The following handle holds various files of this Leiden University dissertation:
http://hdl.handle.net/1887/61134

**Author:** Wisse, P.
**Title:** The synthesis of chemical tools for studying sphingolipid metabolism
**Issue Date:** 2018-01-18
4.1 Introduction

Chapter 2 described the synthesis of carbon-13-labeled glycosphingolipids.\textsuperscript{[1]} Another class of functionalized sphingolipids are the phospholipids, metabolites often encountered together with glycosphingolipids. As well, the metabolism of both sphingolipid families is often interconnected.\textsuperscript{[2,3]} Some major phosphosphingolipids encountered in mammalian cells, and their biosynthetic pathways starting from ceramide (1) are shown in Figure 4.1.
Ceramide kinase (CERK) catalyzes phosphorylation of ceramide (1), using ATP as phosphate source, to form ceramide-1-phosphate (C1P, 3). The reverse reaction, dephosphorylation of C1P (3) to give ceramide is catalyzed by ceramide-1-phosphate phosphatase (C1PP). Alternatively, acid ceramidase (ACase) may take on C1P (3) as substrate to produce, by hydrolysis of the amide bond and concomitant release of the fatty acid, sphingosine-1-phosphate 4 (S1P). S1P, an important signaling lipid, is normally produced from sphingosine (2) by sphingosine kinase (SK) mediated phosphorylation of the primary alcohol. Sphingosine-1-phosphate phosphatase (S1PP) in turn produces sphingosine 2 from S1P (4).

Another occurring phosphosphingolipid is sphingomyelin (5), a zwitterionic species composed of ceramide, the primary alcohol of which carries a choline phosphate group. Sphingomyelin synthase (SMS) converts ceramide to sphingomyelin using phosphatidylcholine as the choline phosphate donor in a transesterification process. Acid sphingomyelinase (aSMase) hydrolyzes sphingomyelin to ceramide and phosphocholine. Inherited, genetic deficiency in aSMase leads to accumulation of sphingomyelin in the lysosomes. This deficiency is caused by mutation of SMPD1 gene, leading to the lysosomal storage disorders, Niemann-Pick disease A and B.[5] Carbon-13-labeled sphingomyelin would be an excellent tool to determine sphingomyelin levels[6] and to identify potential alternative metabolic pathways in these disorders.[7] It has recently become clear that the primary storage material in Niemann-Pick patients, sphingomyelin (5) may be processed by ACase to produce sphingosine-1-cholinephosphatase (6) as a secondary storage material.[8] This situation resembles earlier findings in relation to Gaucher disease (processing of glucosylceramide – the primary storage material – into glucosylsphingo sine) and Fabry disease (globotriaosylceramide as the primary storage material is partially processed to globotriaosylsphingo sine). It has been argued that in Gaucher and Fabry
Synthesis of a Panel of Carbon-$^{13}$-Labeled Phosphosphingolipids

alike, these secondary storage lysolipids may contribute to development of the diseases.$^{[9]}$

As well, very recently it has become clear that glucosylsphingosine, likely produced through the action of ceramidase (ACase), hardly present in healthy individuals and markedly increased in Gaucher patients, can give rise to plasma cells producing antibodies elicited against these lysolipids. Plasma cells that in turn may transform into malignant cells that eventually lead to multiple myeloma.$^{[10]}$ A panel of carbon-$^{13}$-labeled phosphosphingolipids would be highly useful tools to study biosynthesis and degradation pathways of these molecules in health and disease. The synthesis of such a stable isotope metabolite panel is described in this Chapter and builds on the research described in Chapter 2, specifically the synthesis of $^{13}$C-labeled, protected sphingosine as a common building block.

4.2 Results and discussion

Partially protected sphingosine 9a/b (Chapter 2)$^{[1]}$, with the primary alcohol free for modification, bearing protective groups compatible with phosphoramidite chemistry,$^{[12-14]}$ and incorporating either zero carbon-$^{13}$ isotopes (9a) or five carbon-$^{13}$ isotopes (9b) served as the starting point of the synthetic efforts. The required phosphorylating agent, phosphoramidite (8), was synthesized from 2-cyanoethyl $N,N$-diisopropylchlorophosphoramidate 7 using 4-methoxy benzyl alcohol and

**Scheme 4.1** Synthesis of ($^{13}$C$_5$-labeled) sphingosine 1-phosphate 13a/b and ceramide 1-phosphate 14a/b.

Reagents and conditions: (a) PMBOH, DIPEA, DCM, r.t., 1 h, 82%; (b) 8, tetrazole, MeCN, r.t., 1 h; (c) tBuOOH, r.t., 30 min; (d) (i) DBU, r.t., 1 h; (ii) AcOH:H$_2$O (2:1), r.t., 2 days; (e) NaOMe, MeOH, r.t., 3 days; (vi) TFA:DCM (1:1), r.t., 1 h, 13a: 68%, 13b: 76% (over four steps); (f) (i) BSA, r.t., 20 h; (ii) palmitoyl chloride, DIPEA, DCM, 0 °C to r.t., 2 h, 14a: 79%, 14b: 81%.

