The synthesis of chemical tools for studying sphingolipid metabolism

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Chapter 4

Synthesis of a Panel of Carbon-13-Labeled Phosphosphingolipids

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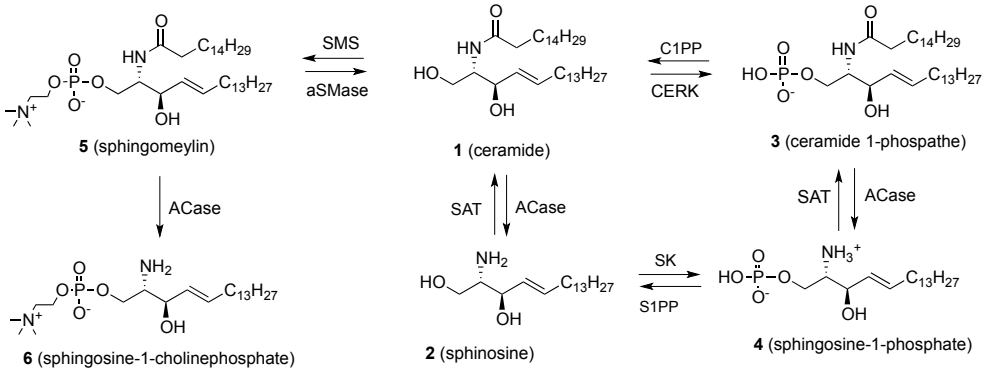
4.1 Introduction

Chapter 2 described the synthesis of carbon-13-labeled glycosphingolipids.[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.[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**).



Scheme 4.1 Partial overview of phosphosphingolipid metabolism in man. ACase: acid ceramidase; aSMase: acid sphingomyelinase; C1PP: ceramide-1-phosphate phosphatase; CERK: ceramide kinase; S1PP: sphingosine-1-phosphate phosphatase; SAT: sphingosine acyl transferase; SMS: sphingomyelin synthase.

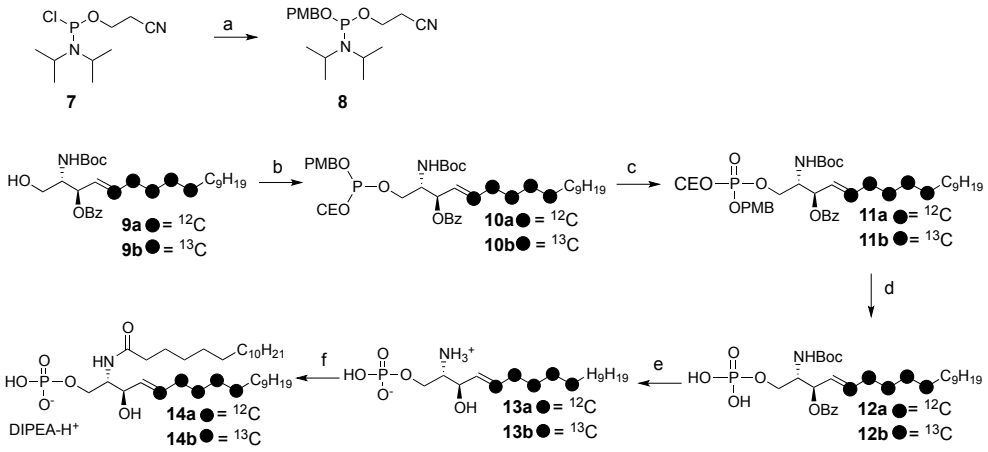
Another occurring phosphosphingolipid is sphingomyelin (**5**), a zwitterionic species composed of ceramide, the primary alcohol of which carries a choline phosphate group. Sphingomyelin synthase (SMS) converts ceramide to sphingomyelin using phosphatidylcholine as the choline phosphate donor in a transesterification 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-cholinephosphate (**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 glucosylsphingosine) and Fabry disease (globotriaosylceramide as the primary storage material is partially processed to globotriaosylsphingosine). It has been argued that in Gaucher and Fabry

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 synthesic 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**.

