyelin (free base form)

Sphingomyelin (100 mg, 0.12 mmol) was dissolved in anhydrous methanolic hydrogen chloride (10 ml, 0.5 M, prepared as described above) in a screw-capped vial. The solution was heated to 50°C in a shaking water bath and monitored by TLC. The plates were visualized with a phosphorus-sensitive molybdate spray. After 4 days, sphingomyelin was consumed and the reaction mixture was concentrated under reduced pressure. The resulting residue was suspended in 1 ml of a solution of chloroform–methanol–water 65:35:5 and chromatographed on silica ($CHCl_3$–methanol–H_2O, 65:35:8; R_f 0.10). The concentrated product was dissolved

TABLE 1. Screening of conditions required to hydrolyze the amide linkage of egg-yolk sphingomyelin

Reagent to Initiate *N*-Acyl Bond Hydrolysis	Temperature	Duration	Result[a]
	°C	*days*	
p-TsOH[b]	RT[b]	14	No product
HCl (aqueous)	RT	14	No product
Sodium methoxide (anhydrous)	RT	14	No product
p-TsOH	40	7	No product
HCl (aqueous)	40	7	No product
Sodium methoxide (anhydrous)	40	7	No product
p-TsOH	50	7	No product
HCl (aqueous)	50	7	Product formed
Sodium methoxide (anhydrous)[c]	50	7	No product
HCl/methanol (anhydrous)	50	5	Starting material consumed in ~3 days
HCl/methanol–H_2O 9:1	50	5	Starting material consumed in ~3 days
HCl/methanol (anhydrous)	50	3	Optimal conditions

[a] The reaction was monitored by TLC with $CHCl_3$–methanol–H_2O 65:35:8 (v/v/v). "No product" indicates that only starting material was detected.
[b] *p*-TsOH, *p*-toluenesulfonic acid. RT, room temperature.
[c] A stoichiometric amount of sodium methoxide was used.

in methanol (3 ml) and passed through a column of Amberlite IRA-400 anion-exchange resin. The solution was filtered through a Cameo filter. The filtrate was concentrated on a rotary evaporator, giving a residue that was lyophilized from benzene to afford 54 mg (90%) of lysosphingomyelin as a fine, slightly yellow powder, whose ^1H and ^{13}C NMR spectra agree with those reported by Sripada et al. (20).

N-Acylation of lysosphingomyelin

The use of methylene chloride as a cosolvent in the *N*-acylation of the free base form of lysosphingomyelin with a *p*-nitrophenyl ester substantially decreased the time required for completion of the reaction.[1] Methylene chloride increases the solubility of the activated fatty acid in DMF.

Semisynthetic lysosphingomyelin (70 mg, 0.14 mmol), *p*-nitrophenyl stearate (113 mg, 0.28 mmol), and anhydrous potassium carbonate (29 mg, 0.21 mmol) were added to a round-bottom flask equipped with a nitrogen inlet. The mixture was suspended in anhydrous DMF (5 ml) and CH_2Cl_2 (2 ml) and stirred at room temperature. After 1 day, TLC analysis indicated that lysosphingomyelin had been consumed, and the reaction mixture was concentrated under reduced pressure. The resulting residue was dissolved in 1 ml of chloroform–methanol–water 65:35:5 (by volume) and chromatographed on silica ($CHCl_3$–methanol–H_2O 65:35:5, by volume; R_f 0.17). The fractions containing the product were combined and concentrated under reduced pressure. A solution of the resulting residue in 20 ml of chloroform was filtered through a Cameo filter and concentrated in a rotary evaporator. The residue was lyophilized from benzene to afford 66 mg (65%) of *N*-stearoylsphingomyelin as a fine colorless powder whose ^1H and ^{13}C NMR spectra agreed with those reported by Bruzik (17) and Bruzik, Salamonczyk, and Sobon (24).

