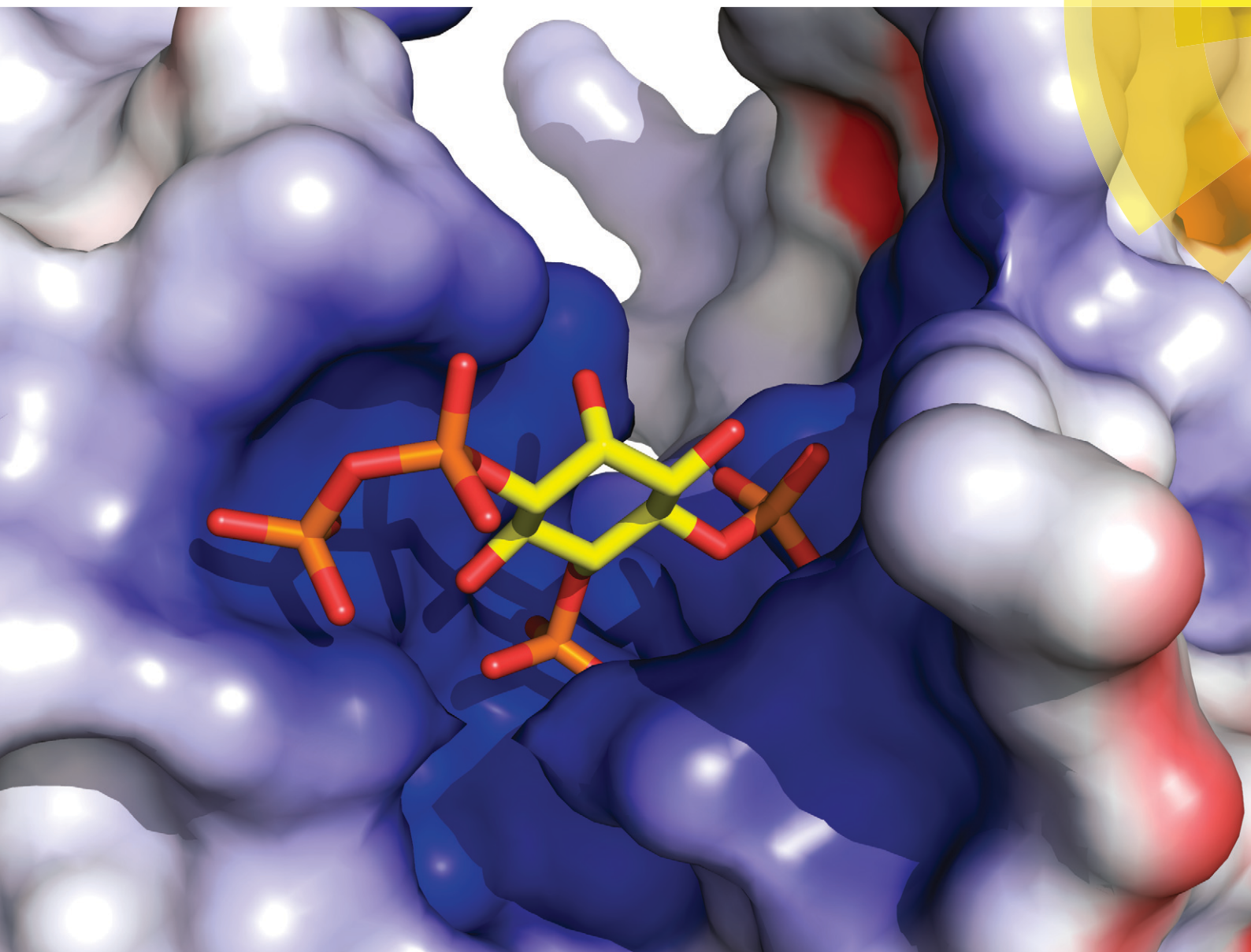


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ISSN 2040-2511



RESEARCH ARTICLE

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 Cite this: *Med. Chem. Commun.*,
 2018, 9, 1105

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-InsP₅ and 5-PP-InsP₅. 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.

 Received 16th March 2018,
 Accepted 1st May 2018

DOI: 10.1039/c8md00149a

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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).¹ InsPs regulate many cellular processes, the best known being the release of Ca²⁺ from intracellular stores by D-*myo*-inositol 1,4,5-trisphosphate (InsP₃), which binds to receptors on the endoplasmic reticulum.² InsP₃ is converted *via* a series of enzymatic phosphorylations³ into InsP₆ (Fig. 1), which can then be further phosphorylated to give highly charged PP-InsPs containing diphosphate (pyrophosphate) groups.^{4,5}

InsP₃ receptors (IP₃Rs) are tetrameric intracellular Ca²⁺ channels, expressed in most animal cells.² When InsP₃ binds to the N-terminal InsP₃-binding core (IBC) of all four IP₃R

subunits,⁶ 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 essential⁷) 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.⁸

Although PP-InsP signalling is thought to be more evolutionarily ancient than InsP₃-mediated mobilisation of Ca²⁺,⁹ 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.^{4,5} Possible receptors for PP-InsPs include the PH (pleckstrin homology) domains^{10,11} and SPX (SYG1/Pho81/XPR1) domains^{12,13} of proteins. PP-InsPs may also exert some of their effects by direct non-enzymatic diphosphorylation of target proteins.¹⁴

