**Synthesis of an α-phosphono-α,α-difluoroacetamide analogue of the diphosphoinositol pentakisphosphate 5-InsP7**

Andrew M. Riley, Huanchen Wang, Stephen B. Shears and Barry V. L. Potter

Diphosphoinositol phosphates (PP-InsPs, “inositol pyrophosphates”) are of fundamental importance to all eukaryotes, with pivotal roles in cellular and organismal metabolic homeostasis (Fig. 1). The acute clustering of monophosphate and diphosphate groups around the hexahydroxycyclohexane ring of myo-inositol (Ins) endows the PP-InsPs with the most concentrated three-dimensional array of phosphate groups found in Nature. The PP-InsPs are formed through the enzymatic phosphorylation of myo-inositol hexakisphosphate (InsP6, Fig. 1) by inositol hexakisphosphate kinases (IP6Ks) and diphosphoinositol pentakisphosphate kinases (PPIP5Ks).

Among the PP-InsPs, 5-diphospho-myoinositol pentakisphosphate (5-PP-InsP5, also known as “5-InsP”) is both the most abundant and the most intensively studied member of this signalling family.

**Introduction**

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Among the PP-InsPs, 5-diphospho-myoinositol pentakisphosphate (5-PP-InsP5, also known as “5-InsP”) is both the most abundant and the most intensively studied member of this signalling family.

**Synthesis of an α-phosphono-α,α-difluoroacetamide analogue of the diphosphoinositol pentakisphosphate 5-InsP7**

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Diphosphoinositol phosphates (PP-InsPs, “inositol pyrophosphates”) are of fundamental importance to all eukaryotes, with pivotal roles in cellular and organismal metabolic homeostasis. As the detailed mechanisms of PP-InsP signalling begin to emerge, synthetic analogues of PP-InsPs containing stabilised mimics of the labile diphosphate group can provide valuable investigational tools. We synthesised 5-PCF2Am-InsP5 (1), a novel fluorinated phosphonate analogue of 5-PP-InsP5, and obtained an X-ray crystal structure of 1 in complex with diphosphoinositol pentakisphosphate kinase 2 (PPIP5K2). 5-PCF2Am-InsP5 binds to the kinase domain of PPIP5K2 in a similar orientation to that of the natural substrate 5-PP-InsP5 and the PCF2Am structure can mimic many aspects of the diphosphate group in 5-PP-InsP5. We propose that 1, the structural and electronic properties of which are in some ways complementary to those of existing phosphonoacetate and methylenebisphosphonate analogues of 5-PP-InsP5, may be a useful addition to the expanding array of chemical tools for the investigation of signalling by PP-InsPs. The PCF2Am group may also deserve attention for wider application as a diphosphate mimic.

An understanding of the molecular actions of PP-InsPs by their addition to cell-free systems can be confounded by the enzymatic and chemical instability of the diphosphate (PP) component. Therefore, we and other workers have developed more stable, synthetic versions of PP-InsPs in which the PP groups are replaced with phosphonoacetate (PA), or methylenebisphosphonate (PCP) groups (Fig. 1). Thus, a tethered version of 5-PCP-InsP5 was recently used to search for novel binding proteins for PP-InsPs. Metabolically-stable PP-InsP analogues can also be informative for structural analysis of enzyme(substrate) crystal complexes. For example, we have used 5-PA-InsP5 to reveal a previously unidentified ligand capture site on PPIP5K2.

While PA analogues are relatively easy to synthesise, the carboxylic ester of the PA group could be prone to chemical hydrolysis at high pH and/or enzymatic cleavage by cellular esterases. In both PA and PCP analogues, the bridging oxygen of the diphosphate group is replaced by a methylene (CH2) group, making the resulting carbon–phosphorus bonds resistant to hydrolysis in comparison to the native oxygen–phosphorus bonds. However, the CH2 group in methylene phosphonates is less electronegative than the bridging oxygen atom of phosphates, causing an increase in the pK<sub>a</sub> value of the methylene phosphonic acid in its second deprotonation. This can mean that a methylene phosphate analogue is less strongly ionised than the phosphate equivalent at physiological pH, potentially leading to a decreased affinity of the
Fig. 1 A. Inositol hexakisphosphate (InsP6) is phosphorylated on P-5 by IP6K to give 5-PP-InsP5, also known as “5-InsP5.” Further phosphorylation at P-1 by PPiPSK gives 1,5-PP-InsP5 (“InsP6”). B. Stabilised analogues of 5-PP-InsP5 containing methylenebisphosphonate (PCP), phosphonooacetate (PA) and phosphonodifluoroacetamide (PCF2Am) mimics of the natural PP group. IP6K, inositol hexakisphosphate kinase; PPiPSK, diphosphoinositol pentakisphosphate kinase.

