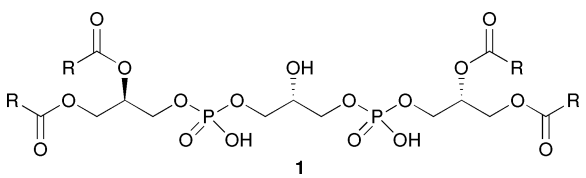
Synthesis and biological evaluation of a novel cardiolipin affinity matrix†

Melloney K. Johns,[a] Meng-Xin Yin,[b] Stuart J. Conway,[a,c] Diane E. J. E. Robinson,[b] Leon S.-M. Wong,[b] Rebecca Bamert,[d,e] Richard E. H. Wettenhall[e] and Andrew B. Holmes*[b]

Received 12th May 2009, Accepted 9th June 2009
First published as an Advance Article on the web 14th July 2009
DOI: 10.1039/b909306k

Cardiolipin (**1**) is a dimeric phospholipid found in the mitochondrial membranes of both plants and animals. In order to understand better its role, we report the preparation of an immobilised analogue (**2**) using phosphoramidite chemistry; the probe has been used successfully to bind a recombinant protein containing a cardiolipin-binding domain.

Cardiolipin (**1**) (Fig. 1) represents a family of dimeric phospholipids that comprise three glycerol molecules connected *via* the primary hydroxyl groups through two phosphodiester linkages.[1] The primary and secondary hydroxyl groups of the two terminal glycerol moieties are acylated, giving rise to its alternative name, diphosphatidylglycerol. This phospholipid is particularly abundant in mammalian heart tissue, although it is found in the mitochondria of all animal and plant tissues, and throughout the prokaryotic kingdom. In general, cardiolipins from mammalian tissues are rich in unsaturated fatty acid side chains whilst those from bacteria contain mostly saturated chains and those from plants have varying proportions of both.[2] The majority of cardiolipin (**1**) is found in membranes that generate an electrochemical potential for substrate transport and ATP synthesis.[3] It comprises around 20% of the phospholipids present in mitochondrial membranes[4] and is located mainly on the inner membrane, where it interacts with a variety of mitochondrial proteins. Cardiolipin (**1**) is believed to be involved in the activation of a number of enzymes, in particular those involved in the production of energy by oxidative phosphorylation.[5,6] It also plays a role in mitochondrial lipid and protein import, programmed cell death and the regulation of gene expression.[3,7] Aberrant cellular levels of cardiolipin have been linked to disease states including autoimmune disorders such as arthritis, antiphospholipid syndrome (APS) and systematic lupus erythematosus (SLE), ischemic heart disease, thyroid dysfunction, Barth's syndrome and ageing.[3,8]



1

Fig. 1 The general structure of cardiolipin **1**. R = fatty acid.

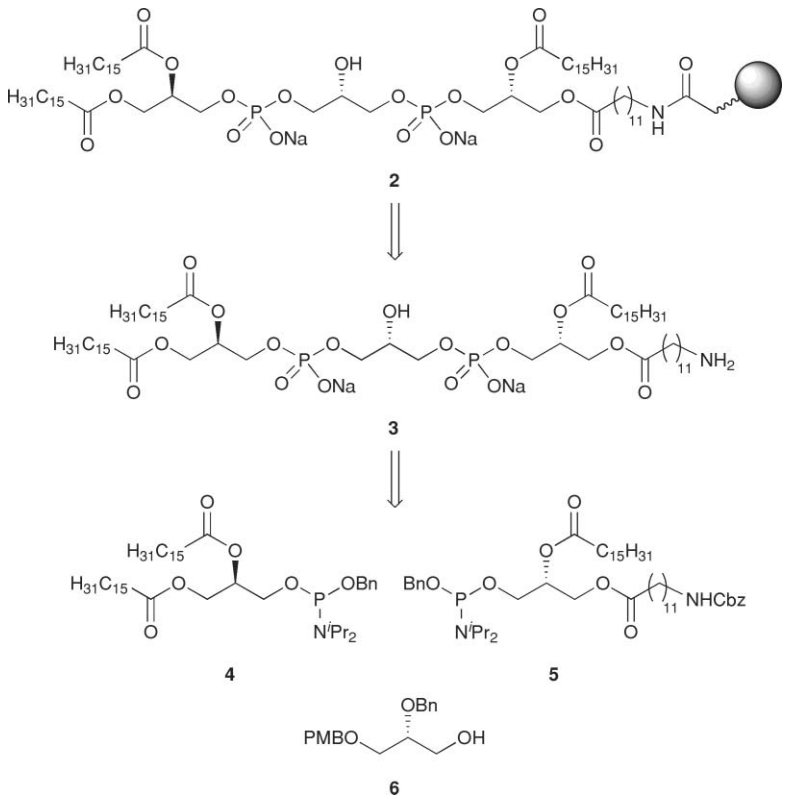
We have previously shown[9–11] that immobilised phospholipid analogues (including the phosphatidylinositol phosphates [PIP*n*s][9–11] and phosphatidic acid [PA][10]) can be used as affinity matrices to identify the intracellular protein targets of the lipid. Applying this technique to cardiolipin (**1**) may assist in elucidating its intracellular role, its involvement in disease and the intracellular proteins with which it interacts. Several probes have been reported for the purification of anticardiolipin antibodies, in which the phospholipid is attached to a solid support *via* non-covalent hydrophobic interactions.[12] However, previous studies have shown that covalent attachment of the phospholipids to the solid phase has a number of advantages.[13] These include the improved removal of the protein from the matrix and the ability to reuse the matrix a number of times. It has also been demonstrated that covalent immobilisation of the lipid allows control over, and estimations of, the number of sites on the solid phase that are occupied, termed the percentage loading.[10] Additionally, covalently immobilising the phospholipid through a functional group at the end of one of the fatty acid chains orients the molecule away from the surface of the bead, in a manner that is designed to mimic its natural orientation in cellular membranes.

Phosphoramidite chemistry has been used by our group in the synthesis of numerous inositol polyphosphates[14,15] and phospholipids.[9,10,15,16] Herein, we report the application of this methodology to the synthesis of the cardiolipin analogue **3** and the subsequent immobilisation of this compound onto an agarose-based solid support, to form a covalently bound cardiolipin affinity matrix **2**.

