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Synthesis of unsaturated phosphatidylinositol 4-phosphates and the effects of substrate unsaturation on *Sop*B phosphatase activity†

Samuel Furse,[a,b] LokHang Mak,[b,c] Edward W. Tate,[a,b] Richard H. Templer,[a,b] Oscar Ces,[a,b] Rüdiger Woscholski*[a,b,c] and Piers R. J. Gaffney*[a,b]

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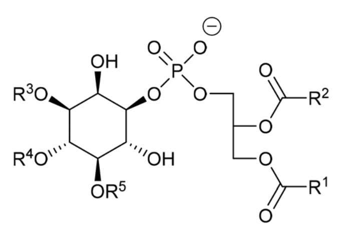
In this paper evidence is presented that the fatty acid component of an inositide substrate affects the kinetic parameters of the lipid phosphatase Salmonella Outer Protein B (*Sop*B). A succinct route was used to prepare the naturally occurring enantiomer of phosphatidylinositol 4-phosphate (PI-4-*P*) with saturated, as well as singly, triply and quadruply unsaturated, fatty acid esters, in four stages: (1) The enantiomers of 2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol were resolved by crystallisation of their di(acetylmandelate) diastereoisomers. (2) The resulting diol was phosphorylated regio-selectively exclusively on the 1-*O* using the new reagent tri(2-cyanoethyl)phosphite. (3) With the 4-OH still unprotected, the glyceride was coupled using phosphate *tri*-ester methodology. (4) A final phosphorylation of the 4-*O*, followed by global deprotection under basic then acidic conditions, provided PI-4-*P* bearing a range of *sn*-1-stearoyl, *sn*-2-stearoyl, -oleoyl, -γ-linolenoyl and arachidonoyl, glycerides. Enzymological studies showed that the introduction of *cis*-unsaturated bonds has a measurable influence on the activity (relative V_{max}) of *Sop*B. *Mono*-unsaturated PI-4-*P* exhibited a five-fold higher activity, with a two-fold higher K_M, over the saturated substrate, when presented in DOPC vesicles. *Poly*-unsaturated PI-4-*P* showed little further change with respect to the singly unsaturated species. This result, coupled with our previous report that saturated PI-4-*P* has much higher stored curvature elastic stress than PI, supports the hypothesis that the activity of inositide phosphatase *Sop*B has a physical role *in vivo*.

Introduction

Inositol phospholipids (inositides) are central to several essential metabolic processes, including insulin signal transduction,[1–3] protein translocation to membranes[4–6] and protein kinase activity.[7,8] In the last decade it has also become apparent that inositides have functions in biological systems beyond specific protein–lipid recognition.[9–12] For instance, there is evidence that there may be a physical role in cell division for phosphatidylinositol (PI, **1**, Fig. 1) because this inositide increases in concentration by an order of magnitude during cytokinesis in HeLa cells[13] but is not known to be directly involved in mammalian signalling systems.



R^1 = Alkyl , R^2 = Alkenyl

1 , PI, $R^3 = R^4 = R^5 = H$

2 , PI-4-*P* , $R^3 = R^5 = H$, $R^4 = OPO_3H^-$

3 , PI-4,5-P_2 , $R^3 = H$, $R^4 = R^5 = OPO_3H^-$

4 , PI-3,4,5-P_3 , $R^3 = R^4 = R^5 = OPO_3H^-$

Fig. 1 Inositide lipids.

Over many years the central role of inositides in intracellular signalling has sustained intense interest in the enzymes that produce and catabolise them.[7,14] Although less widely explored than the corresponding lipid kinases, the roles of

[a]Institute of Chemical Biology, Imperial College London, Exhibition Road, London SW7 2AZ, UK. E-mail: r.woscholski@imperial.ac.uk, p.gaffney@imperial.ac.uk
[b]Department of Chemistry, Imperial College London, Exhibition Road, London SW7 2AZ, UK
[c]Division of Molecular Biology, Imperial College London, South Kensington Campus, London SW7 2AZ, UK

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several of the endogenous phosphatases that mediate the metabolism of inositides in human disease have been characterised in studies of phosphatase inhibition by small molecules.[15,16]

Beyond human metabolism, exogenous phosphatases have been found to play a pivotal role in Salmonella infection.[17–19] Norris *et al*. showed that Salmonella virulence depended on secretion of an inositide 3-phosphatase, Salmonella outer protein B (*Sop*B), and hinted that this enzyme might also be a 4-phosphatase and thus dephosphorylates PI-4-*P* (**2**, Fig. 1).[20] More recent work has shown that *Sop*B exhibits measurable 4- and 5-phosphatase activity *in vitro*, although its activity on the most abundant phosphatidylinositol *bis*-phosphate, PI-4,5-P_2 (**3**, Fig. 1), was relatively low.[15]

The identification of *Sop*B as an inositide phosphatase raises the question of what the advantage to *Salmonella* might be in altering the host cell's inositide profile. It may be anticipated that the introduction of an inositide phosphatase could interfere with inositide-based signalling, since phosphatidylinositol *tris*-phosphate, (PI-3,4,5-P_3, **4**, Fig. 1) and other highly phosphorylated inositides are dephosphorylated by *Sop*B.[15,20,21] However, *in vivo* this phosphatase activity also includes modification of inositides without a direct signalling role, but which through their higher abundance could affect the overall properties of the membrane, *e.g.* PI-4-*P* (**2**). Recent evidence suggests that PI-4-*P*s impart much higher stored curvature elastic stress on membranes under physiological conditions than do PIs (**1**).[9,10,12]

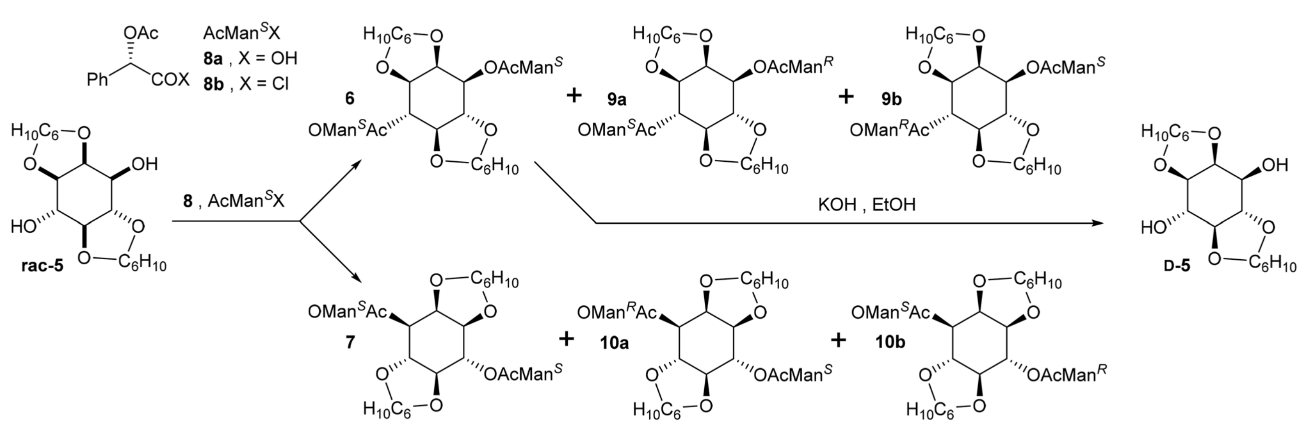
The alkyl fraction (hydrophobic, fatty acid tails) of lipid systems also has an important influence on membrane behaviour. Physical studies of the effect of unsaturated bonds on lipid systems have shown that as the number of olefinic bonds increases, the packing in the alkyl fraction changes such that the transition temperature between gel and fluid lamellar phases falls.[12,22,23] Naturally occurring PI-4-*P*s have a fatty acid profile with a wide variety of fatty acid residues having different numbers of olefin bonds per chain. As PI-4-*P*s are understood to be distributed homogenously at physiological abundance,[9,11,24] the local membrane environment around individual PI-4-*P* head groups may vary considerably. We therefore developed the hypothesis that the kinetic activity of *Sop*B on lipid substrates might be directed by a physical or topological influence of the substrate upon the membrane in which it is located, modulated by the number of double bonds it possesses.

We proposed to test this hypothesis using a relatively abundant phosphorylated inositide that does not have a direct signalling role. As inositides make up around 10% of the cellular phospholipids, with PI (**1**), PI-4-*P* (**2**) and phosphatidylinositol 4,5-*bis*-phosphate (PI-4,5-P_2, **3**), in order of decreasing abundance, typically representing around 90% of this fraction (Fig. 1),[25–28] the obvious candidate was PI-4-*P*. We therefore required a source of PI-4-*P* that allowed us to control the fatty acid profile.