An analogue for protein binding sites. A well-established approach to this problem, originally developed by Blackburn and co-workers, involves replacing the phosphonate CH2 group with difluoromethylene (CF2). The more electronegative CF2 group increases the acidity of the phosphonic acid and, in addition, the CF2 group itself has greater electronic and steric similarity to the bridging oxygen atom of a phosphate than does CH2. The difluoromethylene phosphonate (PCF2) group has been particularly useful as a phosphate mimic in the development of protein tyrosine phosphatase inhibitors.

In nucleotide chemistry, the difluoromethylenebisphosphonate (PCF2P) motif has been used to mimic PP in stable analogues of nucleoside diphosphates and triphosphates. Although the PCF2P group has been proposed as a potential PP mimic in stabilised analogues of PP-InsPs, this was anticipated to present a considerable synthetic challenge, and while several PCP-InsPs have been synthesised, no fluorinated equivalents have yet been disclosed.

In the current report, we explore an alternative approach to a fluorinated isopolar analogue of a PP-InsP, building on our earlier work with phosphonoacetic acid (PA) esters. Although replacing the CH2 group in the β-ketophosphonate fragment of a PA ester with CF2 is possible, the resulting difluoroacetate ester would be very labile to hydrolysis. Therefore, we chose to replace the ester of PA with a stable amide linkage, allowing the inclusion of the electron-withdrawing CF2 group in place of CH2. In the resulting analogue, 5-PCF2Am-InsP5 (1, Fig. 1), the terminal phosphate group should more closely resemble the corresponding β-phosphate group of 5-PP-InsP5 in its electronic properties than in either the PA or the PCP equivalents. The α-phosphono-α,α-difluoroacetamide (PCF2Am) unit lacks a close equivalent to the α-phosphate of PP, but the amide carbonyl retains the potential to accept H-bonds and the rigidity of the amide structure itself may confer advantages at some binding sites. To the best of our knowledge, the PCF2Am motif has not previously been explored as a diphasphate isostere, although it has been used successfully in analogues of 1,3-bisphosphoglyceric acid as inhibitors of phosphoglycerate kinase and in the design of inhibitors of aspartate carbamoyltransferase and protein tyrosine phosphatases.

Results and discussion
Synthesis of 5-PCF2Am-InsP5 (1)
To construct compound 1, we needed to synthesise an appropriately protected 5-deoxy-5-amino-myoinositol intermediate (Scheme 1). We have previously shown that regioselective sulfonylation of butanediactal (BDA) protected myo-inositol 2 (ref. 3, 22 and 23) using triflic anhydride, followed by solvolysis using wet dimethylacetamide, gives inversion of configuration at C-5, to produce the neo-inositol acetate derivative 3 via an iminium ion intermediate. In the present work, we used a second triflate esterification of the free OH group in 3 followed by treatment with sodium azide in DMF to give a second configurational inversion, returning us to the myo-inositol configuration in the 5-deoxy-5-azido-myoinositol derivative 4.

† A recent study has systematically investigated the solvolysis and azidolysis of triflates derived from diol 2.
Compound 4, with its combination of acid- and base-labile protecting groups, is a potentially versatile intermediate in itself. However, to simplify the current synthesis, we sequentially replaced these labile protecting groups with benzyl ethers. Thus, replacement of the 2-O-acetate ester with a 2-O-benzyl ether gave 5, a precursor for the synthesis of analogues of 5-PP-InsP₄. Next, the BDA groups were removed and replaced with benzyl ethers to give pentabenzyl 5-deoxy-5-azido-inositol 6. Reduction of the azide group in 6 using LiAlH₄ now gave the 5-deoxy-5-amino-inositol 7 in quantitative yield. The next step was to introduce the phosphonodifluoroacetamide unit (Scheme 2). Thus, reaction of 7 with diethyl phosphonodifluoroacetic acid gave phosphonodifluoroacetamide 8. The benzyl protecting groups in 8 were removed by catalytic hydrogenolysis over Pd(OH)₂/C to give pentaol 9. Phosphitylation using bisbenzylisodisopropylaminophosphine activated with 5-phenyl-1H-tetrazole then gave the intermediate pentakisphosphate, which was observed by ³¹P NMR, but not isolated. Oxidation of phosphites with mCPBA yielded the fully-protected pentakisphosphate 10. Finally, the benzyl and ethyl protecting groups in 10 were cleanly removed using TMSBr in dichloromethane followed by MeOH.

