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Benzene Polyphosphates as Tools for Cell Signalling: Inhibition of Inositol 1,4,5-Trisphosphate 5-Phosphatase and Interaction with the PH Domain of Protein Kinase B α

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Abstract

Novel benzene polyphosphates were synthesised as inositol polyphosphate mimics and evaluated against both type-I inositol 1,4,5-trisphosphate 5-phosphatase, which only binds soluble inositol polyphosphates, and the PH domain of protein kinase B α (PKB α), which can bind both soluble inositol polyphosphates and inositol phospholipids. The most potent trisphosphate 5-phosphatase inhibitor is benzene 1,2,4-trisphosphate **2**, (IC₅₀ of 14 μ M) a potential mimic of D-myo-inositol 1,4,5-trisphosphate, and the most potent tetrakisphosphate Ins(1,4,5)P₃ 5-phosphatase inhibitor is benzene 1,2,4,5-tetrakisphosphate, with an IC₅₀ of 4 μ M. Biphenyl 2,3',4,5',6-pentakisphosphate **4** was the most potent inhibitor evaluated against type I Ins(1,4,5)P₃ 5-phosphatase (IC₅₀ of 1 μ M). All new benzene polyphosphates are resistant to dephosphorylation by type I Ins(1,4,5)P₃ 5-phosphatase. Unexpectedly, all benzene polyphosphates studied bind to the PH domain of PKB α with apparent higher affinity than type I Ins(1,4,5)P₃ 5-phosphatase. The most potent ligand for PKB α PH domain is biphenyl 2,3',4,5',6-pentakisphosphate **4** (K_i = 27 nM), measured by inhibition of biotinylated diC₈-PtdIns(3,4)P₂ binding. The ca 80-fold enhancement of binding relative to parent benzene trisphosphate is rationalised by the involvement of a cation– π interaction. These new molecular tools will be of potential use in structural and cell signalling studies.

Keywords

Benzene polyphosphate; Cation– π interaction; Ins(1,4,5)P₃ 5-Phosphatase; molecular modelling; PKB α PH domain

Introduction

D-myo-Inositol 1,4,5-trisphosphate, [Ins(1,4,5)P₃ **1**] is a second messenger which releases Ca²⁺ ions from intracellular stores.[1,2] The crystal structure of Ins(1,4,5)P₃ bound in the ligand binding domain of the type I Ins(1,4,5)P₃ receptor [Ins(1,4,5)P₃R] has been reported.

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[3] Many compounds have been synthesised[4] and evaluated at the Ins(1,4,5)P₃R and against the Ins(1,4,5)P₃ metabolising enzymes, Ins(1,4,5)P₃ 3-kinase and Ins(1,4,5)P₃ 5-phosphatase. There are several inositol polyphosphate 5-phosphatase isoenzymes;[5] some can dephosphorylate water soluble inositol polyphosphates and some their lipid counterparts, whilst others can hydrolyse both water soluble and lipid substrates. Some of these enzymes are directly linked with human disease (e.g. the Lowe syndrome),[6] and insulin signalling.[7] Type I Ins(1,4,5)P₃ 5-phosphatase is probably the most characterised of the inositol 5-phosphatase enzymes, and we have a long-standing interest in discovering inhibitors of this enzyme class.[8-10]

Because of the complexities of inositol polyphosphate chemical synthesis we[11-14] and others[15,16] have proposed that surrogate benzene polyphosphates could be useful tools for cell signalling studies. Benzene polyphosphates are compounds which can accommodate phosphate groups around a six membered planar aromatic ring, for example benzene 1,2,4-trisphosphate [Bz(1,2,4)P₃ **2**], has the same regioisomeric arrangement of phosphate groups as in Ins(1,4,5)P₃ **1**. Such compounds are synthetically accessible, achiral derivatives and contrast with the ring-puckered conformation and optical activity of most of their inositol polyphosphate counterparts.

In particular, we showed that such compounds could inhibit PI 3-kinase,[11] Ins(1,4,5)P₃ 5-phosphatase[14] or the SH2 domain of inositol 5-phosphatase (SHIP2)[14] and one such derivative, Bz(1,2,3,4)P₄ **3** binds to the PH domain of PKB α with relatively high affinity and was co-crystallised with the protein and studied by X-ray crystallography even though it is highly structurally diverse from the natural lipid headgroup.[13]

Protein kinase B (PKB also known as Akt) is an enzyme involved in inositol lipid signalling and is comprised of three distinct highly conserved enzymes PKB α , PKB β and PKB γ (also known as Akt1, Akt2 and Akt3) which have their own functions within cells.[17-19] A pleckstrin homology domain (PH domain) binds phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃], which is required for PKB phosphorylation and activation.[20] The kinase activity of PKB has received considerable attention as a drug target, since PKB α is constitutively active in cancerous cells and requires strict regulation. However, inhibition of PtdIns(3,4,5)P₃ binding to the PH domain by small molecules has also become a priority target for anticancer treatment.[21,22]

We report here a structure–activity study of some novel regioisomeric benzene polyphosphates against type I Ins(1,4,5)P₃ 5-phosphatase and PKB α PH domain and demonstrate further their potential as high affinity ligands and emerging signalling tools.