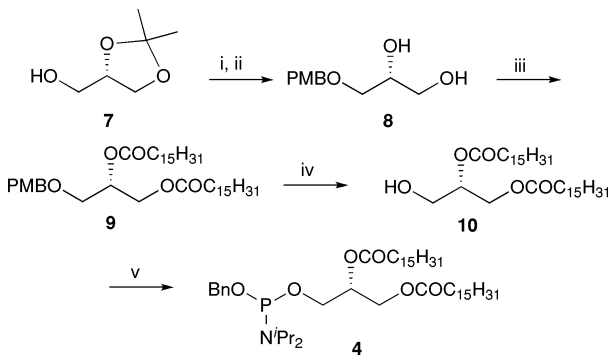
Retrosynthetic analysis of the immobilised phospholipid gives two phosphoramidite fragments **4** and **5**, and a differentially protected glycerol fragment **6** (Scheme 1). PMB protection of (+)-1,2-*O*-isopropylidene-glycerol **7** followed by acid catalysed methanolysis of the acetal afforded, in good yield, diol **8**, a key intermediate for all three fragments (Scheme 2). Esterification of diol **8** with palmitoyl chloride followed by removal of the PMB group with DDQ furnished alcohol **10** in good yield. This compound was then transformed into phosphoramidite fragment, **4**, *via* a 1*H*-tetrazole mediated coupling with (benzyloxy)bis(*N*,*N*-diisopropylamino)phosphine.[17] The synthesis of

[a]*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK*
[b]*School of Chemistry, Bio21 Institute, Building 102, 30 Flemington Road, University of Melbourne, Parkville, VIC 3010, Australia*
[c]*Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford, OX1 3TA, UK*
[d]*Department of Biochemistry and Molecular Biology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Victoria 3800, Australia*
[e]*Bio21 Institute, Building 102, 30 Flemington Road, University of Melbourne, Parkville, VIC 3010, Australia*

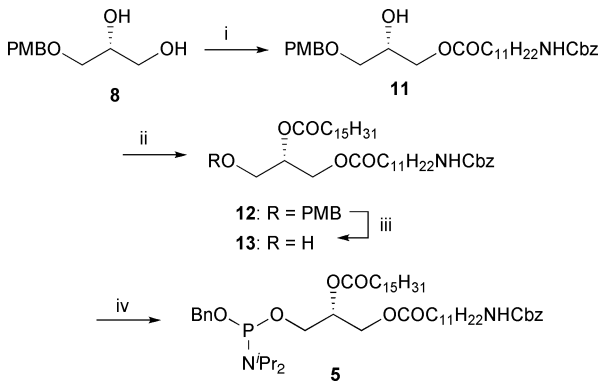
† Electronic supplementary information (ESI) available: NMR spectra of **3**, **4**, **15–19**. See DOI: 10.1039/b909306k



Scheme 1 Retrosynthetic analysis of the affinity matrix.



Scheme 2 Synthesis of fragment **4**. *Reagents and conditions:* i. NaH, PMBCl, DMF, 0 °C → rt, quant.; ii. *p*-TsOH·H_2O, MeOH, reflux, 82%; iii. $C_{15}H_{31}COCl$, pyr., DMAP, CH_2Cl_2, 0 °C → rt, 94%; iv. DDQ, CH_2Cl_2/H_2O (16/1, v/v), rt, 85%; v. $(BnO)P(N^iPr_2)_2$, 1*H*-tetrazole, CH_2Cl_2, rt, 97%.

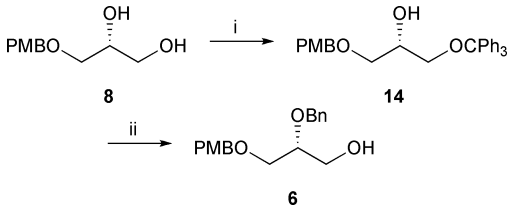


Scheme 3 Synthesis of fragment **5**. *Reagents and conditions:* i. $CbzNHC_{11}H_{22}CO_2H$, DCC, DMAP, CH_2Cl_2, 0 °C → rt, 87%; ii. $C_{15}H_{31}COCl$, pyr., DMAP, CH_2Cl_2, 0 °C → rt, quant.; iii. DDQ, CH_2Cl_2/H_2O (16/1, v/v), rt, 80%; iv. $(BnO)P(N^iPr_2)_2$, 1*H*-tetrazole, CH_2Cl_2, rt, 92%.

fragment **5** (Scheme 3) was conducted in a similar manner and was identical to that reported for the synthesis of immobilised phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and immobilised PA.[10] Thus, selective DCC-mediated coupling of the primary alcohol of **8** with 12-*N*-(benzyloxycarbonyl)aminododecanoic acid and subsequent esterification with palmitoyl chloride afforded diester **12**, which was deprotected and coupled with (benzyloxy)bis(*N,N*-diisopropylamino)phosphine to afford fragment **5**.

The synthesis of the central fragment **6** commenced with protection of the primary alcohol of the diol **8** (Scheme 4) as the corresponding trityl ether **14**. This reaction proceeded in high yield and with complete chemoselectivity, as adjudged by ^1H NMR spectroscopy. A one-pot procedure involving the introduction of the benzyl ether group at the secondary position and subsequent removal of the trityl protecting group by treatment with *p*-toluenesulfonic acid monohydrate afforded the desired alcohol **6** in 97% yield.

With all three fragments in hand, assembly of the cardiolipin analogue was undertaken (Scheme 5). Coupling of bis-protected glycerol moiety **6** with the phosphoramidite **5** in the presence of 1*H*-tetrazole followed by *in situ* oxidation with *m*CPBA proceeded smoothly to afford the protected phosphatidylglycerol **15** in 74% yield. The PMB protecting group was cleaved by



Scheme 4 Synthesis of the central fragment **6**. *Reagents and conditions:* i. Ph_3CCl, pyr., DMAP, CH_2Cl_2, rt, 84%; ii. NaH, BnBr, TBAI, DMF, rt then *p*-TsOH·H_2O, MeOH, rt, 97%.

DDQ oxidation to furnish the alcohol **16** and the fully protected cardiolipin derivative **17** was obtained in 75% yield upon coupling of phosphoramidite **4** with alcohol **16** in a similar manner.[18,19]

All that remained was to remove the four protecting groups in **17**. It is necessary for the benzyl phosphate esters to be removed before the benzyl ether, to prevent a 1,2-migration of the phosphatidyl ester to the secondary hydroxyl. Thus, disodium salt **18** was obtained in 61% yield by treatment of **17** with sodium iodide. Hydrogenolysis of the benzyl ether and *N*-Cbz groups afforded hydroxyamine **3** in 65% yield. It was subsequently found that ammonium salt **19** could be prepared in an improved 91% yield *via* a one-pot hydrogenation strategy (Scheme 5).[20]

The cardiolipin affinity matrix **2** was prepared by coupling amine **3** with *N*-hydroxysuccimidyl (NHS)-activated Affi-Gel® 10 resin (BioRad) in a mixture of chloroform, methanol and water in the presence of an excess of sodium hydrogen carbonate. The matrix was washed thoroughly with the reaction solvent and then with water, and the washings were lyophilised and analysed by ^{1}H NMR. In this manner, the percentage loading of phospholipid on the solid phase was found to be 1.7%, based on the recovery of unreacted cardiolipin analogue **3**, which was quantified through use of an internal standard (benzyloxy-*myo*-inositol orthorformate).

The efficacy of cardiolipin beads as affinity probes was tested using the purified recombinant HR1 domain of PRK2, a protein kinase containing a negative regulatory domain known to be activated seven-fold by cardiolipin in *in vitro* assays.[21] The expressed and purified HR1 domain of PRK2 has been shown to interact with cardiolipin in surface plasmon resonance experiments with an 11 μM affinity.[22] To further confirm these results, the cardiolipin affinity matrix was incubated with purified recombinant PRK2 HR1 domain and the binding was analysed (Fig. 2b) and compared with a control experiment with blank beads (Fig. 2a). As can be seen from Fig. 2b, the majority of PRK2 HR1 domain bound to the immobilised cardiolipin with high affinity with relatively little detected in the unbound fraction. The control experiment revealed that a small amount of the HR1 domain bound to the blank beads, reflecting non-specific (probably hydrophobic) interactions, however the clear band seen in both the washing steps indicates that the majority of the protein was only weakly bound to the beads and easily removed by phosphate buffer solution (W1 and W2, Fig. 2a). These experiments confirm the validity of our approach and that the HR1 domain of PRK2 is involved in the binding of cardiolipin.

