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A synthetic diphosphoinositol phosphate analogue of inositol trisphosphate
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Diphosphoinositol phosphates (PP-InsPs) are inositol phosphates (InsPs) that contain PP (diphosphate) groups. Converting a phosphate group in an InsP into a diphosphate has been reported to enhance affinity for some binding proteins. We synthesised 1-PP-Ins(4,5)P₂, the first diphosphate analogue of the intracellular signalling molecule InsP₃, and examined its effects on InsP₃ receptors, which are intracellular Ca²⁺ channels. 1-PP-Ins(4,5)P₂ was indistinguishable from InsP₃ in its ability to bind to and activate type 1 InsP₃ receptors, indicating that the diphosphate modification of InsP₃ affected neither affinity nor efficacy. Nevertheless, 1-PP-Ins(4,5)P₂ is the most potent 1-phosphate modified analogue of InsP₃ yet identified. PP-InsPs are generally hydrolysed by diphosphoinositol polyphosphate phosphohydrolases (DIPPs), but 1-PP-Ins(4,5)P₂ was not readily metabolised by human DIPPs. Differential scanning fluorimetry showed that 1-PP-Ins(4,5)P₂ stabilises DIPP proteins, but to a lesser extent than naturally occurring substrates 1-PP-Ins₃ and 5-PP-Ins₃. The non-hydrolysable InsP₇ analogues 1-PCP-InsP₅ and 5-PCP-InsP₅ showed comparable stabilising abilities to their natural counterparts and may therefore be promising substrate analogues for co-crystallisation with DIPPs.

Introduction

The myo-inositol phosphates (InsPs) are a family of intracellular signalling molecules containing monophosphate (P) and diphosphate (PP) groups arranged around the hexahydroxycyclohexane ring of myo-inositol (Ins). InsPs regulate many cellular processes, the best known being the release of Ca²⁺ from intracellular stores by myo-inositol 1,4,5-trisphosphate (InsP₃), which binds to receptors on the endoplasmic reticulum. InsP₃ is converted via a series of enzymatic phosphorylations into InsP₆ (Fig. 1), which can then be further phosphorylated to give highly charged PP-InsPs containing diphosphate (pyrophosphate) groups. Subunits, conformational changes propagate to the central pore. The pore then opens, allowing Ca²⁺ to flow into the cytosol, where it regulates many intracellular processes. The vicinal 4,5-bisphosphate structure of InsP₃ is crucial (if not absolutely essential) for activating IP₃Rs because it cross-links the two domains of the clam-like IBC, pulling them together and initiating the conformational changes. The 1-phosphate group has a less direct, but enhancing, effect on activity.

Although PP-Ins₃ signalling is thought to be more evolutionarily ancient than InsP₃-mediated mobilisation of Ca²⁺, much less is known about the functions and protein targets of PP-InsPs. Nevertheless, evidence is accumulating that PP-InsPs play important roles at the interface of cell signalling and metabolism in the regulation of bioenergetic and phosphate homeostasis. Possible receptors for PP-InsPs include the PH (pleckstrin homology) domains of proteins. PP-InsPs may also exert some of their effects by direct non-enzymatic diphosphorylation of target proteins.

Phosphorylating a phosphate monoester in an InsP₃ to give a PP-InsPₙ₋₁ not only increases the overall negative charge of the molecule, but also changes its shape, solvation and metal complexation properties. Unsurprisingly, therefore, a diphosphate group may alter ligand affinity for protein binding sites. For example, some PH domains that bind InsP₃ bind 5-InsP₇ with higher affinity while 1-InsP₇ and InsP₈ are weaker. In contrast, both 1-InsP₅ and 5-InsP₅ stimulate synthesis of inorganic polyphosphate (polyP) by the
vacular transporter chaperone (VTC) by binding to its SPX domain, while InsP6 is inactive and InsP8 is 20-fold more potent.\textsuperscript{13} PP-InsPs can be dephosphorylated back to InsPs by diphosphoinositol polyphosphate phosphohydrolases (DIPPs, Fig. 1), which specifically hydrolyse the diphosphate group, leaving a phosphate monoester and liberating inorganic phosphate.\textsuperscript{3,15}

Given that introducing a diphosphate into an InsP may modify its interaction with proteins, we were interested in the possible effects of converting one of the phosphate groups in InsP\textsubscript{3} into a diphosphate. The 1-phosphate group may enhance affinity.\textsuperscript{17}

