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Modular synthesis of bis(monoacylglycero)phosphate for convenient access to analogues bearing hydrocarbon and perdeuterated acyl chains of varying length

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ABSTRACT

Bis(monoacylglycero)phosphate is an important phospholipid that controls the structure of late endosomes as well as biological activities that occur there. The study of this lipid is complicated by the fact that acyl migrations are known to generate different regioisomers. Herein, we describe a modular synthesis of 3,3′-BMP derivatives that allows for the incorporation of a range of different acyl chains at a late stage. This approach was exploited to produce eight BMP analogues bearing normal saturated and perdeuterated acyl chains of varying length.

1. Introduction

Bis(monoacylglycero)phosphate (BMP), also referred to as lyso-bisphosphatidic acid (LBPA), while a minimal component of cellular phospholipid content, has emerged as an important lipid in the regulation of biological pathways. This lipid primarily exists in late endosomes, where it makes up ~15% of the total membrane composition,[1] and ~70% of the inner membranes of this organelle.[2] As a result, the presence of this lipid has been found to affect the trafficking and degradation of a number of molecules. For instance, membranes presenting BMP are known to enhance the degradation of sphingolipids including glucosylceramide,[3] ceramide,[4] GM1,[5] and GM2.[6] BMP also regulates cholesterol transport,[7,8] which is aberrant in Niemann-Pick type C disease, and the accumulation of anti-BMP antibodies is known to alter cholesterol homeostasis.[9] In addition, BMP is a known antigen of antiphospholipid antibodies associated with antiphospholipid syndrome.[1] Finally, the transport of stomatitus virus to late endosomes, the last step prior to cytoplasmic release of the nucleocapsid and infection, has been shown to require the presence of BMP.[10]

Due to these implications, there has been great interest in understanding the molecular basis for the biological activity of BMP. An important aspect of this is the effect that BMP has on the structure of the inner endosomal membranes in which it exists. This was spurred by studies in which the presence of BMP in model membranes was shown to induce the formation of complex multivesicular liposomes resembling those that exist in late endosomes.[11] Since these initial studies, a number of techniques including electron microscopy, differential scanning calorimetry, fluorescence spectroscopy, and small- and wide-angle X-ray scattering have been used to probe membrane structures and phase transitions in different membranes containing BMP.[12–14] While these studies have provided insights such as the pH-dependence of membrane morphology in different compositions containing BMP, and the effect of BMP stereochemistry on membrane properties, further analysis is needed to understand how BMP affects membrane structure.

The molecular structure of BMP itself yields interesting issues as different regioisomers (see (*S*,*S*)-3,3′-BMP (**1a**), and (*S*,*S*)-2,2′-BMP (**1b**), Fig. 1) are possible depending upon the location of the acyl chains. Understanding the precise composition of BMP in biological systems is complicated by the fact that (*S*,*S*)-2,2′-BMP is readily converted to (*S*,*S*)-3,3′-BMP via *sn*-2-to-*sn*-3 acyl chain migration that occurs under a variety of conditions,[13] including in mildly acidic solution.[15,16] This is important as the late endosomes in which BMP exists are known to be acidic, and optimal BMP hydrolysis by the enzyme phospholipase A2 occurs under acidic conditions.[17] In addition, different stereoisomers have been observed in nature. While the majority of BMP is believed to exist as the somewhat unusual *sn*-1:*sn*-1′ configuration,[18–20,13] the *sn*-3:*sn*-1′ derivative has also been reported.[21]

Previously reported syntheses of BMPs initially utilized chemoenzymatic approaches through reaction of 2 equiv of diacylglycerol with a phosphoryl dichloride, followed by acyl chain removal using

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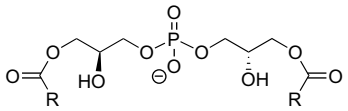
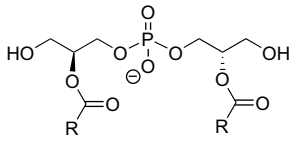


1a

(S,S)-3,3'-BMP



1b

(S,S)-2,2'-BMP

Figure 1. Generalized structures of regioisomers of *sn*-1:*sn*-1′ bis(monoacylglycero)-phosphate (BMP).

phospholipase A_2.[22,23] More recently, chemical synthesis has been employed to access BMPs from simple glycerol precursors.[16,24,15] In these reports, the primary focus has been the synthesis of (*S*,*S*)-2,2′-BMP structures, in which methods were developed to minimize acyl migration to successfully produce this target. In the generation of (*S*,*S*)-3,3′-BMPs, rationally induced acyl migration has been effective for the synthesis of this regioisomer from the corresponding (*S*,*S*)-2,2′-BMP structure.[13]

