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Synthesis of unsaturated phosphatidylinositol 4-phosphates and the effects of substrate unsaturation on SopB phosphatase activity

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In this paper evidence is presented that the fatty acid component of an inositol substrate affects the kinetic parameters of the lipid phosphatase Salmonella Outer Protein B (SopB). 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-dicyclohexylidendey-myoinositol were resolved by crystallisation of their di(acylmaleinate) diastereoisomers. 2) The resulting diol was phosphorylated regio-selectively exclusively on the 1-O using the new reagent tri(2-cyanoethyl)phosphate. 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 SopB. 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 inositol phosphatase SopB has a physical role in vivo.

Introduction

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

Over many years the central role of inositides in intracellular signalling has sustained intense interest in the enzymes that produce and catabolise them\textsuperscript{7,14}. Although less widely explored than the corresponding lipid kinases, the roles of 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\textsuperscript{15,16}.

Beyond human metabolism, exogenous phosphatases have been found to play a pivotal role in Salmonella infection\textsuperscript{17-19}. Norris \textit{et al.} showed that Salmonella virulence depended on secretion of an inositol 3-phosphatase, Salmonella outer protein B (SopB), and hinted that this enzyme might also be a 4-phosphatase and thus dephosphorylated PI-4-P (Fig. 1)\textsuperscript{20}. More recent work has shown that SopB exhibits measurable 4- and 5-phosphatase activity \textit{in vitro}, although its activity on the most abundant phosphatidylinositol bis-phosphate, PI-4,5-P\textsubscript{2}, is relatively low\textsuperscript{15}.

The identification of SopB as an inositol phosphatase raises the question of what the advantage to Salmonella might be in altering the host cell’s inositol profile. It may be anticipated that the introduction of an inositol phosphatase could interfere with inositide-based signalling, since phosphatidylinositol tri-phosphate, (PI-3,4,5-P\textsubscript{3}, Fig. 1) and other highly phosphorylated inositides are dephosphorylated by SopB\textsuperscript{19,20,21}. However, \textit{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-Ps impart much higher stored curvature elastic stress on membranes under physiological conditions than do PIs (1)\textsuperscript{9,10}.

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\textsuperscript{12,22,23}. Naturally occurring PI-4-Ps have a fatty acid profile with a wide variety of...
fatty acid residues having different numbers of olefin bonds per chain. As PI-4-Ps are understood to be distributed homogenously at physiological abundance9, 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 SopB 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-P2, 3), in order of decreasing abundance, typically representing around 90% of this fraction (Figure 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 were not chromatographically separable. We therefore sought a synthetic strategy for preparing unsaturated PI-4-Ps.

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-camphane and di-menthoxycetate 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 yield32, 33.

Mandelic acid reacted cleanly with acetyl chloride to give crystalline acetylmandelic acid (AcMan2OH, 8a) with identical NMR and optical rotation to the published data. Carboxylic acid 8a was converted to the acyl chloride (AcManCl, 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 thiomyl 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 1H 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 hopefully have limited time to racemise.

Fig. 1 Inositide lipids.
Furthermore, assuming that the proton trap/catalyst participated in the racemisation, we selected species with their $pK_a$ as low as possible. 2,6-Dichlorobenzoyl chloride (DcbCl) was added portionwise to a mixture of inositol diol rac-5 and acetylmandelic acid (8a) with $N$-methylimidazole (NMI, $pK_a$ 7.00−7.06)\(^{35}\) acting as both base and catalyst, in dichloromethane\(^{33}\). 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 (Figure 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 (Figure 2, lower trace). The two di(acetylmandelate) diastereoisomers of diol 5 (Scheme 1) are clearly distinguishable by NMR spectroscopy (Figure 2), contrary to previous reports\(^{32, 36}\), with differences in the shift of the $\alpha$-carbonyl protons, and of the resonances of the Ins 1- and 4-CHs 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 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 intramolecular 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 phosphoramidates are sensitive to hydrolysis and oxidation. Additionally, if the phosphorylation reaction gave a mixture of regioisomers, 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-bcyaanoethyl)phosphite [(CneO)\(_3\)P, \((13)\), Scheme 2]. This phosphite was prepared from the reaction of 3·2 equivalents of 3btrimethylsilyloxy propionitrile (\(12\)) with \(\text{PCl}_3\); this is a modification of the simple preparation of the phosphitylating agent di(2-bcyaanoethyl) phosphorochloridite (\(15\)).\(^40\)

Although tri(2-bcyaanoethyl)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).