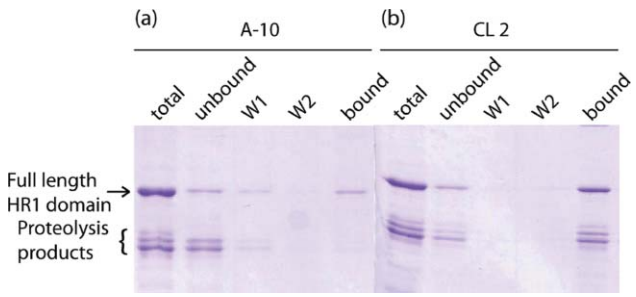
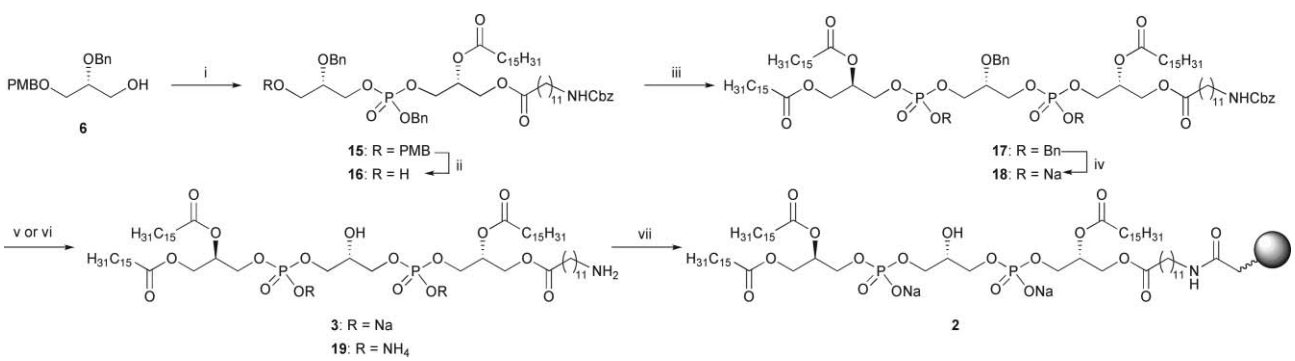


Fig. 2 SDS-PAGE analysis of the binding of recombinant PRK2 HR1 domain with (a) blank beads and (b) immobilised cardiolipin. Total: total amount of protein; unbound: residual protein in supernatant; W1: protein in first wash; W2: protein in second wash; bound: protein bound to beads.

Conclusions

An ω-amine functionalised analogue of cardiolipin has been synthesised and immobilised onto Affi-Gel® 10 beads. The use of these beads as a tool for the isolation of cardiolipin-binding proteins has been demonstrated, which may help improve understanding of the physiological roles of cardiolipin in both healthy and disease states. Furthermore, the HR1 domain of a cardiolipin-activated protein kinase has been proven to bind to cardiolipin. To our knowledge, this is the first example of a cardiolipin affinity probe being used to investigate the HR1 domain of protein kinase C related kinases. Such an affinity matrix may also be used for



Scheme 5 Final coupling of the fragments and immobilisation of the cardiolipin analogue (**3**). *Reagents and conditions:* i. **5**, 1*H*-tetrazole, CH_2Cl_2, rt, then *m*CPBA, −78 °C → rt, 74%; ii. DDQ, CH_2Cl_2/H_2O (16/1, v/v), rt, 82%; iii. **4**, 1*H*-tetrazole, CH_2Cl_2, rt, then *m*CPBA, −78 °C → rt, 75%; iv. NaI, acetone, 90 °C, 3 h, microwave, 61%; v. From **18**: Pd black, H_2 (150 psi), THF, rt, **3**: 65%; vi. From **17**: 10% Pd/C, THF, H_2 (150 psi), rt, then NH_4OH **19**: 91%; vii. From **3**: Affi-Gel® 10, chloroform/methanol/water (4/5/1, v/v/v), 4 °C, 1.7% loading.

diagnostic purposes and for the identification of potential therapeutic agents.

Experimental

General experimental techniques

Unless otherwise specified, all ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AV400 spectrometer at 400 MHz, 100 MHz and 162 MHz respectively, or a Bruker DRX500 spectrometer at 500 MHz, 125 MHz or 202 MHz respectively, using deuterochloroform (or other indicated solvents) as reference ($\delta = 7.26$ ppm and 77.0 ppm for ^{1}H and ^{13}C NMR respectively). Chemical shifts (δ) are measured in ppm. The chemical shift data for ^{31}P signals are reported in ppm relative to an external standard of 85% H_3PO_4 ($\delta = 0.00$ ppm). Coupling constants (J) are quoted in Hz and are recorded to the nearest 0.1 Hz. Infrared spectra were recorded on Perkin-Elmer 1600 series FTIR and Perkin-Elmer Spectrum One ATR-FTIR spectrometers in the region 4000–600 cm^{-1}. The sample was prepared as a solution in the indicated solvent. Mass spectra were recorded at the EPSRC National Mass Spectroscopy Centre, University of Wales, Swansea, the Mass Spectrometry Service at the University of Cambridge Chemical Laboratory and the University of Melbourne Mass Spectrometry Service. Melting points were determined using a Reichert-Koeffler hot stage or Büchi melting point apparatus and are uncorrected. Optical specific rotations were measured using a JASCO DIP-1000 digital polarimeter or a Perkin-Elmer 241 polarimeter, in a microcell of 1 dm path length for 1 mL of solution, units are 10^{-1} deg cm^2 g^{-1} and concentration is expressed in g/100 cm^3. Microwave reactions were carried out in sealed reaction vessels using a Biotage Initiator 2.0 (400 W). Solvents and reagents were purified and dried where necessary by standard techniques. Where appropriate and if not stated otherwise, all reactions were performed in flame-dried apparatus under an inert atmosphere of nitrogen or argon. Petrol refers to the fraction of petroleum ether of boiling point range 40–60 °C.