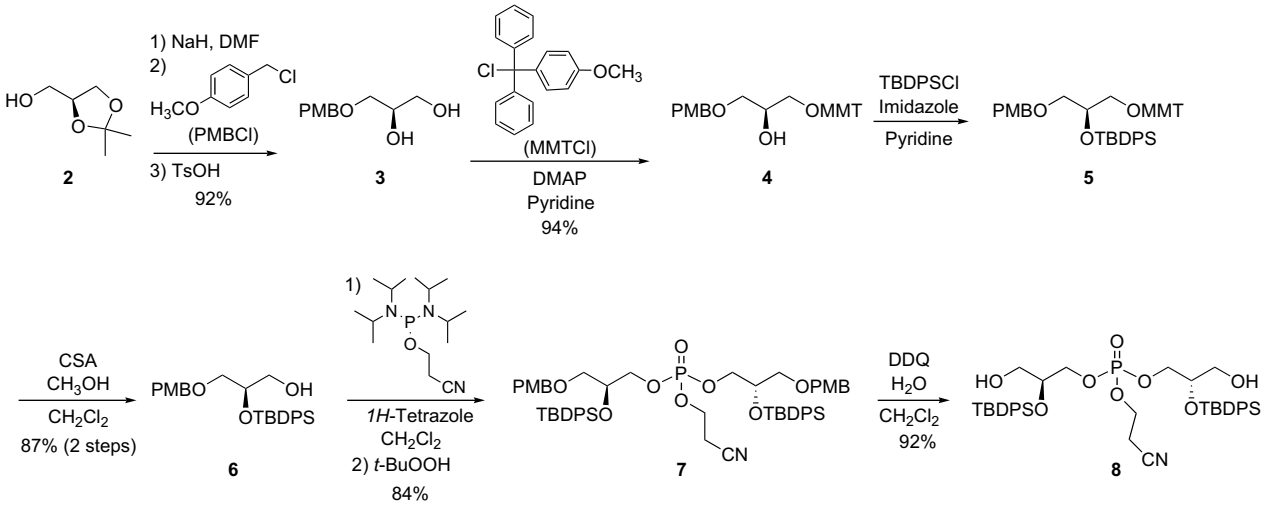
2. Results and discussion

Synthetic analogues of the different BMPs are invaluable as chemical tools for understanding the biological and structural effects of these molecules. In the current study, we sought to develop novel (*S*,*S*)-3,3′-BMP analogues bearing labeled acyl chains for use in characterizing the effect of this lipid on membrane structure. For this purpose, a particularly advantageous structural design involves the incorporation of perdeuterated acyl chains into the BMP structure. Lipid probes with specifically deuterated domains are beneficial for structural studies employing NMR and neutron scattering, and have previously been synthesized.[25] Furthermore, since we are interested in determining how the identities of the acyl chains affect packing within a membrane environment, we set out to synthesize a number of (*S*,*S*)-3,3′-BMP analogues bearing different chain lengths of both normal saturated and perdeuterated lipid tails.

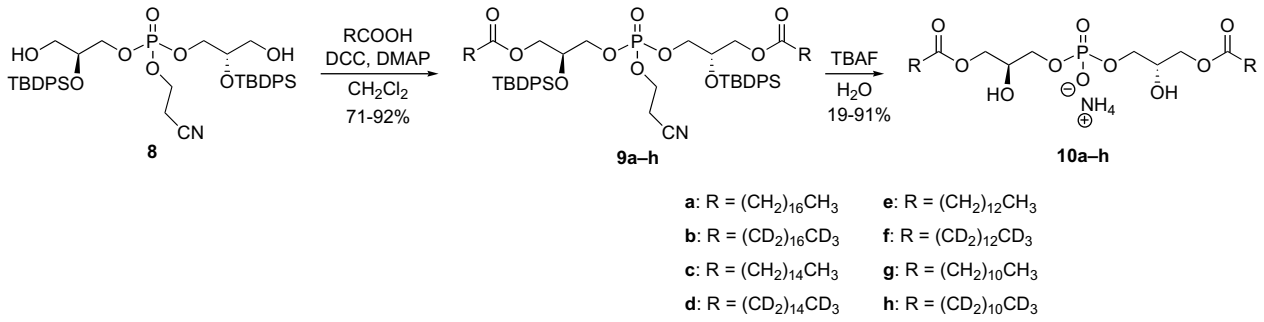
The efficient production of a number of BMP analogues containing diversity in the acyl chains would benefit from a modular synthesis in which the lipid tails are introduced at a late stage. The initial synthetic approach we developed for this purpose is indicated in Scheme 1. This route commenced with commercially available and enantiomerically pure (*S*)-acetonide-protected glycerol (**2**), which contains the appropriate stereochemistry for the targeted (*S*,*S*)-3,3′-BMP derivatives. Protection of the hydroxyl group of **2** as a *para*-methoxybenzyl (PMB) ether was followed by acetonide deprotection to produce diol **3**, as previously described.[26] Next, the primary hydroxyl group of **3** was selectively protected as a mono-methoxytrityl (MMT) ether to generate alcohol **4**, and then a *tert*-butyldiphenylsilyl (TBDPS) group was installed at the remaining secondary hydroxyl to produce fully protected glycerol derivative **5**. The MMT protecting group was next removed and the resulting alcohol, **6**, was converted to phosphodiester **7** using phosphoramidite chemistry. Finally, PMB-deprotection generated diol **8** as a core scaffold for the production of a range of derivatized BMPs.

Compound **8** acts as a convenient modular precursor to BMP analogues as different lipid tails can be appended to this diol, followed only by global deprotection to the final targets (Scheme 2). Here, coupling chemistry was used to introduce all saturated acyl chains of even length between 12 and 18 carbons in both normal saturated and perdeuterated form to produce protected (*S*,*S*)-3,3′-BMP phosphotriester intermediates **9a**–**h**. These specific acyl chain motifs were selected to study the effect of the length of the lipid tail on biological and physiochemical properties, and because the corresponding perdeuterated precursors of these fatty acids are commercially available. Finally, global deprotection using TBAF[15] was achieved to access **10a**–**h**, consisting of a number of (*S*,*S*)-3,3′-BMP probes with acyl chains of varying lengths composed of either perdeuterated or natural hydrocarbon acyl chains. The final deprotection step proceeded in high yield for the formation of certain derivatives, such as bis-palmitoyl derivative **9c** (91%). However, this yield tended to decrease with compounds with shorter acyl chains, likely due to problems in isolating these compounds as a result of their amphiphilic nature. Other than this challenge, the described synthesis is effective, with most steps proceeding in ~85 to 95% yield.

Since BMPs are known to undergo acyl chain migrations, it is important to confirm the identity of the products from the deprotection producing **10a**–**h**. In previous reports of acyl chain migrations, *sn*-2-to-*sn*-3 migration has primarily been noted, suggesting that the primary driving force for this transformation is the relief of



Scheme 1. Synthetic route to modular BMP intermediate **8** for the installation of different derivatized acyl chains.