Treatment of two equivalents of the resolved diol (\(\text{d}-5\)) with (CneO)\(_3\)P (\(13\)) in dichloromethane-pyridine with the mild oxidising agent pyridinium bromide perbromide (\(14\)) at \(-35^\circ\text{C}\) gave the desired 1-O-phosphate triester \(11\) cleanly as the only product in 80% yield with recovery of all of the excess unphosphorylated diol \(\text{d}-5\) (Scheme 3). In the \(^1\text{H}\) 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 \(\text{rac}-5\) with (CneO)\(_3\)P (\(13\)) 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 \(^1\text{H}\) NMR. These data confirmed our earlier assumed structure (see Supplementary Information).

We are uncertain of the mechanism of this phosphorylation, although it is established that the initial phosphate activation will proceed via formation of the corresponding bromophosphonium bromide (\(16\), Scheme 2)\(^41\text{-}43\). However, it is notable that after addition of a slight excess of pyridinium bromide perbromide to a mixture of (CneO)\(_3\)P (\(13\)) with 1 eq. 3bhydroxypropionitrile the \(^1\text{H}\) NMR in \(\text{C}_2\text{D}_2\text{N}^+\text{D}_3\text{CCN} \) exhibited, in addition to peaks for tri(2-bcyaanoethyl)phosphate (\(19\)), new resonances at \(\delta_\text{H} 3.59\) (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 Supplementary information for spectra). Therefore this Arbuzov reaction is assumed to proceed with nucleophilic attack on the cyanoethyl protecting group, not the elimination of acrylonitrile.

**Scheme 2** Preparation of phosphorus reagents.

**Scheme 3** Preparation of phosphatidylinositol 4-phosphate (2). Reagents and conditions: i) \(13\), \(\text{CH}_2\text{Cl}_2\text{-C}_5\text{H}_5\text{N}\), \(\text{C}_5\text{H}_5\text{NH—Br} (14)\), \(-35^\circ\text{C}\); ii) (MeN)\(_2\text{C=N-tBu}, \text{TmsCl, CH}_2\text{Cl}_2–\text{MeCN}, 16\;\text{hr}; iii) \text{AcOH–water, 24}\;\text{hr}; iv) \(21\), \text{DcbCl, NMI, CH}_2\text{Cl}_2, 16\;\text{hr}; v) \text{ClCOCOMe, pyrrole, CH}_2\text{Cl}_2, 2\;\text{min}; vi) \text{EtN—CH}_2\text{Cl}_2—\text{MeCN, 36}\;\text{hr}; vii) 1,1,5-Me_2—\text{C}_2\text{H}_4—\text{COCl}, \text{22}, \text{CH}_2\text{Cl}_2—\text{MeCN—C}_5\text{H}_5\text{N}, 25\;\text{min}; viii) \text{NMI, CH}_2\text{Cl}_2—\text{C}_5\text{H}_5\text{N}, 16\;\text{hr then CneOH then tert-BuOH-H}.\(22\), CH₂Cl₂–MeCN–C₅H₅N, 25 min; x) \(15\), NMI, CH₂Cl₂–C₅H₅N, 16 h then CneOH then tert-BuOH-H.\(22\), CH₂Cl₂–MeCN–C₅H₅N, 25 min; x) \(15\), NMI, CH₂Cl₂–C₅H₅N, 16 h then CneOH then tert-BuOH-H.
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-linolenic acid (21c), or arachidonic acid (21d)] with the 2-OH using DcbCl and NMI\(^{15,44}\), followed by acetyl catalysed deprotection of the 3-OH in the presence of pyrrolyl\(^{45}\) 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-cyanooethyl 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).

Protected phosphatidylinositol 23 may be deprotected to give the parent PI (1), but in order to produce PI-4-Ps 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 phosphate 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 excess diglyceride (see Supplementary Table 1). Therefore the diacylglycerol 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.