Phosphorylating a phosphate monoester in an InsP_n to give a PP-InsP_{n-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.⁴ For example, some PH domains that bind InsP₆ bind 5-InsP₇ with higher affinity,^{10,11} while 1-InsP₇ and InsP₈ are weaker.¹¹ In contrast, both 1-InsP₇ and 5-InsP₇ stimulate synthesis of inorganic polyphosphate (polyP) by the

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† Electronic supplementary information (ESI) available: NMR spectra, additional DSF data and details of molecular docking experiments. See DOI: 10.1039/c8md00149a



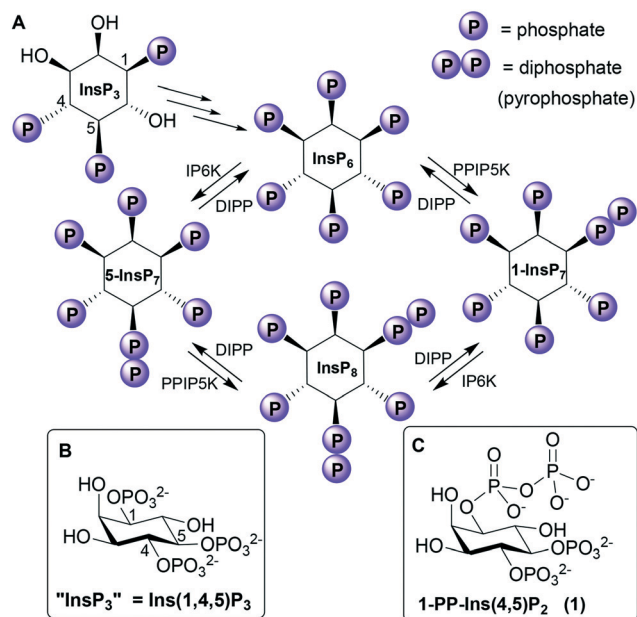


Fig. 1 A. Biosynthetic pathway connecting Ins(1,4,5)P₃ to the PP-InsPs. IP6K, inositol hexakisphosphate 5-kinase; PPIP5K, diphosphoinositol pentakisphosphate kinase; DIPPs, diphosphoinositol polyphosphate phosphohydrolase. “InsP₃”, “5-InsP₇”, “1-InsP₇” and “InsP₈” are alternative names for Ins(1,4,5)P₃, 5-PP-InsP₅, 1-PP-InsP₅ and 1,5-[PP]₂-InsP₄, respectively. B. Structure of Ins(1,4,5)P₃. C. Structure of the synthetic analogue 1-PP-Ins(4,5)P₂ (1).

vacuolar transporter chaperone (VTC) by binding to its SPX domain, while InsP₆ is inactive and InsP₈ is 20-fold more potent.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.^{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 InsP₃ into a diphosphate. The 1-phosphate group of InsP₃ has been a popular target for synthetic elaboration of InsP₃ since early structure–activity studies showed that it is much more tolerant of modification than the 4- or 5-phosphate groups.⁸ Interest in the role of the 1-phosphate group was further stimulated by the discovery in 1993 of the adenophostins, fungal metabolites that are highly potent InsP₃ receptor ligands.¹⁶ The adenophostins contain a glucopyranoside 3,4-bisphosphate structure that mimics the *myo*-inositol 4,5-bisphosphate of InsP₃ but intriguingly, their third phosphate group is located on a separate (ribofuranoside) ring, suggesting that repositioning this phosphate group may enhance affinity.¹⁷

The X-ray structure¹⁸ of the IBC of type 1 InsP₃ receptor bound to InsP₃ confirmed the area of the binding pocket around the 1-phosphate of bound InsP₃ 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 an-

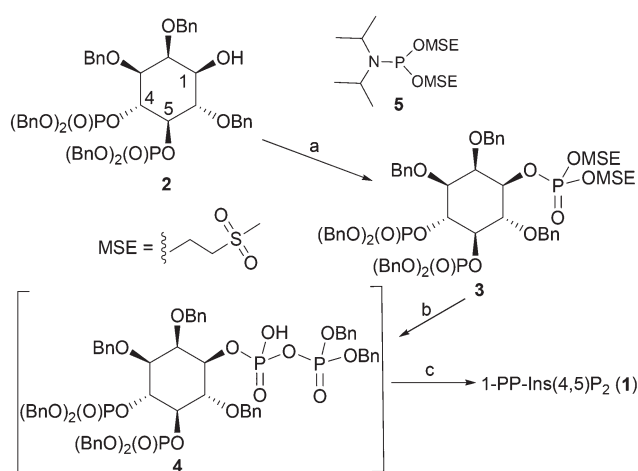
alogue of InsP₃, *i.e.* 1D-diphospho-*myo*-inositol 4,5-bisphosphate [1-PP-Ins(4,5)P₂ (1), Fig. 1] and examine its interaction with InsP₃ receptors.