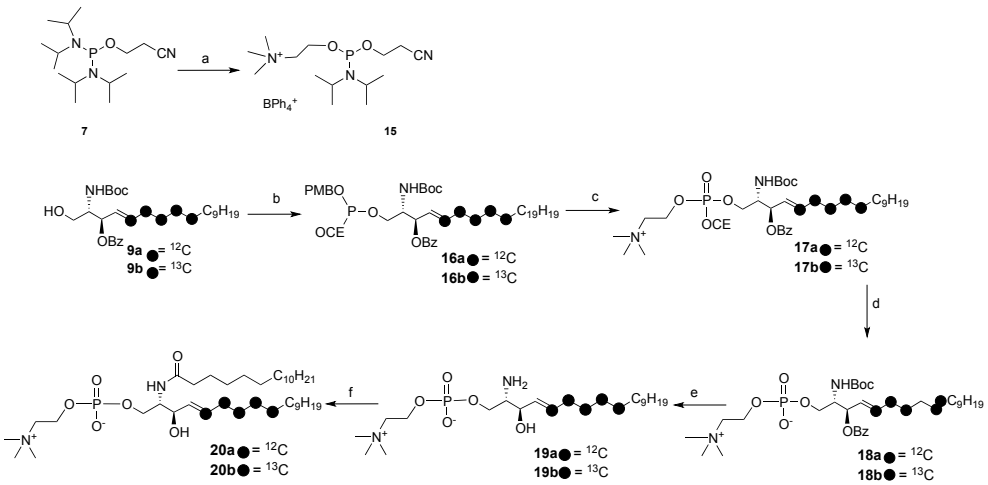


Reagents and conditions: (a) PMBOH, DIPEA, DCM, r.t., 1 h, 82%; (b) **8**, tetrazole, MeCN, r.t., 1 h; (c) tBuOOH, r.t., 30 min; (d) (i) DBU, r.t., 1 h; (ii) AcOH:H_2O (2:1), r.t., 2 days; (e) NaOMe, MeOH, r.t., 3 days; (vi) TFA:DCM (1:1), r.t., 1 h, **13a**: 68%, **13b**: 76% (over four steps); (f) (i) BSA, r.t., 20 h; (ii) palmitoyl chloride, DIPEA, DCM, 0 ^{0}C to r.t., 2 h, **14a**: 79%, **14b**: 81%.

DIPEA in dry DCM in 82% yield. Phosphoramidite **8** and protected sphingosine **9a**/**b** were coupled using tetrazole as the activator, giving the corresponding phosphite triester **10a**/**b**, which was subsequently oxidized (treatment with *t*BuOOH) 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.[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[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.[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**.[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[17] or hexafluorphosphate[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 trimester **16a**/**b** (^{31}P NMR: 140 ppm), tBuOOH was added to oxidize the phosphite triester **16a**/**b** forming **17a**/**b**.

Scheme 4.2 Synthesis of ($^{13}C_5$-labeled) sphingosine 1-cholinephosphate **19a**/**b** and sphingomyelins **20a**/**b**.



Reagents and conditions: (a) choline tetraphenylborane, diisopropylammonium tetrazolide DCM:MeCN (2:1), r.t., 2 h, quant; (b) **15**, tetrazole, MeCN, r.t., 1 h; (c) tBuOOH, r.t., 30 min.; (d) DBU, r.t., 1 h; (e) (i) NaOMe, MeOH, r.t., 3 days; (ii) TFA:DCM (1:1), r.t., 1 h, **19a**: 59%, **19b**: 56% (four steps); (f) (i) BSA, r.t., 20 h; (ii) palmitoyl chloride, DIPEA, DCM, 0 °C to r.t., 2 h, **20a:** 61%, **20b**: 66%.