Scheme 2. Modular synthesis of BMP analogs containing several hydrocarbon and perderteraed acyl chains of varying length using scaffold **8**.

steric strain upon the shift to the less substituted *sn*-3 position. In fact, quantitative *sn*-2-to-*sn*-3 migration has been reported in the synthesis of BMPs and other glycerolipids under certain conditions.[16,27] Based on this evidence, the reverse *sn*-3-to-*sn*-2 migration was not expected to be problematic in the deprotection of **9a**–**h**. This was confirmed via ^{1}H NMR analysis, as the typical chemical shifts for the hydrogens on the (*S*,*S*)-3,3′-BMP isomer (∼δ3.9–δ4.2) dominated the spectra of **10a**–**h** (see ^{1}H NMR spectra in Supplementary data), with minimal of the characteristic peaks of the (*S*,*S*)-2,2′-isomer (∼δ 3.8, δ 5.0), both of which have been previously described.[16] Thus, this approach is effective for accessing a range of analogues of the (*S*,*S*)-3,3′-isomer of BMP.

3. Conclusion

Bis(monoacylglycero)phosphates represent an important but poorly understood class of phospholipid components of cellular membranes. In order to advance studies of the biological and structural ramifications of (*S*,*S*)-3,3′-BMP lipids, we have developed a high-yielding synthesis of analogues of this lipid. This synthesis allows for modular introduction of various acyl chains at a late stage, which we exploited for the synthesis of eight (*S*,*S*)-3,3′-BMP analogues. Currently, we are employing these compounds as probes to understand the effect of these structures on membrane properties using NMR and neutron scattering techniques, studies which will be reported in due course.

4. Experimental section

4.1. General experimental

Generally, reagents were purchased from Acros or Aldrich and used as received. Perdeuterated fatty acids were purchased from Cambridge Isotopes. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Bruker AC250 spectrometer updated with a TecMag data collection system, a Varian Mercury 300 spectrometer, and a Bruker Avance 400 spectrometer. Mass spectra were obtained with either a JEOL DART-AccuTOF spectrometer or a Voyager DE MALDI-TOF spectrometer, each with high resolution capabilities. Optical rotation values were obtained using a Perkin–Elmer 241 polarimeter.

4.2. 3-*O*-(4-Methoxybenzyl)-1-*O*-((4-methoxyphenyl)-diphenylmethyl)-*sn*-glycerol (4)

Enantiomerically pure alcohol **2** was purchased from Acros Organics, converted to diol **3** using a literature procedure,[25] and the product matched previous characterizations.[28,25] Diol **3** (0.080 g, 0.377 mmol) was then dissolved in dry pyridine (5 mL), to which was added 4-methoxytrityl chloride (0.233 g, 0.754 mmol) and 4-dimethylaminopyridine (0.005 g, 0.038 mmol). The solution was stirred at rt for 24 h. The reaction mixture was then quenched with methanol (0.5 mL), and the solvent was removed under reduced pressure. The resulting residue was purified by column chromatography with silica gel and a gradient solvent of 10–40% ethyl acetate/hexanes to yield **4** as a white solid (0.171 g, 94%). $[\alpha]_D^{296K}$ −0.3 (*c* 3.0, $CH_3OH/CHCl_3$ (1:1)); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.41 (d, *J*=6.0 Hz, 4H), 7.16–7.30 (m, 10H), 6.83 (dd, ^1J=12.0 Hz, ^2J=9.0 Hz, 4H), 4.45 (s, 2H), 3.91–4.02 (m, 1H), 3.80 (s, 3H), 3.78 (s, 3H), 3.49–3.60 (AB of ABX, J_{AX}=6 Hz, J_{AB}=12 Hz, J_{BX}=6 Hz, 2H), 3.15–3.24 (AB of ABX, J_{AB}=9 Hz, J_{AX}=6 Hz, J_{BX}=6 Hz, 2H), 2.40 (d, *J*=6.0 Hz, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 159.2, 158.5, 144.3, 135.4, 130.3, 130.0, 129.3, 128.3, 127.8, 126.9, 113.7, 113.1, 86.3, 72.9, 71.2, 69.9, 64.4, 55.2, 55.1; MALDI-HRMS $[M+Na]^+$ calcd: 507.2142, found: 507.2153.

4.3. 2-*O*-(*tert*-Butyldiphenylsilyl)-3-*O*-(4-methoxybenzyl)-1-*O*-((4-methoxyphenyl)diphenylmethyl)-*sn*-glycerol (5)

Alcohol **4** (0.866 g, 2.89 mmol), *tert*-butyldiphenylsilyl chloride (0.984 g, 3.58 mmol) and imidazole (0.393 g, 3.58 mmol) were combined in dry pyridine (60 mL), and the reaction was allowed to stir at rt for 24 h. The reaction mixture was then concentrated and the resulting crude was purified by column chromatography with silica gel and a gradient solvent of 5–15% ethyl acetate/hexanes to yield crude **5** as a colorless oil. Compound **5** was generally passed on to the next step without complete purification due to difficulties in removing all byproducts. However, a sample of this compound was isolated to homogeneity for the purpose of characterization. $[\alpha]_D^{296K}$ −0.85 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (d, *J*=8 Hz, 2H), 7.59 (d, *J*=8 Hz, 2H), 7.18–7.37 (m, 16H), 7.04 (d, *J*=8 Hz, 2H), 6.76 (dd, ^1J=16 Hz, ^2J=8 Hz, 4H), 4.21–4.29 (AB, J_{AB}=12 Hz, 2H), 3.96–4.05 (m, 1H), 3.78 (s, 3H), 3.76 (s, 3H), 3.41–3.58 (AB of ABX, J_{AB}=16 Hz, J_{AX}=8 Hz, J_{BX}=8 Hz, 2H), 3.13–3.22 (m, 2H), 1.02 (s, 9H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 158.9, 158.3, 144.6, 135.9, 135.8, 133.9, 130.6, 130.4, 129.4, 129.0, 128.5, 127.6, 127.4, 126.6, 113.5, 112.9, 86.1, 72.6, 72.0, 71.5, 65.0, 55.2, 55.1, 30.9, 27.0, 19.3; MALDI-HRMS $[M+Na]^+$ calcd: 745.3320, found: 745.3336.