We were also interested to examine the interaction of 1-PP-Ins(4,5)P₂ with DIPPs. Although DIPPs can hydrolyse the PP groups of highly phosphorylated PP-InsPs (Fig. 1), inorganic polyphosphate, 5-phosphoribosyl 1-pyrophosphate and nucleotide dimers,^{3,15} their catalytic mechanisms are poorly understood. 1-PP-Ins(4,5)P₂ contains the target 1-PP structure found in the known DIPP substrate 1-PP-InsP₅, but presented in the context of a molecule with only two phosphate monoester groups. There are no reports in the literature on whether “lower” PP-InsPs such as 1-PP-Ins(4,5)P₂ could be recognised by the active sites of DIPPs.

Results and discussion

Chemistry

The synthesis of 1-PP-Ins(4,5)P₂ (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,^{21,22} in which a temporarily protected phosphate group is introduced and then selectively deprotected to reveal a phosphate monoester. This phosphate is then phosphitylated to give a mixed P(III)–P(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 methylsulfonyl ethyl (MSE)^{23,24} as a temporary phosphate protecting group in this sequence. The MSE group can be removed by β -elimination, similar to the better-known β -cyanoethyl (β -CE)^{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.



Scheme 1 Synthesis of 1-PP-Ins(4,5)P₂ (1). Reagents and conditions: a. i. 5-phenyl-1*H*-tetrazole, 5, CH₂Cl₂; ii. *m*CPBA, CH₂Cl₂, 89%; b. i. DBU, BSTFA, CDCl₃; ii. MeOH, then TFA; iii. 5-phenyl-1*H*-tetrazole, (BnO)₂PNPr₂, CH₂Cl₂; iv. *m*CPBA, CH₂Cl₂, 90%; c. i. H₂, Pd(OH)₂/C, MeOH, H₂O, NaHCO₃; ii. Ion-exchange chromatography on Q-Sepharose Fast Flow resin, 57%. Bn, benzyl.



Thus, the 1-OH group in **2** was reacted with **5** in the presence of 5-phenyl-1*H*-tetrazole to give an intermediate MSE-protected phosphite triester. Oxidation using *m*CPBA 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,^{21,22,25} with slight modifications. The progress of each step was carefully monitored by ³¹P 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)P₂ (**1**) as the triethylammonium salt, which was accurately quantified by total phosphate assay.

Interactions of 1-PP-Ins(4,5)P₂ with type 1 InsP₃ receptors

Both InsP₃ and 1-PP-Ins(4,5)P₂ (**1**) stimulated a concentration-dependent release of Ca²⁺ from the intracellular stores of permeabilised DT40 cells expressing type 1 InsP₃ receptors (Fig. 2A). The maximal Ca²⁺ release evoked by each ligand

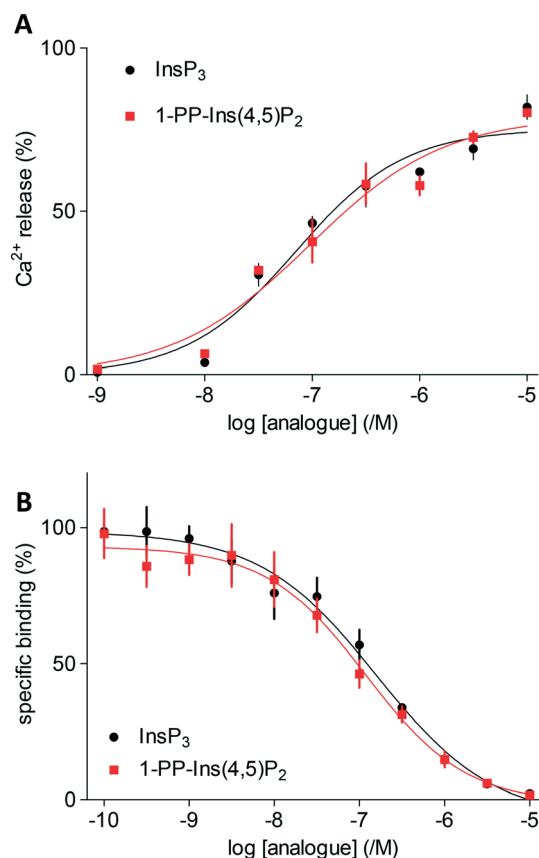


Fig. 2 A. Ca²⁺ release from intracellular stores of DT40 cells expressing type 1 InsP₃ receptors stimulated by InsP₃ and 1-PP-Ins(4,5)P₂ (**1**). Results are shown as % of Ca²⁺ content of intracellular stores. B. Equilibrium competition binding with ³H-InsP₃ and InsP₃ or 1-PP-Ins(4,5)P₂ (**1**) using membranes from Sf9-IP₃R1 cells in CLM containing 1.5 mM MgATP. Results are means ± s.e.m., *n* = 3.

and the half-maximally effective concentration (EC₅₀) were similar for **1** and InsP₃ (Fig. 2A). Membranes from Sf9 cells expressing rat type 1 InsP₃ receptors were used for equilibrium competition binding studies with ³H-InsP₃, because these membranes express full-length type 1 InsP₃ 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 Ca²⁺-release assays.

In agreement with the Ca²⁺-release assays, 1-PP-Ins(4,5)P₂ (**1**) bound with the same affinity as InsP₃ to InsP₃ 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 InsP₃ and **1**, but we saw no evidence that **1** is unstable. The ³¹P NMR spectrum of **1** in D₂O (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 InsP₃ receptor¹⁸ suggested that the diphosphate group in **1** should be well-tolerated by the InsP₃-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 InsP₃ for type 1 InsP₃ receptors.

