An Improved, Versatile, and Easily Scalable Synthesis of Sphingomyelins: Application to Stable Isotope Labeling

Nicolas Philippe*  
Serge Pérard*  
Franck Le Strat  
Jörg Blankenstein  
Sébastien Roy  
Sanofi-Aventis R & D, Integrated Drug Discovery, Isotope Chemistry Department, 13 quai Jules Guesde, 94403 Vitry-sur-Seine, France  
nicolas.philippe-ICMS@sanofi.com  
serge.perard@sanofi.com

Abstract  
With a view to make conveniently labeled mass spectrometry standards available, a set of deuterated sphingomyelins were prepared by a new expedient, flexible, robust, scalable, and high-yielding synthetic scheme starting from 2-azido-3-O-benzoylsphingosine as the key intermediate. Unlike previously published procedures, this work emphasizes the benefit arising from the choice of the azido function as a masking group for the reactive primary amine during the troublesome, though crucial, phosphorylation step.

Key words  
sphingomyelin, sphingosine, stable isotope, deuterium, mass spectrometry standard, chemical synthesis

Sphingomyelins are the main components of mammalian sphingolipids. Indeed, they are found in all cell membranes and especially in the central nervous system at the level of myelin sheath. These complex sphingolipids are made up of both hydrophobic and hydrophilic groups. The nonpolar part comprises the sphingoid base, which is amidified by a fatty acid whereas the polar head consists of a phosphocholine moiety (Figure 1). This amphipathic arrangement allows sphingomyelins to play also a role in various cellular events such as signal transduction and apoptosis. Furthermore, an anomaly in sphingomyelin metabolism triggers severe illnesses such as the rare hereditary Niemann–Pick disease. This well-known example of lysosomal storage disorder (LSD) is the result of a deficiency in the hydrolytic enzyme acid sphingomyelinase (ASM), leading to the accumulation of sphingomyelin in vital organs, including brain, and inflicting, among others, irreversible neurological damages.

Therefore, it is easy to understand that specific quantification of sphingomyelins in biological materials is of paramount importance for any pharmacological study. The use of stable isotope labeled (SIL) standards has been recognized as the method of choice for this purpose. Ideally, SIL standards should hold the very structure of the molecule to be analyzed by mass spectrometry (LC-MS/MS). The synthesis of these isotopologues has been a major concern for the pharmaceutical industry and was highlighted recently. Indeed, the commercial availability of these standards is rather scarce and several strategies have been developed to allow a realistic access to the target molecules.

SIL sphingomyelins can be prepared using conventional organic synthesis. To the best of our knowledge, only a few preparations of deuterated sphingomyelins were spread out over the literature. Byun and Bittman described the total synthesis of 3-deuterio-D-erythro-sphingomyelin, an isotopomer failing to achieve the degree of labeling generally requested by bioanalysis scientists. Bartels et al. very succinctly (neither analytical data nor yield given) mentioned the preparation of N-perdeuteriopalmitoyl-D-erythro-sphingomyelin (d31) by acylation of the corresponding amine (lysosphingomyelin). Matsumori et al. claimed the total synthesis of many kinds of deuterated (d to d3) site-
specific labeled stearoylsphingomyelins\textsuperscript{10} and 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholines\textsuperscript{11} specifically designed for \textsuperscript{2}H NMR biophysical studies. Mehnert et al.\textsuperscript{12} touched upon the preparation of three analogous derivatives (C2-d2-palmitoylsphingomyelin, C3-d2-palmitoylsphingomyelin and d31-palmitoylsphingomyelin). Finally, Cui et al.\textsuperscript{13} were brought to prepare 2-(d9-trimethylammonio)ethylphosphate-stearyl-\textit{o-erythro}-sphingomyelin, as a probe for lipid rafts Raman imaging, by nucleophilic substitution of a 2-bromoethylphosphate-sphingosine precursor.

We now describe a refined, efficient, and more versatile methodology to provide an easy access to any labeled or unlabeled sphingomyelin variant, starting from 2-azido-3-O-benzoylsphingosine (1) and fatty acids, deuterated where needed.

This key intermediate 1 was synthesized in 8 steps from D-arabitol based on the method of Demchenko.\textsuperscript{14} After optimization of the experimental conditions to secure the overall yield and isomeric purity, this compound was obtained at several tens of gram scale.

Our first tested access to labeled sphingomyelin consisted in synthesizing first the ceramide, composed of sphingosine and a labeled fatty acid, before introducing the phosphocholine moiety. The ceramide was obtained in three steps from 1, after deprotection of the secondary alcohol, reduction of the azido group and N-acylation\textsuperscript{15} in good yield.