Compound 1 was isolated as the triethylammonium salt, containing six TEA⁺ ions per molecule of 1. The non-fluorinated phosphonoacetamide (PCH₂Am) equivalent of 1 was also initially synthesised to explore synthetic methods (see ESI† for details). During this synthesis, we observed facile exchange of the CH₂ protons with deuterium from D₂O during NMR spectroscopy of the PCH₂Am-containing pentaol intermediate analogous to compound 9. The deuterium was retained after the subsequent phosphorylation and deprotection steps. Attempts to exchange deuterium back to hydrogen in the final product were unsuccessful, possibly because ionisation of the methylenephosphonate group disfavours the required enolisation (see ESI† for further details). This may suggest a strategy for developing tritiated versions of the non-fluorinated equivalent of 1 and related analogues of PP-InsPs.
The $^{31}$P NMR spectrum of 1 in D$_2$O showed the signal corresponding to the PCF$_2$Am phosphorus atom at $\delta$ 0.64, shifted approximately 13.5 ppm up-field relative to the equivalent signal in the PCH$_2$Am equivalent (ESI† Fig. S1). This reflects the profound effect of the CF$_2$ group on the electronic properties of the phosphonate group in 1. Compound 1 was very stable in aqueous solution; the NMR sample showed no sign of decomposition after >1 year in solution in D$_2$O.

Interaction of 5-PCF$_2$Am-InsP$_5$ with PPIPK2

To determine whether 1 could act as a mimic of 5-PP-InsP$_5$, we examined its interaction with the kinase domain of human PPIPK2. We used a “reverse-kinase” assay that records ATP generated from 0.1 mM ADP during the dephosphorylation of 100 nM 1,5-[PP]$_2$-InsP$_4$. This approach avoids the need to use radiolabelled material and slow-throughput HPLC analysis. Furthermore, the assay of ATP production is inherently more sensitive compared to measuring ATP consumption. Compound 1 inhibited 1,5-[PP]$_2$-InsP$_5$ metabolism by PPIPK2 with an IC$_{50}$ of 375 nM (Fig. 2A). We then obtained an X-ray structure of 5-PCF$_2$Am-InsP$_5$ (1) in complex with PPIPK2 kinase domain and the stable ATP analogue AMPPNP. The structure shows that 5-PCF$_2$Am-InsP$_5$ binds to the catalytic site of PPIPK2 in a similar orientation to 5-PP-InsP$_5$ (Fig. 2B–D). While crystallography previously showed that 5-PA-InsP$_5$ binds to two sites in PPIPK5 (the catalytic site and a surface-located ligand capture site),$^{4,10}$ electron density for 1 was seen only in the catalytic site (Fig. 2B). This is similar to the complex structures of PPIPK2 with 5-PP-InsP$_5$ (ref. 7) or with 5-PP-InsP$_5$ itself.$^{25}$

The electron density for 1 (Fig. 2C) clearly shows that the planar amide unit adopts the Z conformation, and with the amide NH antiperiplanar to H-5 of myo-inositol. The carbonyl oxygen of the amide is orientated similarly to one non-bridging oxygen atom of the $\alpha$-phosphate of bound 5-PP-InsP$_5$ (Fig. 2E and F). Additionally, one of the fluorine atoms in the CF$_2$ group stretches its position to that of the other non-bridging O-atom of an $\alpha$-phosphate, which is expected to bring the negative charge distribution in this region closer to that of 5-PP-InsP$_5$ than occurs with the PA analogue. The terminal phosphonate group in bound 1 is held in an extended conformation (Fig. 2E) although its orientation is different to that of the corresponding $\beta$-phosphate group in 5-PP-InsP$_5$ bound to PPIPK5 (Fig. 2F).

Conclusions

We have described the design and synthesis of a novel type of stable PP-InsP analogue containing a phosphonodifluoroacetamide (PCF$_2$Am) structure, intended to mimic the 5-diphosphate (PP) group of 5-PP-InsP$_5$. As proof of principle, we examined the interaction of the new analogue, 5-PCF$_2$Am-InsP$_5$ (1), with the catalytic domain of the diphosphoinositol pentakisphosphate kinase PPIPK5. A crystallographic study showed that 1 binds to the PP-InsP-binding site of the PPIPK5 kinase domain in a similar orientation to that of the natural substrate 5-PP-InsP$_5$ and that the PCF$_2$Am structure can mimic several functionally important aspects of the diphosphate group (PP) in 5-PP-InsP$_5$.

Recent years have seen a rapid increase in reported functions of PP-InsPs, the identification of their target proteins, and the characterisation of metabolising enzymes. Stabilised analogues of PP-InsPs have often played valuable roles in this work. No single analogue of a PP-InsP can perfectly mimic its steric and electronic features in all environments, and a range of compounds with complementary properties therefore offers a more versatile analytical approach. It is in this context that we propose that 5-PCF$_2$Am-InsP$_5$ (1) brings new opportunities. For example, the presence of the CF$_2$ group in 1 means that its terminal phosphonate group should be more acidic than the equivalent group in 5-PA-InsP$_5$ or 5-PCP-InsP$_5$, making it more similar in this respect to the $\beta$-phosphate of 5-PP-InsP$_5$ and potentially allowing stronger interactions with protein binding sites. In addition, the rigidity of the amide unit in 1 means that the PCF$_2$Am unit contains fewer rotatable bonds than the equivalent PP, PA or PCP structures. A more rigid ligand is less likely to lose entropy on binding than a more flexible one, and thus may bind more tightly to some sites. Note also that the 5-diphosphate in 5-PP-InsP$_5$ participates in complexing a hydrated Mg$^{2+}$ ion, a property that is mimicked in the complex of 5-PCP-InsP$_5$ with PPIPK5.$^7$ This Mg$^{2+}$ ion is not present in the complex with 1 (Fig. 2E). Thus, 5-PCF$_2$Am-InsP$_5$ may be particularly useful for studying ligand/protein interactions that do not involve Mg$^{2+}$. To the best of our knowledge, the PCF$_2$Am group has not been previously employed as a diphosphate mimic; given the large number of biomolecules that contain diphosphate or polyphosphate motifs, it may find wider applications than those outlined here.