Interaction of 1-PP-Ins(4,5)P₂ with DIPP

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)P₂ (**1**) with all four DIPPs in comparison

Table 1 Interactions of InsP₃ and 1-PP-Ins(4,5)P₂ (**1**) with type 1 InsP₃ receptors (*n* = 3)

	Ca ²⁺ release		Binding ^a		
	pEC ₅₀ /M	EC ₅₀ /nM	% release	pK _d /M	K _d /nM
InsP ₃	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 InsP₃ receptors in CLM (pH 7.3) containing 1.5 mM Mg-ATP to match the conditions used in the Ca²⁺ release assay.



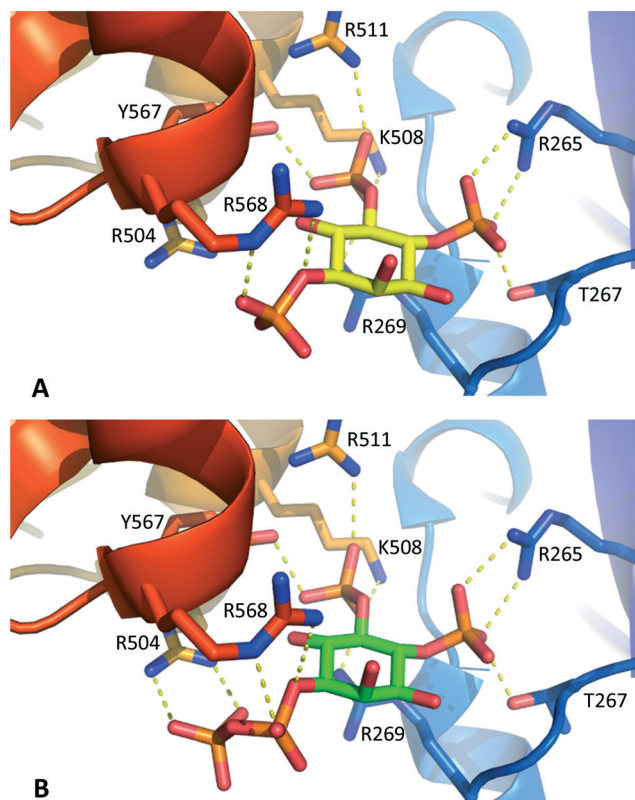


Fig. 3 A. Interactions of InsP₃ with the IBC of type 1 InsP₃ receptors, based on the X-ray crystal structure of IP₃R1 in complex with InsP₃ (ref. 18) (1N4K). B. Model of 1-PP-Ins(4,5)P₂ (1) in the IBC produced by molecular docking (see Experimental section and ESI† for details). For clarity, water molecules are not shown.

with two naturally-occurring substrates 1-PP-InsP₅ and 5-PP-InsP₅ (“1-InsP₇” and “5-InsP₇”, respectively) and also with the alternative substrates diadenosine polyphosphates Ap₃A and Ap₅A. Non-hydrolysable InsP₇ analogues 1-PCP-InsP₅ (ref. 27) and 5-PCP-InsP₅ (ref. 28) were independently synthesised and included as controls.

With Mg²⁺ present in the buffer, 1-PP-InsP₅ and 5-PP-InsP₅ were rapidly metabolised by all four DIPPs (Fig. 4A). The rate of hydrolysis of 1-PP-InsP₅ was significantly higher than that for 5-PP-InsP₅ in each case. This finding is in agreement with a previous study.¹⁵ As expected, the PCP analogues were not metabolised, confirming that DIPPs can hydrolyse only the diphosphate unit and not the phosphate monoesters. Ap₃A and Ap₅A were unaffected by all four enzymes in Mg²⁺-containing buffer, an observation that had been reported for NUDT10 and NUDT11, but not for NUDT3 and NUDT4.²⁹ Perhaps surprisingly, 1-PP-Ins(4,5)P₂ (1) was also not metabolised under these conditions. The presence of a divalent cation is required for the activity of NUDT10 and NUDT11 and also for NUDT3.³ When Mg²⁺ in the buffer was replaced by Mn²⁺, 1 was now hydrolysed by the DIPPs, while 1-PP-InsP₅ and 5-PP-InsP₅ resisted hydrolysis. In addition, Ap₅A 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 pres-

ence of either Mg²⁺ or Mn²⁺-containing buffers. This further supports our conclusion above that 1 was not hydrolysed to InsP₃ during the InsP₃ receptor assays.