According to the literature,\textsuperscript{16} sphingomyelin could be obtained in moderate to good yield from ceramides displaying either a free or protected secondary alcohol function by using different phosphorylation reagents such as 2-chloro-2-oxo-1,3,2-dioxaphospholane or 2-chloro-1,3,2-dioxaphospholane. Unfortunately, our attempts to introduce the phosphocholine moiety under these conditions were unsuccessful as we faced deplorable low (at best 10\%) and inconsistent yields.

Another access to sphingomyelin consists first in performing the delicate step, that is, the primary alcohol phosphorylation, leading to lyso-sphingomyelin before adding the fatty acid chain. In the literature, sphingosin’s phosphorylation required to protect the amine as N-Boc derivatives,\textsuperscript{8,10,11b,13,17} whereas the secondary alcohol function could be left free. However, this strategy suffered the disadvantage of involving additional synthetic steps.

Nevertheless, Cairo et al.\textsuperscript{18} demonstrated that the phosphorylation step can be carried out with an azide acting as a protected amino group, enabling them to synthesize sphingomyelin analogues with modified cholyl headgroups. The latter were obtained using modified phosphocholines as reagents. We choose to adapt this strategy to finally get

\textbf{Scheme 1}

\begin{align*}
\text{Me(CH}_2\text{)}_{12} &\text{} \text{N}_3 \text{Ph} \text{OH} \text{TMEDA, MeCN, rt, 16 h} \\
\text{CH}_2\text{(CH}_2\text{)}_{12} &\text{N}_3 \text{Ph} \text{Cl} \text{MeCN/THF, 50 °C} (79\%) \\
\text{Me(CH}_2\text{)}_{12} &\text{NH}_2 \text{O} \text{O} \text{N}^+ \text{O} \text{O}– \text{NH}_2 \text{Cl} \text{MeOH/CH}_2\text{Cl}_2, \text{rt} (73\%) \\
\text{Me(CH}_2\text{)}_{12} &\text{OH} \text{OH} \text{OH} \text{pyridine, CH}_2\text{Cl}_2, \text{rt} (69–86\%) \\
\text{Cd}_9\text{C}_16\text{O} &\text{O} \text{4-nitrophenol, DCI} \text{DMA, CH}_2\text{Cl}_2, \text{rt} (74–80\%) \\
\text{Cd}_9\text{C}_16\text{O} &\text{O} \text{pyridine, CH}_2\text{Cl}_2, \text{rt} (69–86\%) \\
\text{Cd}_9\text{C}_16\text{O} &\text{O} \text{[D9]-Sphingomyelin} \text{C}_16\text{O} \text{C}_24

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sphingomyelin itself in only three steps featuring a short, versatile, scalable and robust synthetic pathway as depicted in Scheme 1.

The phosphocholine moiety was introduced via a one-pot reaction by reacting 1 and 2-chloro-2-oxo-1,3,2-dioxaphospholane in acetonitrile overnight following by addition of trimethylamine in THF at 50 °C for 2 days to give the azidophosphodiester 3 in 79% yield (Scheme 1). For this reaction, as the dioxaphospholane intermediate 2 is very sensitive to moisture, it was important to work under an inert atmosphere with carefully dried solvents and compounds to ensure an excellent reproducibility. The O-benzoyl derivative 3 was deprotected by sodium methoxide in methanol to give the secondary alcohol 4 nearly quantitatively. The azide 4 was reduced with propanedithiol in the presence of trimethylamine in methanol to afford the lyso-sphingomyelin 5 in 73% yield. In the last step, the amine 5 was reacted with labeled p-nitrophenyl palmitate 6 or lignocerate 7 in pyridine to provide the corresponding sphingomyelins 8 and 9 in greater than 70% yield after crystallization (Scheme 1).

The relative configuration (erythro or threo) of sphingomyelins 8 and 9 can be most conveniently deduced from their 1H NMR spectra, on the basis of data published by Bruzik. Nevertheless, to avoid possible fallacies owing to solvent variation, temperature or concentration effects, we decided to validate this point by conducting a nuclear Overhauser enhancement NMR experiment on a cyclic derivative 10 (oxazolidone) prepared from compound 9 according to Scheme 2. The results are in full agreement with the desired erythro stereochemistry.

We have described a hitherto unpublished and straightforward process leading to any sphingomyelin derivative in four optimized steps with a mean overall yield of 40%, starting from 2-azido-3-O-benzoylsphingosine, readily available at a multi-gram scale. We demonstrated that this azido derivative can be easily transformed into lyso-sphingomyelin, skipping the usual protection/deprotection cycle of the primary amine. Afterwards, a simple acylation of lyso-sphingomyelin by various stable isotope labeled or unlabeled fatty acids will provide any sphingomyelin analogue. Alternatively, this strategy could be applied to the labeling of the choline moiety giving access to molecules such as 2-(d9-trimethylammonio)ethylphosphate-sphingomyelin or the corresponding lyso-sphingosine.