4.4. 2-*O*-(*tert*-Butyldiphenylsilyl)-3-*O*-(4-methoxybenzyl)-*sn*-glycerol (6)

Crude compound **5** from the previous procedure and camphorsulfonic acid (0.102 g, 0.44 mmol) were dissolved in 75 mL methanol/methylene chloride mixture (v/v 2:1), and the solution was allowed to stir at rt for 3 h. The solvent was then removed under reduced pressure, and the residue was purified by column chromatography with silica gel and a gradient solvent of 5–35% ethyl acetate/hexanes to yield **6** as a colorless oil (0.732 g, 87% from **4**). $[\alpha]_D^{296K}$ +28.6 (*c* 1.50, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63–7.69 (m, 4H), 7.32–7.41 (m, 6H), 7.10 (d, *J*=9.0 Hz, 2H), 6.81 (d,

J=9.0 Hz, 2H), 4.24–4.32 (AB, J_{AB}=12 Hz, 2H), 3.88–3.96 (m, 1H), 3.78 (s, 3H), 3.61–3.65 (AB, J_{AB}=6 Hz, 2H), 3.38–3.53 (AB of ABX, J_{AB}=9 Hz, J_{AX}=6 Hz, J_{BX}=6 Hz, 2H), 2.09 (t, J=6.0 Hz, 1H), 1.06 (s, 9H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 159.1, 135.8, 133.4, 130.0, 129.9, 129.8, 129.2, 127.7, 127.6, 113.7, 72.9, 71.9, 71.3, 64.8, 55.2, 27.0, 19.3; MALDI-HRMS $[M+Na]^+$ calcd: 473.2119, found: 473.2093.

4.5. 2-Cyanoethyl bis-(3-*O*-(4-methoxybenzyl)-2-*O*-(*tert*-butyldiphenylsilyl)-*sn*-glycer-1-yl) phosphate (7)

Alcohol **6** (1.19 g, 2.53 mmol) and bis-*N*,*N*-diisopropylamino cyanoethyl phosphine (0.381 g, 1.27 mmol) were stirred in 10 mL dry methylene chloride. 1*H*-tetrazole (5.6 mL of a 0.45 M solution in acetontrile, 2.52 mmol) was added and the solution was allowed to stir at rt for 48 h. To the stirred reaction mixture, *tert*-butylhydroperoxide (0.50 mL, 5.06 mmol) was added. After 1 h, the reaction was quenched by adding 50 mL of saturated sodium thiosulfate aqueous solution. Next, the resulting solution was extracted with methylene chloride (2×80 mL), and the organic layers were combined and dried with magnesium sulfate. The solvent was then removed under reduced pressure, and the resulting residue was purified by column chromatography with silica gel and a gradient solvent of 20–60% ethyl acetate/hexanes to yield **7** as a colorless oil (1.09 g, 84%). $[\alpha]_D^{296K}$ +12.2 (*c* 7.25, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65 (d, J=9.0 Hz, 8H), 7.30–7.43 (m, 12H), 7.09 (d, J=6.0 Hz, 4H), 6.81 (d, J=6.0 Hz, 4H), 4.22–4.29 (m, 4H), 3.88–4.06 (m, 8H), 3.78 (s, 6H), 3.34–3.44 (m, 4H), 2.43 (t, J=6.0 Hz, 2H), 1.05 (s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 159.2, 136.0, 133.7, 133.3, 130.0, 129.3, 127.8 127.7, 113.8, 73.0, 71.0, 70.9, 70.3, 70.0, 69.0, 68.9, 61.6, 60.5, 55.4, 27.0, 19.4, 14.3; ^{31}P NMR: δ −0.534; MALDI-HRMS $[M+Na]^+$ calcd: 1308.4168, found: 1308.4135

4.6. 2-Cyanoethyl bis-(2-*O*-(*tert*-butyldiphenylsilyl)-*sn*-glycer-1-yl) phosphate (8)

Phosphotriester **7** (1.09 g, 1.07 mmol) was dissolved in methylene chloride (20 mL) and water (1 mL), and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 1.21 g, 5.35 mmol) was added. The reaction was then allowed to stir at rt for 12 h. Next, 60 mL of 10% sodium bicarbonate solution was added, which was extracted with methylene chloride (2×80 mL). The organic layers were then combined, concentrated, and the residue was purified by column chromatography with silica gel and a gradient solvent of 5–20% acetone/methylene chloride to give **8** as a colorless oil (0.762 g, 92%). $[\alpha]_D^{296K}$ −12.6 (*c* 13.4, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63–7.68 (m, 8H), 7.32–7.48 (m, 12H), 3.95–4.15 (m, 6H), 3.85 (t, J=6.0 Hz, 2H), 3.48–3.58 (m, 4H), 2.57 (t, J=6.0 Hz, 2H), 2.49 (br s, 1H), 1.06 (s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 135.9, 135.8, 133.2, 130.2, 128.1, 128.0, 116.4, 71.9, 71.8, 67.6, 67.5, 62.5, 62.0, 27.1, 19.4; ^{31}P NMR: δ 0.378; MALDI-HRMS $[M+Na]^+$ calcd: 798.3018, found: 798.2960.