The following compounds were synthesised following literature procedures: (+)-1,2-*O*-isopropylidene-3-*O*-(4′-methoxybenzyl)-*sn*-glycerol,[10] 12-*N*-(benzyloxycarbonyl)aminododecanoic acid,[23] **5**,[10] **8**,[10] **11**,[10] **12**,[10] **13**,[10] **14**.[24]

(+)-3-*O*-(4-Methoxybenzyl)-1,2-di-*O*-hexadecanoyl-*sn*-glycerol 9

To a stirred solution of the diol (–)-**8** (1.50 g, 7.10 mmol, 1 equiv) in dry dichloromethane (25 mL) was added dry pyridine (1.40 mL, 1.40 g, 17.75 mmol, 2.5 equiv) and 4-dimethylaminopyridine (0.043 g, 0.3 mmol, 0.05 equiv). The solution was cooled to 0 °C and palmitoyl chloride (4.70 mL, 4.29 g, 15.62 mmol, 2.2 equiv) was added dropwise. After stirring for 10 minutes at this temperature, the solution was allowed to warm to room temperature and stirred overnight. The reaction was quenched by addition of water (25 mL). The aqueous phase was extracted with dichloromethane (3 × 30 mL) and the combined organic layers were washed with 2 M aqueous hydrochloric acid (20 mL) and water (25 mL), and these washings were back-extracted with dichloromethane (30 mL). The organic phase was washed with brine (30 mL), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. Flash chromatography (50% dichloromethane in petrol) afforded the diester (+)-**9** (4.58 g, 6.67 mmol, 94%) as a white solid with data in agreement with the literature.[25]

(–)-1,2-Di-*O*-hexadecanoyl-*sn*-glycerol 10

To a stirred solution of PMB ether (+)-**9** (1.50 g, 2.18 mmol, 1 equiv) in dry dichloromethane (87 mL) and deionised water (5.5 mL) was added 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (1.01 g, 4.46 mmol, 2.05 equiv) and the reaction mixture was stirred overnight at room temperature. The reaction was diluted with dichloromethane (50 mL) and washed with a saturated aqueous solution of sodium hydrogen carbonate (100 mL) and brine (2 × 75 mL). The combined aqueous layers were extracted with dichloromethane (30 mL). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. Flash chromatography (40% diethyl ether in petrol) afforded the alcohol (–)-**10** (1.06 g, 1.83 mmol, 85%) as a white solid with data in agreement with the literature.[25]

(+)-Benzyloxy(*N*,*N*-diisopropylamino)(1,2-di-*O*-hexadecanoyl-*sn*-glycer-3-yl)phosphine 4

A mixture of the alcohol (–)-**10** (0.50 g, 0.88 mmol, 1 equiv), (benzyloxy)bis(*N*,*N*-diisopropylamino)phosphine (0.75 g, 2.20 mmol, 2.5 equiv) and 1*H*-tetrazole (0.19 g, 2.64 mmol, 3.0 equiv) was placed under high vacuum for 5 minutes and then under an atmosphere of argon. After addition of dry dichloromethane (40 mL), the reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with dichloromethane (30 mL) and quenched by addition of saturated aqueous sodium hydrogen carbonate solution (50 mL). The organic phase was separated and the aqueous phase was extracted with dichloromethane (3 × 30 mL). The combined organic phases were washed with brine (50 mL), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. Flash chromatography (5/15/80 triethylamine/ethyl acetate/hexane) afforded the phosphoramidite (+)-**4** (0.69 g, 0.85 mmol, 97%) as a waxy solid: R_f 0.69 (20% ethyl acetate in hexane); $[\alpha]_D^{24} = +6.2$ (*c* 1.21 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 7.36–7.24 (5 H, m, C_6H_5), 5.19–5.15 (1 H, m, CH_2CHCH_2), 4.75–4.65 (2 H, m, $OCH_2C_6H_5$), 4.36–4.30 (1 H, m, CH_2CHCHH), 4.17–4.10 (1 H, m, CH_2CHCHH), 3.78–3.45 (4 H, m, CH_2CHCH_2, 2 × $CH(CH_3)_2$), 2.28 (4 H, t, *J* 7.4, 2 × $OCOCH_2$), 1.61–1.55 (4 H, m, 2 × $OCOCH_2CH_2$), 1.29–1.16 (48 H, br s, 2 × $C_{15}H_{31}$), 1.02 (12 H, d, *J* 7.1, $CH(CH_3)_2$), 0.87 (6 H, t, *J* 6.8, 2 × CH_2CH_3); δ_P (162 MHz; $CDCl_3$) 151.65, 151.50.

(+)-2-*O*-Benzyl-3-*O*-(4′-methoxybenzyl)-*sn*-glycerol 6

To a stirred solution of the alcohol (–)-**14** (0.050 g, 0.11 mmol, 1 equiv) in dry THF (2 mL) under argon was added sodium hydride (0.006 g, 60% suspension in mineral oil, 0.15 mmol, 1.4 equiv), followed after 15 minutes by tetrabutylammonium iodide (0.004 g, 0.01 mmol, 0.1 equiv) and benzyl bromide (0.016 mL, 0.023 g, 0.13 mmol, 1.2 equiv). After stirring at room temperature for 2 h, the reaction was quenched with methanol (2 mL) and diluted with diethyl ether (10 mL). The solution was washed with saturated aqueous ammonium chloride solution (10 mL) and the aqueous layer was separated and extracted with diethyl ether (2 × 10 mL). The combined organic layers were washed with water (20 mL) and the solvent was removed *in vacuo*. The

residue was taken up in methanol (10 mL) and *p*-toluenesulfonic acid monohydrate (0.002 g, 0.01 mmol, 0.1 equiv) was added. After stirring at room temperature for 1 h, the reaction was quenched with triethylamine (1 mL) and the solvent was removed *in vacuo*. Flash chromatography (30% ethyl acetate in petrol) gave the desired alcohol (+)-**6** (0.032 g, 0.106 mmol, 97%) as a colourless oil with data in agreement with the published literature values.[26]

(+)-1-*O*-(1′-*O*-[12″-*N*-(Benzyloxycarbonylamino)dodecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-benzyloxyphosphoryl)-2-*O*-benzyl-3-*O*-(4′-methoxybenzyl)-*sn*-glycerol 15