PI-4-Ps as substrates for SopB

We had hypothesised that the kinetic activity of SopB 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. However, only the three \(sn\)-2-C\(_n\) (i.e. saturated, singly and triply unsaturated) isomers 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 SopB-mediated hydrolysis, i.e. PI-4-P \(\rightarrow\) PI + P\(_i\). PI-4-Ps 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. 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 Supplementary Table 1). Therefore the diacylglycerol 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.

<table>
<thead>
<tr>
<th>PI-4-P substrate</th>
<th>Activity (% (V_{max}))</th>
<th>(K_M) / (\mu)M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>micelles</td>
<td>vesicles</td>
</tr>
<tr>
<td>2a, C(<em>{18,5})C(</em>{18,9})</td>
<td>64·8 (± 10·9)</td>
<td>19·0 (± 9·1)</td>
</tr>
<tr>
<td>2b, C(<em>{18,5})C(</em>{18,1})</td>
<td>98·6 (± 8·6)</td>
<td>100·0 (± 10·6)</td>
</tr>
<tr>
<td>2c, C(<em>{18,5})C(</em>{18,3})</td>
<td>100·0 (± 7·0)</td>
<td>81·0 (± 10·6)</td>
</tr>
</tbody>
</table>

Table 1. The kinetic parameters of SopB 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 \(\mu\)M (micelles) and 0-120 \(\mu\)M (vesicles) with an assay time of 20 min and 1 \(\mu\)g SopB per measurement, \(n = 3\). Activity calculated as a percentage of the \(V_{max}\) observed for either substrate presentation.

These data indicate that one double bond is enough to change the kinetic parameters of SopB, regardless of the presentation of the lipids. In turn, this means that SopB 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\textsuperscript{22, 23}. Physical studies have shown that the principle difference in physical behaviour between stearoyl, oleoyl and \(\gamma\)-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\textsuperscript{24, 25}. Additionally, at the concentrations of PI\textsubscript{4}-P used here (2\% PI\textsubscript{4}-P, pH 7.4, 5 mM Mg\textsuperscript{2+}), which are similar to physiological values (in fact PI\textsubscript{4}-P concentration can be even higher), the inositol 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\textsuperscript{9, 11, 24}. This suggests that the influence of PI\textsubscript{4}-P on its local environment may be a consequence of its molecular structure alone and not of interactions between individual PI\textsubscript{4}-P molecules.

It is possible that the difference between the kinetics of the substrates with SopB 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 inositol 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\textsubscript{4}-P (2), and used them to characterise the kinetic behaviour of a phosphatase important in the mechanism of *Salmonella* infection.

A novel synthetic strategy, developed for its versatility, was used to produce poly-unsaturated PI\textsubscript{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\textsuperscript{9, 47, 48}. This preparation of unsaturated PI\textsubscript{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 phosphate (13), which is prepared easily and may be stored for protracted periods. The resulting inositol 1-phosphate (11) was then coupled to four different glycides, without the need to protect the 4-OH. Finally, the 4-OH was phosphorylated with dicyanoethyl phosphorochloridite (15) and the fully protected PI\textsubscript{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 SopB 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\textsubscript{4}-P lipids increases they become poorer substrates. Vesicular presentation shows the same trend but with less differentiation between unsaturated substrates. The molecular differences between the synthetically prepared PI\textsubscript{4}-Ps 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\textsuperscript{22, 23}, the more unsaturated lipids, the more fluid bilayer becomes. We therefore conclude that differences in packing of the alkyl fraction near the substrate are responsible for the observed differences in SopB 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\textsubscript{4}-P inositates are beginning to be understood\textsuperscript{12} and have far-reaching implications for interpreting the phosphatase activity of SopB. It is clear that saturated PI\textsubscript{4}-P self-assembles into a phase with pronounced negative curvature at physiological concentrations under model physiological conditions\textsuperscript{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\textsuperscript{10}. This implies that the phosphatase activity of SopB reduces the stored curvature elastic stress in membranes by reducing the concentration of PI\textsubscript{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 inositol phosphatase. The activity of SopB on inositate 4-phosphates provides a valuable insight into the mode of action of externally-mediated, disease-based changes in inositol 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 SopB-inositol 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\textsubscript{6}H\textsubscript{5}N\textsubscript{3}, CH\textsubscript{3}Cl\textsubscript{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\textsubscript{254} glass-backed plates. TLC plates of inositol derivatives were stained with \(p\)-anisaldehyde, glyceride derivatives stained with KMnO\textsubscript{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 SopB were purchased from *Fisher Ltd* (Loughborough, Leicestershire, UK) and *Bio-Rad* (Hemel
Hempstead, Hertfordshire, UK). n-Octyl-β-D-glucopyranoside was purchased from Calbiochem (Beeston, Nottinghamshire, UK).