67
DIPEA in dry DCM in 82% yield. Phosphoramidite 8 and protected sphingosine 9a/b were coupled using tetrazole as the activator, giving the corresponding phosphate triester 10a/b, which was subsequently oxidized (treatment with tBuOOH) without intermediate work-up and purification to give fully protected sphingosine 1-phosphate 11a/b. Treatment with DBU led to removal of the 2-cyanoethyl group in the phosphotriester, after which treatment with mild acid (acetic acid in water) gave phosphate 12a/b. Finally, treatment with sodium methoxide in methanol, followed by treatment with TFA led to methanolysis of the benzoyl ester and removal of the N-Boc group, respectively, to yield the target phosphosphingolipids 13a/b. Purification of crude sphingosine-1-phosphate 13a/b proved complicated due to insolubility in solvent systems normally used in either silica gel chromatography or reverse-phase HPLC. Dissolving crude 13a/b in boiling acetic acid followed by addition of water led to precipitation of the product.\textsuperscript{[12,14]} The precipitate was filtered successively and washed with water (to remove salts), followed by organic solvents (MeOH, DCM, acetone and diethyl ether to remove remaining organic impurities), giving analytically pure sphingosine-1-phosphate 13a/b in 68\% (13a) and 76\% (13b) yield, respectively (for the same reason – insolubility in most organic solvents – NMR spectra of 13a/b were recorded in deuterated acetic acid\textsuperscript{[14]}). Besides the utility as internal standards for metabolomics applications, sphingosine-1-phosphates 13a/b also served as starting point to produce the corresponding ceramide-1-phosphates 14a/b. In the first instance, subjecting 13a/b to Schotten-Bauman conditions (reaction of the free amine in 13a/b with palmitoyl chloride) appeared an unsuitable strategy, again because the starting material does not dissolve in suitable solvent systems. Treatment of 13a/b with N,O-bis(trimethylsilyl)acetamide (BSA – a strong trimethylsilylation reagent) followed by treatment with palmitoyl chloride and aqueous work-up (at which stage the intermediate trimethylsilyl protective groups are removed) gave ceramide-1-phosphates 14a/b as the DIPEA salt in good yields.\textsuperscript{[16]}

The synthesis of sphingosine-1-cholinephosphates 19a/b and sphingomyelins 20a/b followed strategies (see Scheme 4.2) essentially the same as for compounds 13a/b and 14a/b, but now starting from phosphoramidite 15.\textsuperscript{[17,18]} It was decided to introduce the choline moiety already in the phosphoramidite stage and not following the phosphorylation event, to avoid unnecessarily harsh conditions in later stages of the synthesis. Known literature procedures on the synthesis of phosphocholine moieties are based on either tosylate\textsuperscript{[17]} or hexafluorphosphate\textsuperscript{[18]} as counter-ions for the quaternary ammonium ion. However, ammonium salts composed of these cations are often poorly soluble in DCM. For this reason, tetraphenylborate was selected as the counter-ion. For the coupling with sphingosine 9a/b and phosphoramidite 15, tetrazole activation (see Scheme 4.2) conditions were used, but the solvent was switched from DCM to MeCN. After confirming the formation of phosphite triester 16a/b (\textsuperscript{31}P NMR: 140 ppm), tBuOOH was added to oxidize the phosphite triester 16a/b forming 17a/b.
The fully protected sphingosine-1-cholinephosphate 17a/b was deprotected, by first removing the protecting group on the choline phosphate (2-cyanoethyl) (DBU) giving the zwitter-ionic sphingosine 18a/b. Next, the sphingosine part was deprotected. To this end the benzoyl was removed using NaOMe in methanol, followed by acid treatment (TFA) to deprotect the amine giving crude sphingosine 1-cholinephosphate 19a/b. Due to the better solubility compared to sphingosine 1-phosphate 13a/b, the crude mixture was purified by HPLC-MS giving the pure zwitter-ionic sphingosines 19a/b. Schotten-Bauman conditions were tried to get the corresponding ceramides 20a/b. Unfortunately, these conditions were not suitable to acylate the amine. Therefore the same conditions as described for the acylation of sphingosine-1-phosphate were applied giving after silica gel chromatography the corresponding sphingomyelin 20a/b in a yield of 61-66%.[13]

4.3 Conclusion

The synthesis of a comprehensive set of carbon-13-labeled phosphosphingolipids, together with their non-isotopically-enriched form, is described in this Chapter. The synthetic strategy is based on phosphoramidite chemistry to install either a phosphate (as in 11a/b) or a choline phosphate (13a/b). The strategy proved successful, but measures needed to be taken to deal with solubility issues in both syntheses. With these carbon-13-labeled phosphosphingolipids in hand, improved mass spectrometric procedures can be developed for phosphosphingolipids to determine the levels in biological materials and
giving better understanding of the metabolism of phosphosphingolipids.\[6\] For the synthetic procedure that was developed, new phosphorylating agents were introduced. With the new phosphorylating reagents 8 and 15 it is possible to introduce (choline)phosphate diesters under mild conditions and in high yield.