To a stirred solution of the alcohol (+)-**6** (0.043 g, 0.14 mmol, 1 equiv) and 1*H*-tetrazole (0.030 g, 0.42 mmol, 3 equiv) in dry dichloromethane (4 mL) under argon was added a solution of the phosphoramidite (+)-**5** (0.390 g, 0.43 mmol, 3.1 equiv) in dry dichloromethane (6 mL). After stirring at room temperature for 2 h, the reaction mixture was cooled to −78 °C and *m*CPBA (0.072 g, 0.42 mmol, 3.0 equiv) was added. The resulting mixture was stirred at −78 °C for 10 minutes and then for a further 1 h once it had attained room temperature. The reaction was quenched by addition of 10% (w/v) aqueous sodium hydrogen sulfite solution (20 mL), and after stirring for 10 minutes, the aqueous phase was separated and extracted with dichloromethane (3 × 20 mL). The combined organic extracts were washed with aqueous saturated sodium hydrogen carbonate solution (20 mL) and brine (2 × 30 mL), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. Flash chromatography (30% ethyl acetate in hexane) of the resulting oil afforded the lipid (+)-**15** (0.115 g, 0.10 mmol, 74%) as a yellow oil: R_f 0.45 (50% ethyl acetate in hexane); $[\alpha]_D^{25} = +2.6$ (*c* 5.8 in $CHCl_3$); ν_{max} ($CDCl_3$)/cm^{-1} 3452, 2927, 2855, 1732, 1612, 1586, 1514, 1465, 1249, 1173, 1150, 1102, 1035, 1022; δ_H (400 MHz; $CDCl_3$) 7.35–7.19 (17 H, m, C_6H_5, $C_6H_4OCH_3$), 6.87–6.83 (2 H, m, $C_6H_4OCH_3$), 5.16 (1 H, qn, *J* 5.0, CH_2CHCH_2), 5.08 (2 H, br s, HNC(O)O$CH_2C_6H_5$), 5.03 (1 H, d, *J* 8.2, PO$CH_AH_BC_6H_5$), 5.02 (1 H, dd, *J* 8.2 and 1.4, PO$CH_AH_BC_6H_5$), 4.77 (1 H, br s, N*H*), 4.63 (1 H, dd, *J* 11.8 and 4.1, CHO$CH_AH_BC_6H_5$), 4.60 (1 H, dd, *J* 11.8 and 3.8, CHO$CH_AH_BC_6H_5$), 4.44–4.43 (2 H, m, $CH_2C_6H_4OCH_3$), 4.27–4.16 (2 H, m, CH_2CHCH_2OCO), 4.15–4.00 (4 H, m, CH_2CHCH_2OP, $POCH_2CHCH_2$), 3.78–3.77 (3 H, m, $C_6H_4OCH_3$), 3.76–3.72 (1 H, m, CH_2CH(OBn)CH_2), 3.53–3.51 (2 H, m, OCH_2CHCH_2), 3.17 (2 H, dt, *J* 6.7 and 6.7, CH_2NH), 2.29–2.23 (4 H, m, 2 × $OCOCH_2$), 1.58–1.54 (4 H, m, 2 × $OCOCH_2CH_2$), 1.49–1.44 (2 H, m, CH_2CH_2NH), 1.33–1.20 (38 H, m, $C_{15}H_{31}$, $C_{11}H_{22}NH$), 0.87 (3 H, t, *J* 6.9, CH_2CH_3); δ_C (100 MHz; $CDCl_3$) 172.2 (d), 171.8 (d), 158.3, 155.4, 137.1, 135.7 (d), 134.7 (d), 129.0, 128.3, 127.6, 127.5, 127.4, 127.1, 126.9, 126.8, 126.7, 112.8, 75.5 (d), 72.1, 71.2, 68.5 (d), 68.4 (d), 68.4 (d), 68.3 (d), 67.6, 66.3 (d), 66.2 (d), 65.5, 64.5–64.4 (m), 60.6, 54.2, 40.1, 33.1, 33.0, 30.9, 29.0, 28.7, 28.6, 28.5, 28.4, 28.3, 28.2, 28.1, 23.8, 21.7, 13.1; δ_P (162 MHz; $CDCl_3$) −0.37; *m*/*z* (ES^+) 1133.6796 (M + NH_4)$^+$, $C_{64}H_{98}N_2O_{13}P$ requires 1133.6807.

(−)-1-*O*-(1′-*O*-[12″-*N*-(Benzyloxycarbonylamino)dodecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-benzyloxyphosphoryl)-2-*O*-benzyl-3-lyso-*sn*-glycerol 16

To a stirred solution of the PMB ether (+)-**15** (0.114 g, 0.10 mmol, 1 equiv) in dichloromethane (5 mL) and deionised water (0.25 mL) under air was added portionwise DDQ (0.046 g, 0.20 mmol, 2.0 equiv). After stirring at room temperature overnight, the reaction mixture was diluted with dichloromethane (40 mL) and saturated aqueous sodium hydrogen carbonate solution (5 mL) was added. The aqueous phase was extracted with dichloromethane (3 × 20 mL), and the combined organic phases were washed with water (20 mL) and brine (2 × 20 mL), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. The resulting yellow oil was purified by flash chromatography (45% ethyl acetate in hexane) to afford the alcohol (−)-**16** (0.082 g, 0.08 mmol, 82%) as a white solid: mp 34–36 °C (from diethyl ether/dichloromethane); R_f 0.12 (50% ethyl acetate in hexane); $[\alpha]_D^{25} = -4.2$ (*c* 4.0 in $CHCl_3$); ν_{max} ($CDCl_3$)/cm^{-1} 3451, 2926, 2855, 1735, 1515, 1456, 1255, 1147, 1109, 1035, 1024; δ_H (250 MHz; $CDCl_3$) 7.37–7.27 (15 H, m, 3 × C_6H_5), 5.18 (1 H, qn, *J* 5.0, $CH_2CH(OCOC_{15}H_{31})CH_2$), 5.09–5.05 (4 H, m, HNC(O)O$CH_2C_6H_5$, PO$CH_2C_6H_5$), 4.78 (1 H, br s, N*H*), 4.65 (1 H, dd, *J* 11.7 and 6.1, CHO$CH_AH_BC_6H_5$), 4.57 (1 H, dd, *J* 11.7 and 3.7, CHO$CH_AH_BC_6H_5$), 4.30–4.22 (1 H, m, CH_2CHCHHOCO), 4.20–4.01 (5 H, m, $CH_2CHCHHOCO$, 2 × CH_2CHCH_2OP), 3.75–3.68 (1 H, m, CH_2CH(OBn)CH_2), 3.67–3.56 (2 H, m, $HOCH_2CHCH_2$), 3.17 (2 H, dt, *J* 6.4 and 6.4, CH_2NH), 2.44 (1 H, br s, $HOCH_2CHCH_2$), 2.30–2.25 (4 H, m, 2 × $OCOCH_2$), 1.60–1.55 (4 H, m, 2 × $OCOCH_2CH_2$), 1.50–1.45 (2 H, m, CH_2CH_2NH), 1.33–1.21 (38 H, m, $C_{15}H_{31}$, $C_{11}H_{22}NH$), 0.88 (3 H, t, *J* 6.8, CH_2CH_3); δ_C (100 MHz; $CDCl_3$) 173.2, 172.8, 156.4, 137.8, 136.7, 135.5 (2 × d), 128.7, 128.5, 128.1, 128.0, 127.9, 127.8, 77.6 (d), 77.5 (d), 72.1, 69.7 (d), 69.3 (d), 66.5, 66.1 (d), 65.5 (m), 61.6, 61.0 (d), 41.1, 34.1, 34.0, 31.9, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.7, 24.8, 22.7, 14.1; δ_P (162 MHz; $CDCl_3$) 0.17; *m*/*z* (EI^+) 1013.6230 (M + NH_4)$^+$, $C_{56}H_{90}N_2O_{12}P$ requires 1013.6231.

(+)-1-*O*-(1′-*O*-[12″-*N*-(Benzyloxycarbonylamino)dodecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-benzyloxyphosphoryl)-2-*O*-benzyl-3-*O*-(1′,2′-di-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-benzyloxyphosphoryl)-*sn*-glycerol 17

To a stirred solution of the phosphoramidite (+)-**4** (0.08 g, 0.10 mmol, 2.5 equiv) and 1*H*-tetrazole (0.08 g, 0.12 mmol, 3.0 equiv) in dry dichloromethane (2 mL) under argon was added a solution of alcohol (−)-**16** (0.04 g, 0.04 mmol, 1 equiv) in dry dichloromethane (3 mL) and the resulting mixture was stirred overnight at room temperature. The reaction mixture was then cooled to −78 °C and *m*CPBA (0.02 g, 0.12 mmol, 3.0 equiv) was added. After stirring at this temperature for 30 minutes, the reaction mixture was allowed to attain room temperature and stirred for 1 h. The reaction was diluted with dichloromethane (10 mL) and quenched by addition of 10% (w/v) aqueous sodium hydrogen sulfite solution (10 mL). After stirring at room temperature for 10 minutes, the aqueous phase was separated and extracted with dichloromethane (3 × 20 mL). The combined organic extracts were washed with saturated aqueous sodium hydrogen carbonate solution (10 mL) and brine (2 × 20 mL), dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. The resulting residue was purified by flash chromatography (30–50% ethyl acetate in hexane) to afford the product (+)-**17** (0.050 g, 0.03 mmol, 75%) as a colourless oil: R_f 0.22 (50% ethyl acetate in hexane); $[\alpha]_D^{25} = +2.3$ (*c* 2.3 in $CHCl_3$); ν_{max} ($CDCl_3$)/cm^{-1} 3451, 2927, 2855, 1737, 1602, 1515, 1456, 1268, 1159, 1112, 1038, 1024; δ_H (250 MHz; $CDCl_3$) 7.35–7.29 (20 H, m, 4 × C_6H_5),