Animal brain and liver tissues are common natural sources of inositides. However, inositides cannot be isolated from any natural source in significant quantities, and even when they have been obtained, (phospho)inositides with differing fatty acid profiles are not chromatographically separable. We therefore sought a synthetic strategy for preparing unsaturated PI-4-*P*s.

Synthetic strategies for phosphorylated inositides have to control not only the number and position of phosphate *mono*-esters around the *myo*-inositol ring, but also the number and position of olefinic bonds in the fatty acid residues of the glyceride moiety. This is particularly important in preparing the inositides of higher organisms, as these are almost unique amongst phospholipids in the high degree of unsaturation of the *sn*-2-fatty acid ester of the glyceride. However, few synthetic strategies address this point. The strategy described in this paper accounts for all features of naturally occurring phosphorylated inositides.

We chose to start from the established inositide building block 2,3:5,6-*O*-dicyclohexylidene *myo*-inositol, **rac-5** (Scheme 1).[29–31] This building block already exhibits much of the desired regio-control and is compatible with our previously developed synthetic strategy for unsaturated phosphorylated inositides. To advance **rac-5** to an unsaturated PI-4-*P* (**2**) three key manipulations were required: first, a reliable resolution of racemic inositol diol **rac-5**; second, a method to exert regio-



Scheme 1 The resolution of 2,3:5,6-*O*-dicyclohexylidene *myo*-inositol (**rac-5**) using acetylmandelate chiral auxiliary (**8**).

control over phosphorylation, installing two different phosphoryl moieties on the hydroxyls of D-5; third, a global deprotection strategy compatible with the functionality of the target, especially the fatty acid unsaturation. This approach seemed straightforward as diol **rac-5** has previously been resolved as its 1,4-di(*S*-acetylmandelates)[32] (**6** and **7**, Scheme 1).

In this report the syntheses of single enantiomers of PI-4-*P* with saturated, and one, three or four double bonds on the *sn*-2-fatty acid ester are described. We then describe the testing of our hypothesis using a kinetic assay to explore the influence of the glyceride portion of PI-4-*P* on *Sop*B activity. Two methods of substrate presentation (synthetic lipids in detergent micelles and lipid vesicles) were employed, and compared to *Sop*B activity against Ins 1,4-P_2 (the head group of PI-4-*P*).

Results and discussion

Resolution of 2,3:5,6-*O*-dicyclohexylidene *myo*-inositol, rac-5

Racemic 2,3:5,6-*O*-dicyclohexylidene *myo*-inositol (**rac-5**) was prepared directly from commercial *myo*-inositol on multi-gram scales (Scheme 1). A practical multi-gram-scale resolution, requiring no chromatography, was then sought. We found the di-camphanate and di-menthoxyacetate esters of **rac-5** to be inseparable by both crystallisation and chromatography. We therefore set out to apply the method of Sureshan *et al.* to append the acetylmandelate chiral auxiliary (**8**) because both they, and earlier Potter *et al.* with a related inositol building block, reported that one of the two di(*S*-acetylmandelates) could be purified by crystallisation, if only in low yield.[32,33]

Mandelic acid reacted cleanly with acetyl chloride to give crystalline acetylmandelic acid (AcMan^{S}OH, **8a**) with identical NMR and optical rotation to the published data. Carboxylic acid **8a** was converted to the acyl chloride (AcMan^{S}Cl, **8b**) by dissolution in oxalyl chloride at room temperature, before removal of the volatile components under vacuum. Acyl chloride **8b** was used without further purification; since these conditions required neither heating nor distillation, we regarded them as less likely to degrade the chirality of the reagent than the previously reported reaction with refluxing thionyl chloride. However, when racemic diol **rac-5** was treated with 2.5 eq. of *S*-acetylmandeloyl chloride (**8b**) in dichloromethane–pyridine (1 : 1) under the conditions reported by Sureshan *et al.*[32] several chromatographically inseparable acylated species formed in an overall isolated yield of *ca.* 75%. Due to the many similarities between the desired species and the by-products, the contaminants are thought to derive from racemisation of the mandelate stereo-centre. The proportion of racemisation of **9** and **10** was estimated from ^{1}H NMR by comparison of their integrals to those of the signals from **6** and **7**.

We suspected that if the racemisation could be reduced, then a greater yield of a single di-acetylmandelate would be isolated, and requiring fewer crystallisations. We therefore generated the acylating agent *in situ*, so that it would be consumed as it was generated, and thus have limited time to racemise.

Furthermore, assuming that the proton trap/catalyst participated in the racemisation, we selected species with a pK_a as low as possible. 2,6-Dichlorobenzoyl chloride (DcbCl) was added portion-wise to a mixture of inositol diol **rac-5** and acetylmandelic acid (**8a**) with *N*-methylimidazole (NMI, pK_a 7.00–7.06[34]) acting as both base and catalyst, in dichloromethane.[35] The proportion of racemisation fell substantially under these conditions. These benefits were enhanced both by increasing the reaction concentration 4-fold and reducing the temperature (5 °C) so that racemisation fell to almost negligible amounts.

The crude mixture of *bis*-acylated inositols **6** and **7** was crystallised three times from ethyl acetate and a mixture of petroleum spirit *and* cyclohexane (7 : 3). This returned virtually all of one diastereoisomer (**6**) in 48% yield (Fig. 2, upper trace); in contrast to previous reports[36] we observed that mixtures of ethyl acetate and petroleum spirit or hexanes alone removed traces of **9** and **10** and other reagent debris, but did not fractionate diastereoisomers **6** and **7**. The inositol diastereoisomer that remained in solution (**7**) was recovered from the mother liquor and also found to be virtually pure (Fig. 2, lower trace). The two di(acetylmendelate) diastereoisomers of diol **5** (Scheme 1) are clearly distinguishable by NMR spectroscopy (Fig. 2), contrary to previous reports,[32,36] with differences in the shift of the α-carbonyl protons, and of the resonances of the Ins 1- and 4-C*H*s of **6** and **7**.

The *S*-acetylmandelate auxiliary from **6** and **7** has previously been removed using a butylamine in methanol at reflux for 30 min,[36] but this also produces neutral amide by-products that must then be separated from the crude resolved diol (D-5). Instead, we used a large excess of ethanolic sodium hydroxide so that, after quenching with ammonium chloride, all reagent debris could be removed simply by aqueous partition and achieved an almost quantitative yield of diol D-5 requiring no further purification. Notably, air had to be excluded rigorously

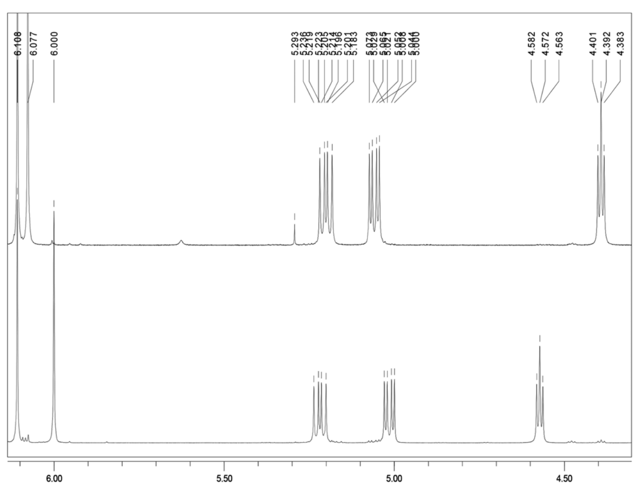
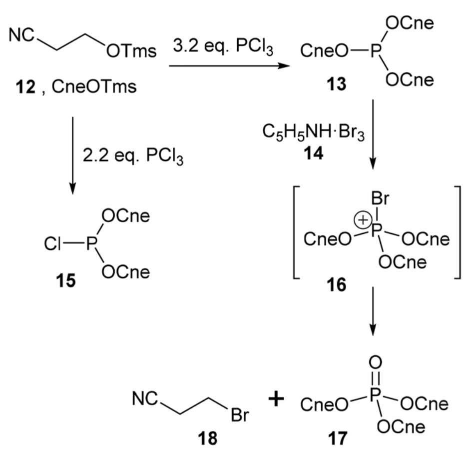


Fig. 2 ^{1}H NMR of the mandelate α-carbonyl C*H* (6.0–6.5 ppm) and the inositol 1- and 4-C*H*s (4.5–6.0 ppm) of **6** and **7**: upper trace, crystalline di(*S*-acetylmandelate) **6**; lower trace, amorphous di(*S*-acetylmandelate) **7** from mother liquor after crystallisation.

in order to avoid a large proportion of the saponification product partitioning into the aqueous phase; presumably this is due to reaction with atmospheric carbon dioxide.