The X-ray structure\textsuperscript{18} of the IBC of type 1 InsP\textsubscript{3} receptor bound to InsP\textsubscript{3} confirmed the area of the binding pocket around the 1-phosphate of bound InsP\textsubscript{3} to be relatively open. Our molecular docking experiments using this structure suggested that a 1-diphosphate should bind well to this region. We therefore set out to synthesise the 1-diphosphate analogue of InsP\textsubscript{3}, \textit{i.e.} 1-diphospho-

Results and discussion

Chemistry

The synthesis of 1-PP-Ins\textsubscript{(4,5)}P\textsubscript{2} (1) begins from the known alcohol 2 (ref. 19 and 20) (Scheme 1). To construct the diphosphate unit at O-1, we employed a modification of a recently described strategy,\textsuperscript{21,22} in which a temporarily protected phosphate group is introduced and then selectively deprotected to reveal a phosphate monoester. This phosphate is then phosphorylated to give a mixed $P(\text{iii})-P(\text{v})$ anhydride, which is oxidised to a partially protected pyrophosphate unit. Removal of all protecting groups by catalytic hydrogenolysis then yields the target PP-InsP. We reasoned that it might be possible to employ methylsulfonylpyrophosphate (MSE)\textsuperscript{23,24} as a temporary phosphate protecting group in this sequence. The MSE group can be removed by $\beta$-elimination, similar to the better-known $\beta$-cyanoethyl ($\beta$-CE)\textsuperscript{22,25} group. However, the MSE group is unaffected by catalytic hydrogenation, affording greater synthetic versatility, and the required phosphitylating reagent, phosphoramidite 5, is a stable crystalline solid.

![Synthesis of 1-PP-Ins\textsubscript{(4,5)}P\textsubscript{2} (1) begins from the known alcohol 2 (ref. 19 and 20) (Scheme 1).](image-url)
Thus, the 1-OH group in 2 was reacted with 5 in the presence of 5-phenyl-1H-tetrazole to give an intermediate MSE-protected phosphate triester. Oxidation using mCPBA then gave 3, containing the MSE-protected phosphate triester at O-1. The diphosphate unit at O-1 was then constructed using a sequence of transformations carried out as described previously, with slight modifications. The progress of each step was carefully monitored by $^{31}$P NMR spectroscopy (see Experimental section and ESI†). The protected diphosphate 4 was found to be rather unstable and was immediately deprotected by catalytic hydrogenolysis at atmospheric pressure. A final purification step by gradient elution anion exchange chromatography on Q-Sepharose Fast Flow resin gave 1-PP-Ins(4,5)P$_2$ (1) as the triethylammonium salt, which was accurately quantified by total phosphate assay.

Interactions of 1-PP-Ins(4,5)P$_2$ with type 1 InsP$_3$ receptors

Both InsP$_3$ and 1-PP-Ins(4,5)P$_2$ (1) stimulated a concentration-dependent release of Ca$^{2+}$ from the intracellular stores of permeabilised DT40 cells expressing type 1 InsP$_3$ receptors (Fig. 2A). The maximal Ca$^{2+}$ release evoked by each ligand and the half-maximally effective concentration (EC$_{50}$) were similar for 1 and InsP$_3$ (Fig. 2A). Membranes from SF9 cells expressing rat type 1 InsP$_3$ receptors were used for equilibrium competition binding studies with $^3$H-InsP$_3$, because these membranes express full-length type 1 InsP$_3$ receptors at ∼20-fold higher levels than cerebellar membranes, the richest endogenous source. The experiments were carried out in cytosol-like medium (CLM, pH 7.3) containing 1.5 mM Mg-ATP to match the conditions used for Ca$^{2+}$-release assays.

In agreement with the Ca$^{2+}$-release assays, 1-PP-Ins(4,5)P$_2$ (1) bound with the same affinity as InsP$_3$ to InsP$_3$ receptors (Fig. 2B). Thus, the two compounds were essentially indistinguishable in both functional and binding assays (Table 1). Rapid chemical hydrolysis of 1 could in principle explain the similar behaviour of InsP$_3$ and 1, but we saw no evidence that 1 is unstable. The $^{31}$P NMR spectrum of 1 in D$_2$O (see ESI†) was unchanged after the sample solution had been kept for several days at room temperature, followed by one year at 4 °C.