RESULTS AND DISCUSSION

Reaction conditions

Because the known procedures for acid-mediated hydrolysis of sphingomyelins in aqueous alcohol at elevated temperatures result in a high extent of epimerization, our initial efforts focused on identifying the most mild reaction conditions required to achieve efficient hydrolysis of the amide linkage. Conditions (basic as well as acidic) under which a variety of amides are known to undergo hydrolysis (25) were applied to egg-yolk sphingomyelin, and the reactions were analyzed by TLC (**Table 1**). The strongly basic conditions known to effect the hydrolysis of the *N*-acyl linkage in cerebrosides at high temperatures (26) are not likely to be applicable to sphingomyelin, whose phosphocholine moiety would not survive these conditions. Because we sought to avoid possible base-induced displacement of the phosphocholine moiety, we focused our attention primarily on acidic conditions. All attempts to hydrolyze sphingomyelin at ambient temperature failed to provide lysosphingomyelin. The reaction temperature was incrementally increased and the progress of reaction was monitored for 7 days at each temperature. When the reaction temperature was increased to 50°C, methanolic hydrogen chloride was found to provide hydrolyzed material within 7 days (as observed by TLC; the plates were developed with $CHCl_3$–methanol–H_2O 65:35:8; R_f 0.1). The use of hydrogen chloride at 50°C was, therefore, determined to be the mildest condition under which the reaction occurs. On further investigation, the reaction time necessary to consume the starting material was found to be significantly shorter than originally found, requiring only 3–4 days.

Quantification of the extent of epimerization at C-3

Figure 1 shows that two C-5 signals appear in the ^{13}C NMR spectrum of lysosphingomyelin obtained by acid hydrolysis of sphingomyelin in refluxing aqueous methanol (14, 20). The signals at δ 128.2 and δ 129.4 ppm (Fig. 1A) are assigned to the D-*erythro* and L-*threo* stereoisomers, respectively. The integrated ratio of the peak areas of these C-5 signals is 1.3:1.0, respectively. (Both spectra in Fig. 1 have a small unidentified peak at ~δ 129.5 ppm.) In the

[1] The *N*-acylation of lysosphingomyelin with *p*-nitrophenyl stearate in DMF without CH_2Cl_2 as the cosolvent required 3 days to reach completion.

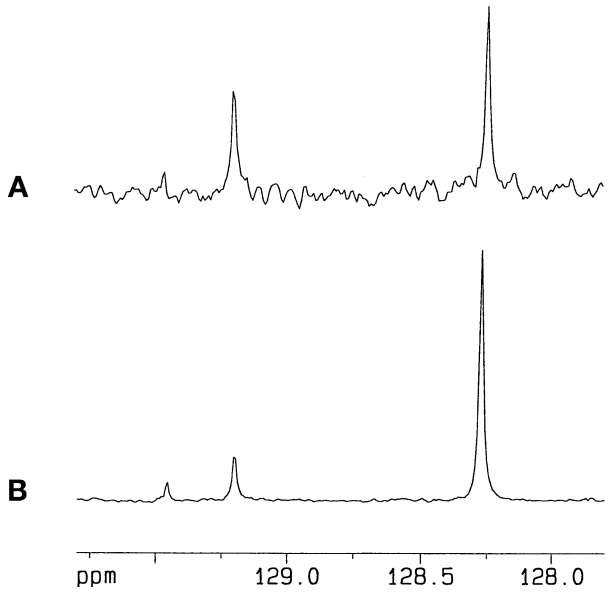

Fig. 1. The C-5 region of the ^{13}C NMR spectrum of lysosphingomyelin. (A) Spectrum of the product obtained by the Gaver and Sweeley (14) protocol. (B) Spectrum of the product obtained by the method presented in this article. The representative trace shown here gave a ratio of 5.5:1.0; the average ratio of four experiments was ~10:1.

HPLC traces presented by Bünemann et al. (15) and Liliom et al. (16), the ratio of the integrated peak area ratios of the D-*erythro* and L-*threo* lysosphingomyelin stereoisomers [also obtained by the method of Gaver and Sweeley (14)] is 57:43, or 1.33:1.00, in excellent agreement with our ^{13}C NMR analysis in Fig. 1A. Figure 1B shows the C-5 NMR signals of lysosphingomyelin obtained by using the anhydrous methanolic hydrogen chloride method we present in this article; the integrated peak ratio of these C-5 signals is ~10:1. This observation demonstrates the superiority of our anhydrous methanolic hydrogen chloride method (with a reaction temperature of 50°C and a reaction duration of 3 to 4 days).