Phosphorylation of 2,3:5,6-*O*-dicyclohexylidene *myo*-inositol, D-5

With the resolved diol **D-5** now readily available, the next step was to attach two different phosphoryl groups, the 1-*O*-phosphatidate and the 4-*O*-phosphate *mono*-ester (Scheme 3). Various inositols with a free 1-*OH* have undergone regio-selective phosphorylation with alkyl phosphites[37,38] but the resulting protected phosphate esters are not readily compatible with global deprotection of poly-unsaturated phosphorylated inositide precursors. Although a temporary protecting group could be used to differentiate the 1- and 4-*O*, it is more concise to phosphorylate diol **D-5** regio-selectively. The 1-*OH* is well known to be more reactive than the 4-*OH* due to an intra-molecular hydrogen bond to the 2-*O*. However, the product from direct attachment of phosphatidate[38,39] has a diasterotopic phosphate *tri*-ester centre (*e.g.* **25**), making it harder to assess its purity unambiguously compared with a single species. Also, the requisite glyceryl phosphoramidites are sensitive to hydrolysis and oxidation. Additionally, if the phosphorylation reaction gave a mixture of regio-isomers, four *mono*-phosphorylated products would form instead of two, resulting from two phosphorus-centred diastereoisomers for each regio-isomer, making product fractionation more difficult. Thus, we elected first to phosphorylate the inositol building block (to give *myo*-inositol 1-phosphate, **11**) before appending the glyceride. Notably, in this strategy the coupling of valuable inositol and glyceride moieties (Scheme 1) is not a regio-selective reaction. Thus a smaller excess of the non-limiting component can be used under forcing conditions, minimising the excess of both building blocks, to provide a valuable saving in intermediates that require several steps to prepare.[38] In preparing **11** regio-selectively, we made use of a novel phosphorylating agent tri(2-cyanoethyl)phosphite [$(CneO)_3P$, **13**, Scheme 2]. This phosphite was prepared from the reaction of 3.2 equivalents of 3-trimethylsilyloxy propionitrile (**12**) with PCl_3; this is a modification of the simple preparation of the phosphitylating agent di(2-cyanoethyl) phosphorochloridite (**15**).[40] Although tri(2-cyanoethyl)phosphite (**13**) cannot be distilled reliably, the crude material can be used without further purification because the small excess of **12** can be evaporated under high vacuum, and the desired reagent stored indefinitely under nitrogen and in solution (DCM).



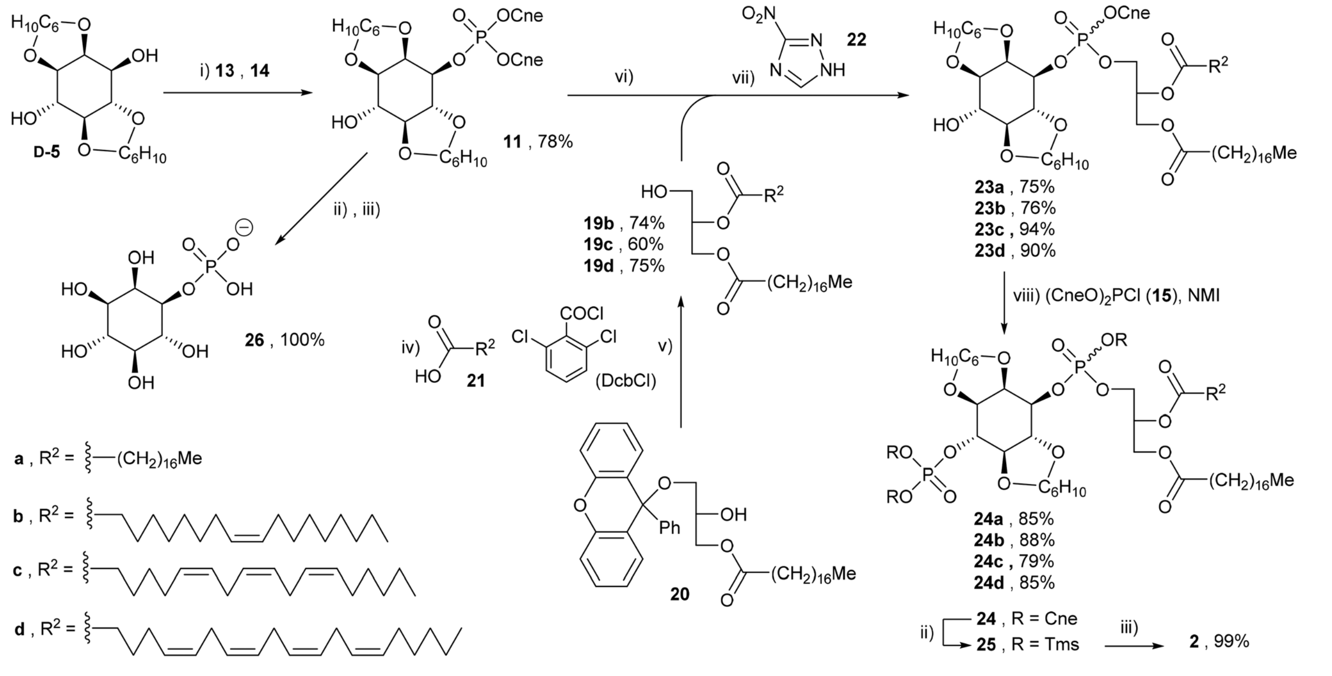
Scheme 2 Preparation of phosphorus reagents.

Treatment of two equivalents of the resolved diol (**D-5**) with $(CneO)_3P$ (**13**) in dichloromethane–pyridine with the mild oxidising agent pyridinium bromide perbromide (**14**) at −35 °C gave the desired 1-*O*-phospho *tri*-ester **11** cleanly as the only product in 80% yield with recovery of all of the excess unphosphorylated diol **D-5** (Scheme 3). In the ^{1}H NMR of **11** all the resonances of each of the inositol ring protons were fully resolved, allowing full assignment of the spectrum. However, to verify that the phosphorylation of inositol diol **rac-5** with $(CneO)_3P$ (**13**) had given the desired regio-selectivity unambiguously, the resulting 1-*O*-phosphate was deprotected fully using our basic-then-acidic two-step procedure. The chair conformation of the resulting inositol 1-phosphate (**26**) is no longer distorted by the ring-fusions of the two cyclic acetals in **11** and so it is possible to determine the substitution pattern of this compound precisely by inspection of its 1D ^{1}H NMR. These data confirmed our earlier assumed structure (see ESI†).

We are uncertain of the mechanism of this phosphorylation, although it is established that the initial phosphite activation will proceed *via* formation of the corresponding bromophosphonium bromide (**16**, Scheme 2).[41–43] However, it is notable that after addition of a slight excess of pyridinium bromide perbromide to a mixture of $(CneO)_3P$ (**13**) with 1 eq. 3-hydroxypropionitrile the ^{1}H NMR in $C_5D_5N–D_3CCN$ exhibited, in addition to peaks for tri(2-cyanoethyl)phosphate (**19**), new resonances at δ_H 3.59 (t, J = 6.1 Hz) and 3.09 (t, J = 6.2 Hz) ppm, assumed to be 3-bromo propionitrile (**18**), but no alkene resonances for acrylonitrile (see ESI† for spectra). Therefore this Arbuzov reaction is assumed to proceed with nucleophilic attack on the cyanoethyl protecting group, not the elimination of acrylonitrile.

Preparation of protected PIs and deprotection of PI-4-*P* precursors

sn-1,2-Distearoyl glycerol (**19a**) was prepared from *sn*-3-benzyl glycerol and stearic acid using established manipulations. The *sn*-2-unsaturated diglycerides (**19b–d**) were prepared from *sn*-1-stearoyl-3-(9-phenylxanthen-9-yl) glycerol (**20**), by condensing excess fatty acid [oleic acid (**21b**), γ-linolenoic acid (**21c**), or arachidonic acid (**21d**)] with the 2-*OH* using DcbCl and NMI,[35,44] followed by acid catalysed deprotection of the 3-OH in the presence of pyrrole[45] to force the reaction to completion (Scheme 3). The 1-*O*-phosphate of inositol building block **11** was partially deprotected over night with triethylamine–MeCN. Condensation between the resulting inositol *mono*-cyanoethyl phosphate salt and excess diglyceride (**19**) was effected by mesitylenesulfonyl chloride and 3-nitro-1,2,4-triazole (**22**, generating MSNT *in situ*)[46] and required no protection of the 4-*OH* (Scheme 3).