Molecular docking experiments (see Experimental section and ESI† for details) using the X-ray crystal structure of the IBC of type 1 InsP$_3$ receptor$^{18}$ suggested that the diphosphate group in 1 should be well-tolerated by the InsP$_3$-binding pocket and may be capable of forming additional hydrogen bonds with residues in the binding site (Fig. 3). Nevertheless, it is well known that attempts to optimise drug candidates by adding polar groups may fail because the expected enthalpic gains from new polar interactions are opposed by ligand desolvation penalties and unfavourable entropic effects, resulting in no gain in binding affinity.$^{26}$ Such compensatory effects may underlie the similar affinities of 1 and InsP$_3$ for type 1 InsP$_3$ receptors.

Interaction of 1-PP-Ins(4,5)P$_2$ with DIPPs

The dephosphorylation of PP-InsPs is catalysed by diphosphoinositol polyphosphate phosphohydrolases (DIPPs), which selectively cleave the diphosphate (PP) to give a phosphate monoester and inorganic phosphate (Pi).$^3$ Humans express four DIPP types: DIPP-1 is the product of the NUDT3 gene; DIPP-2 (of which there are two isoforms, DIPP-2α and DIPP-2β, produced by alternative splicing) is the product of NUDT4; DIPP-3α is the product of NUDT10 and DIPP-3β is the product of NUDT11.$^3$ We examined the interaction of 1-PP-Ins(4,5)P$_2$ (1) with all four DIPPs in comparison

| Table 1. Interactions of InsP$_3$ and 1-PP-Ins(4,5)P$_2$ (1) with type 1 InsP$_3$ receptors (n = 3) |
|-----------------|-----------------|-----------------|-----------------|
|                | Ca$^{2+}$ release | EC$_{50}$/nM | Binding$^a$ |
| **InsP$_3$**   | pEC$_{50}$/M     | EC$_{50}$/nM  | % release | pK$_D$/M | K$_D$/nM |
| 1              | 7.21 ± 0.08      | 62            | 82 ± 4    | 6.89 ± 0.07 | 128     |
| 1-PP-Ins(4,5)P$_2$ (1) | 7.17 ± 0.11 | 68            | 80 ± 1    | 6.96 ± 0.05 | 110     |

$^a$ Binding was done using SF9 cell membranes overexpressing rat type 1 InsP$_3$ receptors in CLM (pH 7.3) containing 1.5 mM Mg-ATP to match the conditions used in the Ca$^{2+}$ release assay.
with two naturally-occurring substrates 1-PP-InsP$_5$ and 5-PP-InsP$_5$ (“1-InsP$_7$” and “5-InsP$_7$”, respectively) and also with the alternative substrates diadenosine polyphosphates Ap$_3$A and Ap$_5$A. Non-hydrolysable InsP$_2$ analogues 1-PCP-InsP$_5$ (ref. 27) and 5-PCP-InsP$_5$ (ref. 28) were independently synthesised and included as controls.

With Mg$^{2+}$ present in the buffer, 1-PP-InsP$_5$ and 5-PP-InsP$_5$ were rapidly metabolised by all four DIPPs (Fig. 4A). The rate of hydrolysis of 1-PP-InsP$_5$ was significantly higher than that for 5-PP-InsP$_5$ in each case. This finding is in agreement with a previous study. As expected, the PCP analogues were not metabolised, confirming that DIPPs can hydrolyse only the di-phosphate unit and not the phosphate monoesters. Ap$_3$A and Ap$_5$A were unaffected by all four enzymes in Mg$^{2+}$-containing conditions. The presence of a divalent cation is required for the activity of NUDT10 and NUDT11 and also for NUDT3. When Mg$^{2+}$ in the buffer was replaced by Mn$^{2+}$, 1 was now hydrolysed by the DIPPs, while 1-PP-InsP$_5$ and 5-PP-InsP$_5$ resisted hydrolysis. In addition, Ap$_3$A now also behaved as a substrate for all four DIPPs (Fig. 4B). In the absence of enzyme none of the compounds, including 1, showed any sign of hydrolysis during the time course of the experiment in the presence of either Mg$^{2+}$ or Mn$^{2+}$-containing buffers. This further supports our conclusion above that 1 was not hydrolysed to InsP$_3$ during the InsP$_3$ receptor assays.