Next, we used differential scanning fluorimetry (DSF) to measure the ability of the compounds to stabilise NUDT3 (DIPP1). While the effects of Ap₃A and Ap₅A were not significantly different from control (Fig. 5A), 1-PP-Ins(4,5)P₂ (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-InsP₅ 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. Ap₃A 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-InsP₅ and 1-PP-Ins(4,5)P₂ (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-InsP₅ and 5-PCP-InsP₅ (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.^{10–13} Structure-activity studies have previously shown that the 1-phosphate group of InsP₃ 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 InsP₃ receptor. We therefore synthesised 1-PP-Ins(4,5)P₂ (1), the first PP-containing analogue of InsP₃. Using assays of Ca²⁺-release through type 1 InsP₃ receptors, we found that 1 was equipotent to InsP₃ and in binding assays its affinity was indistinguishable from that of InsP₃. Thus, the 1-diphosphate modification of InsP₃ does not affect its affinity for or activity at type 1 InsP₃ receptors. Nevertheless, 1 is the first Ca²⁺-releasing PP-InsP and also the most potent P-1 modified ligand of InsP₃ receptors yet identified.‡

The novel diphosphate compound 1 was not metabolised by DIPPs in the presence of Mg²⁺-containing buffer, while the naturally-occurring InsP₇ isomers, 5-PP-InsP₅ and 1-PP-InsP₅

‡ A synthetic InsP₃ derivative featuring 4-carboxy-malachite green conjugated to the 1-phosphate group was reported to have ~170-fold higher affinity than InsP₃ for an N-terminal fragment of type 1 InsP₃ receptors.⁴³ In our hands, this compound was ~5-fold less potent than InsP₃ at each InsP₃ receptor subtype and had an affinity ~7-fold less than InsP₃ for type 1 InsP₃ receptors.⁴⁴



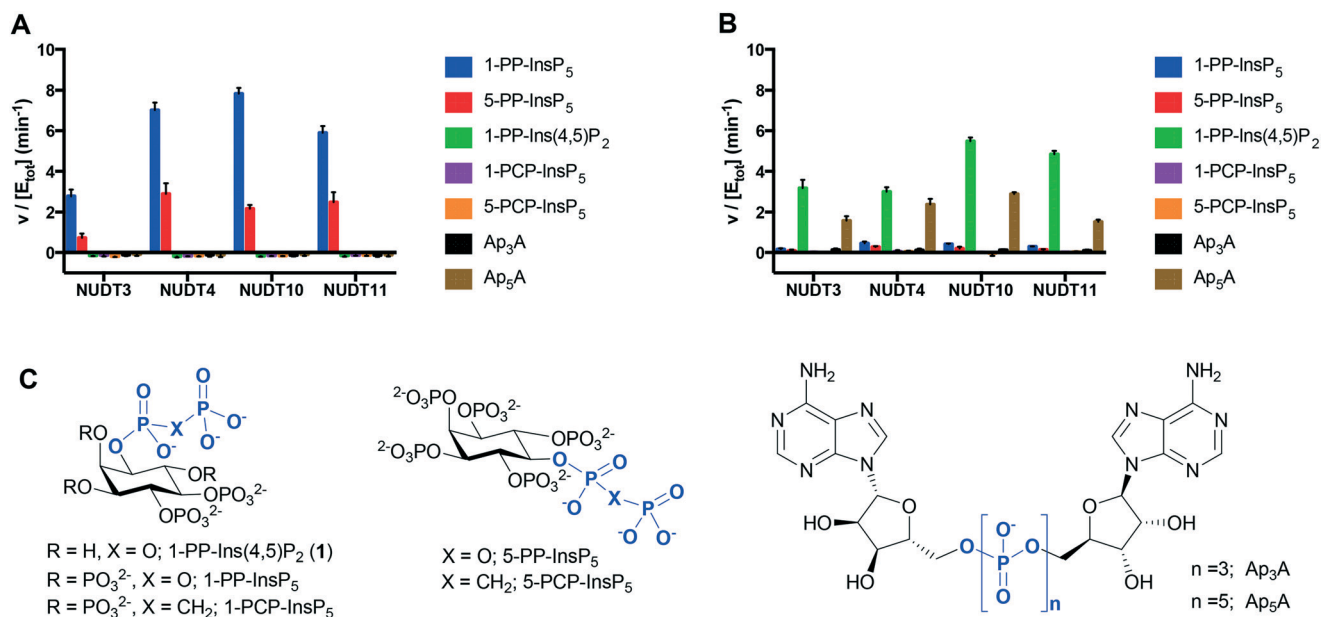


Fig. 4 Specific activities of DIPP with 1-PP-Ins(4,5)P₂ (1), known substrates 1-PP-InsP₅ and 5-PP-InsP₅ and controls. Experiments were conducted in buffer containing Mg²⁺ (A) or Mn²⁺ (B). Data shown represent the formed concentration of Pi (micromolar) per enzyme concentration (micromolar) per minute. A630 was converted to Pi concentration (micromolar) using the equation A630 = 0.01897[P_i] - 0.5877 (Mg²⁺ containing buffer) or A630 = 0.01923[P_i] + 0.1053 (Mn²⁺ containing buffer). C. Structures of compounds examined, including methylenebisphosphonate (PCP) analogues of InsP₇, and diadenosine polyphosphates Ap₃A and Ap₅A.

were rapidly hydrolysed. Conversely, in the presence of Mn²⁺, 1 was hydrolysed while the two InsP₇ isomers were unaffected. Synthetic PCP-containing analogues of the InsP₇s 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.