Experimental

General chemistry methods

All reagents and solvents were of commercial quality and were used without further purification. Petroleum ether used for chromatography and crystallisations was of fractions 40–60 °C. Alcohol 3 was synthesised as previously reported.$^{22}$ Diethyl phosphonodifluoroacetic acid was synthesised according to a literature procedure.$^{24}$ Thin-layer chromatography (TLC) was performed on pre-coated aluminium plates (Merck, silica gel 60, F254). Chromatograms were visualised under UV light and by dipping plates into either phosphomolybdic acid in EtOH, vanillin in acidic EtOH, or alkaline aqueous KMnO$_4$, followed by heating. Flash column chromatography was performed on an ISCO CombiFlash RF automated flash chromatography system using RediSep RF disposable flash columns. NMR spectra were recorded on Bruker Avance III 400 and 500 MHz NMR spectrometers. Proton chemical shifts are reported in ppm ($\delta$) relative to internal tetramethylsilane (TMS, 0.0 ppm) or with the solvent resonance relative to TMS employed as the internal standard (D$_2$O, 4.79 ppm). The following abbreviations are used to
describe resonances: br, broad; s, singlet; d, doublet; dd, doublet; q, quartet; m, multiplet; t, triplet. $^{13}$C chemical shifts are reported relative to internal TMS (TMS, 0.0 ppm) or with the solvent resonance relative to TMS employed as the internal standard. The assignments of the proton and carbon atoms are based on 2D-NMR experiments ($^1$H−$^1$H-COSY, HSQC). $^{31}$P chemical shifts are reported in ppm ($\delta$) relative to an 85% H$_3$PO$_4$ external standard (H$_3$PO$_4$, 0.0 ppm). $^{19}$F chemical shifts are reported in ppm ($\delta$) relative to a CFCl$_3$ external standard (CFCl$_3$, 0.0 ppm). Melting points were determined using a Stanford Research Systems Optimelt MPA100 automated melting point system and are uncorrected. High resolution time-of-flight mass spectra were obtained on a Bruker Daltonics micrOTOF mass spectrometer using electrospray ionisation (ESI$^+$.)

2-O-Acetyl-5-deoxy-5-azido-1,6,3,4-bis-O-(2,3-dimethoxybutane-2,3-diyl))-myo-inositol (4)

To a stirred solution of alcohol 3 (1.15 g, 2.55 mmole) in dry dichloromethane (10 mL) and dry pyridine (1 mL) under N$_2$ at −78 °C was added trifluoromethanesulfonic anhydride (4.0 mL of a 1.0 mol dm$^{-3}$ solution in dichloromethane, 4.0 mmol) dropwise over 15 min. The cooling bath was removed and the solution was allowed to warm to room temperature. Stirring was continued overnight (16 h) after which time TLC (ethyl acetate : petroleum ether 2 : 1) showed complete conversion of alcohol ($R_f$ 0.32) into a less polar product ($R_f$ 0.70). Excess triflic anhydride was destroyed by careful addition of water and the solution was allowed to warm to room temperature. Excess NaH was destroyed by careful addition of deionised water and the mixture was concentrated. The residue was taken up in ethyl acetate (50 mL) and washed with water (2 × 50 mL), dried (MgSO$_4$) and concentrated to give the alcohol as a white solid (484 mg). This crude alcohol was purified by flash chromatography on silica (ethyl acetate in petroleum ether, 0 to 50% gave 5 as a white solid (541 mg, 1.03 mmol, 99% over two steps); $R_f$ 0.34 (ethyl acetate : petroleum ether 1 : 3); crystals from petroleum ether, m.p. 175−176.5 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.31 (6H, s, 2 × CH$_3$), 1.32 (6H, s, 2 × CH$_3$), 3.23 (6H, s, OCH$_3$), 3.29 (6H, s, OCH$_3$), 3.49 (1H, t, J 10.0 Hz, H-5), 3.57 (2H, dd, J 10.0, 2.5 Hz, H-4 and H-6), 5.41 (1H, t, J 10.0 Hz, H-1), 5.50 (1H, t, J 10.0 Hz, H-3); $^13$C NMR (100 MHz, CDCl$_3$) $\delta$ 17.41 (CH$_3$), 17.62 (CH$_3$), 47.92 (OCH$_3$), 48.00 (OCH$_3$), 61.52 (C-5), 68.46 (C-4 and C-6), 69.70 (C-2), 7.34 (2H, m, para H of Ph), 7.48−7.51 (2H, m, ortho H of Ph); $^{31}$P NMR (100 MHz, CDCl$_3$) $\delta$ 17.61 (CH$_3$), 17.70 (CH$_3$), 47.92 (OCH$_3$), 48.00 (OCH$_3$), 61.52 (C-5), 68.46 (C-4 and C-6), 69.70 (C-1 and C-3), 73.94 (OCH$_3$Ph), 76.02 (C-2), 99.51 (BDA quaternary C), 99.71 (BDA quaternary C), 127.02 (BDA quaternary C), 170.23 (C=O); HRMS (m/z) [M + Na]$^+$ calcd. for C$_{20}$H$_{32}$N$_3$O$_{10}$, 498.2058; found 498.2035.