Next, we used differential scanning fluorimetry (DSF) to measure the ability of the compounds to stabilise NUDT3 (DIPPI1). While the effects of Ap$_3$A and Ap$_5$A were not significantly different from control (Fig. 5A), 1-PP-Ins(4,5)P$_2$ (1) raised the melting temperature ($T_m$) of NUDT3 by approx. 5 °C at a concentration of 0.1 mM. As expected, the more highly phosphorylated 1-PP-InsP$_5$ had much stronger effects, resulting in a $T_m$-shift of 20–25 °C. Similar DSF experiments were then carried out for NUDT4, NUDT10 and NUDT11. Ap$_3$A did not stabilise any of the DIPPs, which supports our results for the activity assay. The results are summarised in Fig. 5B.

We obtained further DSF data over a range of ligand concentrations for 1-PP-InsP$_5$ and 1-PP-Ins(4,5)P$_2$ (1), constructing dose–response curves for the two compounds (Fig. 6). It is interesting to note that the effect of 1 on NUDT10 was significantly lower compared to the other DIPPs and especially compared to NUDT11 (Fig. 6B). NUDT10 and NUDT11 have identical protein sequences apart from residue 89, which is either proline (NUDT10) or arginine (NUDT11).

Noting the strong stabilisation of all the proteins by the PCP analogues, we obtained further DSF data over a range of ligand concentrations for 1-PCP-InsP$_5$ and 5-PCP-InsP$_5$ (ESI† Fig. S4 and S5) and calculated $K_d$ values from these curves (ESI† Tables S1 and S2). We found that, in some cases, the PCP analogues had binding affinities comparable to those of their natural PP-containing ligands.

Conclusions

Replacing a phosphate group in an inositol phosphate ligand with a diphosphate (PP) group can modify the interaction of the ligand with target proteins. Structure–activity studies have previously shown that the 1-phosphate group of InsP$_3$ is amenable to synthetic modification, and molecular docking experiments suggested that a 1-diphosphate group should be well-tolerated by the binding site of the InsP$_3$ receptor. We therefore synthesised 1-PP-Ins(4,5)P$_2$ (1), the first PP-containing analogue of InsP$_3$. Using assays of Ca$^{2+}$-release through type 1 InsP$_3$ receptors, we found that 1 was equipotent to InsP$_3$ and in binding assays its affinity was indistinguishable from that of InsP$_3$. Thus, the 1-diphosphate modification of InsP$_3$ does not affect its affinity for or activity at type 1 InsP$_3$ receptors. Nevertheless, 1 is the first Ca$^{2+}$-releasing PP-InsP and also the most potent P-1 modified ligand of InsP$_3$ receptors yet identified.†

The novel diphosphate compound 1 was not metabolised by DIPPs in the presence of Mg$^{2+}$-containing buffer, while the naturally-occurring InsP$_3$ isomers, 5-PP-InsP$_5$ and 1-PP-InsP$_5$, 1,2 0 1 8 , 1 1 0 5 - 1 1 1 3 T h i s j o u r n a l i s © T h e R o y a l S o c i e t y o f C h e m i s t r y 2 0 1 8
were rapidly hydrolysed. Conversely, in the presence of Mn$^{2+}$, 1 was hydrolysed while the two InsP$_7$ isomers were unaffected. Synthetic PCP-containing analogues of the InsP$_7$s were not hydrolysed under any conditions examined, but when evaluated for their ability to stabilise DIPP proteins using differential scanning fluorimetry (DSF), they gave temperature shifts comparable to their natural PP-containing equivalents.

Fig. 4 Specific activities of DIPPs with 1-PP-Ins(4,5)P$_2$ (1), known substrates 1-PP-InsP$_5$ and 5-PP-InsP$_5$ and controls. Experiments were conducted in buffer containing Mg$^{2+}$ (A) or Mn$^{2+}$ (B). Data shown represent the formed concentration of Pi (micromolar) per enzyme concentration (micromolar) per minute. A630 was converted to Pi concentration (micromolar) using the equation A630 = 0.01897[Pi] − 0.5877 (Mg$^{2+}$ containing buffer) or A630 = 0.01923[Pi] + 0.1053 (Mn$^{2+}$ containing buffer). C. Structures of compounds examined, including methylenebisphosphonate (PCP) analogues of InsP$_7$, and diadenosine polyphosphates Ap$_n$A and Ap$_n$A.

Fig. 5 A. Effect of 1-PP-Ins(4,5)P$_2$ (1) and other compounds shown in Fig. 4C on the melting temperature ($T_m$) of NUDT3, measured using differential scanning fluorimetry (DSF). B. Comparison of melting temperature shifts (delta $T_m$) induced by all compounds for all four DIPPs examined.