5.19–5.15 (2 H, m, 2 × $CH_2CH(OCOC_{15}H_{31})CH_2$), 5.09 (2 H, s, $HNC(O)OCH_2C_6H_5$), 5.05–5.02 (4 H, m, 2 × $POCH_2C_6H_5$), 4.75 (1 H, br s, N*H*), 4.60–4.59 (2 H, m, $CHOCH_2C_6H_5$), 4.27–4.22 (2 H, m, CH_2CHCH_2OCO), 4.16–4.01 (10 H, m, CH_2CHCH_2OCO, 4 × CH_2CHCH_2OP), 3.75–3.71 (1 H, m, $CH_2CH(OBn)CH_2$), 3.18 (2 H, dt, *J* 6.3 and 6.3, CH_2NH), 2.27 (8 H, t, *J* 7.4, 4 × $OCOCH_2$), 1.58–1.56 (8 H, m, 4 × $OCOCH_2CH_2$), 1.50–1.46 (2 H, m, CH_2CH_2NH), 1.33–1.21 (86 H, br s, 3 × $C_{15}H_{31}$, $C_{11}H_{22}NH$), 0.88 (9 H, t, *J* 6.9, 3 × CH_2CH_3); δ_C (100 MHz; $CDCl_3$) 173.2, 172.8, 156.4, 137.4 (d), 136.7, 135.5 (2 × d), 128.7, 128.5, 128.4, 128.1, 128.0, 127.8, 77.2 (m), 72.3, 69.6 (d), 69.2 (2 × d) 68.4, 66.6, 65.8, 65.5 (2 × m), 61.6, 41.1, 34.1, 34.0, 31.9, 30.0, 29.7, 29.5, 29.4, 29.3, 29.1, 26.7, 24.8, 22.7, 14.1; δ_P (162 MHz; $CDCl_3$) –0.28, –0.33; *m/z* (ES^+) 1739.0874 $(M + Na)^+$, $C_{98}H_{159}NNaO_{19}P_2$ requires 1739.0841].

(+)-1-*O*-(1′-*O*-[12″-*N*-(Benzyloxycarbonylamino)dodecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-2-*O*-benzyl-3-*O*-(1′,2′-di-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-*sn*-glycerol disodium salt 18

To a stirred solution of sodium iodide (0.011 g, 0.073 mmol, 6.0 equiv) in dry acetone (0.2 mL) was added a solution of lipid (+)-**17** (0.021 g, 0.012 mmol, 1 equiv) in dry acetone (0.1 mL) under nitrogen and the resulting mixture was heated at 90 °C for 3 h in a microwave. The reaction mixture was allowed to cool to room temperature, diluted with acetone (10 mL) and centrifuged (3000 rpm, 15 minutes). The yellow supernatant was removed and the remaining white solid was washed with acetone (2 × 10 mL) by centrifugation. The resulting white solid was taken up in chloroform (2 mL) and precipitated with acetone (10 mL), the supernatant was removed by centrifugation (3000 rpm, 15 minutes). The solid was dried under vacuum, affording the disodium salt (+)-**18** (0.011 g, 0.007 mmol, 61%) as a white amorphous solid: R_f 0.44 (65/25/4 $CHCl_3$/MeOH/H_2O); $[\alpha]_D^{25}$ = +4.5 (*c* 0.74 in $CHCl_3$); ν_{max} ($CDCl_3$)/cm^{-1} 2927, 2854, 1732, 1603, 1516, 1456, 1259, 1223, 1169, 1106, 1078, 1015; δ_H (400 MHz; $CDCl_3$) 7.35–7.27 (10 H, m, 2 × C_6H_5), 5.21 (2 H, br s, 2 × $CH_2CH(OCOC_{15}H_{31})CH_2$), 5.08 (2 H, s, $HNC(O)OCH_2C_6H_5$), 4.78 (1 H, br s, N*H*), 4.59 (2 H, s, $CHOCH_2C_6H_5$), 4.38–4.35 (2 H, m, glycerol CH_2), 4.16–4.12 (2 H, m, glycerol CH_2), 4.01 (4 H, br s, 2 × glycerol CH_2), 3.90 (4 H, br s, 2 × glycerol CH_2), 3.66 (1 H, m, $CH_2CH(OBn)CH_2$), 3.18 (2 H, dt, *J* 6.4 and 6.4, CH_2NH_2), 2.27–2.21 (8 H, m, 4 × $OCOCH_2$), 1.57–1.48 (10 H, m, 4 × $OCOCH_2CH_2$, CH_2CH_2NH), 1.25 (86 H, br s, 3 × $C_{15}H_{31}$, $C_{11}H_{22}NH_2$), 0.87 (9 H, t, *J* 6.9, 3 × CH_2CH_3); δ_C (125 MHz; $CDCl_3$) 173.5, 156.3, 138.0, 136.7, 128.5, 128.3, 128.1, 128.0, 127.6, [A signal was expected at around 77 ppm but it was obscured by the chloroform peaks], 71.2, 70.6, 66.5, 63.5, 62.8, 41.1, 34.3, 34.1, 31.9, 30.0, 29.8, 29.7, 29.4, 29.2, 26.8, 24.9, 24.8, 22.7, 14.1; δ_P (162 MHz; $CDCl_3$) 1.56; *m/z* (ES^+) 1580.9754 $(M + H)^+$, $C_{84}H_{146}NNa_2O_{19}P_2$ requires 1580.9740.

(+)-1-*O*-(1′-*O*-[12″-Aminododecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-3-*O*-(1′,2′-di-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-*sn*-glycerol diammonium 19