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-choline phosphate **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 synthesic 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 phosphorylation 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: [$^{13}C_2$]-acetic acid (99.95% isotopically pure, product code CLM-105), potassium [^{13}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. CH_2Cl_2 and THF were freshly distilled before use, over P_2O_5 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/CH_2Cl_2, 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 [$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_6{\cdot}2H_2O$ (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, λ = 589 nm). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AV 400 MHz spectrometer at 400.2 (^{1}H) and 100.6 (^{13}C) MHz, or with a Bruker AV 600 MHz spectrometer at 600.0 (^{1}H) and 151.1 (^{13}C) MHz. Chemical shifts are reported as δ values (ppm), and were referenced to tetramethylsilane (δ = 0.00 ppm) directly in $CDCl_3$, or using the residual solvent peak (D_2O). 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: H_2O, 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.

4-methoxybenzyl-2-cyanoethyl *N,N*-diisopropylphosphoramidate (8). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidate **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. $NaHCO_3$ (100 mL) and brine (100 mL). The aqueous layers were extracted with EtOAc (100 mL) and the combined organics dried (Na_2SO_4), filtered and concentrated *in vacuo.* Purification by column chromatography (10% DCM, 1% NEt_3, in pentane to 10% DCM, 10% EtOAc, 1% NEt_3, in pentane) giving titled compound **8** as a clear oil (1.11 g, 3.28 mmol, 82%). R_f = 0.2 (10% DCM, 10% EtOAc, 1% NEt_3, in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, 2 H, J = 4.4 Hz, 2x H_{PMB}), 6.87 (d, 2 H, J = 4.4 Hz, 2x H_{PMB}), 4.69 (dd, 1 H, J = 9.0, 8.4 Hz, $CH_{2\text{-}PMB\text{-}a}$), 4.60 (dd, 1 H, J = 9.2, 9.0 Hz, $CH_{2\text{-}PMB\text{-}b}$), 3.83 (m, 2 H, -$OCH_2$-, 3.81 (s, 3 H, OMe_{PMB}), 3.60 (m, 2 H, 2x $CH_{diisopropyl}$), 2.61 (t, 2 H, J = 6.4 Hz, -CH_2-CN), 1.19 (t, 12 H, J = 7.2 Hz, 4x $CH_{2\text{-}diisopropyl}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.1 ($C_{q\text{-}PMB}$), 131.2 ($C_{q\text{-}PMB}$), 128.7 ($CH_{arom\text{-}}$

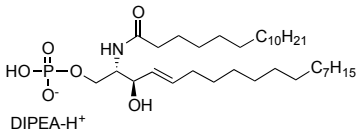
$_{PMB}$), 117.7 (CN), 113.8 ($CH_{arom\text{-}PMB}$), 65.2 (d, *J* = 1.8 Hz, $CH_{2\text{-}PMB}$), 58.4 (d, *J* = 1.9 Hz, $CH_{2\text{-}OCE}$), 55.3 ($OMe_{\text{-}PMN}$), 43.2, 43.1 (2x $CH_{Diisopropyl}$), 24.72, 24.65, 24.62, 24.55 (4x $CH_{3\text{-}Diisopropyl}$), 20.3 (*$CH_2$*CN); ^{13}P NMR (162 MHz, $CDCl_3$) δ 148.5.

1-Phosphate-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-diisopropylamino-4-methoxy-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 ^{31}P 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 ^{31}P 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:H_2O (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 repeted two times with toluene (10 mL), before concentration to dryness. The residue was dissolved in hot galcial acetic acid followed by addition of water. The precipitate was filtered and washed with water, acetone and diethylether giving pure sphingosine-1-phosphate **13a** as white powder (29 mg, 0.07 mmol, 76%). ^{1}H NMR (600 MHz, Acetic acid-*d_3*) 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); ^{13}C NMR (151 MHz, Acetic acid-*d_3*) δ 137.6 (C-5), 126.8 (C-4), 70.7 (C-3), 62.8 (C-1), 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 CH_2 C-7 to C-17), 14.4 (C-18); ^{31}P NMR (162 MHz, Acetic acid-*d_3*) δ -0.51; IR (neat): 3427, 2950, 2847, 1634, 1543, 1460, 1246, 1066, 1029, 925 cm^{-1}; HRMS calculated for $[C_{18}H_{38}NO_5P + H]^+$: 380.2568, found 380.2575.