Fig. 6 Dose–response curves showing stabilisation of all four DIPPs by A. 1-PP-InsP$_5$ and B. 1-PP-Ins(4,5)P$_2$ (1). Note the different y-axis scales in A and B.
This strongly suggests that 1-PCP-InsP₅ and 5-PCP-InsP₅ could be promising ligands for co-crystallisation studies with DIPPs.

Could 1-PP-Ins(4,5)P₂ be an endogenous molecule? The mammalian enzymes known to synthesise PP-InsPs are 5-diphosphoinositol pentakisphosphate kinases (PPIP5Ks) and inositol hexakisphosphate kinases (IP6Ks). Inositol phosphate multikinase (IPMK) has also been reported to be recrystallized from dichloromethane/ether; m.p. 75.5 °C. Phosphate groups. The present work shows that this criterion may not be determined. Later work confirmed that InsP₃ was not phosphorylated. Converting the 1-phosphate of InsP₃ into a diphosphate group. Notwithstanding the evidence for PP-InsPs playing physiological roles, the present work indicates that a physiological function for 1-PP-Ins₅P₂, at least in relation to the regulation of InsP₃ receptor-mediated Ca²⁺ release, may be unlikely. Converting the 1-phosphate of InsP₃ into a diphosphate group that is suitable for activation of InsP₃R. As the first example of a diphosphate analogue of a second messenger, however, the results add a new component to structure-activity relationships. Co-crystallisation studies with DIPPs using some of the non-hydrolysable substrate analogues discussed here are currently in progress.

## Experimental

### General chemistry methods

General methods were as previously reported. Alcohol 2 = 1,2,3,6-tri-O-benzyl-myoo-inositol 4,5-bis-O-(dibenzyolphosphate) was synthesised according to the literature and crystallised from diethyl ether/light petroleum; m.p. 90–91 °C; Lit. 90–91 °C; [α]D²⁰ = −18.2 (c = 2, CHCl₃). N,N-Diisopropylamino-bis-[2-(methylsulfonyl)ethyl]phosphine (5) was synthesised according to the literature and recrystallized from dichloromethane/ether; m.p. 75.5–77.0 °C; ¹H NMR (CDCl₃, 400 MHz) δ 1.20 (20 H, d, ²JHF 6.8 Hz, 4 x CH₂Ph), 3.01 (6 H, s, 2 x SCH₃), 3.22–3.34 (4 H, m, 2 x OCH₂CH₃), 3.59 (2 H, d, ²JHF 10.4 Hz, ²JHH 6.8 Hz, 2 x CH₂Ph), 4.01–4.15 (4 H, m, 2 x OCH₂CH₃), 4.15 (9 H, m, 4 x OCH₂CH₃), 5.02 (2 x CH₃, 2 x CH₂CH₂), 5.31 (²JCP 12.4 Hz, 2 x CH₃), 5.67 (²JCP 8.3 Hz, 2 x OCH₂CH₃), 5.78 (²JCP 20.0 Hz, 2 x OCH₂CH₃), ¹³C NMR (CDCl₃, 162 MHz, ¹H-decoupled) δ 148.98; HRMS [m/z] [M + H]calcd. for C₁₂H₂₀O₆P₈S₅: 378.1168; found 378.1167. 5-PP-Ins₅P, 1-PP-Ins₅P and their PCP analogues were synthesised using similar methods to those previously described.