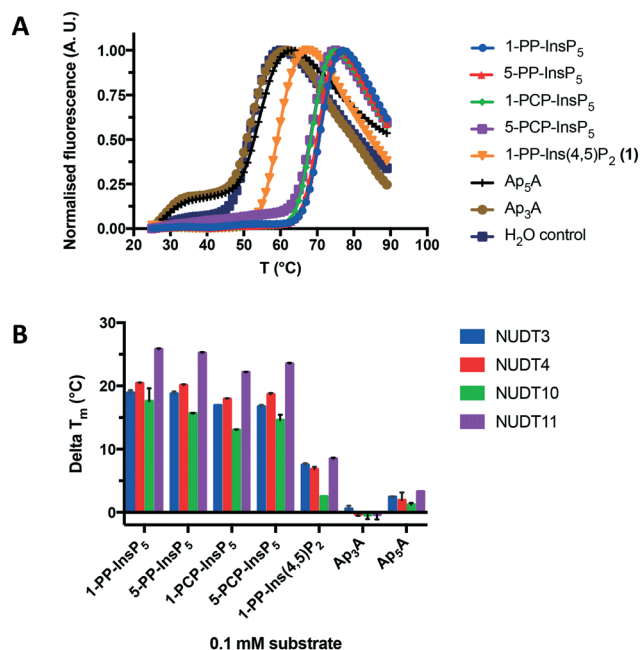


Fig. 5 A. Effect of 1-PP-Ins(4,5)P₂ (1) and other compounds shown in Fig. 4C on the melting temperature (T_m) of NUDT3, measured using differential scanning fluorimetry (DSF). B. Comparison of melting temperature shifts (delta T_m) induced by all compounds for all four DIPP compounds examined.

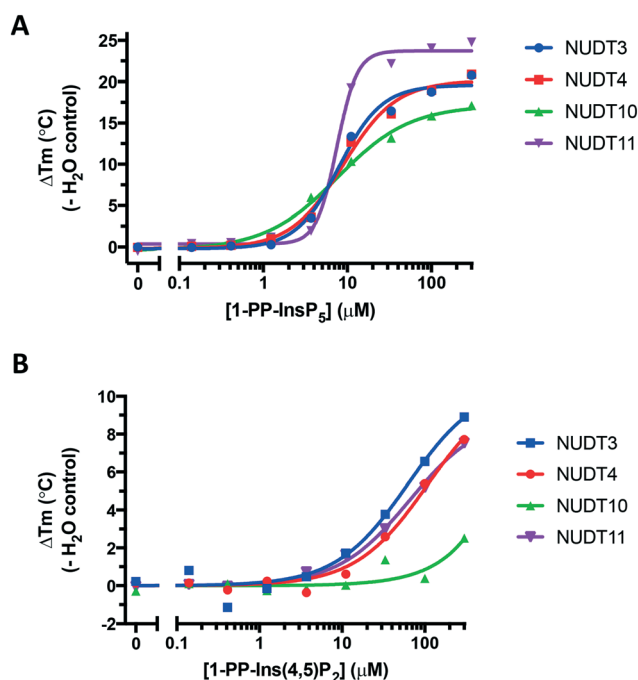


Fig. 6 Dose-response curves showing stabilisation of all four DIPP proteins by A. 1-PP-InsP₅ and B. 1-PP-Ins(4,5)P₂ (1). Note the different y-axis scales in A and B.



This strongly suggests that 1-PCP-InsP₅ and 5-PCP-InsP₅ could be promising ligands for co-crystallisation studies with DIPP₅.

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 synthesise PP-InsP₄ from InsP₅ *in vitro*,³⁰ but the products of InsP₃ phosphorylation by IPMK are Ins(1,3,4,5)P₄ and/or Ins(1,4,5,6)P₄.³¹ Phosphorylation of lower InsPs by PPIP5Ks seems unlikely, considering the constraints of the catalytic site³² and the recently discovered capture site;²² even Ins(1,3,4,5,6)P₅ is not phosphorylated.³² Recombinant Kcs1p, a yeast homologue of IP6K1, was reported to phosphorylate InsP₃ slowly, although the identities of the products could not be determined.³³ Later work confirmed that InsP₃ was phosphorylated by Kcs1 and the product was identified as Ins(1,3,4,5)P₄ (*i.e.* in this case, Kcs1 functioned as a 3-kinase).³⁴ More recently, a study found that EhIP6KA, an IP6K homologue from *Entamoeba histolytica*, was capable of slowly phosphorylating InsP₃, although the products were identified as Ins(1,4,5,6)P₄ and Ins(1,2,4,5)P₄.³⁵ On this basis, naturally occurring 1-PP-Ins(4,5)P₂ seems unlikely. However, in both studies where the identities of the enzyme products were assigned,^{34,35} resistance to hydrolysis by DIPP1 was used to exclude the possibility that the products contained diphosphate groups. The present work shows that this criterion may not always be valid; in our hands, 1-PP-Ins(4,5)P₂ was not metabolised in the presence of Mg²⁺ by any of the DIPP₅, yet it does contain a diphosphate group.

Notwithstanding the evidence for PP-InsPs playing physiological roles,^{4,5} the present work indicates that a physiological function for 1-PP-Ins(4,5)P₂, 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 neither attenuates nor enhances the ability of the ligand to activate 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 DIPP₅ using some of the non-hydrolysable substrate analogues discussed here are currently in progress.