2-O-Benzyl-5-deoxy-5-azido-1,6,3,4-bis-O-(2,3-dimethoxybutane-2,3-diyl))-myo-inositol (5)

To a solution of 4 (530 mg, 1.11 mmol) in THF (12 mL) was added a solution of LiOH·H$_2$O (420 mg, 10 mmol) in deionised water (3 mL) and methanol (12 mL). The mixture was stirred vigorously at room temperature for 2 h, after which time TLC (ethyl acetate : petroleum ether 1 : 1) showed complete conversion of 4 ($R_f$ 0.54) into a more polar product ($R_f$ 0.28). The solution was concentrated and the residue was taken up in ethyl acetate (50 mL). The solution was washed with water (2 × 50 mL), dried (MgSO$_4$) and concentrated to give the alcohol as a white solid (484 mg). This crude alcohol was taken up in DMF (10 mL). The solution was stirred at 0 °C and sodium hydride (60% suspension in mineral oil, 67 mg, 1.67 mmole) was added. After 30 min, benzyl bromide (0.16 mL, 1.3 mmol) was added and the mixture was stirred overnight (16 h) at room temperature. Excess NaH was destroyed by careful addition of water and the solution was concentrated. The residue was taken up in ethyl acetate (50 mL) and washed with water and brine (50 mL each), dried (MgSO$_4$) and concentrated to give a solid residue. Purification by flash chromatography on silica (ethyl acetate in petroleum ether, 0 to 50% gave 5 as a white solid (541 mg, 1.03 mmol, 99% over two steps); $R_f$ 0.34 (ethyl acetate : petroleum ether 1 : 3); crystals from petroleum ether, m.p. 175−176.5 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.31 (6H, s, 2 × CH$_3$), 1.32 (6H, s, 2 × CH$_3$), 3.23 (6H, s, OCH$_3$), 3.29 (6H, s, OCH$_3$), 3.49 (1H, t, J 10.0 Hz, H-5), 3.57 (2H, dd, J 10.0, 2.5 Hz, H-1 and H-3), 3.80 (1H, t, J 2.7 Hz, H-2), 4.03 (2H, t, J 10.1 Hz, H-4 and H-6), 4.84 (2H, s, OCH$_2$Ph), 7.22−7.27 (1H, m, meta-H of Ph), 7.29−7.34 (2H, m, meta-H of Ph), 7.48−7.51 (2H, m, ortho-H of Ph); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 17.41 (CH$_3$), 17.70 (CH$_3$), 47.92 (OCH$_3$), 48.00 (OCH$_3$), 61.52 (C-5), 68.46 (C-4 and C-6), 69.70 (C-1 and C-3), 73.94 (OCH$_3$Ph), 76.02 (C-2), 99.51 (BDA quaternary C), 99.71 (BDA quaternary C), 127.02 (para-C of Ph), 127.62 (CH of Ph), 127.90 (CH of Ph), 139.49 (ipso-C of Ph); HRMS (m/z) [M + Na]$^+$ calcd. for C$_{20}$H$_{32}$N$_3$O$_{10}$, 546.2422; found 546.2406.