**Protein Preparation**

SopB was produced using IPTG-mediated over-expression in DH5α E. coli bacteria as described before.5 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 assay, with BSA (Thermo Scientific BSA standard, Product number 23209; 2.0 mg/mL in saline, supplemented with sodium azide) for calibration. Bradford’s reagent was purchased from Sigma and used as directed.

**SopB kinetics assays**

The colorimetric malachite green endpoint assay was used to determine the concentration of released inorganic phosphate (P<sub>0</sub>) after exposure of PI-4-P to SopB. Control samples were used to determine the non-enzymatic contribution to [P<sub>0</sub>]. In control samples, enzyme was added after stopping the reaction with the malachite green reagent (enzyme dead end).

Micelles were prepared by sonication (12 min, benchtop Branson 1200) of a known mass of the substrate suspended in a solution of OGPS (stock concentration 4% v/v) in chloroform-methanol-water (70:30:1) that were then dried down together before re-dissolution in 200 mM Tris.

**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, 2.0 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 CH2Cl2 (15 mL) and N-methylimidazole (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 (Na2SO4), and the solvent evaporated in vacuo to leave an off-white solid (5.5 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<sub>t</sub> (EtOAc) 0.83; [δ<sub>4</sub>]<sup>25</sup>, 36.6° (c 4.70, CH2Cl2); δ<sub>7</sub> (400 MHz, CDCl3 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), 7.09 (1H, s), 6.06 (1H, s) (2 × α-CH), 5.18-5.14 (1H, dd, J 6, 9, 11, 2 × Ins 4-CH), 4.50 (1H, dd, J 4.3, 10.5, 10 × Ins 1-CH), 4.37 (1H, t, J 4.6, 10 × Ins 2-CH), 4.06 (1H, t, J 10.0, 10 × Ins 6-CH), 3.80 (1H, dd, J 4.9, 6.7, 3 × Ins-3-CH), 3.43 (1H, dd, J 9.6, 11.0, 11 × Ins-5-CH), 2.17 (3H, s), 2.14 (3H, s), 2 × (CH<sub>3</sub>) at 1.69-1.12 (20H, m, 10 × cyclohexyl CH<sub>3</sub>); δ<sub>2</sub> (125 MHz, CDCl3 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 CH), 113.9, 111.0 (2 × acetal C), 78.6 (Ins 3-CH), 76.0 (Ins 4-CH), 75.2 (Ins 5-CH), 74.4 (Ins 2-CH), 74.2 (2C, 2 × α-CH), 74.1 (Ins 6-CH), 71.8 (Ins 1-CH), 37.2, 36.30, 36.20, 34.6, 24.9, 24.7, 23.62 (2C), 23.57, 23.2 (10 × cyclohexyl CH<sub>2</sub>), 20.73, 20.66 (2 × CH<sub>3</sub>); HRMS (ESI+) m/z: found [M+H<sup>+</sup>] = 693.2911, C<sub>38</sub>H<sub>48</sub>O<sub>2</sub> 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 of the mother liquor that remained in vacuo to leave a white solid (46%). R<sub>t</sub> (EtOAc) 0.83; [δ<sub>4</sub>]<sup>25</sup>, 56.98° (c 2.65, CH2Cl2); δ<sub>7</sub> (400 MHz, CDCl3 with 0.01 M triethylamine) 7.53-7.50 (2H, m), 7.49-7.46 (2H, m), 7.37-7.33 (6H, m) (10 × Ph CH), 6.11 (1H, s), 6.00 (1H, s) (2 × α-CH), 5.22 (1H, dd, J 6.8, 11.1, 11 × Ins 6-CH), 5.01 (1H, dd, J 9.6, 10.4, 10 × Ins 4-CH), 4.34 (2H, CH<sub>2</sub>), 2.15 (3H, s), 2.13 (3H, s) (2 × CH<sub>3</sub>), 1.78-1.33 (20H, m, 10 × cyclohexyl CH<sub>2</sub>); δ<sub>2</sub> (125 MHz, CDCl3 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 CH), 134.0, 133.5, 2 × (acetal C), 78.3, 76.1, 75.7, 74.6 (2C), 74.1 (2C), 71.8 (6 × Ins CH) (2 × α-CH), 37.4, 36.1, 36.0, 34.8, 24.8 (2C), 23.8, 23.5, 23.4, 23.4 (10 × cyclohexyl CH<sub>2</sub>).
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(+)-2,3:5,6-Dicyclohexylidene-2,3,5,6-dicyclohexylidene-myo-inositol, 11. (+)-2,3:5,6-Dicyclohexylidene-2,3,5,6-dicyclohexylidene-myo-inositol (5, 1.00 g, 2.94 mmol, 1 eq) was evaporated from MeCN (3 × 3 mL), dissolved in CHCl₃-pyridine (9:1, 30.0 mL) and tricyanoophosphate (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, 6.0 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 × 1 L), dried (Na₂SO₄), and the solvent removed in vacuo to give a colourless foam-gum (620 mg, 91%). Rₚ (diethyl ether-MeOH, 9:1) 0.60; [α]D 25 +16.12° (c 2.74, CH₂Cl₂); δH (400 MHz, D₂O) 4.03 (6H, q, J6,4, 3 × POCH₂), 2.86 (6H, t, J 5.9, 3 × CH₂CN); δP (202 MHz, d₂-MeNO) 1376; δC (125 MHz, d₂-MeNO) 119.2 (3 × CN), 58.1 (d, J11,10, 3 × 20), 20.1 (d, Jp, 4.6, 3 × CH₂CN); HRMS (ESI+) m/z found [M+Na]+ = 264.0513, C₆H₁₂O₃NP Na requires 264.0514.