### 4.4 Experimental Section

**General Remarks:** \[^{14}C_2\]acetic acid (99.95% isotopically pure, product code CLM-105), potassium \[^{15}C\]-cyanide (99% isotopically pure, product code CLM-297), and \[1,2,3-{^{13}C}_3\]-myristic acid (99% isotopically pure, product code CLM-3665) were purchased from Cambridge Isotope Laboratories, Inc., and were used as received. Commercially available reagents and solvents (Acros, Fluka, or Merck) were used as received, stated otherwise stated. CH₂Cl₂ and THF were freshly distilled before use, over P₂O₅ and Na/benzophenone, respectively. Triethylamine was distilled from calcium hydride and stored over potassium hydroxide. Traces of water were removed from starting compounds by coevaporation with toluene. All moisture-sensitive reactions were carried out under an argon atmosphere. Molecular sieves (3 Å) were flame-dried before use. Column chromatography was carried out using airflow of the indicated solvent systems on Screening Devices Silica gel 60 (40–63 μm mesh). Size-exclusion chromatography was carried out on Sephadex LH20 (MeOH/CH₂Cl₂, 1:1). Analytical TLC was carried out on aluminum sheets (Merck, silica gel 60, F254). Compounds were visualized by UV absorption (254 nm), or by spraying with ammonium molybdate/cerium sulphate solution [\((NH_4)_2MoO_4\cdot4H_2O (25 g/L), (NH_4)_2Ce(SO_4)_3\cdot2H_2O (10 g/L), 10 % sulphuric acid in ethanol] or phosphormolybdic acid in EtOH (150 g/L), followed by charring (ca. 150 °C). IR spectra were recorded with a Shimadzu FTIR-8300 instrument and are reported in cm⁻¹. Optical rotations were measured with a Propol automatic polarimeter (sodium D-line, \(\lambda = 589 nm\)). \(^1H\) and \(^{13}C\) NMR spectra were recorded with a Bruker AV 400 MHz spectrometer at 400.2 (\(^1H\)) and 100.6 (\(^{13}C\) MHz, or with a Bruker AV 600 MHz spectrometer at 600.0 (\(^1H\)) and 151.1 (\(^{13}C\) MHz. Chemical shifts are reported as \(\delta\) values (ppm), and were referenced to tetramethylsilane (\(\delta = 0.00 \text{ ppm}\)) directly in CDCl₃, or using the residual solvent peak (D₂O). Coupling constants (\(J\)) are given in Hz, and all \(^{13}C\) spectra were proton decoupled. NMR assignments were made using COSY and HSQC, and in some cases TOCSY experiments. LC–MS analysis was carried out with an LCQ Advantage Max (Thermo Finnigan) instrument equipped with a Gemini C18 column (Phenomenex, 50, 4.6 mm, 3 μm), using the following buffers: A: H₂O, B: acetonitrile, and C: aq. TFA (1.0 %). HPLC–MS purifications were carried out with an Agilent Technologies 1200 Series automated HPLC system with a Quadrupole MS 6130, equipped with a semi-preparative Gemini C18 column (Phe-nomenex, 250×10.00, 5μm). Products were eluted using the following buffers: A: aq. TFA (0.2 %), B: acetonitrile (HPLC-grade), 5 mL/min. Purified products were lyophilized with a CHRIST ALPHA 2–4 LDPLUS apparatus to remove water and traces of buffer salts.