1,2,3,4,6-Penta-O-benzyl-5-deoxy-5-azido-myosinositol (6)

To a stirred solution of 5 (507 mg, 0.968 mmol) in dichloromethane (5 mL) was added 95% aqueous TFA (5 mL). A yellow colour (butanedione) appeared within 30 s. After 30 min, the solution was concentrated to leave the crude tetrrol as a white solid (325 mg). This solid was taken up in dry DMF (10 mL) and the solution was cooled to 0 °C, before sodium hydride (60% suspension in mineral oil, 240 mg, 6.0 mmole) was added. The stirred suspension was allowed to warm to room temperature and then cooled again to 0 °C before benzyl bromide (0.60 mL, 5.1 mmol) was added, dropwise. The cooling bath was removed and the mixture...
was stirred overnight (16 h) at room temperature. Excess NaH was destroyed by careful addition of water and the solution was concentrated. The residue was taken up in dichloromethane (50 mL) and washed with water and brine (50 mL each), dried (MgSO4) and concentrated to give an oily residue. Purification by flash chromatography on silica (ethyl acetate in petroleum ether, 0 to 50%) gave 6 as a colourless oil, which slowly crystallised (576 mg, 0.878 mmol, 91% over two steps); Rf 0.40 (ethyl acetate:petroleum ether 1:4); crystals from boiling ethanol, m.p. 89.0–90.5 °C; 1H NMR (400 MHz, CDCl3) δ 3.33 (2H, dd, J 9.7, 2.2 Hz, H-1 and H-3), 3.35 (1H, t, J 9.9 Hz, H-5), 3.85 (2H, t, J 9.8 Hz, H-4 and H-6), 4.00 (1H, t, J 2.4 Hz, H-2), 4.57, 4.62 (4H, AB quartet, JAB 11.7 Hz, 2 × OCH2Ph), 4.81, 4.87 (4H, AB quartet, JAB 10.5 Hz, 2 × OCH2Ph), 4.85 (2H, s, OCH2Ph), 7.25–7.41 (25H, m, Ph); 13C NMR (100 MHz, CDCl3) δ 67.68 (2H, t, J 1.2 Hz, C-5), 72.77 (2 × OCH2Ph), 74.25 (OCH2Ph), 74.31 (C-2), 75.73 (2 × OCH2Ph), 79.77 (C-4 and C-6), 81.20 (C-1 and C-3), 127.46, 127.57, 127.70, 127.72, 127.78, 128.20, 128.30, 128.34 and 128.41 (CH of Ph), 138.12 (2 × ipso-C of Ph), 138.24 (2 × ipso-C of Ph), 138.79 (ipso-C of Ph); HRMS [m/z] [M + Na]+ c. for C41H41N3O5, 678.2938; found 678.2966.

1,2,3,4,6-Penta-O-benzyl-5-deoxy-5-amino-myo-inositol (7)

To dry THF (3 mL) under argon was added LiAlH4 (1.0 mL of a 1.0 mol dm−3 solution in THF, 1.0 mmol). The solution was stirred at 0 °C under argon and a solution of the azide (620 mg, 0.448 mmol) in dry dichloromethane (2 mL) was added. The solution was stirred overnight (16 h) at room temperature. Excess LiAlH4 was destroyed by careful addition of water and the solution was concentrated. The residue was taken up in dichloromethane (50 mL) and washed with water and brine (50 mL each), dried (MgSO4) and concentrated to give an oily residue. Purification by flash chromatography on silica (ethyl acetate in petroleum ether, 0 to 50%) gave 8 as a colourless oil, which slowly crystallised (164 mg, 0.194 mmol, 87%); Rf 0.40 (ethyl acetate:petroleum ether 1:4); crystals from boiling diisopropyl ether, m.p. 132.0–133.5 °C; 1H NMR (400 MHz, CDCl3) δ 1.24 (6H, td, J 7.1, 0.6 Hz, POCH2CH3), 3.46 (2H, dd, J 9.0, 2.2 Hz, H-1 and H-3), 3.98 (2H, t, J 9.1 Hz, H-4 and H-6), 4.03 (1H, t, J 2.2 Hz, H-2), 4.10 (1H, q, J 9.2 Hz, H-5), 4.13–4.21 (4H, m, POCH2CH3), 4.56, 4.60 (4H, AB quartet, JAB 11.8 Hz, 2 × OCH2Ph), 4.73, 4.80 (4H, AB quartet, JAB 11.8 Hz × OCH2Ph), 4.84 (2H, s, OCH2Ph), 6.66 (1H, d, J 9.2 Hz, NH), 7.20–7.33 (23H, m, Ph), 7.39–7.42 (2H, m, Ph); 13C NMR (100 MHz, CDCl3) δ 16.22 (1JC 5.6 Hz, POCH2CH3), 54.68 (C-5), 65.65 (1JC 6.6 Hz, POCH2CH3), 72.77 (2 × OCH2Ph), 74.12 (OCH2Ph), 74.55 (C-2), 74.76 (2 × OCH2Ph), 78.31 (C-4 and C-6), 81.34 (C-1 and C-3), 111.92 (td, 1JC 271.6 Hz, 1JC 197.9 Hz, CF3), 127.40, 127.49, 127.66, 127.69, 127.83, 127.92, 128.19, 128.23 and 128.36 (CH of Ph), 138.11 (2 × ipso-C of Ph), 138.35 (2 × ipso-C of Ph), 138.77 (ipso-C of Ph), 161.40 (td, 1JC 24.6 Hz, 1JC 17.3 Hz, C=O), 31P NMR (CDCl3, 162 MHz, 1H-decoupled) δ 3.47 (1P, t, 1JC 96.1 Hz); 19F NMR (CDCl3, 471 MHz) δ −116.11 (2 F, d, 2JC 96.1 Hz); HRMS [m/z] [M]+ c. for C42H43F2NO5P, 842.3275; found 842.3264.