To a stirred suspension of the protected cardiolipin analogue (+)-**17** (0.050 g, 0.030 mmol, 1.0 equiv) in dry tetrahydrofuran (0.5 mL) was added 10% Pd on activated charcoal (0.008 g, 0.008 mmol, 0.3 equiv). The reaction vessel was then pressurised and vented with hydrogen (10 times) then finally pressurised to 150 psi and the reaction stirred overnight at room temperature. The catalyst was removed by filtration through Celite® and the filtrate treated with 28% aqueous ammonia solution (0.1 mL). This mixture was allowed to stir for 1 h before the solvent was removed *in vacuo*. The residue was taken up in chloroform and the product precipitated with acetone. The resulting suspension was centrifuged (10 minutes, 3000 rpm) and the supernatant was removed. The remaining solid was taken up in chloroform, precipitated with acetone and the supernatant removed by centrifuging (10 minutes, 3000 rpm). Drying the solid under vacuum afforded the desired diammonium cardiolipin salt (+)-**19** (0.037 g, 0.011 mmol, 91%) as a white amorphous solid: $[\alpha]_D^{25}$ = +4.4 (*c* 1.0 in $CHCl_3$); ν_{max} (neat)/cm^{-1} 3368, 2919, 2851, 1738, 1641, 1467, 1215, 1171, 1088, 1064, 835, 758, 721, 664; δ_H (500 MHz; $CDCl_3$) 5.20–5.18 (2 H, m, 2 × $CH_2CH(OCOC_{15}H_{31})CH_2$), 4.46–4.43 (1H, m, one CH_2CH), 4.39–4.36 (1 H, m, one CH_2CH), 4.16–4.07 (2 H, m, CH_2CH), 3.95–3.80 (9 H, m, 4 × CH_2CH, $CH_2CH(OH)CH_2$), 2.87 (2 H, br s, CH_2NH_2), 2.34–2.27 (8 H, m, 4 × $OCOCH_2$), 1.85 (8 H, br s, 2 × NH_4), 1.70–1.53 (10 H, m, 4 × $OCOCH_2CH_2$, $CH_2CH_2NH_2$), 1.40–1.25 (89 H, m, O*H*, NH_2, 3 × $C_{15}H_{31}$, $C_{11}H_{22}NH_2$), 0.88 (9 H, t, *J* 7.0, 3 × CH_2CH_3); δ_C (125 MHz; $CDCl_3$) 173.4, 173.3, 173.0, 172.9(5), 70.3, 69.6, 66.9, 63.6, 63.5, 62.6, 62.2, 39.5, 34.2(7), 34.2(5), 34.1, 33.9, 31.9, 29.7(2), 29.6(7), 29.6(0), 29.5(8), 29.4(1), 29.3(6), 29.2(2), 29.1(9), 24.9(4), 24.8(9), 22.7, 14.1; δ_P (202 MHz, $CDCl_3$) 1.30, 0.98; MS (ESI^+) *m/z* 1312.9280 $(M - 2NH_3 + H)^+$, $C_{69}H_{136}NO_{17}P_2$ requires, 1312.9278; 1334.9097 $(M - 2NH_3 + Na)^+$, $C_{69}H_{135}NO_{17}P_2Na$ requires 1334.9098; MS (ESI^-) *m/z* 1310.9126 $(M - 2NH_3 - H)^-$, $C_{69}H_{134}NO_{17}P_2$ requires 1310.9133.

(–)-1-*O*-(1′-*O*-[12″-Aminododecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-3-*O*-(1′,2′-di-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-*sn*-glycerol disodium salt 3

To a stirred suspension of protected cardiolipin analogue (+)-**18** (0.020 g, 0.013 mmol, 1.0 equiv) in dry tetrahydrofuran (5 mL) was added palladium black (0.014 g, 0.13 mmol, 10.0 equiv). The reaction vessel was then pressurised and vented with hydrogen (10 times) then finally pressurised to 150 psi and stirred for 2 days at room temperature. After degassing, the reaction mixture was centrifuged (3 minutes, 3000 rpm) and the supernatant was removed. The palladium pellet was washed with THF (15 mL) and centrifuged again. After removing the supernatant, this washing process was repeated with more THF (10 mL). The combined supernatants were passed through a plug of Celite® and concentrated *in vacuo*. The residue was then taken up in chloroform and precipitated with acetone. The resulting suspension was centrifuged (10 minutes, 3000 rpm) and the supernatant was removed. The remaining solid was redissolved in chloroform, precipitated with acetone and the supernatant was removed by centrifuging (10 minutes, 3000 rpm). The solid was dried under vacuum to give the desired cardiolipin analogue (–)-**3** (0.011 g, 0.008 mmol, 65%) as a white amorphous solid: $[\alpha]_D^{25}$ = –2.0 (*c* 0.2 in $CHCl_3$); ν_{max} (neat)/cm $^{-1}$ 3384, 2957, 2918, 2851, 1737, 1664, 1467, 1260, 1221, 1170, 1092, 1040, 801, 758, 721, 662; δ_H (500 MHz; $CDCl_3$) 5.22 (2 H, br s, 2 × $CH_2CH(OCOC_{15}H_{31})CH_2$), 4.48–4.35 (2H, m, CH_2CH), 4.20–4.10 (2H, m, CH_2CH), 4.10–3.82 (9 H, m, N*H*, 4 × CH_2CH, $CH_2CH(OH)CH_2$), 3.09 (3 H, br s,

CH_2NH_2, O*H*), 2.89–2.82 (2 H, m, CH_2NH), 2.35–2.26 (8 H, m, 4 × OCOCH_2), 1.59 (10 H, br s, 4 × OCOCH_2CH_2, CH_2CH_2NH$_2$), 1.26 (86 H, br s, 3 × $C_{15}H_{31}$, $C_{11}H_{22}$NH), 0.88 (9 H, t, *J* 7.0, 3 × CH_2CH_3); δ_C (125 MHz; $CDCl_3$) 173.5, 173.3, 70.5, 69.6, 66.7, 63.5, 62.8, 34.3, 34.1, 31.9, 30.9, 29.8, 29.7, 29.4, 29.3, 24.9, 22.7, 14.1; δ_P (202 MHz; $CDCl_3$) 1.87; *m/z* (ES^+) 1356.8917 $(M + H)^+$, $C_{69}H_{134}NNa_2O_{17}P_2$ requires 1356.8922.

1-*O*-(1′-*O*-[12″-*N*-Affi-Gel-10-aminododecanoyl]-2′-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-3-*O*-(1′,2′-di-*O*-hexadecanoyl-*sn*-glycer-3′-yl-*O*-phosphoryl)-*sn*-glycerol disodium salt 2

Affi-Gel® 10 resin (1 mL, 0.015 mmol) was washed with cold chloroform/methanol/water (4/5/1). A solution of the cardiolipin analogue (–)-**3** (0.002 g, 0.0015 mmol) and $NaHCO_3$ (0.006 g, 0.071 mmol) in chloroform/methanol/water (1 mL, 4/5/1) was shaken at room temperature for 10 minutes and then incubated with Affi-Gel® 10 beads at 4 °C in chloroform/methanol/water (3 mL, 4/5/1) for 2 days. The beads were washed successively with cold chloroform/methanol/water (4/5/1), cold methanol, and cold water by centrifugation (2000 rpm, 5 minutes). The beads were blocked by incubation with 0.1 mL of 1 M ethanolamine (pH = 8.0) in chloroform/methanol/water (2 mL, 4/5/1) for 2 h at room temperature. The beads were then washed with cold chloroform/methanol/water (4/5/1), cold methanol, and cold water by centrifugation (2000 rpm, 5 minutes). The beads were stored (at 4 °C) and used as a 50% slurry in water. The loading of cardiolipin on the beads was 1.7% as determined by ^{1}H NMR analysis of the residual cardiolipin in the supernatant after lyophilisation, using benzyloxy *myo*-inositol orthorformate (0.0074 mmol) as an internal standard.