**N,N-diisopropylphosphoramidate (8).** 2-Cyanoethyl N,N-diisopropylphosphoramidate 7 (0.89 mL, 4.0 mmol, 1.0 eq) was dissolved in dry DCM (12 mL) under protected atmosphere and DIPEA (1.04 mL, 6.0 mmol, 1.5 eq) was added, followed by the addition of 4-methoxybenzyl alcohol (0.5 mL, 4.0 mmol, 1 eq). The reaction was stirred at room temperature for 1 hour. The reaction mixture was then transferred to an extraction funnel with EtOAc (100 mL) and washed twice with sat. aq. NaHCO₃ (100 mL) and brine (100 mL). The aqueous layers were extracted with EtOAc (100 mL) and the combined organics dried (Na₂SO₄), filtered and concentrated in vacuo. Purification by column chromatography (10% DCM, 1% NEt₃, in pentane to 10% DCM, 10% EtOAc, 1% NEt₃, in pentane) giving titled compound 8 as a clear oil (1.11 g, 3.28 mmol, 82%). \(R_t = 0.2 \text{ (10% DCM, 10% EtOAc, 1% NEt₃, in pentane)}\); \(^1H\) NMR (400 MHz, CDCl₃) \(\delta\) 7.27 (d, 2 H, \(J = 4.4 \text{ Hz, CH₃-PMB}, 4.60 \text{ (dd, 1 H, J = 9.0, 8.4 Hz, CH₂-PMB)}, 4.60 \text{ (dd, 1 H, J = 9.2, 9.0 Hz, CH₃-PMB)}, 3.83 \text{ (m, 2 H, -OCH₂-)}, 3.81 \text{ (s, 3 H, OMe-PMB)}, 3.60 \text{ (m, 2 H, 2x CH₃-diisopropyl), 2.61 \text{ (t, 2 H, J = 6.4 Hz, -CH₂-CN), 1.19 \text{ (t, 12 H, J = 7.2 Hz, 4x CH₂-diisopropyl)}, \(^{13}C\) NMR (101 MHz, CDCl₃) \(\delta\) 159.1 (C₉-PMB), 131.2 (C₉-PMB), 128.7 (CHarom-
1-Phospho-Sphingosine (13a). Sphingosine acceptor (9a) (50 mg, 0.1 mmol, 1.0 eq) was co-evaporated twice in toluene (2.5 mL) and then dissolved in anhydrous MeCN (4 mL) under protected atmosphere. 2-Cyanoethoxy-diisopropylaminomethyl-benzyloxy-phosphine 8 (40 mg, 0.12 mmol, 1.2 eq) was added followed by the addition of 4,5-dicyanoimidazole (23.6 mg, 0.2 mmol, 2.0 eq). The reaction was stirred until TLC showed complete conversion of the sphingosine acceptor 9a (~1 hour) and with 31P NMR (140 ppm). Anhydrous t-BuOOH (~5.5 M in nonane) (91 µL, 0.5 mmol, 5.0 eq) was added and the reaction was stirred (~30 minutes) at room temperature until 31P NMR (-1.7 ppm) showed full conversion. DBU (76 µL, 0.5 mmol, 5.0 eq) was added and stirred (~1 hour) at room temperature until HPLC-MS showed full removal of 2-Cyanoethoxy group. The reaction was concentrated in vacuo and coevaporated twice with toluene (20 mL). The crude reaction mixture was then dissolved in AcOH:H2O (95:5) (5 mL) and stirred (~2 days) at room temperature until LC-MS showed full removing of 4-methoxy-benzyloxy group. The crude reaction mixture was concentrated in vacuo and coevaporated twice with toluene (10 mL). The residu was dissolved in methanol (2.5 mL) and sodium methoxide (30% in methanol) (0.26 mL, 2.0 mmol, 20 eq) was added. The reaction was stirred for 3 days at room temperature and the progression of the reaction was followed by HPLC-MS. The reaction was neutralized with Amberlite H+ resin and the reaction mixture was filtered and concentrated in vacuo. The crude reaction mixture was coevaporated in toluene (10 mL) and put on ice-bath before addition of DCM (1 mL) and TFA (1 mL). The reaction mixture was stirred for 5 minutes at 0°C. The solution was diluted with toluene (10 mL) and concentrated to about 2 mL in vacuo. The coevaporation was repeated two times with toluene (10 mL), before concentration to dryness. The residue was dissolved in hot gacial acetic acid followed by addition of water. The precipitate was filtered and washed with water, acetone and diethyl ether giving pure sphingosine 13a as white powder (29 mg, 0.07 mmol, 76%). 1H NMR (600 MHz, Acetic acid-d6) δ 5.92 (dt, 1 H, J = 14.3, 6.7 Hz, H-5), 5.55 (dd, 1 H, J = 15.5, 7.0 Hz, H-4), 4.50 (t, 1 H, J = 6.1 Hz, H-3), 4.25 (m, 2 H, H-1), 3.70 (m, 1 H, H-2), 2.09 (q, 2 H, J = 7.5 Hz, H-6), 1.41 (m, 2 H, H-7), 1.36-1.23 (m, 20 H, H-8 to H-17), 0.89 (t, 3 H, J = 7.2 Hz, H-18); 13C NMR (151 MHz, Acetic acid-d6) δ 137.6 (C-7), 126.8 (C-8), 70.7 (C-3), 65.2 (C-5), 57.2 (C-2), 33.6 (C-6), 32.7, 30.58, 30.56 x3, 30.52, 30.51, 30.42, 30.23, 30.16, 29.68, 23.47 (11x CH2-C7 to C17), 14.4 (C-18); 31P NMR (162 MHz, Acetic acid-d6) δ -0.51; IR (neat): 3427, 2950, 2847, 1634, 1543, 1460, 1246, 1066, 1029, 925 cm⁻¹; HRMS calculated for [C18H35NO3P+H]+: 380.2568, found 380.2575.

1-Phospho-[5,6,7,8,9-13C5]-sphingosine (13b). Sphingosine acceptor (9b) (35 mg, 69 µmol, 1.0 eq) was co-evaporated twice in toluene (2.5 mL) and then dissolved in anhydrous MeCN (3 mL) under protected atmosphere. 2-Cyanoethoxy-diisopropylaminomethyl-benzyloxy-phosphine 8 (28 mg, 83 µmol, 1.2 eq) was added followed by the addition of 4,5-dicyanoimidazole (16.2 mg, 0.14 mmol, 2.0 eq). The reaction was stirred until TLC showed complete conversion of the sphingosine acceptor (~1 hours) and with 31P NMR (140 ppm). Anhydrous t-BuOOH (~5.5 M in nonane) (63 µL, 0.35 mmol, 5.0 eq) was added and the reaction was stirred (~30 minutes) at room temperature until 31P NMR (-1.7 ppm) showed full conversion. DBU (52 µL, 0.34 mmol, 5.0 eq) was added and stirred (~1 hour) at room temperature until HPLC-MS showed full removing of 2-Cyanoethoxy group. The reaction was concentrated in vacuo and coevaporated twice with toluene (20 mL). The crude reaction mixture was then dissolved in AcOH:H2O (95:5) (4 mL) and stirred (~2 days) at room temperature until HPLC-MS showed full removing of 4-methoxy-benzyloxy group. The crude reaction mixture was concentrated in vacuo and coevaporated twice with toluene (10 mL). The residu was dissolved in methanol (2.0 mL) and sodium methoxide (30% in methanol) (0.18 mL, 1.4 mmol, 20 eq) was added. The reaction was stirred for 3 days at room temperature and the progression of the reaction was followed by HPLC-MS. The reaction was neutralized with Amberlist H+ resin and the reaction mixture was filtered and concentrated in vacuo. The crude reaction mixture was coevaporated in toluene (10 mL) and put on ice-bath before addition of DCM (1 mL) and TFA (1 mL). The reaction mixture was stirred for 5 minutes at 0°C. The
solution was diluted with toluene (10 mL) and concentrated to about 2 mL in vacuo. The coevaporation was repeated two times with toluene (10 mL), before concentration to dryness. The residue was dissolved in hot galicic acid followed by addition of water. The precipitate was filtered and washed with water, acetone and diethylether giving pure sphingosine-1-phosphate as white powder (20 mg, 0.05 mmol, 76%). $^1$$H$ NMR (600 MHz, Acetic acid-d$_4$) δ 5.91 (dm, 1 H, J = 152.9 Hz, H-5, $^{11}$C), 5.55 (m, 1 H, H-4), 4.49 (q, 1 H, J = 5.4 Hz, H-3), 4.25 (m, 2 H, H-1), 3.70 (m, 1 H, H-2), 2.09 (dm, 2 H, J = 126.9 Hz, H-6, $^{13}$C), 1.56-1.13 (m, 22 H, H-7 to H-17, 3x $^{13}$C), 0.89 (t, 3 H, J = 7.2 Hz, H-18); $^{13}$C NMR (150 MHz, Acetic acid-d$_4$) δ 137.7 (d, J = 45.0 Hz, C-5, $^{11}$C), 128.5 (dd, J = 72.4, 3.5 Hz C-4), 70.8 (d, J = 5.1 Hz, C-3), 62.8 (C-2), 57.2 (C-1), 33.1 (dd, J = 42.4, 32.3 Hz, C-6, $^{13}$C), 30.8-29.2 (m, 10x CH$_2$, 3x $^{13}$C), 23.5 (CH$_3$), 14.3 (C-18); $^{31}$P NMR (Acetic acid-d$_4$) δ -0.51; IR (neat): 3428, 2949, 2847, 1634, 1542, 1460, 1246, 1066, 1029, 925 cm$^{-1}$; HRMS calculated for [C$_{31}$H$_{50}$NO$_5$P + H]$^+$: 385.2213, found 385.2215.