4.7. General procedure for the synthesis of compounds of type 9

Fatty acid (4 equiv for normal fatty acid; 2.5 equiv for deuterated fatty acid) was dissolved in methylene chloride. *N*,*N*′-Dicyclohexylcarbodiimide (4 equiv for normal fatty acid or 3 equiv for deuterated fatty acid) and 4-dimethylaminopyridine (4 equiv for normal fatty acid or 3 equiv for deuterated fatty acid) were added to the solution. The reaction mixture was allowed to stir for 10 min, followed by the addition of phosphotriester **8**. The solution was then allowed to stir for 12 h at rt, at which point the precipitate that formed was filtered off and the solvent was removed under reduced pressure. The residue was purified by column chromatography with silica gel and a gradient solvent of 30–40% ethyl acetate/hexanes to yield **9a**–**h**.

*4.7.1. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-stearyl-sn-glycer-1-yl) phosphate (**9a**)*

Phosphotriester **8** (0.114 g, 0.147 mmol) and stearic acid (0.167 g, 0.588 mmol) yielded **9a** as a colorless oil (0.171 g, 89%). $[\alpha]_D^{296K}$ +1.66 (*c* 9.4, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.60–7.70 (m, 8H), 7.34–7.47 (m, 12H), 3.87–4.12 (m, 12H), 2.51 (t, J=6.0 Hz, 2H), 2.07–2.18 (m, 4H), 1.46–1.55 (m, 4H), 1.25 (s, 56H), 1.05 (s, 18H), 0.88 (t, J=6.0 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.1, 135.6, 135.5, 132.9, 132.7, 129.8, 129.7, 127.6, 127.5, 115.8, 69.5, 67.9, 64.2, 61.5, 29.5, 29.1, 29.0, 26.6, 24.6, 22.5, 19.2, 19.1, 13.9; ^{31}P NMR: δ −0.759; MALDI-HRMS $[M+Na]^+$ calcd: 1330.8237, found: 1330.8277.

*4.7.2. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-stearyl-D35-sn-glycer-1-yl) phosphate (**9b**)*

Phosphotriester **8** (0.200 g, 0.258 mmol) and deuterated stearic acid D35 (0.212 g, 0.663 mmol) yielded **9b** as a colorless oil (0.280 g, 71%). $[\alpha]_D^{296K}$ +1.44 (*c* 1.9, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.66 (t, J=6.0 Hz, 8H), 7.34–7.47 (m, 12H), 3.88–4.14 (m, 12H), 2.51 (t, J=6.0 Hz, 2H), 1.05 (s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.4, 135.8, 135.7, 133.1, 132.9, 130.0, 129.9, 127.8, 127.7, 116.0, 69.8, 69.7, 68.1, 68.0, 64.3, 61.7, 28.6, 28.4, 28.2, 28.0, 27.8, 26.8, 19.4, 19.3; ^{31}P NMR: δ −0.752; MALDI-HRMS $[M+Na]^+$ calcd: 1401.2636, found: 1401.2641.

*4.7.3. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-palmitoyl-sn-glycer-1-yl) phosphate (**9c**)*

Phosphotriester **8** (0.200 g, 0.258 mmol) and palmitic acid (0.265 g, 1.03 mmol) yielded **9c** as a colorless oil (0.266 g, 82%). $[\alpha]_D^{296K}$ +0.14 (*c* 13.3, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.67 (t, J=6.0 Hz, 8H), 7.34–7.48 (m, 12H), 3.82–4.14 (m, 12H), 2.51 (t, J=6.0 Hz, 2H), 2.06–2.19 (m, 4H), 1.46–1.57 (m, 4H), 1.26 (s, 48H), 1.05 (s, 18H), 0.88 (t, J=6.0 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.8, 136.3, 136.2, 133.6, 133.5, 133.4, 130.5, 130.4, 128.3, 128.2, 116.6, 70.3, 70.1, 68.6, 68.5, 64.9, 64.8, 62.2, 30.2, 30.0, 29.9, 29.8, 29.6, 27.3, 25.3, 23.2, 19.8, 14.6; ^{31}P NMR: δ −0.752; MALDI-HRMS $[M+Na]^+$ calcd: 1274.7611, found: 1274.7509.

*4.7.4. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-palmitoyl-D31-sn-glycer-1-yl) phosphate (**9d**)*

Phosphotriester **8** (0.125 g, 0.161 mmol) and deuterated paltimic acid D31 (0.116 g, 0.403 mmol) yielded **9d** as a colorless oil (0.180 g, 85%). $[\alpha]_D^{296K}$ +1.56 (*c* 6.2, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.66 (t, J=6.0 Hz, 8H), 7.34–7.45 (m, 12H), 3.87–4.15 (m, 12H), 2.51 (t, J=6.0 Hz, 2H), 1.05 (s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.6, 136.1, 136.0, 133.3, 133.2, 133.1, 130.2, 128.0, 116.3, 70.0, 69.9, 68.3, 64.6, 64.5, 62.0, 61.9, 28.6, 28.5, 28.4, 28.3, 27.0, 19.7, 19.6, 19.5; ^{31}P NMR: δ −0.752; MALDI-HRMS $[M+Na]^+$ calcd: 1337.1168, found: 1337.1210.

*4.7.5. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-myristoyl-sn-glycer-1-yl) phosphate (**9e**)*

Phosphotriester **8** (0.128 g, 0.165 mmol) and myristic acid (0.151 g, 0.660 mmol) yielded **9e** as a colorless oil (0.177 g, 90%). $[\alpha]_D^{296K}$ +1.64 (*c* 2.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.66 (t, J=6.0 Hz, 8H), 7.33–7.47 (m, 12H), 3.89–4.16 (m, 12H), 2.52 (t, J=6.0 Hz, 2H), 2.12–2.16 (m, 4H), 1.50–1.54 (m, 4H), 1.26 (s, 40H), 1.05 (s, 18H), 0.88 (t, J=6.0 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.3, 135.8, 135.7, 132.9, 130.0, 129.9, 127.8, 127.7, 116.1, 69.8, 69.6, 68.1, 68.0, 64.4, 64.3, 61.7, 33.9, 31.9, 29.1, 26.8, 24.8, 22.7, 19.3, 14.1; ^{31}P NMR: δ −0.752; MALDI-HRMS $[M+Na]^+$ calcd: 1218.6985, found: 1218.6965.