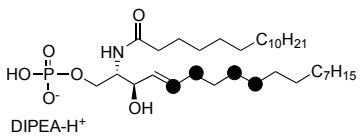
1-Phosphate-[5,6,7,8,9-$^{13}C_5$]-sphingosine (13b). Sphingosine acceptor (**9b**) (35 mg, 69 µmol, 1.0 eq) was co-evaporated twice in toluene (2.5 mL) and then dissolved in anhydrous MeCN (3 mL) under protected atmosphere. 2-Cyanoethoxy-diisopropylamino-4-methoxy-benzyloxy-phosphine **8** (28 mg, 83µmol, 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 ^{31}P 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 ^{31}P 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:H_2O (95:5) (4 mL) and stirred (~2 days) at room temperature until HPLCS-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

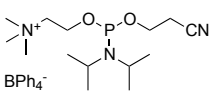
solution was diluted with toluene (10 mL) and concentrated to about 2 mL *in vacuo*. The coevaporation was repeted two times with toluene (10 mL), before concentration to dryness. The residue was dissolved in hot galcial acetic acid followed by addition of water. The precipitate was filtered and washed 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*$_3$) δ 5.91 (dm, 1 H, *J* = 152.9 Hz, H-5, ^{13}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*$_3$) δ 137.7 (d, *J* = 45.0 Hz, C-5, ^{13}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_2), 14.3 (C-18); ^{31}P NMR (Acetic acid-*d*$_3$) δ - 0.51; IR (neat): 3428, 2949, 2847, 1634, 1542, 1460, 1246, 1066, 1029, 925 cm^{-1}. HRMS calculated for $[C_{13}{}^{13}C_5H_{38}NO_5P + H]^+$: 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 temperatue 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 gaves 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-1$_a$), 4.10 (t, 1 H, *J* = 7.2 Hz, H-3), 3.95-3.92 (m 2 H, H-1$_b$ 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, 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, 30.35, 30.31, 27.03, (25x CH_2 C-6 to C17 and C-3* to C-15*), 18.7, 17.2 (CH_3 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 $[C_{34}H_{68}NO_6P + H]^+$: 618.4864, found 618.4865.

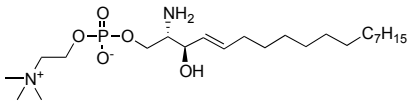


1-Phosphate-2-*N*-(hexadecanoyl)-[5,6,7,8,9-^{13}C$_5$]-sphingosine (14b). Sphingosine **13b** (12 mg, 0.03 mmol, 1 eq) was mixted with BSA (0.4 mL) under protected atmosphere and stirred over night at room temperature. The mixture was concentrated under high vacuum and the silylated phosphosphingosine 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 temperatue 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 gaves 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-1$_a$), 4.09 (m, H-3), 3.94-3.91 (m, 2 H, H-1$_b$ 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 C-17 and C-3* to C15*), 18.7, 17.2 (CH_3 DIPEA), 14.4 (C-18 and C-16*); ^{13}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 $[C_{29}{}^{13}C_5H_{68}NO_6P + H]^+$: 619.4509, found 619.4512.

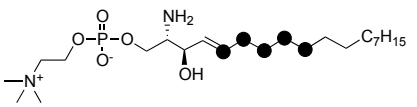


Choline-2-cyanoethyl *N,N*-diisopropylphosporamidate tetraphenylborate (15). 