Experimental

General chemistry methods

General methods were as previously reported.³⁶ Alcohol 2 = 1*D*-2,3,6-*tri-O*-benzyl-*myo*-inositol 4,5-*bis-O*-(dibenzylphosphate) was synthesised according to the literature¹⁹ and crystallised from diethyl ether/light petroleum; m.p. 90–91 °C; Lit.¹⁹ 90–91 °C; [α]_D²⁰ = –18.2, (*c* = 2, CHCl₃), Lit.¹⁹ [α]_D²⁵ = –15.6, (*c* = 1, CHCl₃); Lit.²⁰ [α]_D²⁰ = –17.8, (*c* = 1.7, CHCl₃). *N,N*-Diisopropylamino-bis-[2-(methylsulfonyl)ethoxy]-phosphine (5) was synthesised according to the literature²⁴ and recrystallized from dichloromethane/ether; m.p. 75.5–77.0 °C;

¹H NMR (CDCl₃, 400 MHz) δ 1.20 (12 H, d, ³J_{HP} 6.8 Hz, 4 × CHCH₃), 3.01 (6 H, s, 2 × SCH₃), 3.22–3.34 (4 H, m, 2 × OCH₂CH₂S), 3.59 (2 H, dh, ³J_{HP} 10.4 Hz, ³J_{HH} 6.8 Hz, 2 × CHCH₃), 4.01–4.15 (4 H, m, 2 × OCH₂CH₂S); ¹³C NMR (CDCl₃, 101 MHz) δ 24.62 (³J_{CP} 7.3 Hz, 4 × CHCH₃), 42.85 (2 × SCH₃), 43.31 (²J_{CP} 12.4 Hz, 2 × CHCH₃), 56.17 (³J_{CP} 8.3 Hz, 2 × OCH₂CH₂S), 57.58 (²J_{CP} 20.0 Hz, 2 × OCH₂CH₂S); ³¹P NMR (CDCl₃, 162 MHz, ¹H-decoupled) δ 148.98; HRMS (*m/z*) [*M* + H]⁺ calcd. for C₁₂H₂₈O₆NPS₂; 378.11684; found 378.11687. 5-PP-InsP₅, 1-PP-InsP₅ and their PCP analogues were synthesised using similar methods to those previously described.^{21,22,27,28,36}

D-2,3,6-tri-*O*-Benzyl-*myo*-inositol-4,5-bis(dibenzylphosphate)-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-1*H*-tetrazole (64 mg, 0.44 mmol) and *N,N*-diisopropylamino-bis-[2-(methylsulfonyl)ethoxy]-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 (*R*_f 0.56) into a more polar product (*R*_f 0.24). The mixture was then cooled to –78 °C, before *m*CPBA (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 × 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): *R*_f = 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, OCH₂CHCHS), 2.92–3.00 (1 H, m, OCH₂CHCHS), 3.03–3.13 (2 H, m, 2 × OCH₂CHCHS), 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 × OCH₂CH₂CHS), 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 × OCH₂Ph), 5.09, 5.11 (1 H, ²J_{AB} 11.9 Hz, ³J_{HP} 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, 100 MHz) δ 42.34 (2 × CH₃), 54.03 (³J_{CP} 7.7 Hz, POCH₂CH₂S), 54.20 (³J_{CP} 7.7 Hz, POCH₂CH₂S), 61.16–61.24 (overlapping signals with ²J_{CP} couplings, POCH₂CH₂S), 69.14–69.55 (overlapping signals with ²J_{CP} couplings, POCH₂Ph), 72.64 (OCH₂Ph), 74.75 (OCH₂Ph), 75.08 (OCH₂Ph), 75.19 (C-2), 77.78–78.12 (overlapping signals with *J*_{CP} couplings, C-1, C-3, C-4 and C-6), 78.81 (C-5), 127.32–128.36 (CH of Ph), 135.51 (³J_{CP} 7.4 Hz, *ipso*-C of POCH₂Ph), 135.98–136.06 (overlapping signals with ³J_{CP} couplings, 3 × *ipso*-C of POCH₂Ph), 137.53 (*ipso*-C of OCH₂Ph), 138.13 (*ipso*-C of OCH₂Ph), 138.19 (*ipso*-C of OCH₂Ph); ³¹P NMR (CDCl₃, 162 MHz) δ –3.37 (1 P), –1.94 (1 P), –1.59 (1 P); HRMS (*m/z*) [*M* + Na]⁺ calcd. for C₆₁H₆₉O₁₉P₃S₂; 1285.2980; found 1285.3011.

D-1-Diphospho-*myo*-inositol 4,5-bisphosphate (1). Compound 3 (63 mg, 50 μ mol) was dissolved in dry CDCl₃ (1.5 mL) and the solution was transferred to an NMR tube. A ³¹P NMR spectrum (¹H decoupled) showed three peaks as