myo-Inositol-1-phosphate, 26. 1-O-Di(2-cyanoethoxy)phosphoryl2,3:5,6-Dicyclohexylidene-myo-inositol (11, 112 mg, 0.213 mmol) was dissolved in MeCN-CH₂Cl₂ (1:1, 2 mL) and TmsCl (156 µL, 6.0 eq) was added, followed by N,N,N’,N’-tetramethyl-N’-tert-butyloguanidine (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 δp 19-0 confirmed complete exchange of the cyanoethyl phosphate esters. The filtrate was evaporated to dryness and the residue stirred in 1M 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). δH (400 MHz, D₂O) 4.13 (1H, t, J 2.7, Ins 2-CH₂), 3.82 (1H, dt, J 2.7, 9.1 Hz, Ins 1-CH₂), 3.63 (1H, t, J 9.6, Ins 6-CH₂), 3.53 (1H, t, J 9.6, Ins 4-CH₂), 3.45 (1H, dd, J 2.8, 10.0, Ins 3-CH₂), 3.22 (1H, t, J 9.3, Ins 5-CH₂); δP (162 MHz, D₂O) 0.43; δC (125 MHz, D₂O) 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]+ = 529.0210, C₁₀H₁₆O₄N₃P requires 529.0219.

1-O-[(Cyaenoethoxy)(sn-1-O-stearoyl-2-O-arachidonoylgluceryl)-phosphoryl]-2,3:5,6-Dicyclohexylidene-myo-inositol, 23d. To 1-O-(dicynoethoxyphosphoryl)-2,3:5,6-Dicyclohexylidene-myo-inositol (11, 203 mg, 0.354 mmol) were added CH₂Cl₂ (3 mL), MeCN (1 mL) and triethylamine (3 mL). After stirring the solution for 36 h, the solvents were 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 CH₃Cl₂-MeCN-pyridine (2:2:1, 5 mL) and a solution of mesitylene sulfon 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 (MgSO₄) 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%). Rₚ (EtOAc) 0.90; δH (400 MHz, CDCl₃) 5.45-5.36 (8H, m, 4 × CH₃); 3.54-2.56 (1H, m, Gly 2-CH₂), 4.80-4.72 (1H, m, Ins 1-CH₂), 4.61-4.58 (1H, m, Ins 2-CH₂), 4.41-4.25 (6H, m, 2 × POCH₂) + Gly 1-CH₂), 4.23-4.15 (1H, m, Ins 3-CH₂), 4.08 (1H, t, J 6.0, Ins 3-CH₂), 4.06-4.01 (2H, m, Gly, Ins 3-CH₂), 3.91 (1H, dd, J 10.5, 6.5, Ins 4-CH₂), 3.39 (1H, t, J 10.0, Ins 6-CH₂), 2.85 (8H, m, CH₂CN + (3 × CH₂CHCH₂CHCH₂CN), 2.38 (2H, t, J 7.5, CH₂CO₂), 2.33 (2H, t, J 7.5, CH₂CO₂), 2.14 (2H, q, J 7.5, CH₂CH₂CH₂CH₃), 2.07 (2H, q, J 7.0, CH₂CH₂CH₂CH₃), 1.74-1.25 (64H, m, 32 × CH₂), 0.90 (6H, 2 × t, J 7.0, 2 × CH₃); δP (162 MHz, CDCl₃) -2.81 (0.5P), -2.94 (0.5P); δC (125 MHz, CDCl₃) 173.3, 172.6 (2 × C-0), 130.5, 129.1, 128.7, 128.6, 128.34, 128.07,