5-Deoxy-5-(diethylphosphonodifluorooacetamido)-myo-inositol (9)

To a solution of 8 (130 mg, 0.154 mmol) in methanol (8 mL), THF (2 mL), deionised water (2 mL) and acetic acid (1 mL) was added palladium hydroxide on activated charcoal (20%, 50% water, 50 mg). The suspension was shaken in a Parr hydrogenator under H2 (50 p.s.i.) for 24 h. The catalyst was removed by filtration through a PTFE syringe filter and the resulting colourless solution was concentrated, then dried under vacuum to give pentaol 9 as a white solid (61 mg, 0.154 mmol, 100%); TLC (dichloromethane : methanol 2 : 1): Rf 0.40; 1H NMR (400 MHz, D2O) δ 1.38 (6H, broad t, J ~7 Hz, POCH2CH3), 3.61 (2H, broad d, J ~9 Hz, H-1 and H-3), 3.71–3.81 (3H, m, H-4, H-5 and H-6), 4.10 (1H, broad s, H-2), 4.35–4.42 (4H, m, POCH2CH3); 13C NMR (100 MHz, D2O) δ 15.64 (1JC 5.2 Hz, POCH2CH3), 56.28 (C-5), 67.37 (1JC 7.1 Hz, POCH2CH3), 70.05 (C-4 and C-6), 71.91 (C-1 and C-3), 72.04 (C-1 and C-3), 111.71 (td, 1JC 271.8 Hz, 1JC 208.7 Hz, CF3), 163.33 (td, 1JC 24.5 Hz, 1JC 16.2 Hz, C=O); 31P NMR (162 MHz, D2O, 1H-decoupled) δ 4.39 (t, 1JC 101.5 Hz); 19F NMR (471 MHz, D2O) δ −117.62 (d, 2JC 101.4 Hz); HRMS [m/z] [M + Na]+ c. for C43H32F2NO5P, 416.0892; found 416.0876.
5-Deoxy-5-(diethylphosphonodifluoroacetamido)-myo-inositol 1,2,3,4,6-O-pentakis(dibenzyloxylphosphoryl) (10)

To a stirred suspension of pentaol 9 (30 mg, 0.076 mmol) and 5-phenyl-1H-tetrazole (83 mg 0.57 mmol) in dry dichloromethane (3 mL) under N2 at room temperature was added bis(benzoxyl)disisopropylaminophosphine (0.20 mL, 0.60 mmol). The mixture was stirred under N2 at room temperature for 3 h and then cooled to ~78 °C, before mCPBA (70%, 187 mg, 0.760 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 Na2SO4 solution (3 × 25 mL), dried over MgSO4 and concentrated. The residue was purified by flash chromatography (EtOAc in petroleum ether, 0 to 100%) to give 10 as a colourless oil (85 mg, 0.050 mmole, 66%); TLC (EtOAc : petroleum ether, 4 : 1): Rf 0.56; 1H NMR (CDCl3, 400 MHz) δ 1.27 (6H, t, J 7.1, 0.5 Hz, POCH2CH3), 4.20-4.33 (5H, m, 2 × POCH2CH2 and H-5), 4.39 (2H, broad t, J ∼9.5 Hz, H-1 and H-3), 4.82 (2H, q, J ∼10 Hz, H-2 and H-4), 4.89-5.11 (18H, POCH2Ph), 5.15–5.19 (2H, POCH2Ph), 5.63 (1H, broad d, J ∼9 Hz, H-2), 7.11–7.28 (50H, m, Ph), 7.52 (1H, broad d, J ∼9 Hz, NH); 13C NMR (101 MHz, CDCl3) δ 16.31 (d, JCP 15.3 Hz, C-1 and C-3), 16.87 (d, JCP 176.6 Hz, 1C), 52.70 (broad, C-5), 65.35 (d, JCP 6.4 Hz, POCH2CH3), 69.75–70.06 (overlapping signals with JCP couplings, POCH2Ph), 73.72 (with JCP couplings, C-4 and C-6), 74.28 (with JCP couplings, C-1 and C-3), 75.93 (with JCP couplings, C-2), 112.1 (JCP 205.0 Hz, JCP 100 Hz, unreacted through noise, C-7F), 127.83, 127.95, 128.12, 128.16, 128.21, 132.82, 132.86, 132.89, 132.45, 132.49 (CH of Ph), 135.54–135.74 (overlapping signals with JCP couplings, ipso-C of Ph), 162.85 (JCP ∼18 Hz, JCP 100 Hz, unreacted through noise, C=O); 31P NMR (162 MHz, CDCl3, 1H-decoupled) δ −2.30 (1P, P-2), −1.64 (2P), −0.11 (2P), 3.20 (1P, t, JPP 93.6 Hz); 19F NMR (471 MHz, CDCl3) δ −113.48 (d, JPP 93.4 Hz); HRMS (m/z) [M + Na]+ calcd. for C82H92F2NO32P6, 1716.3904; found 1716.3965; [M + H]+ calcd. for C82H92F2NO32P6, 1694.4085; found 1694.4130.