Scheme 3 Preparation of phosphatidylinositol 4-phosphate isoforms (**2**). Reagents and conditions: (i) **13**, CH_2Cl_2–C_5H_5N, $C_5H_5NH·Br_3$ (**14**), −35 °C; (ii) $(Me_2N)_2C{=}Nt$-Bu, TmsCl, CH_2Cl_2–MeCN, 16 h; (iii) AcOH–water, 24 h; (iv) **21**, DcbCl, NMI, CH_2Cl_2, 16 h; (v) $Cl_2CHCOOH$, pyrrole, CH_2Cl_2, 2 min; (vi) Et_3N–CH_2Cl_2–MeCN, 36 h; (vii) 1,3,5-Me_3–$C_6H_2SO_2Cl$, **22**, CH_2Cl_2–MeCN–C_5H_5N, 25 min; (viii) **15**, NMI, CH_2Cl_2–C_5H_5N, 16 h then CneOH then *tert*-BuO_2H.

Protected phosphatidylinositol **23** may be unblocked to give the parent PI (**1**), but in order to produce PI-4-*P*s the installation of a phosphate group on the 4-OH was required. Treatment of **23** with dicyanoethyl phosphorochloridite (**15**), followed by oxidation of the intermediate phosphite *tri*-ester with *tert*-butyl hydroperoxide, provided fully protected PI-4-*P* precursor **24**. A careful two-step purification was performed, first using reverse phase fractionation through a column of silanised silica to remove phosphorylating reagent debris, then normal phase chromatography to remove any traces of lipid-related contaminants. In this way, so long as the final unblocking causes no decomposition of the lipid, then the resulting PI-4-*P* (**2**), which is very awkward to fractionate, requires only simple work-up to purify it completely from other uncharged organic debris.

Global deprotection of **24** was effected by initial treatment with *N*,*N*,*N*′,*N*′-tetramethyl-*N*″-*tert*-butyl-guanidine (Barton's base) and trimethylsilyl chloride to exchange cyanoethyl for *tri*-methylsilyl phosphate esters. Excess reagents and protecting group (acrylonitrile) were removed *in vacuo* whilst chloride salts were separated by trituration with trimethylsilyl chloride–petroleum spirit (1 : 19 v/v). The distinctive up-field shift and sharp signals in the ^{31}P NMR of the silyl phosphate esters of intermediate **25** were used to verify complete ester exchange prior to completing the deprotection. The silyl esters were then cleaved rapidly by methanolysis, and the acetal protecting groups were hydrolysed with aqueous acetic acid to give the crude lipid, requiring only trituration in MeCN and diethyl ether to give pure PI-4-*P* (**2**) in quantitative yield.

Self-assembly of amphiphilic species such as phospholipids **14** in solution makes acquisition of NMR data difficult. NMR solvent systems based on DMF, designed for mono-dispersion of high concentrations of lipid(s)[27] were unsuitable for our purposes as they introduced non-volatile impurities. Instead, other trace solvents and water were driven off by initial co-evaporation of the lipids several times *in vacuo* from $CDCl_3$ and CD_3OD. NMR spectra were then acquired in a mixture of $CDCl_3$ and CD_3OD, in which both the hydrophobic and hydrophilic regions of the molecule are solvated, allowing it to become fully mono-dispersed in solution, giving sharper signals.

PI-4-*P*s as substrates for *Sop*B

We had hypothesised that the kinetic activity of *Sop*B on lipid substrates might be directed by a physical influence of the substrate upon the membrane in which it is located, modulated by the number of double bonds it possessed. This was tested using our synthetic single enantiomers of naturally occurring PI-4-*P* **2a–c**. Only the three *sn*-2-C_{18} (*i.e.* saturated, singly and triply unsaturated) isoforms of PI-4-*P* were examined as the longer length of the arachidonoyl fatty acid ester (C_{20}, **2d**) would confound interpretation of the kinetic results. The colorimetric malachite green 'endpoint' assay was used to determine the concentration of phosphate liberated from PI-4-*P* by *Sop*B-mediated hydrolysis, *i.e.* PI-4-*P* (**2**) → PI (**1**) + P_i. PI-4-*P*s were presented in detergent (*n*-octyl-β-D-glucopyranoside, OGPS) micelles or *sn*-1,2-dioleoyl phosphatidylcholine (DOPC) vesicles.[15] It was noted that the presence of OGPS caused a slight, systematic increase in the background optical density.

Table 1 The kinetic parameters of *Sop*B in detergent-based (OGPS) micelles and lipid-based (DOPC) vesicles with PI-4-*P* substrates **2a–c**, varying the fatty acids of the glyceride. Substrate concentrations 0–200 μM (micelles) and 0–120 μM. (vesicles) with an assay time of 20 min and 1 μg *Sop*B per measurement, n = 3. Activity calculated as a percentage of the V_{max} observed for either substrate presentation

PI-4-*P* substrate	Activity (% V_{max})		K_M/μM	
	Micelles	Vesicles	Micelles	Vesicles
2a, $C_{18:0}$,$C_{18:0}$	64.8 (±10.9)	19.0 (±9.1)	18.2 (±6.5)	13.1 (±3.6)
2b, $C_{18:0}$,$C_{18:1}$	98.6 (±8.6)	100.0 (±6.9)	26.1 (±4.9)	28.6 (±6.4)
2c, $C_{18:0}$,$C_{18:3}$	100.0 (±7.0)	81.0 (±10.6)	50.6 (±10.7)	25.7 (±7.3)

The kinetic properties of the lipid substrates were compared by calculation of their activity, defined as the relative V_{max}, with the substrate of the highest activity designated 100% (Table 1).

There was significant phosphatase activity against each synthetic PI-4-*P* (Table 1), but no activity on *myo*-inositol 1,4-diphosphate (see ESI Table 1†). Therefore the diacylglyceryl moiety must be essential for the phosphatase activity of this enzyme. As it is energetically unfavourable for the diglyceride to leave the membrane, we probed the effects of the structure of the lipid membrane anchor by varying the saturation of the fatty acid chains. We observed the same increase in activity when one double bond was introduced with both micelles and vesicles (Table 1). On moving from saturated to mono-unsaturated substrates, the slight increase in K_M in detergent micelles was not significant, but there was a significant two-fold increase in K_M for vesicle presentation. The introduction of two additional double bonds gave rise to a further two-fold increase in K_M in detergent micelles, but not in vesicles.

These data indicate that one double bond is enough to change the kinetic parameters of *Sop*B, regardless of the presentation of the lipids. In turn, this means that *Sop*B responds to the single olefin bond, either directly or indirectly. We suggest that the latter could be due to a difference in the physical properties of the micelles and vesicles.

There is much evidence that the addition of an unsaturated component to a lipid assembly with a saturated alkyl fraction gives rise to a considerable change in packing.[22,23] Physical studies have shown that the principle difference in physical behaviour between stearoyl, oleoyl and γ-linoleoyl residues in lipids is the transition temperatures for the gel-to-fluid transition of hydrated lipid systems that is the result of the presence of the double bonds.[22,23] Additionally, at the concentrations of PI-4-*P* used here (2% PI-4-*P*, *p*H 7.4, 5 mM Mg^{++}), which are similar to physiological values (in fact PI-4-*P* concentration can be even higher), the inositide head groups represent about 1 in 50 of those present. This means they are not in direct contact with one another but some distance apart.[9,11,24] This suggests that the influence of PI-4-*P* on its local environment may be a consequence of its molecular structure alone and not of interactions between individual PI-4-*P* molecules.

It is possible that the difference between the kinetics of the substrates with *Sop*B may therefore be a result of the difference in fluidity of the self-assembled systems in which the substrates are located, with a more fluid system giving rise to lower enzyme activity. It is noteworthy that this difference between inositide substrates is the result of one fatty acid residue only, and that the difference between these is solely the number of double bonds, and thus the packing of the alkyl fraction of the membrane.

Conclusions

In this paper we have described the preparation of single enantiomers of saturated and unsaturated PI-4-*P* (**2**), and used them to characterise the kinetic behaviour of a phosphatase essential in the mechanism of *Salmonella* infection.