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1-O-[(Cyanoethoxy)sn-1-O-stearoyl-2-O-arachidonoylglycerol oxy]phosphoryl-4-O-(dicaproxyloxyphosphoryl)-2,3,5,6-O-die cyclolhexylidine-myo-inositol, 24d. 1-O-[(Cyanoethoxy)(sn-1-O-stearoyl-2-O-arachidonoylglycerol oxy)phosphoryl]-4-O-(dicaproxyloxyphosphoryl)-2,3,5,6-O-dicyclohexylidine-myo-inositol (23d, 225 mg, 0.2 mmol) was evaporated from pyridine (3 2 mL) then dissolved in CHCl3-pyridine (3:2, 2.5 mL) to which dicyclohexylideneb2b

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127·8, 127·5 (4 × H=CH=C-H), 116·4 (50C), 116·1 (50C) (CH=N), 113·8, 111·4 (2 × acetal C), 81·5, 77·5, 76·0, 75·4, 74·96, 74·83 (6 × Ins-CH₃), 69·3 (3 Gly-2-CH₃), 66·2 (d, Gly-3-CH₃, J₃,6 = 6·3), 62·1-61·7 (2C, Gly-1-CH₃ + P-O-CH₂), 37·8, 36·36 (2 × CH₂COOCHR), 36·28, 35·12, 35·07, 34·0, 33·6, 32·1, 31·9, 31·5, 29·7 (6C), 29·5 (2C), 29·38, 29·32, 29·16, 27·2, 25·6 (4C), 24·8 (3C), 24·79, 24·72, 23·99, 23·79, 23·72, 23·65, 22·67 (2C), 22·59, 19·4 (24 × fatty acid CH₃) + (10 × cyclohexyldiene CH₃) + CH₃CN), 14·15, 14·10 (2 × CH₃); HRMS (EI+) m/z found [M+Na]+ = 1,122-7013, C₃₀H₆₀O₁₃PNa requires 1,122-6987.

127.8, 127.5 (4 × H=CH=CH), 116.4 (50C), 116.1 (50C) (CH=N), 113.8, 111.4 (2 × acetal C), 81.5, 77.5, 76.0, 75.4, 74.96, 74.83 (6 × Ins-CH₃), 69.3 (3 Gly-2-CH₃), 66.2 (d, Gly-3-CH₃, J₃,6 = 6.3), 62.1-61.7 (2C, Gly-1-CH₃ + P-O-CH₂), 37.8, 36.36 (2 × CH₂COOCHR), 36.28, 35.12, 35.07, 34.0, 33.6, 32.1, 31.9, 31.5, 29.7 (6C), 29.5 (2C), 29.38, 29.32, 29.16, 27.2, 25.6 (4C), 24.8 (3C), 24.79, 24.72, 23.99, 23.79, 23.72, 23.65, 22.67 (2C), 22.59, 19.4 (24 × fatty acid CH₃) + (10 × cyclohexyldiene CH₃) + CH₃CN), 14.15, 14.10 (2 × CH₃); HRMS (EI+) m/z found [M+Na]+ = 1,122-7013, C₃₀H₆₀O₁₃PNa requires 1,122-6987.
Notes and References