*4.7.6. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-myristoyl-D27-sn-glycer-1-yl) phosphate (**9f**)*

Phosphotriester **8** (0.120 g, 0.155 mmol) and deuterated myristic acid D27 (0.099 g, 0.387 mmol) yielded **9f** as a colorless oil (0.164 g,

85%). $[\alpha]_D^{296K}$ +1.46 (*c* 1.6, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 7.66 (t, *J*=6.0 Hz, 8H), 7.33–7.47 (m, 12H), 3.86–4.15 (m, 12H), 2.51 (t, *J*=6.0 Hz, 2H), 1.06 (s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.6, 136.1, 136.0, 133.3, 133.2, 133.1, 130.3, 130.2, 128.0, 116.3, 70.0, 69.9, 68.3, 64.5, 62.0, 34.2, 28.3, 25.2, 19.7, 19.6, 19.5; ^{31}P NMR: δ −0.759; MALDI-HRMS $[M+Na]^+$ calcd: 1273.0084, found: 1,273.0082.

4.7.7. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-lauroyl-sn-glycer-1-yl) phosphate (**9g**)

Phosphotriester **8** (0.122 g, 0.157 mmol) and lauric acid (0.126 g, 0.629 mmol) yielded **9g** as a colorless oil (0.158 g, 88%). $[\alpha]_D^{296K}$ +1.86 (*c* 5.3, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 7.66 (t, *J*=6.0 Hz, 8H), 7.32–7.44 (m, 12H), 3.87–4.16 (m, 12H), 2.51 (t, *J*=6.0 Hz, 2H), 2.06–2.18 (m, 4H), 1.50–1.54 (m, 4H), 1.26 (s, 32H), 1.05 (s, 18H), 0.88 (t, *J*=6.0 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.6, 136.1, 136.0, 133.3, 133.1, 130.3, 130.2, 128.0, 116.3, 70.0, 69.9, 68.3, 64.6, 62.0, 61.9, 29.9, 29.7, 29.6, 29.5, 29.4, 27.0, 25.0, 22.9, 19.5, 14.4; ^{31}P NMR: δ −0.759; MALDI-HRMS $[M+Na]^+$ calcd: 1162.6359, found: 1162.6335.

4.7.8. 2-Cyanoethyl bis-(2-O-(tert-butyldiphenylsilyl)-3-lauroyl-D23-sn-glycer-1-yl) phosphate (**9h**)

Phosphotriester **8** (0.122 g, 0.157 mmol) and deuterated lauric acid D23 (0.088 g, 0.393 mmol) yielded **9h** as a colorless oil (0.172 g, 92%). $[\alpha]_D^{296K}$ +1.60(*c* 1.9, $CHCl_3$); 1H NMR (300 MHz, $CDCl_3$): δ 7.66 (t, *J*=6.0 Hz, 8H), 7.33–7.46 (m, 12H), 3.86–4.18 (m, 12H), 2.51 (t, *J*=6.0 Hz, 2H), 1.05 (s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.6, 136.1, 136.0, 133.1, 130.2, 128.0, 116.3, 70.0, 69.9, 68.3, 64.5, 62.0, 61.9, 34.2, 28.5, 28.3, 28.0, 27.0, 19.7, 19.6, 19.5; ^{31}P NMR: δ −0.759; MALDI-HRMS $[M+Na]^+$ calcd: 1208.9000, found: 1208.9042.

4.8. General procedure for the synthesis of compound 10

Phosphotriester **9** (1 equiv) and tetrabutylammonium fluoride trihydrate (TBAF, 15–20 equiv) were dissolved in 5 mL of THF, and the reaction was allowed to stir at rt overnight. The solvent was then removed under reduced pressure, and the resulting residue was dissolved in 100 mL of methanol/chloroform mixture (v/v 1:4), and extracted with 40 mL of 8 mM aqueous ammonium acetate solution. Next, the organic layer was concentrated, and the resulting residue was purified by column chromatography with 20 g silica gel and a gradient solvent of 5–16% methanol/methylene chloride to yield a yellowish solid as the crude product. The crude product was then washed with water and then methanol to provide a white solid, which was dissolved in methanol/chloroform mixture (v/v 1:4) and filtered. The filtrate was then concentrated under reduced pressure to yield **9** as a white powder.

4.8.1. Bis-(3-stearyl-sn-glycer-1-yl) phosphate ammonium salt (**10a**)

Phosphotriester **9a** (0.094 g, 0.0719 mmol) was treated with tetrabutylammonium fluoride trihydrate (0.340 g, 1.08 mmol) in 5 mL THF and yielded **10a** as a white powder (0.046 g, 80%). $[\alpha]_D^{296K}$ +0.33 (*c* 1.2, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.75–4.35 (m, 10H), 2.30–2.40 (m, 4H), 1.58–1.66 (m, 4H), 1.27 (s, 56H), 0.83–0.92 (m, 6H); ^{13}C NMR (100.6 MHz, $CD_3OD/CDCl_3$): δ 174.1, 68.4, 66.6, 64.7, 33.8, 31.7, 29.5, 29.4, 29.3, 29.1, 29.0, 24.6, 22.4, 13.7; ^{31}P NMR: δ −2.483; MALDI-HRMS $[C_{42}H_{83}O_{10}P+Na]^+$ calcd: 801.5616, found: 801.5597.