ν-2,3,6-tri-O-Benzyl-myoo-inositol-4,5-bis(dibenzyolphosphate)-1-bis[2-(methylsulfonyl)ethyl]phosphate (3). To a solution of alcohol 2 (194 mg, 0.200 mmol) in dry dichloromethane (3 mL) was added 5-phenyl-1H-tetrazole (64 mg, 0.44 mmol) and N,N-diisopropylamino-bis-[2-(methylsulfonyl)ethyl]phosphine (5) (130 mg, 0.344 mmol). The suspension was stirred under N₂ at room temperature for 2 h, after which time TLC (dichloromethane: ethyl acetate 1:1) showed total conversion of 2 (Rf 0.56) into a more polar product (Rf 0.24). The mixture was then cooled to −78 °C, before mpCPBA (70%, 100 mg, 0.406 mmol) was added. The mixture was allowed to warm to room temperature and then diluted with EtOAc (30 mL). The clear, colourless solution was washed with 10% aq. Na₂SO₃ solution (2 x 30 mL) and 1.0 mold per m³ HCl (30 mL), then dried over MgSO₄ and concentrated. The residue was purified by flash chromatography on silica, eluting with methanol in ethyl acetate (0 to 15%) to give 3 as a colourless oil (225 mg, 0.178 mmole, 89%); TLC (ethyl acetate: methanol 10:1) Rf = 0.50; [α]D²⁰ = −10.3, (c = 1.4, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ 2.76 (3 H, s, SCh₃), 2.82 (3 H, s, SCh₃), 2.79–2.87 (1 H, m, OCH₂CH₃), 2.90–3.00 (12 H, d, 3JHP 7.0 Hz, 0.5 ABX system of POCOCH₂H), 3.01 (6 H, s, 2 x OCH₂CH₃), 3.03–3.13 (2 H, m, 2 x OCH₂CH₂CHS), 3.62 (1 H, dd, J 9.8, 1.9 Hz, H-3), 4.10 (1 H, dd, J 9.5, 9.5 Hz, H-6), 4.07–4.40 (6 H, m, H-1, H-2 and 2 x OCH₂CH₃), 4.58–4.73 (6 H, m, H-5 and 2.5 AB systems of OCH₂Ph), 4.82–5.03 (9 H, m, H-4 and 4 x OCH₂Ph), 5.09, 5.11 (1 H, ²JHH 11.9 Hz, ²JHF 7.0 Hz, 0.5 ABX system of POCH₂Ph), 6.95–6.97 (2 H, m, Ph), 7.09–7.40 (33 H, m, Ph); ¹³C NMR (CDCl₃, 162 MHz) δ 42.34 (2 x CH₃), 54.03 (²JCP 7.7 Hz, POCH₂CH₃), 54.20 (²JCP 7.7 Hz, POCH₂CH₃), 61.16–61.24 (overlapping signals with ²JCP couplings, POCH₂CH₃), 69.14–69.55 (overlapping signals with ²JCP couplings, POCH₂Ph), 72.64 (OCH₂Ph), 74.75 (OCH₂Ph), 75.08 (OCH₂Ph), 75.19 (C-2), 77.78–78.12 (overlapping signals with ²JCP couplings, C-1, C-3, C-4 and C-6), 78.81 (C-5), 127.32–128.36 (CH of Ph), 135.51 (²JCP 7.4 Hz, ipso-C of POCH₂Ph), 135.98–136.06 (overlapping signals with ²JCP couplings, 3 x ipso-C of POCH₂Ph), 137.53 (ipso-C of OCH₂Ph), 138.13 (ipso-C of OCH₂Ph), 138.19 (ipso-C of OCH₂Ph), ¹³C NMR (CDCl₃, 162 MHz) δ −3.37 (1 P), −1.94 (1 P), −1.59 (1 P); HRMS (m/z) [M + Na]calcd. for C₆H₁₀O₁₀P₃S₅: 1285.2980; found 1285.3011.

ν-1-Diphasopho-myoo-inositol 4,5-bisphosphate (1). Compound 3 (63 mg, 50 μmol) was dissolved in dry CDCl₃ (1.5 mL) and the solution was transferred to an NMR tube. A ¹³C NMR spectrum (¹H decoupled) showed three peaks as
described above. DBU (30 μL, 200 μmol), followed by BSTFA (53 μL, 200 μmol) was added and the sample was shaken to mix the liquids. A 31P NMR spectrum taken after 1 h now showed three peaks: δ −1.84 (1 P), −2.20 (1 P) and −18.10 (1 P), this last signal corresponding to the bis-silylated phosphate triester at O-1. Methanol (100 μL) was added and the tube was shaken again. After 10 min, TFA (15 μL, 200 μmol) was added and the solution was concentrated by evaporation under reduced pressure, then thoroughly dried under vacuum. A 31P NMR spectrum (CDCl3) of the residue showed that the silyl groups were completely cleaved, with three peaks at δ −0.18 (1 P, P-1), −2.23 (1 P) and −2.45 (1 P). To this residue was added 5-phenyl-1H-tetrazole (20 mg, 137 μmol). Then, under argon, dry dichloromethane (2 mL) followed by bis(benzylxoy)disopropylaminophosphine (30 μL, 89 μmol) were added. The mixture was stirred under argon for 45 min, after which a 31P NMR spectrum of a sample (CDCl3 added) showed major peaks at δ 127.24 (J_Pp 4.2 Hz, P-1p), 7.59 (H-phosphonate by-product from hydrolysis of excess P(ν) reagent), −2.22 and −2.41 (P-4 and P-5) and −10.26 (J_Pp 4.2 Hz, P-1o). The solution was cooled to −78 °C and mCPBA (70%, 25 mg, 100 μmol) was added. After 5 min, the solution was allowed to warm to room temperature, then concentrated under reduced pressure (no heat). A 31P NMR spectrum of the residue now showed peaks at δ 7.62 (H-phosphonate by-product), −2.28 and −2.52 (P-4 and P-5) −11.97 (d, J_Pp 14.7 Hz, P-1p) and −13.69 (d, J_Pp 14.7 Hz, P-1o). This residue was purified by flash chromatography on silica (methanol in ethyl acetate, 0 to 20%) giving 4 as a colourless oil (59 mg, 45 μmol, 90%); TLC (ethyl acetate: methanol 10:1); Rf = 0.30; 31P NMR (CDCl3, 162 MHz, 1H-decoupled) δ −1.75 (1 P, s), −2.35 (1 P, s), −11.08 (1 P, broad s, P-1p), −12.32 (1 P, broad s, P-1o); HRMS (m/z) [M + Na]+ caleld. for C_{12}H_{10}O_{12}P_{4}; 1333.3405; found 1333.3377. In earlier trials, this material had been found to be unstable after flash chromatography; a portion of it was therefore deprotected immediately as follows.