5-Deoxy-5-(phosphonodifluoroacetamido)-myo-inositol 1,2,3,4,6-pentakisphosphate (1)

A stirred solution of 10 (68 mg, 40 μmole) in dry dichloromethane (2 mL) was cooled to 0 °C under N2 and dimethylsilyl bromide (1 mL) was added dropwise over 5 min. The solution was allowed to warm gradually to room temperature and stirring was continued for 48 h. The solution was concentrated and methanol (5 mL) was added to the residue. The resulting colourless solution was stirred at room temperature for a further 1 h, and then concentrated to give a white gum. The gum was washed with diethyl ether (3 × 2 mL), then taken up in aqueous triethylammonium bicarbonate (1.0 mol dm−3, pH 7.6, 5 mL). This solution was then washed with diethyl ether (3 × 5 mL) and concentrated. The residue was re-dissolved in MilliQ water and lyophilised to give the triethylammonium salt of the title compound 1 as a colourless solid (47 mg, 34 μmole, 85%); 1H NMR (500 MHz, D2O) δ 1.28 (approx. 57 H, t, J 7.3 Hz, CH3 of TEA+), 3.20 (approx. 38 H, q, J 7.3 Hz, CH2 of TEA+), 4.16 (1H, broad t, J ∼10 Hz, H-5), 4.30 (2H, tt, J 9.6, 2.0 Hz, H-1 and H-3), 4.55 (2H, q, J 9.7 Hz, H-4 and H-6), 4.87 (1H, dt, J 9.8, 2.4 Hz, H-2); 13C NMR (126 MHz, D2O) δ 8.19 (CH3 of TEA+), 46.54 (CH2 of TEA+), 53.91 (C-5), 74.44 (C-1, C-3, C-4 and C-6), 75.86 (C-2), 114.65 (dt, JCP 176.6 Hz, JCP 268.7 Hz, CF2), 166.30 (td, JCP 25.3 Hz, JCP 15.3 Hz, C=O); 31P NMR (162 MHz, D2O, 1H-decoupled) δ −0.75 (1 P, P-2), −0.32 (2 P), 0.15 (2 P), 0.64 (1 P, t, JPP 87.9 Hz, P-5); 19F NMR (471 MHz, D2O) δ −118.86 (d, JPP 85.5 Hz, CF2); HRMS (m/z) [M − H]+ calcd. for C82H92F2NO32P6, 735.8618; found 735.8654.

Protein expression, purification, crystallisation and structure determination

The kinase domain of human PPIP5K2 (residues 41–366) was sub-cloned, expressed and purified as before.25 PPIP5K2 kinase domain was crystallised by hanging drop vapour diffusion against a well buffer of 12% (w/v) PEG 3350, 20 mM MgCl2, 0.1 M HEPES, pH 7.0, 2 mM CdCl2, 1 mM AMPPNP at 4 °C. The crystals were transferred to a stabilising buffer containing 22% (w/v) PEG 3350, 10 mM MgCl2, 0.1 M sodium acetate, pH 5.2 at 4 °C and the crystals were then soaked under the above stabilising buffer for three days with 2 mM compound 1. Cryosolvent was prepared by adding 33% ethylene glycol into the soaking buffer. Diffraction data were collected using APS beamlines 22-ID. All data were processed with the program HKL2000. The structure was determined using rigid body and direct Fourier synthesis, and refined with the equivalent and expanded test sets. The structure was further manually rebuilt with COOT and refined with REFMAC from the CCP4 package. Ligand topology and parameter files were prepared using the program PyMOL (Schrödinger, LLC). Atomic coordinates and structure factors have been deposited with the Protein Data Bank with accession code 6N5C.

PPIP5K2 kinase assay

Human PPIP5K2 kinase domain (residues 1–366, 2.5 μg mL−1) was incubated at 24 °C for 30 min with buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 0.1 mM ADP, 100 nM 1,5-[PP]2-InsP4 and various concentration of compound 1 in a 20 μL assay. The generated ATP was measured using a Molecular Probes ATP Determination kit (Thermo Fisher Scientific catalog number A22066). The IC50 value for 1 was calculated using GraphPad Prism.

Conflicts of interest

There are no conflicts to declare.

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