The bulk synthesis of 2-azido-3-O-benzoylsphingosine was done in collaboration with SynCom (Groningen, The Netherlands). Hexadecanoic acid and tetracosanoic acid were purchased from C/D/N Isotopes Inc. (Quebec, Canada). All reagents and solvents were obtained from commercial suppliers and used without further purification. The solution of 1.0 M Me3N in THF from Thermo Fisher Scientific was dried by adding a Trap-PakTM bag (medium) from Applied Biosystems. The 99.9% anhydrous MeCN solution was purchased from Sigma-Aldrich.

Air and moisture sensitive reactions were conducted under an inert atmosphere of argon and were magnetically stirred. Reactions were monitored by TLC performed on 60 F254 silica gel plates. To locate spots, plates were sprayed with 10% phosphomolybdic acid in ETOH followed by heating.

1H NMR spectra were recorded on a Bruker Avance 500 spectrometer and 31P NMR spectra on a Bruker Avance 400 spectrometer in the stated deuterated solvents. Proton decoupled 13C NMR spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 13C selective cryoprobe so that several deuterium coupled carbons became detectable. These 13C-31P and 13C-2H coupling constants are thus reported. The chemical shift data for each signal are given in units of δ relative to CD3OD (δ = 4.99 for 1H NMR spectra and δ = 50.41 for 13C NMR spectra) or to CHCl3 (δ = 7.26 for 1H NMR spectra and δ = 77.00 for 13C NMR spectra) and to phosphoric acid (δ = 6.00 for 31P NMR spectra). Data for 1H NMR are reported as follows: chemical shift (δ, ppm), multiplicity (standard abbreviations), coupling constants (J, Hz), and integration.

High-resolution mass spectra were recorded on a Shimadzu hybrid ion Trap/Time of Flight spectrometer (IT-TOF). The molecular formula determination was performed using Shimadzu’s Formula Predictor software.

Melting points were determined on a Büchi B-545 apparatus, optical rotations on a PerkinElmer 341 polarimeter at 589 nm, and IR spectra were acquired with a Thermo Scientific (Nicolet) i550 FT-IR spectrophotometer.

(E,25,3R)-2-Azido-3-(benzoyloxy)octadec-4-en-1-yl 2-(Trimethylammonio)ethyl Phosphate (3)

In a 25 mL flask fitted with a rubber septum under argon, a stirred solution of (E,25,3R)-2-azido-1-hydroxyoctadec-4-en-3-yl benzoate (1; 1.0 g, 2.3 mmol) in anhyd MeCN (10 mL) was treated with a solution of TMEDA (0.3 mL, 2.0 mmol) in anhyd MeCN (0.5 mL) at 0 °C. A solution of 2-chloro-1,3,2-dioxaphospholane-2-oxide (0.32 mL, 3.5 mmol) in anhyd MeCN (0.5 mL) was slowly added at 0 °C and the mixture was stirred at rt overnight. The reaction was monitored by TLC (SiO2, CHCl3). A solution of 1.0 M anhyd Me3N in THF (20 mL, 25 mmol) was added dropwise at rt. The mixture was heated at 50 °C for 2 days. The reaction was monitored by TLC (SiO2, CHCl3 and

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treated with 1,3-propanedithiol (4.0 mL, 39.5 mmol) and Et_3N (7.5 mL) for 5 min and filtered. The resin was washed with CH_2Cl_2 and the filtrate was monitored by TLC (SiO_2, CHCl_3/MeOH/H_2O, 70:30:4). Amberlite IR-120(H) resin (900 mg) was added and the mixture was stirred for 5 min and filtered. The filtrate was cooled to 0 °C before 4-nitrophenol (440 mg, 3.2 mmol), DMAP (3 mg), and 1,3-diisopropylcarbodiimide (0.535 mL, 5.2 mmol) were added. The mixture was stirred at rt overnight and filtered through a pad of Celite and washed with CHCl_3. The filtrate was concentrated under vacuum and the residue purified by chromatography using a silica gel column eluted with CHCl_3/n-pentane (7:3, v/v) to afford 6 as a white solid; yield: 1.6 g (80%).