Compound 4 (37 mg, 28 μmol) was dissolved in methanol (4 mL) and deionised water (1 mL). Powdered NaHCO3 (14 mg, 168 μmol) was added followed by Pd(OH)$_2$/C (30 mg). The suspension was stirred vigorously under H₂ (balloon) for 24 h, after which time more water (4 mL) was added. A fresh balloon of H₂ was attached and stirring was continued for a further 72 h. The catalyst was then removed by filtration through a PTFE filter, giving a colourless solution, which was concentrated under reduced pressure to give a solid white residue. Analysis of this residue by 31P and 1H NMR in D$_2$O showed that deprotection was complete. The residue was purified by anion-exchange chromatography on Q-Sepharose Fast Flow resin, eluting with a gradient of 0 to 1.5 M triethylammonium bicarbonate (TEAB). The target compound 1 eluted at 70 to 77% 1.5 M TEAB. Fractions containing the target were identified using the Briggs phosphate assay, combined and evaporated under reduced pressure. De-ionised water was repeatedly added and evaporated until the triethylammonium salt of 1 remained as a colourless glassy solid (14 mg, 16 μmol, 57%). This material was accurately quantified using total phosphate assay before biological evaluation. For 31P and 1H NMR analysis of 1, a small amount of EDTA (sodium salt, approx. 0.1 mg) was added to a sample of 1 (20 μmol in 0.4 mL D$_2$O) to give sharper signals. This NMR sample containing EDTA was kept as the solution in D$_2$O for &gt;1 year at 4 °C with no sign of deterioration. 1H NMR (D$_2$O, 500 MHz, EDTA added) δ 3.77 (1 H, dd, J 9.8, 2.9 Hz, H-3), 3.95 (1 H, t, J 9.6 Hz, H-6), 4.08 (1 H, apparent q, J 9.1 Hz, H-5), 4.15 (1 H, ddd, J 9.9, 8.3, 2.8 Hz, H-1), 4.32 (1 H, apparent q, J 9.4 Hz, H-4), 4.35 (1 H, apparent t, J 2.8 Hz, H-2); 13C NMR (D$_2$O, 101 MHz) δ 70.15 and 70.31 (C-2 and C-3), 70.88 (C-5), 76.28 (JCP 5.6 Hz, C-1), 76.82 (JCP, C-4) and 78.11 (JCP couplings, C-5); 31P NMR (D$_2$O, 202 MHz, EDTA added, 1H-coupled) δ 1.11 (1 P, 0.45 (1 P), 10.48 (1 P, d, J 20.9 Hz, P-1p), −11.96 (d, J 20.9 Hz, P-1o); 31P NMR (D$_2$O, 162 MHz, EDTA added, 1H-coupled) δ 1.13 (1 P, d, J_{C}P 8.8 Hz), 0.47 (1 P, d, J_{C}P 8.9 Hz), −10.46 (1 P, d, J_{C}P 20.5 Hz, P-1p), −11.94 (1 P, d, J_{C}P 20.5, J_{CP} 8.3 Hz, P-1o); HRMS (m/z) M$^+$ caleld. for C$_{12}$H$_{10}$O$_{12}$P$_{4}$; 498.9209; found 498.9214.