4.8.2. Bis-(3-stearyl-D35-sn-glycer-1-yl) phosphate ammonium salt (**10b**)

Phosphotriester **9b** (0.062 g, 0.0450 mmol) was treated with tetrabutylammonium fluoride trihydrate (0.213 g, 0.675 mmol) in 5 mL THF and yielded **10b** as a white powder (0.034 g, 87%). $[\alpha]_D^{296K}$ +2.03 (*c* 3.2, MeOH/$CHCl_3$); 1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.78–4.28 (m, 10H), 1.27 (s, 7H), 0.83–0.92 (m, 2H); ^{31}P NMR: δ 2.813; MALDI-HRMS $[C_{42}H_{12}D_{70}O_{10}PNa+Na]^+$ calcd: 893.9848, found: 893.9797.

4.8.3. Bis-(3-palmitoyl-sn-glycer-1-yl) phosphate ammonium salt (**10c**)

Phosphotriester **9c** (0.266 g, 0.213 mmol) was treated with tetrabutylammonium fluoride trihydrate (1.00 g, 3.19 mmol) in 5 mL THF and yielded phosphate **10c** as a white powder (0.143 g, 91%). $[\alpha]_D^{296K}$ +0.36 (*c* 1.4, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.70–4.30 (m, 10H), 2.28–2.42 (m, 4H), 1.55–1.68 (m, 4H), 1.27 (s, 48H), 0.80–0.95 (m, 6H); ^{13}C NMR (100.6 MHz, $CD_3OD/CDCl_3$): δ 174.0, 66.5, 66.4, 64.7, 33.7, 31.7, 29.5, 29.3, 29.1, 29.0, 28.7, 24.6, 22.4, 13.6; ^{31}P NMR: δ 2.806; MALDI-HRMS $[C_{38}H_{75}O_{10}P+Na]^+$ calcd: 745.4990, found: 745.4944.

4.8.4. Bis-(3-palmitoyl-D31-sn-glycer-1-yl) phosphate ammonium salt (**10d**)

Phosphotriester **9d** (0.180 g, 0.137 mmol) was treated with tetrabutylammonium fluoride trihydrate (0.648 g, 2.05 mmol) in 5 mL THF and yielded phosphate **10d** as a white powder (0.051 g, 46%). $[\alpha]_D^{296K}$ +0.27 (*c* 4.1, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.85–4.28 (m, 10H), 1.18–1.35 (m, 2H); ^{31}P NMR: δ 2.483; MALDI-HRMS $[C_{38}H_{12}D_{62}O_{10}PNa+Na]^+$ calcd: 829.8367, found: 829.8643.

4.8.5. Bis-(3-myristoyl-sn-glycer-1-yl) phosphate ammonium salt (**10e**)

Phosphotriester **9e** (0.177 g, 0.148 mmol) was treated with tetrabutylammonium fluoride trihydrate (0.700 g, 2.22 mmol) in 5 mL THF and yielded phosphate **10e** as a white powder (0.046 g, 45%). $[\alpha]_D^{296K}$ +0.96 (*c* 1.2, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.85–4.18 (m, 10H), 2.36 (t, *J*=9.0 Hz, 4H), 1.55–1.70 (m, 4H), 1.27(s, 40H), 0.89 (t, *J*=6.0 Hz, 6H); ^{13}C NMR (100.6 MHz, $CD_3OD/CDCl_3$): δ 174.4, 68.7, 66.7, 65.0, 34.1, 32.0, 29.6, 29.3, 24.9, 22.7, 14.0; ^{31}P NMR: δ −1.804; MALDI-HRMS $[C_{34}H_{67}O_{10}P+Na]^+$ calcd: 689.4370, found: 689.4360.

4.8.6. Bis-(3-myristoyl-D27-sn-glycer-1-yl) phosphate ammonium salt (**10f**)

Phosphotriester **9f** (0.265 g, 0.212 mmol) was treated with tetrabutylammonium fluoride trihydrate (0.969 g, 3.07 mmol) in 5 mL THF and yielded phosphate **10f** as a white powder (0.030 g, 19%). $[\alpha]_D^{296K}$ +0.31 (*c* 2.3, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.85–4.25 (m, 10H), 1.20–1.36 (m, 5H); ^{31}P NMR: δ −1.931; MALDI-HRMS $[C_{34}H_{12}D_{54}O_{10}PNa+Na]^+$ calcd: 765.7282, found: 765.7543.

4.8.7. Bis-(3-lauroyl-sn-glycer-1-yl) phosphate ammonium salt (**10g**)

Phosphotriester **9g** (0.158 g, 0.139 mmol) was treated with tetrabutylammonium fluoride trihydrate (0.875 g, 2.77 mmol) in 5 mL THF and yielded phosphate **10g** as a white powder (0.039 g, 45%). $[\alpha]_D^{296K}$ +0.23 (*c* 2.0, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.82–4.25 (m, 10H), 2.36 (t, *J*=9.0 Hz, 4H), 1.55–1.70 (m, 4H), 1.28 (s, 32H), 0.89(t, *J*=6.0 Hz, 6H); ^{13}C NMR (100.6 MHz, $CD_3OD/CDCl_3$): δ 173.9, 68.2, 66.2, 64.6, 33.6, 31.5, 29.1, 28.8, 24.4, 22.2, 13.3; ^{31}P NMR: δ 2.413; MALDI-HRMS $[C_{30}H_{58}O_{10}PNa+Na]^+$ calcd: 655.3558, found: 655.3541.

4.8.8. Bis-(3-lauroyl-D23-sn-glycer-1-yl) phosphate ammonium salt (**10h**)

Phosphotriester **9h** (0.260 g, 0.219 mmol) was treated with tetrabutylammonium fluoride trihydrate (1.38 g, 4.38 mmol) in 5 mL THF and yielded phosphate **10d** as a white powder (0.029 g, 20%). $[\alpha]_D^{296K}$ +0.60 (*c* 2.0, MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CD_3OD/CDCl_3$): δ 3.85–4.25 (m, 10H), 1.20–1.38 (m, 15H); ^{31}P NMR: δ 2.490;

MALDI-HRMS $[C_{30}H_{12}D_{46}O_{10}PNa+Na]^+$ calcd: 701.6450, found: 701.6435.