1H NMR (500 MHz, CDCl_3; δ = 8.27 (d, J = 9.1 Hz, 2 H), 7.27 (d, J = 9.1 Hz, 2 H), 2.59 (t, J = 7.5 Hz), 1.76 (q, J = 7.4 Hz, 2 H), 1.41 (m, 2 H), 1.38–1.22 (m, 16 H).

13C NMR (125 MHz, CDCl_3; δ = 171.32, 155.54, 145.26, 125.19, 122.42, 34.35, 30.59 (quint, J = 16 Hz), 29.69, 29.67, 29.65, 29.64, 29.58, 29.42, 29.21, 29.04, 28.28 (quint, J = 19 Hz), 24.75, 21.37 (quint, J = 18 Hz), 12.94 (hept, J = 18.8 Hz).

4-Nitrophenyl [21,21,22,23,22,23,24,24,24-2H_9]Tetracosanoate (7)
[21,21,22,23,22,23,24,24,24-2H_9]Tetracosanoic acid (1.0 g, 2.7 mmol) was dissolved in CHCl_3 (15 mL) under argon. The solution was cooled at 0 °C, then 4-nitrophenol (440 mg, 3.2 mmol), DMAP (3 mg), and N,N-diisopropylcarbodiimide (0.535 mmol, 5.2 mmol) were added. The mixture was stirred at rt overnight, filtered through a pad of Celite, and washed with CHCl_3. The filtrate was concentrated under vacuum and the residue purified by chromatography using a silica gel column eluted with CHCl_3/n-pentane (7:3, v/v) to afford 7 as a white solid; yield: 980 mg (74%).

1H NMR (500 MHz, CDCl_3; δ = 8.27 (d, J = 9.1 Hz, 2 H), 7.27 (d, J = 9.1 Hz, 2 H), 2.59 (t, J = 7.5 Hz), 1.76 (q, J = 7.4 Hz, 2 H), 1.41 (m, 2 H), 1.37–1.22 (m, 32 H).

13C NMR (125 MHz, CDCl_3; δ = 171.31, 155.54, 145.26, 125.18, 122.42, 34.35, 30.60 (quint, J = 16 Hz), 29.70, 29.68, 29.64, 29.58, 29.49, 29.43, 29.41, 29.28, 29.21, 29.05, 28.27 (quint, J = 19 Hz), 24.75, 21.37 (quint, J = 18 Hz), 13.08 (hept, J = 19 Hz).

(E,25,3R)-2-Amino-3-hydroxyoctadeca-4-en-1-yl-2-(Trimethylammonio)ethyl Phosphate (5)
A stirred solution of 4 (780 mg, 1.6 mmol) in MeOH (40 mL) was treated with 1,3-propanediol (4.0 mL, 39.5 mmol) and Et_3N (7.5 mL, 53.5 mmol) under argon and the mixture was stirred at rt for 3 days. The reaction was monitored by TLC (SiO_2, CHCl_3/MeOH/H_2O, 70:30:4) and after completion, the mixture was concentrated under vacuum. The residue purified by flash chromatography using a silica gel column (elution successively with CHCl_3/MeOH/H_2O, 90:10:1, 70:30:4, 50:50:4, then with MeOH/H_2O, 100:4) to afford 5 as a white solid; yield: 540 mg (73%).

1H NMR (500 MHz, CDCl_3 + CD_2OD 50:50); δ = 5.91 (dt, J = 15.0, 6.9 Hz, 1 H), 5.63 (dtt, J = 15.3, 7.6, 1.4 Hz, 1 H), 4.42 (br, m, 2 H), 4.12 (m, 3 H), 3.77 (br, m, 2 H), 3.37 (s, 9 H), 2.95 (br, m, 2 H), 2.23 (qd, J = 6.9, 1.1 Hz, 2 H), 1.56 (m, 2 H), 1.51–1.38 (br, m, 20 H), 1.04 (t, J = 6.8 Hz, 3 H).

13C NMR (125 MHz, CDCl_3 + CD_2OD 50:50); δ : 137.01, 131.39, 75.39, 68.69 (d, J = 5.8 Hz), 68.51 (m), 61.09 (d, J = 4.9 Hz), 57.77, 55.94, 55.91, 55.88, 34.44, 33.95, 31.69, 31.66, 31.65, 31.54, 31.37, 31.34, 31.28, 24.66, 15.73.


4-Nitrophenyl [13,13,14,14,15,15,16,16-2H_9]Hexadecanoate (6)
[13,13,14,14,15,15,16,16-2H_9]Hexadecanoic acid (1.4 g, 5.2 mmol) was dissolved in CHCl_3 (10 mL) under argon. The solution was cooled at 0 °C before 4-nitrophenol (440 mg, 3.2 mmol), DMAP (3 mg), and N,N-diisopropylcarbodiimide (0.535 mmol, 5.2 mmol) were added. The mixture was stirred at rt overnight and filtered through a pad of Celite and washed with CHCl_3. The filtrate was concentrated under vacuum and the residue purified by chromatography using a silica gel column eluted with CHCl_3/n-pentane (7:3, v/v) to afford 6 as a white solid; yield: 1.1 g (79%).