Ceramide-1-phosphate (DIPEA salt) (14a). Sphingosine 13a (9 mg, 0.025 mmol, 1 eq) was mixed with BSA (0.3 mL) under protected atmosphere and stirred overnight at room temperature. The mixture was concentrated in high vacuum and the silylated phosphosphingosine was dissolved in dry DCM (0.3 mL) under a protected atmosphere and cooled to 0 °C. To the solution was added DIPEA (13 µL, 0.075 mmol, 3.0 eq) followed by palmitoyl chloride (9 µL, 0.03 mmol, 1.2 eq). The reaction was left stirring, reaching room temperature over 2 h. The reaction mixture was then concentrated in vacuo and the residue was dissolved in a small amount of MeOH/DCM. Precipitated with acetone gave ceramide-1-phosphate 14a (12 mg, 0.02 mmol, 79%). $^1$$H$ NMR (600 MHz, MeOD-d$_4$) δ 5.70 (dt, 1 H, J = 15.6, 8.4 Hz, H-5), 5.45 (dd, 1 H, J = 15.0, 8.8 Hz, H-4), 4.18 (m, 1 H, H-1a), 4.10 (t, 1 H, J = 7.2 Hz, H-3), 3.95-3.92 (m, 2 H, H-1b and H-2), 3.68 (m, 1 H, DIPEA), 3.17 (m, 2 H, DIPEA), 2.18 (m, 2 H, H-2*), 2.02 (m, 2 H, H-6), 1.58 (m, 2 H, H-7), 1.38-1.28 (m, 48 H, H-8 to H-17, H-3* to H-15* and DIPEA), 0.89 (t, 6 H, J = 7.2 Hz, H-18 and H-16*); $^{13}$C NMR (150 MHz, MeOD-d$_4$) δ 175.9 (C=O), 134.9 (C-5), 130.9 (C-4), 72.3 (C-3), 65.4 (C-1), 55.7 (C-2), 55.2 (CH$_2$, DIPEA), 37.3 (C-2*), 43.67 (CH$_2$, DIPEA), 33.4, 33.0, 30.76, 30.72 x7, 30.71, 30.69 x3, 30.68, 30.67, 30.65, 30.59, 30.52, 30.39, 30.37, 30.35, 30.31, 27.03, (25x CH$_2$, C-6 to C17 and C-3* to C-15*), 18.7, 17.2 (CH$_2$, DIPEA), 14.4 (C-18 and C-16*); $^{31}$P NMR (162 MHz, MeOD-d$_4$) δ -0.53; IR (neat): IR (neat): 3429, 2916, 2847, 1738, 1633, 1543, 1460, 1247, 1065, 1029, 925 cm$^{-1}$; HRMS Calculated for [C$_{34}$H$_{56}$NO$_5$P + H]$^+$: 618.4864, found 618.4865.