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Supplementary data

This includes ^1H, ^{13}C and ^{31}P NMR spectra of new compounds. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.06.085.

References and notes

1. Kobayashi, T.; Stang, E.; Fang, K. S.; de Moerloose, P.; Parton, R. G.; Gruenberg, J. *Nature* **1998**, *392*, 193–197.
2. Kobayashi, T.; Beuchat, M. H.; Chevallier, J.; Makino, A.; Mayran, N.; Escola, J. M.; Lebrand, C.; Cosson, P.; Gruenberg, J. *J. Biol. Chem.* **2002**, *277*, 32157–32164.
3. Wilkening, G.; Linke, T.; Sandhoff, K. *J. Biol. Chem.* **1998**, *273*, 30271–30278.
4. Linke, T.; Wilkening, G.; Sadeghlar, F.; Mozcall, H.; Bernardo, K.; Schuchman, E.; Sandhoff, K. *J. Biol. Chem.* **2001**, *276*, 5760–5768.
5. Wilkening, G.; Linke, T.; Uhlhorn-Dierks, G.; Sandhoff, K. *J. Biol. Chem.* **2000**, *275*, 35814–35819.
6. Werth, N.; Schuette, C. G.; Wilkening, G.; Lemm, T.; Sandhoff, K. *J. Biol. Chem.* **2001**, *276*, 12685–12690.
7. Kobayashi, T.; Beuchat, M. H.; Lindsay, M.; Frias, S.; Palmiter, R. D.; Sakuraba, H.; Parton, R. G.; Gruenberg, J. *Nat. Cell Biol.* **1999**, *1*, 113–118.
8. Chevallier, J.; Chamoun, Z.; Jiang, G. W.; Prestwich, G.; Sakai, N.; Matile, S.; Parton, R. G.; Gruenberg, J. *J. Biol. Chem.* **2008**, *283*, 27871–27880.
9. Delton-Vandenbroucke, I.; Bouvier, J.; Makino, A.; Besson, N.; Pageaux, J. F.; Lagarde, M.; Kobayashi, T. *J. Lipid Res.* **2007**, *48*, 543–552.
10. Le Blanc, I.; Luyet, P. P.; Pons, V.; Ferguson, C.; Emans, N.; Petiot, A.; Mayran, N.; Demaurex, N.; Faure, J.; Sadoul, R.; Parton, R. G.; Gruenberg, J. *Nat. Cell Biol.* **2005**, *7*, 653–664.
11. Matsuo, H.; Chevallier, J.; Mayran, N.; Le Blanc, I.; Ferguson, C.; Faure, J.; Blanc, N. S.; Matile, S.; Dubochet, J.; Sadoul, R.; Parton, R. G.; Vilbois, F.; Gruenberg, J. *Science* **2004**, *303*, 531–534.
12. Holopainen, J. M.; Soderlund, T.; Alakoskela, J. M.; Sailya, M.; Eriksson, O.; Kinnunen, P. K. J. *Chem. Phys. Lipids* **2005**, *133*, 51–67.
13. Hayakawa, T.; Hirano, Y.; Makino, A.; Michaud, S.; Lagarde, M.; Pageaux, J. F.; Doutheau, A.; Ito, K.; Fujisawa, T.; Takahashi, H.; Kobayashi, T. *Biochemistry* **2006**, *45*, 9198–9209.
14. Hayakawa, T.; Makino, A.; Murate, M.; Sugimoto, I.; Hashimoto, Y.; Takahashi, H.; Ito, K.; Fujisawa, T.; Matsuo, H.; Kobayashi, T. *Biophys. J.* **2007**, *92*, L13–L15.
15. Jiang, G. W.; Xu, Y.; Prestwich, G. D. *J. Org. Chem.* **2006**, *71*, 934–939.
16. Chevallier, J.; Sakai, N.; Robert, F.; Kobayashi, T.; Gruenberg, J.; Matile, S. *Org. Lett.* **2000**, *2*, 1859–1861.
17. Ito, M.; Tchoua, U.; Okamoto, M.; Tojo, H. *J. Biol. Chem.* **2002**, *277*, 43674–43681.
18. Brotheru, J.; Renkonen, O. *Chem. Phys. Lipids* **1974**, *13*, 178–182.
19. Joutti, A.; Brotherus, J.; Renkonen, O.; Laine, R.; Fischer, W. *Biochim. Biophys. Acta* **1976**, *450*, 206–209.
20. Joutti, A.; Renkonen, O. *J. Lipid Res.* **1979**, *20*, 840–847.
21. Joutti, A. *Biochim. Biophys. Acta* **1979**, *575*, 10–15.
22. Dang, Q. Q.; Rogalle, P.; Salvayre, R.; Dousteblazy, L. *Lipids* **1982**, *17*, 798–802.
23. Dang, Q. Q.; Rogalle, P.; Salvayre, R.; Dousteblazy, L. *Biochim. Biophys. Acta* **1985**, *834*, 124–129.
24. Jiang, G. W.; Xu, Y.; Falguieres, T.; Gruenberg, J.; Prestwich, G. D. *Org. Lett.* **2005**, *7*, 3837–3840.
25. Perly, B.; Dufourc, E. J.; Jarrell, H. C. *J. Labelled Compd. Radiopharm.* **1984**, *21*, 1–13.
26. Qin, D. H.; Byun, H. S.; Bittman, R. *J. Am. Chem. Soc.* **1999**, *121*, 662–668.
27. Smith, M. D.; Gong, D. H.; Sudhahar, C. G.; Reno, J. C.; Stahelin, R. V.; Best, M. D. *Bioconjugate Chem.* **2008**, *19*, 1855–1863.
28. De Medeiros, E. F.; Herbert, J. M.; Taylor, R. J. K. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2725–2730.