Molecular docking of 1-PP-Ins(4,5)P$_2$ (1) into type 1 InsP$_3$ receptor. Molecular docking experiments were carried out using the X-ray crystal structure of the N-terminal IBC of type 1 InsP$_3$ receptor in complex with Ins(1,4,5)P$_3$ (1N4K). Docking methods were optimised by docking flexible models of Ins(1,4,5)P$_3$ into the 1N4K structure using GOLD (version 5.6, CCDC). In the most successful protocol, the binding site was defined as a sphere of 6 Å radius centred on the centroid of bound Ins(1,4,5)P$_3$ and two water molecules (waters 1139 and 1198) were included in the docking protocol. These water molecules were toggled on and off and allowed to spin in the docking runs. The ligand was docked 100 times using the GoldScore scoring function, and genetic algorithm settings for very flexible ligands were used. This method accurately reproduced the observed pose of bound Ins(1,4,5)P$_3$ in 1N4K; the ten highest scoring poses all closely resembled the conformation of bound Ins(1,4,5)P$_3$ (mean RMSD 0.58 Å). When 1-PP-Ins(4,5)P$_2$ (1) was docked using the same protocol, the highest-scoring poses were very similar to the bound conformation of Ins(1,4,5)P$_3$ but often showed additional interactions of the 1-beta-phosphate group with residues in the binding site. More details are given in the ESI.

Assays of InsP$_3$ receptor activity. Ca$^{2+}$ release from the intracellular stores of permeabilised DT40 cells expressing rat type 1 InsP$_3$ receptors was measured in cytosol-like medium (CLM) using a low-affinity fluorescent Ca$^{2+}$ indicator trapped within the endoplasmic reticulum as previously reported. Equilibrium competition binding of [H]-InsP$_3$ (1.5 nM, 19.3 Ci mmol$^{-1}$) to membranes prepared from insect Sf9 cells expressing rat type 1 InsP$_3$ receptors was determined in CLM at 4 °C. Bound and free ligand were separated by centrifugation and non-specific binding was determined by addition of 10 μM InsP$_3$.

DIPP purification. cDNAs for all DIPPs were kind gifts from the Structural Genomics Consortium, Stockholm. cDNAs were
modified as necessary in order to represent the full-length constructs, cloned into pET28a (+) and expressed as N-terminally His-tagged proteins. All proteins were expressed in BL21 (DE3) T7R pRARE2 at 18 °C overnight and purified by the Protein Science Facility (PSF) at the Karolinska Institute, Stockholm. Briefly, the proteins were first purified over a HisTrap HP column (GE Healthcare), followed by thrombin cleavage of the N-terminal His-tag. After removal of the His-tag through a second run over a HisTrap HP column, the proteins were further purified by gel filtration using a HiLoad 16/60 Superdex 75 column (GE Healthcare).

**Enzyme activity assay (DIPPs).** Activity of DIPPs with a panel of potential substrates (1-PP-InsP₅, 5-PP-InsP₅, 1-PP-Ins(4,5)P₂, P₃, Ap₃A, and Ap₅A (Sigma Aldrich)) and control compounds (1-PCP-InsP₅ and 5-PCP-InsP₅) was assessed in technical triplicates in reaction buffer (100 mM Tris acetate, pH 7.5, 40 mM NaCl, 1 mM DTT) containing either 1 mM Mg acetate or MnCl₂. Following an incubation time of 20 min at room temperature with shaking, the formed inorganic phosphate was detected through addition of malachite green reagent. After an additional 15 min incubation with shaking, absorbance at 630 nm was read using a Hitex Sense plate reader.

**Differential scanning fluorimetry (DSF).** DSF was performed with 5 μM purified protein in 25 mM HEPES pH 7.5, 100 mM NaCl, 0.5 mM TCEP and 5× Sypro Orange added per well of a 96-well PCR plate. Substrates and substrate analogues were dissolved in water and diluted 1:10 in the assay mixture. The heat denaturation curves with a temperature increase of 1 °C min⁻¹ from 25 °C to 95 °C were recorded on a CFX96 real-time PCR machine (Bio-Rad) by measuring the fluorescence of Sypro Orange with excitation and emission wavelengths of 470 and 570 nm, respectively. The Boltzmann equation was used to analyse the denaturation curves in GraphPad Prism. The determined melting temperature (T_m) is the inflection point of the sigmoidal denaturation curve.

**Conflicts of interest**

There are no conflicts to declare.

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**Notes and references**