A novel synthetic strategy, developed for its versatility, was used to produce *poly*-unsaturated PI-4-*P*. For its success it depended on global deprotection conditions that have already been demonstrated to be compatible with redox-sensitive *poly*-unsaturated phosphorylated inositides indistinguishable from those found in nature.[35,47,48] This preparation of unsaturated PI-4-*P* started from a reliable and scalable resolution of the widely used building block 2,3:5,6-*O*-dicyclohexylidene inositol (**rac-5**), *via* separation of its diastereomeric acetylmandelates by crystallisation, to provide almost quantitative yields of both enantiomeric inositol diols (D-**5** and L-**5**). This procedure is a significant improvement on previous reports of this process as we have minimised racemisation of the chiral auxiliary during the critical esterification reaction. Regio-selective phosphorylation of D-**4** on the 1-*O* was effected using the novel phosphorylating agent tricyanoethyl phosphite (**13**), which is prepared easily and may be stored for protracted periods. The resulting inositol 1-phosphate (**11**) was then coupled to four different glycerides, without the need to protect the 4-OH. Finally, the 4-OH was phosphorylated with dicyanoethyl phosphorochloridite (**15**) and the fully protected PI-4-*P* phospholipids unblocked in a mild two-step, first basic then acidic, global deprotection procedure that is compatible with *poly*-unsaturation of the *sn*-2-fatty acid ester.

We report for the first time that the activity of *Sop*B is reliant upon the presence and type of glyceride in the lipid substrate. When presented in detergent micelles, as the degree of unsaturation of the synthetic PI-4-*P* lipids increases they become poorer substrates (two-fold increase in K_M). Vesicular presentation shows the same trend but with less differentiation between unsaturated substrates. The molecular differences between the synthetically prepared PI-4-*P*s reside in only a single fatty acid chain (*sn*-2). We note that as the number of unsaturated bonds in fatty acid residues is associated with a decrease in the temperature of the phase transition between crystal or gel lamellar and fluid lamellar,[22,23] the more unsaturated lipids, the more fluid the bilayer becomes. We therefore conclude that differences in packing of the alkyl fraction near the substrate are responsible for the observed differences in

*Sop*B activity. Furthermore, we assert that the greater fluidity of the membrane around the substrate restricts enzyme activity in a concentration-dependent manner.

The difference in phase behaviour of PI and PI-4-*P* inositides are beginning to be understood[12] and have far-reaching implications for interpreting the phosphatase activity of *Sop*B. It is clear that saturated PI-4-*P* self-assembles into a phase with pronounced negative curvature at physiological concentrations under model physiological conditions.[9] By contrast, the product of phosphatase action, PI, does not drive the formation of negatively-curved phases under similar conditions in this concentration range.[10] This implies that the phosphatase activity of *Sop*B reduces the stored curvature elastic stress in membranes by reducing the concentration of PI-4-*P* and increasing that of PI.

The evidence from this study supports the hypothesis that the fluidity of the membrane adjacent to its substrate influences the kinetics of a soluble inositide phosphatase. The activity of *Sop*B on inositide 4-phosphatates provides a valuable insight into the mode of action of externally-mediated, disease-based changes in inositide signalling and the physical properties of membranes in which they occur. The results presented here also demonstrate that there is a strong physical aspect to *Sop*B-inositide interactions.

Experimental

Reagents

All solvents used were HPLC grade and bought from Sigma Aldrich Ltd (Gillingham, Dorset, UK). Reactions were typically carried out under anhydrous conditions with a nitrogen atmosphere. C_5H_5N, CH_2Cl_2, MeCN, *N*-methylimidazole and triethylamine were distilled from calcium hydride; THF and diethyl ether were distilled from sodium metal and benzophenone; all, except triethylamine, were stored over 4 Å molecular sieves. Phosphorus trichloride and trimethylsilyl chloride were distilled before use. Flash chromatography was carried out using silica from British Drug Houses for normal phase, and silanised silica gel 60 from Merck for reverse phase. Thin layer chromatography was carried out using Merck silica gel 60 F_{254} glass-backed plates. TLC plates of inositol derivatives were stained with *p*-anisaldehyde, glyceride derivatives stained with $KMnO_4$. COSY spectra were used to assign ^{1}H signals, with DEPT and HSQC used to assign carbon signals. Growth broths and consumables for the preparation of *Sop*B were purchased from Fisher Ltd (Loughborough, Leicestershire, UK) and Bio-Rad (Hemel Hempstead, Hertfordshire, UK). *n*-Octyl-β-D-glucopyranoside was purchased from Calibiochem (Beeston, Nottinghamshire, UK).

Protein preparation

*Sop*B was produced using IPTG-mediated over-expression in DH5α *E. coli* bacteria as described before.[15] The N-terminal GST tag of the full-length enzyme was used to affinity purify the protein. The GST tag was not removed in order to preserve activity of the enzyme. Protein concentration was determined using Bradford's assay, with BSA (Thermo Scientific BSA standard, Product number 23209; 2.0 mg mL^{-1} in saline, supplemented with sodium azide) for calibration. Bradford's reagent was purchased from Sigma and used as directed.

*Sop*B kinetics assays

The colorimetric malachite green endpoint assay was used to determine the concentration of released inorganic phosphate (P_i) after exposure of PI-4-*P* to *Sop*B. Control samples were used to determine the non-enzymatic contribution to [P_i]. In control samples, enzyme was added after stopping the reaction with the malachite green reagent (enzyme dead control).

Micelles were prepared by sonication (12 min, bench top sonicator, Branson 1200) of a known mass of the substrate suspended in a solution of OGPS (stock concentration 4% v/v) and then freeze–thawing (5 × 40 °C to −20 °C). Vesicles were prepared by dissolving the appropriate masses of PI-4-*P* and DOPC (2 : 98 mole/mole) from stocks in chloroform–methanol–water (70 : 30 : 1) that were then dried down together before re-suspension in 200 mM Tris. The re-suspended material was allowed to hydrate for 16 h before freeze–thawing (5 × 40 °C to −20 °C) to give vesicles of consistent size.[49]

Incubation conditions were optimised such that values for V_{max} were within the linearity range of stock malachite green reagent solutions with known concentrations of sodium phosphate (Ordinate: intensity of emission at 625 nm. Abscissa: phosphate concentration, range 200–1700 pmol over a range of optical density of 0.05–0.70). Final conditions: 4 mM Mg^{++}, 200 mM Tris, pH 7.4, 20 min at 37 °C, 1 μg *Sop*B per well (OGPS micelles) or 0.1–0.2 μg per well (DOPC vesicles). Assay volume: 80 μL per well. Controls were carried out by incubation of substrate with magnesium ions and Tris base. Final concentration of OGPS/DOPC was 0.25%; this is the critical micelle concentration for OGPS and gave the highest activity for concentrations of OGPS in the range 0–2% (v/v). Results are calculated from the experimental samples (n = 3) less the average of the duplicate controls, with error = +/− standard deviation. The activity of the enzyme was assumed to follow standard Michaelis–Menten kinetics and was calculated using Grafit (courtesy of Professor Robin Leatherbarrow and Erithacus Software). An example of this fitting is shown in the ESI.†

Nuclear magnetic resonance

Chemical shifts (δ) are expressed in parts per million (ppm), and are referenced with respect to residual solvent signals, ^{1}H NMR δ_H ($CHCl_3$) 7.25, ^{1}H NMR δ_H (DMSO) 2.50, ^{13}C NMR δ_C ($CHCl_3$) 77.50, ^{13}C NMR δ_C (DMSO) 39.43, or an external reference, ^{31}P NMR δ_P (H_3PO_4) 0.00 ppm.