1-Phosphate-2-N-(hexadecanoyl)-[5,6,7,8,9-$^{13}$C]sphingosine (14b). Sphingosine 13b (12 mg, 0.03 mmol, 1 eq) was mixed with BSA (0.4 mL) under protected atmosphere and stirred over night at room temperature. The mixture was concentrated under high vacuum and the silylated phosphosphingosine was dissolved in dry DCM (0.4 mL) under a protected atmosphere and cooled to 0 °C. To the solution was added DIPEA (16 µL, 0.1 mmol, 3.0 eq) followed by palmitoyl chloride (12 µL, 0.04 mmol, 1.2 eq). The reaction was left stirring, reaching room temperature over 2 h. The reaction mixture was then concentrated in vacuo and the residue was dissolved in a small amount of MeOH/DCM and precipitated with acetone gives 1-phosphate-ceramide 14b (16 mg, 0.03 mmol, 81%); $^1$$H$ NMR (600 MHz, MeOD-d$_4$) δ 5.66 (dm, 1 H, J = 156 Hz, H-5), 5.44 (m, 1 H, H-4), 4.18 (m, 1 H, H-1a), 4.09 (m, H-3), 3.94-3.91 (m, 2 H, H-1b and H-2), 3.68 (m, 1 H, DIPEA), 3.17 (m, 2 H, DIPEA), 2.17 (t, 2 H, J = 7.2 Hz, H-2*), 2.01 (dm, 2 H, J = 126.0 Hz, H-6), 1.57-1.17 (m, 48 H, H-7 to H-17, H-3* to H-15* and DIPEA), 0.88 (t, 6 H, J = 6.6 Hz, H-18 and H-16*); $^{13}$C NMR (150 MHz, MeOD-d$_4$) δ 175.6 (C=O), 135.9 (d, J = 42.0 Hz, C-5), 130.4 (d, J = 72.0 Hz, C-4), 72.1 (d, J = 4.5 Hz, C-3), 65.3 (d, J = 4.5 Hz, H-1), 55.4 (C-2), 37.1 (C-2*), 33.5-32.9 (m, 30.6-29.8 (m), 26.8 (25x CH$_2$, C-6 to C17 and C-3* to C15*), 18.7, 17.2 (CH$_2$, DIPEA), 14.4 (C-18 and C-16*); $^{31}$P NMR (162 MHz, MeOD-d$_4$) δ -0.51; IR (neat): 3430, 2914, 2847, 1726, 1636, 1543, 1458, 1246, 1066, 1029, 925 cm$^{-1}$; HRMS Calculated for [C$_{34}$H$_{56}$NO$_5$P + H]$^+$: 619.4509, found 619.4512.
showed complete conversion of the sphingosine acceptor (~2 hours). Anhydrous N,N,N,N'-tetraisopropylphosphoramide diisopropylphosphoramidite 7 (0.30 g, 1.0 mmol, 1.0 eq) was dissolved in DCM:MeCN (2:1, 3 mL) under protected atmosphere and diisopropyl ammonium tetrazolate (0.17 g, 0.5 mmol, 0.5 eq) was added. Choline tetraphenylborate (0.42 g, 1.0 mmol, 1.0 eq)\(^{119}\) was added and the reaction was stirred at room temperature for 2 hours. The reaction mixture was diluted with DCM (10 mL) and was washed twice with sat. aq. NaHCO\(_3\) (10 mL). The aqueous layers were extracted with DCM (10 mL) and the combined organics dried (Na\(_2\)SO\(_4\)), filtered and concentrated in vacuo giving a brown solid which is used without further purification. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.54-7.45 (m, 8 H, H\(_{\text{arom}}\)), 7.04 (t, 8 H, \(J = 7.6\) Hz, H\(_{\text{arom}}\)), 6.88 (t, 4 H, \(J = 7.2\) Hz, H\(_{\text{arom}}\)), 3.70 (m, 1 H, \(-\text{OCH}_2\text{&-OC}\)), 3.56 (m, 1 H, \(-\text{OCH}_2\text{&-OC}\)), 3.50 (m, 2 H, 2x CH\(_{\text{diisopropyl}}\)), 3.24 (m, 2 H, \(-\text{OCH}_2\text{-choline}\)), 2.44 (t, 2 H, \(J = 6.0\) Hz, \(-\text{CH}_2\text{CN}\)), 1.71 (m, 2 H, \(-\text{CH}_2\text{NMe}_2\)), 1.66 (s, 9 H, 3x Me\(_{\text{choline}}\)), 1.17, 1.15, 1.14, 1.13 (4x s, 3 H, CH\(_3\text{diisopropyl}\)); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 165.3, 164.8, 164.3, 163.8 (4x C\(_{\text{arom}}\)), 136.2 (CH\(_{\text{arom}}\)), 126.1 (CH\(_{\text{arom}}\)), 122.1 (CH\(_{\text{arom}}\)), 118.0 (CN), 65.5 (\(-\text{CH}_2\text{CN}\)), 58.0 (\(-\text{OCH}_2\text{&-OC}\)), 57.7 (\(-\text{OCH}_2\text{-choline}\)), 53.2, 43.4, 43.4, (2x CH\(_{\text{isopropyl}}\)), 24.8, 2x 24.71, 24.64 (4x CH\(_{3\text{isopropyl}}\)), 20.4 (\(-\text{CH}_2\text{CN}\)); \(^{31}\)P (162 MHz, CDCl\(_3\)) \(\delta\) 148.1.