Binding experiment

The control blank beads were first blocked by incubation with 0.10 mL of 1 M ethanolamine (pH = 8.0) in chloroform/methanol/water (2 mL, 4/5/1) for 2 h at room temperature. The *E. coli* expressed and purified recombinant HR1 domain from rat PRK2 at a concentration of 0.2 g/mL was then incubated with either the blank Affi-Gel® 10 beads or cardiolipin-conjugated matrix at 37 °C for 10 minutes. The mixture was then centrifuged at 5000 rpm at 4 °C for 5 minutes and the supernatant fraction (containing unbound protein) carefully removed. The sedimented matrix was resuspended in ice-cold phosphate buffer solution (PBS) and centrifuged again, prior to removal of the supernatant. This was repeated and both PBS washes retained for SDS-PAGE analysis. The final protein bound matrix was then resuspended in PBS. Equal loadings of the pre-incubation HR1 protein, unbound fraction and resuspended bound matrix, as well as a 1/10 loading of the washes were analysed on SDS-PAGE and stained with Coomassie Blue.

Acknowledgements

We thank the BBSRC and EPSRC for financial support and for provision of the Swansea Mass Spectrometry Service. SJC thanks Hughes Hall (Cambridge) for a Research Fellowship. This work was supported in part by the Australian Research Council, Discovery Project, Grant DP0770668. General support was provided by CSIRO and VESKI (Victorian Endowment for Science, Knowledge and Innovation). We also thank Daiso Co., Ltd, Japan for the generous gift of (+)-1,2-*O*-isopropylideneglycerol.

References

1 M. Schlame, S. Brody and K. Y. Hostetler, *Eur. J. Biochem.*, 1993, **212**, 727; J. LeCocq and C. E. Ballou, *Biochemistry*, 1964, **3**, 976.
2 P. V. Ioannou and B. T. Golding, *Prog. Lipid Res.*, 1979, **17**, 279.
3 M. Schlame, D. Rua and M. L. Greenberg, *Prog. Lipid Res.*, 2000, **39**, 257.
4 S. Fleischer, G. Rouser, B. Fleischer, A. Casu and G. Kritchevsky, *J. Lipid, Res.*, 1967, **8**, 170.
5 T. H. Haines and N. A. Dencher, *FEBS Lett.*, 2002, **528**, 35.
6 V. M. Gohil, P. Hayes, S. Matsuyama, H. Schagger, M. Schlame and M. L. Greenberg, *J. Biol. Chem.*, 2004, **279**, 42612.
7 J. B. McMillin and W. Dowhan, *Biochim. Biophys. Acta*, 2002, **1585**, 97.
8 G. M. Hatch, *Biochem. Cell Biol.*, 2004, **82**, 99.
9 G. F. Painter, J. W. Thuring, Z. Y. Lim, A. B. Holmes, P. T. Hawkins and L. R. Stephens, *J. Chem. Soc., Chem. Commun.*, 2001, 645.
10 Z.-Y. Lim, J. W. Thuring, A. B. Holmes, M. Manifava and N. T. Ktistakis, *J. Chem. Soc., Perkin Trans. 1*, 2002, 1067.
11 B. Catimel, M.-X. Yin, C. Schieber, M. Condron, H. Patsiouras, J. Catimel, D. E. J. E. Robinson, L. S.-M. Wong, E. C. Nice, A. B. Holmes and A. W. Burgess, *J. Proteome Res.*, 2009, **8**, 3712; B. Catimel, C. Schieber, M. Condron, H. Patsiouras, L. Connolly, J. Catimel, E. C. Nice, A. W. Burgess and A. B. Holmes, *J. Proteome Res.*, 2008, **7**, 5295–5313.
12 T. Exner, N. Sahman and B. Trudinger, *Biochem. Biophys. Res. Commun.*, 1988, **155**, 1001; V. Pengo and A. Biasiolo, *Thromb. Res.*, 1993, **72**, 423; S. P. Walton, S. S. Pierangeli, A. Campbell, E. Klein, B. Burchitt and E. N. Harris, *Lupus*, 1995, **4**, 263; A. B. Zborovsky, I. P. Gontar, S. V. Levkin, G. F. Sycheva and B. V. Zavodovsky, *Sov. Med.*, 1991, **6**, 20.
13 S. Krugman, K. E. Anderson, S. H. Ridley, N. Risso, A. McGregor, J. Coadwell, K. Davidson, A. Eguinoa, C. D. Ellson, P. Lipp, M. Manifava, N. T. Ktistakis, G. F. Painter, J. W. Thuring, M. A. Cooper, Z. Y. Lim, A. B. Holmes, S. K. Dove, R. H. Michell, A. Grewal, A. Nazarian, H. Erdjument-Bromage, P. Tempst, L. R. Stephens and P. T. Hawkins, *Mol. Cell*, 2002, **9**, 95.
14 N. S. Keddie, G. Bultynck, T. Luyten, A. M. Z. Slawin and S. J. Conway, *Tetrahedron: Asymmetry*, 2009, **20**, 857; D. Bello, T. Aslam, G. Bultynck, A. M. Z. Slawin, H. L. Roderick, M. D. Bootman and S. J. Conway, *J. Org. Chem.*, 2007, **72**, 5647; S. J. Conway, J. W. Thuring, S. Andreu, B. T. Kvinlaug, H. L. Roderick, M. D. Bootman and A. B. Holmes, *Aust. J. Chem.*, 2006, **59**, 887.
15 S. J. Conway and G. J. Miller, *Nat. Prod. Rep.*, 2007, **24**, 687.
16 G. F. Painter, S. J. A. Grove, I. H. Gilbert, A. B. Holmes, P. R. Raithby, M. L. Hill, P. T. Hawkins and L. R. Stephens, *J. Chem. Soc., Perkin Trans. 1*, 1999, 923.
17 Synthesised from phosphorus trichloride by the method of W. Bannwarth and A. Trzeciak, *Helv. Chim. Acta*, 1987, **70**, 175.
18 All new compounds were characterised by ^{1}H, ^{13}C, ^{31}P NMR spectroscopy, IR, mp and mass spectrometry.
19 As expected, two signals were observed in the ^{31}P NMR spectrum (δ_P –0.28 and –0.33 ppm), corresponding to the diastereomers arising from the two stereogenic phosphate ester groups. Only one signal is observed in the ^{31}P NMR of sodium salts **18** and **3**.
20 U. M. Krishna, M. U. Ahmad, S. M. Ali and I. Ahmad, *Lipids*, 2004, **39**, 595; M. U. Ahmad, U. M. Krishna, S. M. Ali, S. Choudhury and I. Ahmad, *Lipids*, 2007, **42**, 291.
21 W. Yu, J. Liu, N. A. Morrice and R. E. H. Wettenhall, *J. Biol. Chem.*, 1997, **272**, 10030.
22 R. Bamert, and R. E. H. Wettenhall, unpublished results.
23 D' Aléo, J.-L. Pozzo, K. Heuzé, F. Vögtle and F. Fages, *Tetrahedron*, 2007, **63**, 7482.
24 D. Qin, H.-S. Byun and R. Bittman, *J. Am. Chem. Soc.*, 1999, **121**, 662.
25 C. Vilchtze and R. Bittman, *J. Lipid, Res.*, 1994, **35**, 734.
26 K. Fukase, T. Matsumoto, N. Ito, T. Yoshimura, S. Kotani and S. Musumoto, *Bull. Chem. Soc. Jpn.*, 1992, **65**, 2643.