Organic synthesis

1,4-*O*-Di(*S*-acetylmandelyl)-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol, 6, and 3,6-*O*-di(*S*-acetylmandelyl)-1,2:4,5-*O*-dicyclohexylidene-*myo*-inositol, 7. Racemic 2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**rac-5**, 2.00 g, 5.88 mmol) and *S*-acetyl mandelic acid (**8a**, 3.422 g, 17.6 mmol, 3.0 eq.) were evaporated from MeCN (3 × 4 mL). The residue was re-dissolved in CH_2Cl_2 (15 mL) and *N*-methyl imidazole (4.68 mL, 58.8 mmol, 10.0 eq.) was added. The stirred mixture was cooled to 0 °C after which portions of 2,6-dichlorobenzoyl chloride (10 × 210 μL, 14.7 mmol, 2.5 eq.) were added three minutes apart. After stirring for a further 20 min, water (2 mL) then diethyl ether (400 mL) were added. The solution was washed with water (2 × 100 mL), dried (Na_2SO_4), and the solvent evaporated *in vacuo* to leave an off-white solid (5.0 g). This was dissolved in ethyl acetate and an equal volume of a mixture of hexane–cyclohexane (7 : 3, v/v) was added. The solution was cooled to 5 °C for 48 h whereupon white crystals formed. Three successive crystallisations afforded 1,4-*O*-di(*S*-acetylmandelyl)-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**6**) in 48% yield. R_f (EtOAc) 0.83; $[\alpha]_D^{25}$ +36.65° (*c* 4.70, CH_2Cl_2); δ_H (400 MHz, $CDCl_3$ with 0.01 M triethylamine) 7.50–7.47 (2H, m), 7.45–7.42 (2H, m), 7.35–7.32 (6H, m) (10 × Ph ***H***), 6.09 (1H, s), 6.06 (1H, s) (2 × α-C***H***), 5.18 (1H, dd, *J* 6.9, 11.2, Ins 4-***H***), 5.04 (1H, dd, *J* 4.3, 10.5, Ins 1-***H***), 4.37 (1H, t, *J* 4.6, Ins 2-***H***), 4.06 (1H, t, *J* 10.0, Ins 6-***H***), 3.80 (1H, dd, *J* 4.9, 6.7, Ins 3-***H***), 3.43 (1H, dd, *J* 9.6, 11.0, Ins 5-***H***), 2.17 (3H, s), 2.14 (3H, s) (2 × C***H₃***), 1.69–1.12 (20H, m, 10 × cyclohexyl C***H₂***); δ_C (125 MHz, $CDCl_3$ with 0.01 M triethylamine) 170.1, 169.9, 168.1, 167.8 (4 × ***C***=O), 134.0, 133.5, (2 × Ph ***C***), 129.20, 129.10, 128.7 (2C), 128.6 (2C), 128.2 (2C), 127.8 (2C) (10 × Ph ***C***H), 113.9, 111.0 (2 × acetal ***C***), 78.6 (Ins 3-***C***H), 76.0 (Ins 4-***C***H), 75.2 (Ins 5-***C***H), 74.4 (Ins 2-***C***H), 74.2 (2C, 2 × **α-*C***H), 74.1 (Ins 6-***C***H), 71.8 (Ins 1-***C***H), 37.2, 36.30, 36.20, 34.6, 24.9, 24.7, 23.62 (2C), 23.57, 23.2 (10 × cyclohexyl ***C***H₂), 20.73, 20.66 (2 × ***C***H₃); HRMS (ESI+) *m*/*z* found $[M + H]^+$ = 693.2911, $C_{38}H_{45}O_{12}$ requires 693.2900.

After the more crystalline diastereoisomer had been collected, 3,6-*O*-di(*S*-acetylmandelyl)-1,2:4,5-*O*-dicyclohexylidene-*myo*-inositol (**7**) was isolated by evaporation *in vacuo* of the mother liquor that remained to leave a white solid (46%). R_f (EtOAc) 0.83; $[\alpha]_D^{25}$ +56.98° (*c* 2.65, CH_2Cl_2); δ_H (400 MHz, $CDCl_3$ with 0.01 M triethylamine) 7.53–7.50 (2H, m), 7.46–7.43 (2H, m), 7.37–7.33 (6H, m) (10 × Ph C***H***), 6.11 (1H, s), 6.00 (1H, s) (2 × **α-C*H***), 5.22 (1H, dd, *J* 6.8, 11.1, Ins 6-C***H***), 5.01 (1H, dd, *J* 4.3, 10.6, Ins 3-C***H***), 4.57 (1H, t, *J* 4.6, Ins 2-C***H***), 4.14 (1H, dd, *J* 4.9, 6.8, Ins 1-C***H***), 4.05 (1H, dd, *J* 9.7, 10.4, Ins 4-C***H***), 3.24 (1H, dd, *J* 9.5, 11.1, Ins 5-C***H***), 2.18 (3H, s), 2.17 (3H, s) (2 × C***H₃***), 1.78–1.33 (20H, m, 10 × cyclohexyl C***H₂***); δ_C (125 MHz, $CDCl_3$ with 0.01 M triethylamine) 170.2, 169.9, 168.3, 167.7 (4 × ***C***=O), 134.0, 133.3 (2 × Ph ***C***), 129.2, 129.1, 128.7 (2C), 128.6 (2C), 128.1 (2C), 127.8 (2C) (10 × Ph ***C***H), 113.4, 111.5 (2 × acetal ***C***), 78.3, 76.1, 75.7, 74.6 (2C), 74.1 (2C), 71.8 [(6 × Ins ***C***H) + (2 × α-***C***H)], 37.4, 36.1, 36.0, 34.8, 24.8 (2C), 23.8, 23.5, 23.4, 23.4 (10 × cyclohexyl ***C***H₂), 20.7, 20.6 (2 × ***C***H₃); HRMS (ESI+) *m*/*z* found $[M + H]^+$ = 693.2911, $C_{38}H_{45}O_{12}$ requires 693.2927.

(+)-2,3:5,6-*O*-Dicyclohexylidene-*myo*-inositol, D-5 from 6. 1,4-*O*-Di(*S*-acetylmandelyl)-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**6**, 1.39 g, 2.00 mmol) and potassium hydroxide (3.36 g, 60.0 mmol, 30 eq.) were dissolved in ethanol (96%, 100 mL). After stirring for 2 h, ammonium chloride (3.21 g, 60.0 mmol, 30 eq.) then diethyl ether (300 mL) were added. This was extracted with water (3 × 1 L), dried (Na_2SO_4), and the solvent removed *in vacuo* to give a colourless foam-gum (620 mg, 91%). R_f (diethyl ether–MeOH, 9 : 1) 0.60; $[\alpha]_D^{25}$ +16.12° (*c* 2.74, CH_2Cl_2); ^1H NMR and ^{13}C NMR were indistinguishable from those of the racemic mixture; HRMS (ESI+) *m*/*z* found $[M + H]^+$ = 341.1956, $C_{18}H_{29}O_6$ requires 341.1964.

Tricyanoethyl phosphite, 13. Cyanoethyloxytrimethylsilane (**12**, 24.0 mL, 151 mmol, 3.2 eq.) was placed in a 100 mL bulb sealed with a fitted PTFE tap. To this were added phosphorus trichloride (4.00 mL, 45.9 mmol) and MeCN (40 mL). After stirring for 72 h the volatile components were removed *in vacuo* (oil pump, 60 °C) to leave a colourless, viscous oil (11.0 g) containing *ca.* 92 mol% of the desired product by ^{31}P NMR. This was used without further purification and may be stored for several months in the same flask under nitrogen. δ_H (400 MHz, $CDCl_3$) 3.95 (6H, q, *J* 6.4, 3 × POC***H₂***), 2.57 (6H, t, *J* 5.9, 3 × C***H₂***CN); δ_P (202 MHz, $CDCl_3$) 139.0; δ_C (125 MHz, $CDCl_3$) 119.2 (3 × ***C***N), 58.1 (d, *J* 11.0, 3 × PO***C***H₂), 20.11 (d, J_{P-C} 4.6, 3 × ***C***H₂CN); HRMS (ESI+) *m*/*z* found $[M + Na]^+$ = 264.0513, $C_9H_{12}O_3N_3PNa$ requires 264.0514.

(−)-1-*O*-(Dicyanoethyloxy)phosphoryl-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol, 11. (+)-2,3:5,6-*O*-Dicyclohexylidene-*myo*-inositol (D-**5**, 1.00 g, 2.94 mmol, 1.7 eq.) was evaporated from MeCN (3 × 2 mL), dissolved in CH_2Cl_2–pyridine (9 : 1, 30.0 mL) and tricyanoethyl phosphite (424 mg, 1.76 mmol) was added. Once cooled to −40 °C using a MeCN-dry ice slush bath, pyridinium bromide perbromide (90% tech. grade, 702 mg, 2.06 mmol) was added and the mixture stirred for 3 h. On reaching −20 °C water (10 mL) was added, then ethyl acetate (100 mL). The organic layer was washed with water (3 × 500 mL), dried (Na_2SO_4), and the solvent removed *in vacuo* to leave a foam (1.3 g). The crude material was adsorbed onto silica and fractionated by flash chromatography using a gradient of diethyl ether–methanol (1 : 0–7 : 3, v/v), to afford starting material **d-5** (220 mg), and the title compound as a white foam/gum (600 mg, 78%). R_f (diethyl ether–methanol 9 : 1 v/v) 0.40; $[\alpha]_D^{25}$ −12.68° (*c* 1.98, CH_2Cl_2); δ_H (400 MHz, d_6-DMSO) 5.62 (1H, d, *J* 5.3, Ins 4-O***H***), 4.84 (1H, ddd, *J* 4.4, 8.3, 10.2, Ins 1-C***H***), 4.47 (1H, t, *J* 4.7, Ins 2-C***H***), 4.30–4.20 (4H, m, 2 × OC***H₂***CH₂CN), 4.00 (1H, dd, *J* 5.1, 6.3, Ins 3-C***H***), 3.85 (1H, t, *J* 9.8, Ins 6-C***H***), 3.60 (1H, dt, *J* 5.2, 11.2, Ins 4-C***H***), 3.46 (1H, t, *J* 10.0, Ins 5-C***H***), 3.00 (4H, m, 2 × C***H₂***CN), 1.75–1.20 (20H, m, 10 × cyclohexyl C***H₂***); δ_P (162 MHz, d_6-DMSO) −3.20; δ_C (125 MHz, d_6-DMSO) 118.54, 118.50 (2 × ***C***N), 113.0, 112.3 (2 × acetal ***C***), 82.2, 77.9, 76.1, 75.4, 73.8 (2C), (6 × Ins ***C***H), 63.1, 62.9 (2 × O***C***H₂CH₂CN), 37.8, 36.4, 36.3 (2C), 35.2, 24.9 (2C), 24.1, 23.9 (2C) (10 × cyclohexyl ***C***H₂), 19.9 (2 × CH₂***C***H₂CN); HRMS (ESI+) *m*/*z* found $[M + H]^+$ = 527.2141, $C_{24}H_{36}O_9N_2P$ requires 527.2158.