described above. DBU (30 μ L, 200 μ mol), followed by BSTFA (53 μ L, 200 μ mol) was added and the sample was shaken to mix the liquids. A ^{31}P 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 μ L) was added and the tube was shaken again. After 10 min, TFA (15 μ L, 200 μ mol) was added and the solution was concentrated by evaporation under reduced pressure, then thoroughly dried under vacuum. A ^{31}P NMR spectrum (CDCl_3) 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 μ mol). Then, under argon, dry dichloromethane (2 mL) followed by bis(benzyloxy)diisopropylaminophosphine (30 μ L, 89 μ mol) were added. The mixture was stirred under argon for 45 min, after which time a ^{31}P NMR spectrum of a sample (CDCl_3 added) showed major peaks at δ 127.24 ($^2J_{\text{PP}}$ 4.2 Hz, P-1 $_{\beta}$), 7.59 (H-phosphonate by-product from hydrolysis of excess P(III) reagent), -2.22 and -2.41 (P-4 and P-5) and -10.26 ($^2J_{\text{PP}}$ 4.2 Hz, P-1 $_{\alpha}$). The solution was cooled to -78 $^{\circ}\text{C}$ and *m*CPBA (70%, 25 mg, 100 μ mol) was added. After 5 min, the solution was allowed to warm to room temperature, then concentrated under reduced pressure (no heat). A ^{31}P 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, $^2J_{\text{PP}}$ 14.7 Hz, P-1 $_{\beta}$) and -13.69 (d, $^2J_{\text{PP}}$ 14.7 Hz, P-1 $_{\alpha}$). 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 μ mol, 90%); TLC (ethyl acetate:methanol 10:1): R_f = 0.30; ^{31}P NMR (CDCl_3 , 162 MHz, ^1H -decoupled) δ -1.75 (1 P, s), -2.35 (1 P, s), -11.08 (1 P, broad s, P-1 $_{\beta}$), -12.32 (1 P, broad s, P-1 $_{\alpha}$); HRMS (m/z) [$M + \text{Na}$] $^{+}$ calcd. for $\text{C}_{69}\text{H}_{70}\text{O}_{18}\text{P}_4$; 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 μ mol) was dissolved in methanol (4 mL) and deionised water (1 mL). Powdered NaHCO_3 (14 mg, 168 μ mol) was added followed by $\text{Pd}(\text{OH})_2/\text{C}$ (30 mg). The suspension was stirred vigorously under H_2 (balloon) for 24 h, after which time more water (4 mL) was added. A fresh balloon of H_2 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 ^{31}P and ^1H NMR in D_2O 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 μ mol, 57%). This material was accurately quantified using total phosphate assay³⁷ before biological evaluation. For ^{31}P and ^1H NMR analysis of 1, a small amount of EDTA (sodium salt, approx. 0.1 mg) was added to a sample of 1 (2.0 μ mole in 0.4 mL D_2O) to give sharper signals. This NMR sample containing EDTA was kept as the solution in D_2O for >1 year at 4 $^{\circ}\text{C}$ with no sign of deterioration. ^1H NMR (D_2O , 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); ^{13}C NMR (D_2O , 101 MHz) δ 70.15 and 70.31 (C-2 and C-3), 70.88 (C-6), 76.28 ($^2J_{\text{CP}}$ 5.6 Hz, C-1), 76.82 (with J_{CP} couplings, C-4) and 78.11 (with J_{CP} couplings, C-5); ^{31}P NMR (D_2O , 202 MHz, EDTA added, ^1H -decoupled) δ 1.11 (1 P), 0.45 (1 P), -10.48 (1 P, d, J 20.9 Hz, P-1 $_{\beta}$), -11.96 (d, J 20.9 Hz, P-1 $_{\alpha}$); ^{31}P NMR (D_2O , 162 MHz, EDTA added, ^1H -coupled) δ 1.13 (1 P, d, $^3J_{\text{HP}}$ 8.8 Hz), 0.47 (1 P, d, $^3J_{\text{HP}}$ 8.9 Hz), -10.46 (1 P, d, $^2J_{\text{PP}}$ 20.5 Hz, P-1 $_{\beta}$), -11.94 (1 P, dd, $^2J_{\text{PP}}$ 20.5, $^3J_{\text{HP}}$ 8.3 Hz, P-1 $_{\alpha}$); HRMS (m/z) M^{-} calcd. for $\text{C}_6\text{H}_{16}\text{O}_{18}\text{P}_4$; 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 $\text{Ins}(1,4,5)\text{P}_3$ (1N4K).¹⁸ Docking methods were optimised by docking flexible models of $\text{Ins}(1,4,5)\text{P}_3$ into the 1N4K structure using GOLD³⁸ (version 5.6, CCDC). In the most successful protocol, the binding site was defined as a sphere of 6 \AA radius centred on the centroid of bound $\text{Ins}(1,4,5)\text{P}_3$ 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.³⁹ 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 $\text{Ins}(1,4,5)\text{P}_3$ in 1N4K; the ten highest scoring poses all closely resembled the conformation of bound $\text{Ins}(1,4,5)\text{P}_3$ (mean RMSD 0.58 \AA). 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 $\text{Ins}(1,4,5)\text{P}_3$ 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.⁴⁰ Equilibrium competition binding of [^3H]- 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 $^{\circ}\text{C}$. 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) T1R 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 (1), 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.⁴¹ After an additional 15 min incubation with shaking, absorbance at 630 nm was read using a Hidex Sense plate reader.

Differential scanning fluorimetry (DSF). DSF⁴² 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.

Acknowledgements

BVLP (grant 101010) and CWT (grant 101844) are Wellcome Trust Senior Investigators. We also acknowledge funding by the Swedish Pain Relief Foundation and the Swedish Cancer Society (TH). We thank Dr Stephen B. Shears for helpful discussions on the enzymes that are known to synthesise PP-InsPs.

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