2-cyanoethyl *N,N,N,N'*-tetraisoproppylphosphordiamidite **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 tetrazolide (0.17 g, 0.5 mmol, 0.5 eq) was added. Choline tetraphenylborate (0.42 g, 1.0 mmol, 1.0 eq)[19] 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 aqoeous layers were extracted with DCM (10 mL) and the combined organics dried (Na_2SO_4), filtered and concentrated *in vacuo* giving a brownish solid which is used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54-7.45 (m, 8 H, H_{arom}), 7.04 (t, 8 H. *J* = 7.6 Hz, H_{arom}), 6.88 (t, 4 H, *J* = 7.2 Hz, H_{arom}), 3.70, (m, 1 H, -$OCH_{2\text{-a-OCE}}$), 3.56 (m, 1 H, -$OCH_{2\text{-b-OCE}}$), 3.50 (m, 2 H, 2x $CH_{diisopropyl}$), 3.24 (m, 2 H, -$OCH_{2\text{-choline}}$), 2.44 (t, 2 H, *J* = 6.0 Hz, -CH_2CN), 1.71 (m, 2 H, -CH_2NMe_3), 1.66 (s, 9 H, 3x $Me_{choline}$), 1.17, 1.15, 1.14, 1.13 (4x s, 3 H, $CH_{3\text{-diisopropyl}}$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.3, 164.8, 164.3, 163.8 (4x $C_{q\text{-arom}}$), 136.2 (CH_{arom}), 126.1 (CH_{arom}), 122.1 (CH_{arom}), 118.0 (CN), 65.5 (-CH_2CN), 58.0 (-$OCH_{2\text{-OCE}}$), 57.7 (-$OCH_{2\text{-choline}}$), 53.2, (3x $CH_{3\text{-choline}}$), 43.4, 43.4, (2x $CH_{isopropyl}$), 24.8, 2x 24.71, 24.64 (4x $CH_{3\text{-isopropyl}}$), 20.4 (-CH_2CN); ^{31}P (162 MHz, $CDCl_3$) δ 148.1.

Sphingosine-1-cholinephosphate (19a) 

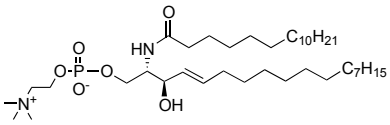
Sphingosine acceptor (**9a**) (0.213 g, 0.42 mmol, 1.0 eq) was co-evaporated twice in toluene (10 mL) and then dissolved in anhydrous MeCN (17 mL) under protected atmosphere. 2-Choline-2-cyanoethyl *N,N*-diisopropylphosporamidate tetraphenylborate (**15**) (530 mg, 0.85 mmol, 2.0 eq) was added followed 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 coevaporated 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 repeted two times with toluene (40 mL), before concentration to dryness. The reaction progess 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) δ 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, $CH_{3\text{-Choline}}$), 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) δ 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 ($CH_{3\text{-Choline}}$ x3), 43.67 (CH_2 DIPEA), 33.4 (C-6), 33.1, 30.80 x4, 30.76, 30.74, 30.47, 30.44, 30.16, 23.7 (CH_2 C-7 to C-17), 14.4 (C-18); ^{31}P NMR (162 MHz, MeOD-d_4) δ -0.37; IR (neat): 3429, 2948, 2846, 1635, 1541, 1461, 1248, 1067, 1030, 928 cm^{-1}; HRMS calculated for $[C_{23}H_{49}N_2O_5P + H]^+$: 465.3459, found 465.3475.

[5,6,7,8,9-$^{13}C_5$]-Sphingosine-1-cholinephosphate (19b). 