***myo*-Inositol-1-phosphate, 26.** 1-*O*-Di(2-cyanoethyloxy)phosphoryl-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**11**, 112 mg, 0.213 mmol) was dissolved in MeCN–CH_2Cl_2 (1 : 1, 2 mL) and TmsCl (156 μL, 6.0 eq.) was added, followed by *N*,*N*,*N*′,*N*′-tetramethyl-*N*″-*tert*-butylguanidine (Barton's base, 86 μL, 5.0 eq.) and the mixture was stirred for 16 h. The solution was evaporated *in vacuo* (oil pump) and the residue triturated with pet.

spirit–TmsCl (9 : 1) under nitrogen; a single signal at δ_P −19.0 confirmed complete exchange of the cyanoethyl phosphate esters. The filtrate was evaporated to dryness and the residue stirred in 1 M methanolic ammonia (5 mL) for 20 min. The solvents were again evaporated *in vacuo*, and the residue taken up in acetic acid–water (2 : 3, 3 mL). After stirring for 24 h, the solution was diluted with water and freeze-dried to give a white solid (60 mg, 102% assuming mono-ammonium salt). δ_H (400 MHz, D_2O) 4.13 (1H, t, *J* 2.7, Ins 2-C***H***), 3.82 (1H, dt, *J* 2.7, 9.1, Ins 1-C***H***), 3.63 (1H, t, *J* 9.6, Ins 6-C***H***), 3.53 (1H, t, *J* 9.6, Ins 4-C***H***), 3.45 (1H, dd, *J* 2.8, 10.0, Ins 3-C***H***), 3.22 (1H, t, *J* 9.3, Ins 5-***H***); δ_P (162 MHz, D_2O) 0.43; δ_C (125 MHz, D_2O) 75.2 (d, *J* 4.2), 74.0, 72.2, 71.7 (d, *J* 4.2), 71.3, 70.7; HRMS (ESI−) *m/z* found $[M - H]^-$ = 259.0210, $C_6H_{12}O_9P$ requires 259.0219.

1-*O*-[(Cyanoethyloxy)(*sn*-1-*O*-stearoyl-2-*O*-arachidonoylglyceryloxy)phosphoryl]-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol, 23d. To 1-*O*-(dicyanoethyloxyphosphoryl)-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**11**, 203 mg, 0.354 mmol) were added CH_2Cl_2 (3 mL), MeCN (1 mL) and triethylamine (3 mL). After stirring the solution for 36 h, the solvent was removed *in vacuo* to give the putative phosphodiester salt as a white solid (169 mg). To this were added *sn*-1-*O*-stearoyl-2-*O*-arachidonoylglycerol (**19d**, 683 mg, 1.06 mmol) and 3-nitro triazole (**22**, 323 mg, 2.83 mmol, 8.0 eq.), and the mixture was co-evaporated from pyridine (3 × 2 mL). The residue was dissolved in CH_2Cl_2–MeCN–pyridine (2 : 2 : 1, 5 mL) and a solution of mesitylene sulfonyl chloride (309 mg, 1.41 mmol, 4.0 eq.) in pyridine (1 mL) was added dropwise over 25 min. The reaction mixture was stirred for a further 2 h after which water (2 mL) was added. The mixture was diluted with ethyl acetate (100 mL), washed with water (3 × 300 mL), dried ($MgSO_4$) and flash silica was added before stripping off the solvent. The silica was poured onto a flash column that was eluted with a gradient of EtOAc–pet. spirit (0 : 1 → 1 : 0) to afford the title compound as a white solid (351 mg, 90%). R_f (EtOAc) 0.90; δ_H (400 MHz, $CDCl_3$) 5.45–5.36 (8H, m, 4 × ***H***C=C***H***), 5.34–5.26 (1H, m, Gly 2-C***H***), 4.80–4.72 (1H, m, Ins 1-C***H***), 4.61–4.58 (1H, m, Ins 2-C***H***), 4.41–4.25 [6H, m, (2 × POC***H₂***) + Gly 1-C***H₂***], 4.23–4.15 (1H, m, Ins 3-C***H***), 4.08 (1H, t, *J* 6.0, Ins 3-C***H***), 4.06–4.01 (2H, m, Gly 3-C***H₂***), 3.91 (1H, dd, *J* 10.5, 6.5, Ins 4-C***H***), 3.39 (1H, t, *J* 10.0, Ins 6-C***H***), 2.85 [8H, m, C***H₂***CN + (3 × (CH=CH)₂C***H₂***)], 2.38 (2H, t, *J* 7.5, C***H₂***CO₂), 2.33 (2H, t, *J* 7.5, C***H₂***CO₂), 2.14 (2H, q, *J* 7.0, CH₂C***H₂***CH=CH), 2.07 (2H, q, *J* 7.0, CH₂C***H₂***CH=CH), 1.74–1.25 (64H, m, 32 × C***H₂***), 0.90 (6H, 2 × t, *J* 7.0, 2 × C***H₃***); δ_P (162 MHz, $CDCl_3$) −2.81 (0.5P), −2.94 (0.5P); δ_C (125 MHz, $CDCl_3$) 173.3, 172.6 (2 × ***C***=O), 130.5, 129.1, 128.7, 128.6, 128.34, 128.07, 127.8, 127.5 (4 × H***C***=***C***H), 116.4 (0.5*C*), 116.1 (0.5*C*) (***C***≡N), 113.8, 111.4 (2 × acetal ***C***), 81.5, 77.5, 76.0, 75.4, 74.96, 74.83 (6 × Ins-***C***H), 69.3 (Gly 2-***C***H₂), 66.2 (d, J_{C-P} 6.3, Gly 3-***C***H₂), 62.1–61.7 (2C, Gly 1-***C***H₂ + P–O–***C***H₂), 37.8, 36.36 (2 × ***C***H₂COORs), 36.28, 35.12, 35.07, 34.0, 33.6, 31.9, 31.5, 29.7 (6*C*), 29.5 (2*C*), 29.38, 29.32, 29.16, 27.2, 26.5, 25.6 (4*C*), 24.8 (3*C*), 24.79, 24.72, 23.99, 23.79, 23.72, 23.65, 22.71 (2*C*), 22.59, 19.4 [(24 × fatty acid ***C***H₂,) + (10 × cyclohexylidene ***C***H₂,) + ***C***H₂CN], 14.15, 14.10 (2 × ***C***H₃); HRMS (ESI+) *m/z* found $[M + Na]^+$ = 1,122.7013, $C_{62}H_{102}NO_{13}PNa$ requires 1,122.6987.