1H NMR (500 MHz, CDCl_3; δ = 8.27 (d, J = 9.1 Hz, 2 H), 7.27 (d, J = 9.1 Hz, 2 H), 2.59 (t, J = 7.5 Hz), 1.76 (q, J = 7.4 Hz, 2 H), 1.41 (m, 2 H), 1.38–1.22 (m, 16 H).

13C NMR (125 MHz, CDCl_3; δ = 171.32, 155.54, 145.26, 125.19, 122.42, 34.35, 30.59 (quint, J = 16 Hz), 29.69, 29.67, 29.65, 29.64, 29.58, 29.42, 29.21, 29.04, 28.28 (quint, J = 19 Hz), 24.75, 21.37 (quint, J = 18 Hz), 12.94 (hept, J = 18.8 Hz).
CHCl₃/MeOH/H₂O, 90:10:1, then with CHCl₃/MeOH/H₂O, 70:30:4). The compound was dissolved in a minimum amount of a CHCl₃/MeOH mixture, then crystallized by addition of acetone to give 8 as a white solid; yield: 690 mg (87%); mp 213 °C; δ = 5.86 (dt, J = 15.2, 6.6 Hz, 1 H), 5.60 (ddt, J = 15.3, 7.6, 1.3 Hz, 1 H), 4.40 (m, 2 H), 4.32 (m, 1 H), 4.22 (t, J = 8.0 Hz, 1 H), 4.08 (m, 1 H), 4.05 (m, 1 H), 3.74 (br t, J = 6.8 Hz, 2 H), 3.36 (s, 9 H), 2.32 (m, 2 H), 2.17 (br q, 2 H), 1.73 (m, 2 H), 1.58 (m, 2 H), 1.48–1.38 (m, 38 H), 1.04 (t, J = 6.8 Hz, 3 H).

IR (ATR): 3282 (br), 2916, 2850, 1642, 1547, 1467, 1378, 1228, 1086, 720, 489 cm⁻¹ (br).

1H NMR (500 MHz, CDCl₃ + CD3OD 50:50): δ = 73.18, 68.42 (m), 66.58 (d, J = 15.3, 7.6, 1.3 Hz, 1 H), 4.40 (m, 2 H), 4.32 (m, 1 H), 4.22 (t, J = 8.0 Hz, 1 H), 4.08 (m, 1 H), 4.05 (m, 1 H), 3.74 (br t, J = 6.8 Hz, 2 H), 3.36 (s, 9 H), 2.32 (m, 2 H), 2.17 (br q, 2 H), 1.73 (m, 2 H), 1.58 (m, 2 H), 1.48–1.38 (m, 38 H), 1.04 (t, J = 6.8 Hz, 3 H).

13C NMR (125 MHz, CDCl₃ + CD3OD 50:50): δ = 176.59, 136.33, 131.36, 73.17, 68.41 (m), 66.57 (d, J = 5.8 Hz, 1 H), 61.02 (d, J = 5.1 Hz), 55.98, 55.92, 55.89, 55.86, 38.41, 34.36, 33.88, 32.52 (quin, J = 19.3 Hz), 31.67, 31.65, 31.61, 32.56, 31.52, 31.45, 31.39, 31.35, 31.31, 31.25, 30.21 (quin, J = 19.7 Hz), 27.99, 24.59, 23.25 (quin, J = 20 Hz), 15.71, 15.45 (hept, J = 15.7 Hz).

31P NMR (162 MHz, CDCl₃ + CD3OD 50:50): 73.17, 68.41 (m), 66.57 (d, J = 15.3, 7.6, 1.3 Hz, 1 H), 4.40 (m, 2 H), 4.32 (m, 1 H), 4.22 (t, J = 8.0 Hz, 1 H), 4.08 (m, 1 H), 4.05 (m, 1 H), 3.74 (br t, J = 6.8 Hz, 2 H), 3.36 (s, 9 H), 2.32 (m, 2 H), 2.17 (br q, 2 H), 1.73 (m, 2 H), 1.58 (m, 2 H), 1.48–1.38 (m, 38 H), 1.04 (t, J = 6.8 Hz, 3 H).


Supporting Information
Supporting information for this article is available online at https://doi.org/10.1055/s-0039-1690863.

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