**Sphingosine-1-cholinephosphate (19a)** Sphingosine acceptor (9a) (0.213 g, 0.42 mmol, 1.0 eq) was co-evaporated twice in toluene (10 mL) and then dissolved in anhydrous MeCN (17 mL) under protected atmosphere. 2-Choline-2-cyanoethyl N,N-diisopropylphosphoramidate tetraphenylborate (15) (530 mg, 0.85 mmol, 2.0 eq) was added followed by the addition of tetrazole (0.45 M in MeCN) (0.93 mL, 0.42 mmol, 1.0 eq). The reaction was stirred until TLC showed complete conversion of the sphingosine acceptor (~2 hours). Anhydrous t-BuOOH (~5.5 M in nonane) (0.38 mL, 2.10 mmol, 5.0 eq) was added and the reaction was stirred (~ 30 minutes) at room temperature until \(^{31}\)P NMR showed full conversion. DBU (0.31 mL, 2.10 mmol, 5.0 eq) was added and stirred (~ 1 hour) at room temperature until HPLC-MS showed full removal of 2-cyanoethoxy group. The reaction was concentrated in vacuo and coevaporated twice with toluene (20 mL). The crude reaction mixture was then dissolved in methanol (10 mL) and sodium methoxide (30% in methanol) (1.1 mL, 8.4 mmol, 20 eq) was added. The reaction was stirred for 3 days at room temperature and the progression of the reaction was followed by HPLC-MS. The reaction was neutralized with Amberlite H\(^+\) resin and the reaction mixture was filtered and concentrated in vacuo. The crude reaction mixture was co-evaporated in toluene (10 mL) and put on ice-bath before addition of DCM (5 mL) and TFA (5 mL). The reaction mixture was stirred for 5 minutes at 0°C. The solution was diluted with toluene (50 mL) and concentrated to about 10 mL in vacuo. The coevaporation was repeated two times with toluene (40 mL), before concentration to dryness. The reaction progress was monitored by HPLC-MS and purified with HPLC-MS as well yielding 19a (0.11 g, 0.25 mmol, 59%). \(^1\)H NMR (600 MHz, MeOD-d\(_4\)) \(\delta\) 5.89 (dt, 1 H, \(J = 15.2\), 6.7 Hz, H-5), 5.49 (dd, 1 H, \(J = 15.2\), 6.9 Hz, H-4), 4.29 (m, 3 H, H-3 and CH\(_2\text{choline}\)), 4.12 (m, 1 H, H-1\(_a\)), 4.03 (m, 1 H, H-1\(_b\)), 3.65 (m, 2 H, CH\(_2\text{choline}\)), 3.37 (m, 1 H, H-2), 3.23 (s, 9 H, 3x CH\(_3\text{choline}\)), 2.11 (q, 2 H, \(J = 7.5\) Hz, H-6), 1.43 (m, 2 H, H-7), 1.37-1.20 (m, 20 H, H-8 to H-17), 0.89 (t, 3 H, \(J = 6.8\) Hz, H-18); \(^{13}\)C NMR(151 MHz, MeOD-d\(_4\)) \(\delta\) 137.2 (C-5), 128.2 (C-4), 70.7 (C-3), 67.4 (m, CH\(_2\text{choline}\)), 63.6 (d, \(J = 5.1\) Hz, C-1), 60.6 (d, \(J = 5.0\) Hz, CH\(_2\text{choline}\)), 57.2 (d, \(J = 7.2\) Hz, C-2), 55.2 (CH, DIPEA), 54.6 (CH\(_2\text{choline}\) X3), 43.67 (CH\(_2\text{DIPEA}\)), 33.4 (C-6), 33.1, 30.80 x4, 30.76, 30.74, 30.47, 30.44, 30.16, 23.7 (CH\(_2\text{C-7 to C-17}\)), 14.4 (C-18); \(^{31}\)P NMR (162 MHz, MeOD-d\(_4\)) \(\delta\) -0.37; IR (neat): 3429, 2948, 2846, 1635, 1541, 1461, 1248, 1067, 1030, 928 cm\(^{-1}\); HRMS calculated for [C\(_{24}\text{H}_{35}\text{N}_3\text{O}_3\text{P} + \text{H}]^+\): 465.3459, found 465.3475.

**[5,6,7,8,9-\(^{13}\)C]Sphingosine-1-cholinephosphate (19b).** \(^{13}\)C\(_{\text{s}}\)-Sphingosine acceptor (9b) (50 mg, 98 \mu\text{mol}, 1.0 eq) was co-evaporated twice in toluene (5 mL) and then dissolved in anhydrous MeCN (4 mL) under protected atmosphere. 2-Cyanoethoxy-diisopropylamino-2-trimethylammonium-ethoxy-phosphine 15 (125 mg, 0.2 mmol, 2.0 eq) was added followed by the addition of tetrazole (0.45 M in MeCN) (0.22 mL, 0.1 mmol, 1.0 eq). The reaction was stirred until TLC showed complete conversion of the sphingosine acceptor (~2 hours). Anhydrous t-BuOOH (~5.5 M in nonane) (91
μL (0.5 mmol, 5.0 eq) was added and the reaction was stirred (~30 minutes) at room temperature until $^{31}$P NMR showed full conversion. DBU (73 μL, 0.5 mmol, 5.0 eq) was added and stirred (~1 hour) at room temperature until HPLC-MS showed full removal of 2-cyanoethoxy group. The reaction was concentrated in vacuo and coevaporated twice with toluene (5 mL). The crude reaction mixture was then dissolved in methanol (2.5 mL) and sodium methoxide (30% in methanol) (0.26 mL, 2.0 mmol, 20 eq) was added. The reaction was stirred for 3 days at room temperature and the progression of the reaction was followed by HPLC-MS. The reaction was neutralized with Amberlite H+ resin and the reaction mixture was filtered and concentrated in vacuo. The crude reaction mixture was coevaporated in toluene (2.5 mL) and put on ice and sodium methoxide (30% in methanol) (0.26 mL, 2.0 mmol, 20 eq) was added. The reaction mixture was stirred for 5 minutes at 0°C. The solution was diluted with toluene (10 mL) and concentrated to about 2 mL in vacuo. The coevaporation was repeated two times with toluene (10 mL), before concentration to dryness. The completion of the reaction was confirmed as well purified by HPLC-MS producing [5,6,7,8,9-$^{13}$C$_5$]-sphingosine-1-cholinephosphate 19b (25 mg, 55 μmol, 56%). $^1$H NMR (600 MHz, MeOD-d$_4$) δ 5.89 (dm, 1 H, J = 151.0 Hz, H-5), 5.49 (m, 1 H, H-4), 4.30 (m, 3 H, H-3 and CH$_2$-Choline), 4.13 (m, 1 H, H-1a), 4.04 (m, 3 H, H-1b), 3.66 (m, 2 H, CH$_2$-Choline), 3.37 (m, 1 H, H-2), 3.23 (s, 9 H, CH$_3$-Choline x3), 2.11 (dm, 2 H, J = 126.7 Hz, H-6), 1.57-1.15 (m, 22 H, H-7 to H-17), 0.90 (t, 3 H, J = 7.2 Hz, H-18); $^{13}$C NMR (151 MHz, MeOD-d$_4$) 137.1 (d, J = 42.6 Hz, C-5), 128.2 (d, J = 72.0 Hz, C-4), 70.7 (d, J = 5.3 Hz, C-3), 67.4 (m, CH$_2$-Choline), 63.4 (d, J = 5.1 Hz, C-1), 60.6 (d, J = 5.0 Hz, CH$_2$-Choline), 57.2 (d, J = 7.2 Hz, C-2), 54.7 (CH$_3$-Choline x3), 33.8 – 32.9 (m, C$_{sp}$ and CH$_2$-sp), 30.9 – 29.8 (m, CH$_{sp}$ x10), 23.7 (CH$_3$-sp), 14.4 (C-18*); $^{31}$P NMR (162 MHz, MeOD-d$_4$) δ -0.51; IR (neat): 3429, 2948, 2846, 1635, 1541, 1461, 1248, 1067, 1030, 928 cm$^{-1}$; HRMS Calculated for [C$_{31}$H$_{49}$N$_2$O$_3$P$^+$]$: 470.3104$, found 465.3475.