1-*O*-[(Cyanoethyloxy)(*sn*-1-*O*-stearoyl-2-*O*-arachidonoylglyceryloxy)phosphoryl]-4-*O*-(dicyanoethyloxyphosphoryl)-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol, 24d. 1-*O*-[(Cyanoethyloxy)(*sn*-1-*O*-stearoyl-2-*O*-arachidonoylglycerol)phosphoryl]-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**23d**, 225 mg, 0.2 mmol) was evaporated from pyridine (3 × 2 mL) then dissolved in CH_2Cl_2–pyridine (3 : 2, 2.5 mL) to which *N*-methyl imidazole (81 μL, 1.00 mmol, 5.0 eq.) and 0.34 M dicyanoethylphosphorochloridite in CH_2Cl_2 (**15**, 2.36 mL, 0.80 mmol, 4.0 eq.) were added. After 16 h cyanoethanol (41 μL, 0.67 mmol, 3.1 eq.) was added and the mixture stirred for 30 min. The solution was then cooled to 0 °C and 5 M *tert*-butyl hydroperoxide in decanes (200 μL, 1.00 mmol, 5.0 eq.) was added. After 12 h water (5 mL) was added and the solution was concentrated *in vacuo*. The resulting mixture was suspended in MeCN–water (1 : 9, 100 mL) and fractionated through a column of silanised silica, eluting with a gradient of MeCN–water (1 : 4 → 7 : 3, and flushed with ethyl acetate). The appropriate fractions were combined, dried ($MgSO_4$), and adsorbed onto flash silica. This was poured onto a column of silica and fractionated, eluting with a gradient of first diethyl ether–pet. spirit (0 : 1 → 1 : 0) then methanol–ethyl acetate (0 : 1 → 1 : 1), to afford the title compound as a white greasy solid (219 mg, 85%). R_f (EtOAc) 0.26; δ_H (400 MHz, $CDCl_3$) 5.39–5.28 (8H, m, 4 × ***H***C=C***H***), 5.28–5.20 (1H, m, Glyc 2-C***H***), 4.76–4.69 (1H, m, Ins 1-C***H***), 4.64–4.56 (2H, m, Ins 4-C***H*** + Ins 2-C***H***), 4.38–4.00 [10H, m, (3 × OC***H₂***CH₂CN) + Glyc 3-C***H₂*** + Ins 3-C***H*** + Ins 6-C***H***], 3.46 (1H, t, *J* 10.4, Ins 5-C***H***), 2.75 [12H, m, (3 × C***H₂***CN) + (3 × (CH=CH)₂C***H₂***)], 2.32 (2H, t, *J* 7.5, C***H₂***CO₂), 2.27 (2H, t, *J* 7.5 Hz, C***H₂***CO₂), 2.09 (2H, q, *J* 7.0, CH₂C***H₂***CH=CH), 2.04 (2H, q, *J* 7.0, CH₂C***H₂***CH=CH), 1.70–1.17 (68H, m, 34 × C***H₂***), 0.83 (6H, 2 × t, *J* 7.0, 2 × C***H₃***); δ_P (202 MHz, $CDCl_3$) −2.4 (1P, 4-***P***), −2.83 (0.5P, 1-***P***), −3.0 (0.5P, 4-***P***); δ_C (125 MHz, $CDCl_3$) 173.1, 172.4 (2*C*, ***C***=O), 130.4, 128.9, 128.58, 128.50, 128.2, 127.93, 127.69, 127.40 (4 × H***C***=***C***H), 116.18, 116.08, 116.00 (3 × ***C***≡N), 114.3, 111.9 (2 × acetal ***C***), 80.8 (d, Ins 4-***C***H, J_{C-P} 5.0), 79.2, 75.97, 75.63, 75.0, 74.5 (5 × Ins-***C***H), 69.2 (Gly 2-***C***H), 66.1 (Gly 3-***C***H₂), 62.5–62.0 (3 × P–O–***C***H₂), 61.5–61.3 (m, Gly 1-***C***H₂), 37.4, 36.1 (2 × ***C***H₂CO₂), 35.0, 33.8, 33.4, 31.8, 31.4, 30.3, 29.58 (7*C*), 29.54 (3*C*), 29.38, 29.24 (2*C*), 29.20, 29.18, 29.01, 27.1, 26.3, 25.5, 24.71, 24.59, 23.88, 23.81, 23.74, 23.68, 23.63, 22.57, 22.45, 19.44, 19.38, 19.32 [(24 × fatty acid ***C***H₂,) + (10 × cyclohexylidene ***C***H₂,) + (3 × ***C***H₂CN)], 14.07, 14.01 (2 × ***C***H₃,); HRMS (ESI+) *m/z* found $[M + H]^+$ = 1,286.7357, $C_{68}H_{110}N_3O_{16}P_2$ requires 1,286.7361.

sn*-1-Stearoyl-2-arachidonoyl phospatidylinositol 4-phosphate, triethylammonium salt, 2d.** 1-*O*-(*sn*-1-*O*-Stearoyl-2-arachidonoyl glycer-3-yloxy)(2-cyanoethyloxy)phosphoryl-4-*O*-di(2-cyanoethyloxy)phosphoryl-2,3:5,6-*O*-dicyclohexylidene-*myo*-inositol (**26d**, 148 mg, 0.115 mmol) was evaporated from MeCN (3 × 2 mL), and dissolved in CH_2Cl_2–MeCN (1 : 1, 6 mL). To this was added trimethylsilyl chloride (1 mL) then *N*,*N*,*N*′,*N*′-tetramethyl-*N*″-*tert*-butyl-guanidine (90%, 774 μL, 5.92 mmol, 30.0 eq.). After 16 h, the volatile components were evaporated *in vacuo* and the residue triturated with TmsCl–pet. spirit (1 : 19) under N_2. The filtrate was evaporated to dryness *in vacuo*, when ^{31}P NMR [−11.2 (1P), −19.5 (1P) ppm] demonstrated complete exchange of the cyanoethyl esters. The filtrate was redissolved in Et_3N–MeOH (3 : 2, 5 mL) and stirred for 20 min before again stripping off the solvent *in vacuo*. The residue was next dissolved in AcOH–water (2 : 1, 6 mL) and after stirring for 48 h the mixture was freeze dried. The off-white solid was triturated with MeCN, then diethyl ether to afford the title compound as an off-white solid (108 mg, 99%). δ_H (500 MHz $CDCl_3$–CD_3OD 3 : 1 323 K) 5.40–5.35 (8H, m, 4 × ***H*C═C*H), 5.25 (1H, m, Gly 2-C***H***), 4.40–4.35 (1H, ddd, *J* 4.0, 7.5, 10.0, Ins 1-C***H***), 4.15–4.00 (3H, m, obscured by HOD, Gly 3-C***H₂*** + Ins 4-C***H***), 4.10–3.90 (2H, m, Gly 1-C$\boldsymbol{H_2}$), 3.85–3.80 (2H, m, Ins 2-C***H*** + Ins 6-C***H***), 3.50 (1H, dd, *J* 4.0, 10.0, Ins 3-C***H***), 3.37 (1H, t, *J* 10.0, Ins 5-C***H***), 3.30 (12H, NC$\boldsymbol{H_2}$), 3.10–2.90 (6H, m, O***H*** + Et_3N***H***), 2.80–2.75 (6H, m, 3 × (CH═CH)$_2$C$\boldsymbol{H_2}$), 2.29 (2H, t, *J* 5.0, C$\boldsymbol{H_2}$COOR), 2.26 (t, *J* 5.0, C$\boldsymbol{H_2}$COOR), 2.10–2.05 (2H, m, arach γ-C$\boldsymbol{H_2}$), 1.68–1.60 (2H, m, arach β-C$\boldsymbol{H_2}$), 1.58–1.53 (2H, m, stear β-C$\boldsymbol{H_2}$), 1.50–1.10 (32H, m, 16 × C$\boldsymbol{H_2}$), 0.90–0.75 (24H, m, arach + stearoyl + NEt C$\boldsymbol{H_3}$); δ_P (162 MHz $CDCl_3$–CD_3OD [3 : 1], 298 K) 3.26 (1P), 1.35 (1P); δ_C (125 MHz, $CDCl_3$) 174.1, 173.4 (2 × ***C***═O), 130.7, 129.19, 129.14, 128.88, 128.57, 128.43, 128.15, 127.87 (4 × H***C***═***C***H), 78.3, 77.1, 74.7, 72.3, 71.85, 71.60 (6 × Ins ***C***H), 71.0 (Gly 2-***C***H), 64.1, 63.0 (Gly 1-***C***H_2 + 3-***C***H_2), 46.4 (6C, N***C***H_2), 34.3, 34.0 (2 × ***C***H_2COORs), 32.1 (2C), 31.78 (2C), 30.33, 30.22, 29.89 (2C), 29.80, 29.73, 29.54, 29.40, 27.45, 27.28, 26.8, 25.9, 25.13, 25.09, 22.85, 22.80, 22.74, 20.9 (24 × ***C***H_2), 15.1 (NCH_2***C***H_3), 14.16, 14.04 (2 × ***C***H_3); HRMS (ESI+) *m*/*z* found $[M + Na]^+$ = 989.5085, $C_{47}H_{85}O_{16}P_2Na$ requires 989.5143.

Author contributions

The manuscript was produced through the contributions of all authors. PRJG and RW conceived the research questions and strategy. SF and PRJG wrote the manuscript. PRJG, RW, OC and RHT wrote the original grant application. Laboratory experiments were designed and carried out by SF, EWT and LHM. Data analyses were carried out by SF. All authors have given approval to the final version of the manuscript.

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