Epimerization at C-3 of lysosphingomyelin: effect of water

Because the epimerization of the allylic stereocenter requires water or some aggregate to form, the effect of water on the reaction was next examined. When two identical reactions were monitored side by side, one containing a known amount of water and the other anhydrous, the anhydrous reaction appeared to require shorter reaction times to consume the sphingomyelin. To ensure that the product contained only one salt form and existed as the free base, the product was passed through an anion-exchange resin (Amberlite IRA-400). Integration of the C-5 signal in the ^{13}C NMR spectrum was used to quantify the extent of epimerization. The amount of the epimerized product obtained from the reaction containing water was higher than that from the anhydrous reaction (**Table 2**). When the methanolysis was carried out in anhydrous methanolic hydrogen chloride, the reaction was found to be complete in 3–4 days, and typically provided >75% chemical yields. The hygroscopic nature of anhydrous alcohols and the water inherent to sphingomyelins (tightly bound to the phosphocholine head group) (4, 5) are expected to introduce water into the methanolysis reaction and, therefore, enhance the degree of epimerization. When the anhydrous acid solutions were stored and used at a later time, the degree of epimerization in the product increased. Efforts to consume the accumulated water as well as the water inherent to sphingomyelin by treatment with trimethyl orthoformate (which can function as a dehydrating agent) (27) resulted in poorer yields without significantly improving the ratio of epimers. When molecular sieves were used to remove water from these systems, the acidic solutions were neutralized, preventing the methanolysis.

TABLE 2. Effect of reaction conditions on the degree of epimerization

Reaction Conditions	Duration	Temperature	Ratio of Epimers[a]
	days	*°C*	
1 M methanolic HCl	1	65	1.3:1
1 M methanolic HCl	3	50	3.7:1
Anhydrous 0.5 M methanolic HCl	3	50	10:1[b]
Anhydrous 0.5 M methanolic HCl with 10% water by volume	3	50	5.5:1
Anhydrous 0.5 M methanolic HCl	1	65	~1:1
Anhydrous 1.1 M methanolic HCl	1	50	3:1

[a] Determined by integration of the resonances at δ 128.2 and 129.4 ppm (Fig. 1). In each reaction, the δ 128.2 ppm signal was the larger peak.

[b] The average ratio obtained from four independent reactions.

Effects of temperature and reaction time on epimerization

In efforts to decrease the reaction duration, we increased both the temperature and acid concentration. When the temperature was increased to 65°C the reaction was complete within 1 day; however, ^{13}C NMR analysis showed that the product contained a nearly equal ratio of epimers. When the concentration of hydrogen chloride was increased to 1.1 M, the starting material was also consumed within 1 day. Unfortunately, analysis of the product once again revealed that a greater degree of epimerization had occurred compared with the reaction carried out in 0.5 M hydrogen chloride. If the epimerization occurs through a carbocation intermediate or by an S_N2-type displacement of a hydroxyl group that has been activated by acid, an increase in reaction temperature or acid concentration would be expected to result in increased epimerization.

In summary, these studies indicate that the optimized conditions for methanolysis require an ~3- to 4-day exposure of sphingomyelin to 0.5 M anhydrous methanolic hydrogen chloride at 50°C.

Microbial and enzymatic approaches to D-*erythro*-sphingosylphosphocholine

In contrast to the acid methanolysis of sphingomyelin, lysosphingomyelin can be produced without epimeriza-

tion at C-3 by using a marine bacterium (*Shewanella alga* NS-589) as a biocatalyst (28). Furthermore, a partially purified sphingolipid ceramide *N*-deacylase (SCDase) from *Pseudomonas* sp. TK4 catalyzed the cleavage of the *N*-acyl linkage of sphingomyelin, albeit in low yield (29, 30). Further studies are needed for these novel approaches to be developed into a practical method that can be used in many laboratories for the preparation of lysosphingomyelin on a large scale. However, they offer the means for producing the epimer-free product.

CONCLUSIONS

We have developed an improved method for the acid-catalyzed methanolysis of commercial sphingomyelin for the objective of preparing chemically defined sphingomyelins with a minimal degree of epimerization. Admittedly, this methodology does not address the issue of the minor structural variation in the sphingoid backbone, but it represents the most efficient approach yet reported to prepare chemically defined sphingomyelins with a high extent of retention of the natural configuration at C-3. This study shows the influence of water, temperature, and acid concentration on the epimerization process. The "perfect" strategy for removing the amide side chain would undoubtedly require the complete absence of water, yet given the hydrophilic nature of sphingomyelin, achieving these reaction conditions seems unlikely.

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