Sphingomyelin (20a). Sphingosine 19a (15 mg, 0.032 mmol, 1 eq) was mixed with BSA (0.4 mL) under protected atmosphere and stirred overnight at room temperature. The mixture was concentrated in high vacuum and the silylated phosphosphingosine was dissolved in dry DCM (0.4 mL) under a protected atmosphere and cooled to 0°C. To the solution was added DIPEA (17 μL, 0.096 mmol, 3.0 eq) followed by palmitoyl chloride (12 μL, 0.04 mmol, 1.2 eq). The reaction was left stirring, reaching room temperature over 2 h. The reaction mixture was then concentrated in vacuo and product was purified by silica column chromatography (9:1 Chloroform/MeOH to 70:27.3 Chloroform:MeOH:H$_2$O) giving the title product 20a as white solid (14 mg, 0.02 mmol, 61%). R$_f$ = 0.18 (70:27.3 Chloroform:MeOH:H$_2$O); $^1$H NMR (600 MHz, MeOD-d$_4$) δ 5.70 (dt, 1 H, J = 15.2, 6.8 Hz, H-5), 5.44 (dd, 1 H, J = 15.2, 7.6 Hz, H-4), 4.28 (m, 2 H, -OCH$_2$-choline), 4.11 (m, 1 H, H-1a), 4.04 (t, 1 H, J = 8.0 Hz, H-3), 3.99-3.94 (m, 2 H, H-1b and H-2), 3.64 (m, 2 H, -CH$_2$N-choline), 3.30 (s, 9 H, 3x CH$_3$-choline), 2.18 (t, 2 H, J = 7.2 Hz, H-2*), 2.04 (m, 2 H, H-6), 1.59 (m, 2 H, H-7), 1.42-1.20 (m, 48 H, H-8 to H-17 and H-3* to H-15*), 0.90 (t, 6 H, J = 6.8 Hz, H-18 nd H-16*); $^{13}$C NMR (150 MHz, MeOD-d$_4$) δ 175.8 (C=O), 135.0 (C-5), 130.8 (C-4), 76.3 (C-3), 70.7 (-CH$_2$N-choline), 63.6 (d, J = 4.5 Hz, C-1), 60.6 (d, J = 4.5 Hz, -OCH$_2$-choline), 57.2 (C-2), 54.7 (3x CH$_3$-choline), 37.3, 33.9, 32.9, 30.76, 30.72, 30.69, 30.68, 30.67, 30.65, 30.59, 30.52, 30.39, 30.37, 30.35, 30.31, 27.03, 23.63 (25x CH$_3$ C-6 to C-17 and C-3* to C15*), 14.4 (C-18 and C-16*); $^{31}$P NMR (162 MHz, MeOD-d$_4$) δ -0.51; IR (neat): 3429, 2915, 2846, 1724, 1633, 1545, 1459, 1250, 1067, 1030, 928 cm$^{-1}$; HRMS Calculated for [C$_{39}$H$_{57}$N$_2$O$_3$P$^+$]$: 702.5657$, found 702.5655.

1-Cholinephosphate-2-N-(hexadecanoyl)-[5,6,7,8,9-$^{13}$C$_5$]-sphingosine (20b). Sphingosine 19b (10 mg, 0.021 mmol, 1 eq) was mixed with BSA (0.3 mL) under protected atmosphere and stirred overnight at room temperature. The mixture was concentrated in high vacuum and the silylated phosphosphingosine was dissolved in dry DCM (0.3 mL) under a protected atmosphere and cooled to 0°C. To the solution was added DIPEA (12 μL, 0.063 mmol, 3.0 eq) followed by palmitoyl chloride (9 μL, 0.032 mmol, 1.2 eq). The reaction was left stirring, reaching room temperature over 2 h. The reaction mixture was then concentrated in vacuo and the product was purified by silica column chromatography (9:1 Chloroform/MeOH to 70:27:3
4.5 References and notes