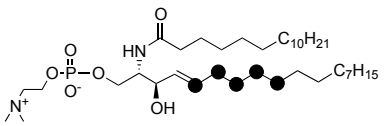
$^{13}C_5$-Sphingosine acceptor (**9b**) (50 mg, 98 µmol, 1.0 eq) was co-evaporated twice in toluene (5 mL) and then dissolved in anhydrous MeCN (4 mL) under protected atmsophere. 2-Cyanoethoxy-diisopropylamino-2-trimethylammonium-ethoxy-phosphine **15** (125 mg, 0.2 mmol, 2.0 eq) was added followed 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-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 repeted 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,-$^{13}C_5$]-sphingsine-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 $CH_{2\text{-Choline}}$), 4.13 (m, 1 H, H-1_a), 4.04 (m, 1 H, H-1_b), 3.66 (m, 2 H, $CH_{2\text{-Choline}}$), 3.37 (m, 1 H, H-2), 3.23 (s, 9 H, $CH_{3\text{-Choline}}$ 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, $CH_{2\text{-Choline}}$), 63.4 (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), 54.7 ($CH_{3\text{-Choline}}$ x3), 33.8 – 32.9 (m, C-6_{Sp} and $CH_{2\text{-Sp}}$), 30.9 – 29.8 (m, $CH_{2\text{-Sp}}$ x10), 23.7 ($CH_{2\text{-Sp}}$), 14.4 (C-18_{Sp}); ^{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 $[C_{23}H_{49}N_2O_5P$ +H$]^+$: 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 atmospere 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_2O) giving the titeld product **20a** as white solid (14 mg, 0.02 mmol, 61%). R_f = 0.18 (70:27:3 Chloroform:MeOH:H_2O); ^{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, -$OCH_{2\text{-choline}}$), 4.11 (m, 1 H, H-1_a), 4.04 (t, 1 H, *J* = 8.0 Hz, H-3), 3.99-3.94 (m, 2 H, H-1_b and H-2), 3.64 (m, 2 H, -$CH_2N_{choline}$), 3.30 (s, 9 H, 3x $CH_{3\text{-choline}}$), 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 (-$CH_2N_{choline}$), 63.6 (d, *J* = 4.5 Hz, C-1), 60.6 (d, *J* = 4.5 Hz, -$OCH_{2\text{-choline}}$), 57.2 (C-2), 54.7 (3x $CH_{3\text{-choline}}$), 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_2 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 $[C_{39}H_{79}N_2O_6P$ +H$]^+$: 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 temperatue 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



Chloroform:MeOH:H_2O) giving the titeld product **20b** as white solid (10 mg, 0.014 mmol, 66%). R_f = 0.18 (70:27:3 Chloroform:MeOH:H_2O); ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.68 (dm, 1 H, *J* = 151 MHz, H-5), 5.44 (m, 1 H, H-4), 4.28 (m, 2 H, -$OCH_{2\text{-choline}}$), 4.12 (m, 1 H, H-1_a), 4.03 (m, 1 H, H-3), 3.98-3.94 (m, 2 H, H-1_b and H-2), 3.65 (m, 2 H, -$CH_2N_{choline}$), 3.29 (s, 9 H, 3x $CH_{3\text{-choline}}$), 2.17 (t, 2 H, *J* = 7.2 Hz, H-2*), 2.03 (m, 2 H, H-6), 1.56-1.14 (m, 48 H, H-7 to H-17 and H-3* to H-15*), 0.88 (t, 6 H, *J* = 6.8 Hz, H-18 and H-16*); ^{13}C NMR (151 MHz, MeOD-d_4) δ 175.6 (C=O), 135.6 (d, *J* = 42.4 Hz, C-5), 128.2 (d, *J* = 72 Hz, C-4), 76.2 (d, *J* = 4.5 Hz, C-3), 70.6 (-$CH_2N_{choline}$), 63.4 (d, *J* = 4.5 Hz, C-1), 60.3 (d, *J* = 4.5 Hz, -$OCH_{2\text{-choline}}$), 57.1 (C-2), 54.6 (3x $CH_{3\text{-choline}}$), 37.1 (C-2*), 33.6-33.0 (m) 30.5-29.8 (m), 26.8 (25x CH_2 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 [$C_{34}{}^{13}C_5H_{79}N_2O_6P$ +H]$^+$: 707.5602, found 707.5599.

4.5 References and notes

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