Results and Discussion

We describe the preparation of a new series of benzene polyphosphates designed to interact with the same proteins as inositol polyphosphates and their lipids. Benzene 1,2,3,4-tetrakisphosphate **3**[13] and biphenyl 2,3',4,5',6-pentakisphosphate **4**[14] have been used in other studies and were synthesised according to published procedures.

Chemistry

Benzene 1,2-bisphosphate **7**, benzene 1,2,3-trisphosphate **10** and benzene 1,3,5-trisphosphate **13**, were synthesised by phosphitylating the appropriate polyphenol with diethylchlorophosphite to give the corresponding bis- or trisphosphite (Scheme 2). The intermediate was oxidised with *m*-chloroperoxybenzoic acid (*m*CPBA) to afford the corresponding polyphosphorylated derivatives (**6**, **9** and **12**) in approx 50% yield for each compound. The ethyl groups of the protected phosphates were removed using

bromotrimethylsilane to give the corresponding silylphosphate derivative. This reaction was monitored by ^1H NMR spectroscopy, since the ethyl groups from these compounds (**6**, **9** and **12**) were replaced by the corresponding trimethylsilyl groups; the corresponding ^1H NMR shifts moved upfield and the extent of the reaction was easily observed. ^{31}P NMR spectroscopy was however, not always a reliable method to monitor the upfield shift of phosphorus peaks, due to the broad nature of the resonances for some of the crude silylphosphate intermediates. Methanol removed the temporary trimethylsilyl protecting groups to give the phosphate monoesters **7**, **10** and **13** in high yield and final purification of each benzene polyphosphate was accomplished over a column of Q-Sepharose Fast Flow using a gradient of triethylammonium bicarbonate (TEAB) buffer and each compound was isolated as its glassy triethylammonium salt.

3,4,5-Trihydroxybenzaldehyde monohydrate **14** was benzylated using caesium carbonate and benzyl bromide to provide **15** (Scheme 3). 3,4,5-Tribenzyloxybenzaldehyde **15** was oxidised to the formate ester **16** using *m*CPBA and the formate ester was removed using acid in methanol to give 3,4,5-tribenzyloxyphenol **17** and the remaining hydroxyl group was benzylated to give the fully protected 1,2,3,5-tetraphenzyloxy derivative **18**. The benzyl groups were removed using $\text{Pd}(\text{OH})_2$ catalysed hydrogenation to provide 1,2,3,5-tetrahydroxybenzene **19**. Compound **19** was added to the phosphitylating mixture at room temperature; ultrasound-aided dissolution of the solid was required, and the colour of the solution turned a deeper yellow/orange as phosphitylation occurred. The phosphitylated mixture was then added to a cooled solution of *m*CPBA in CH_2Cl_2 and compound **20** was isolated in 51% yield after chromatography. Treatment with bromotrimethylsilane followed by methanolysis and addition of TEAB to the residue provided **21** as the triethylammonium salt. Compound **21** was purified by ion exchange chromatography to give the target compound in 80% yield.

2,4,5-Trihydroxybenzaldehyde **22**, was benzylated in the presence of benzyl bromide and caesium carbonate to give 2,4,5-tribenzyloxybenzaldehyde **23** (Scheme 4). Aldehyde **23** was oxidised to give ester **24** using *m*CPBA and acidic methanolysis gave the phenol **25** in 73% for two steps. Palladium hydroxide was added to a solution of 2,4,5-tribenzyloxyphenol dissolved in warm ethanol. The mixture was then stirred over an atmosphere of hydrogen at room temperature, the remaining yellow solution was filtered over a bed of Celite and the solvent was evaporated to give 1,2,4,5-tetrahydroxybenzene **26** in high yield.

Phosphorylation of compound **26** was accomplished using the P(III) reagent diethylchlorophosphite. 1,2,4,5-Tetrahydroxybenzene **26** was added in small portions to the P(III) solution and ultrasound-aided dissolution of the solid was accompanied by a number of colour changes during phosphitylation and the P(III) intermediate was oxidised using *m*CPBA under cooling conditions. The reaction mixture was immediately purified by flash chromatography and 1,2,4,5-tetrakis(diethoxyphosphoryloxy)benzene **27** was isolated as a yellow oil in 66% yield. Tetraol **26** was a difficult compound to phosphorylate; however, the reaction proceeded smoothly when the polyphenol was added to the phosphitylating reagent followed by oxidation to give **27**. The ethyl groups were removed in the presence of bromotrimethylsilane to give the corresponding symmetrical octatrimethylsilyl derivative, as a single peak by ^{31}P NMR at $\delta = -22.53$ ppm, (*cf* -5.96 ppm for the octaethyl derivative **27**). The temporary protective groups were removed using methanol and final purification of $\text{Bz}(1,2,4,5)\text{P}_4$ **28** was achieved by ion exchange chromatography to give the pure compound in 86% yield.

Biochemistry

5-Phosphatase Inhibition—The IC_{50} values of the benzene polyphosphates against 5-phosphatase were derived from a minimum of three experiments for each compound and were measured using an inositol phosphatase assay with $[^3H]Ins(1,4,5)P_3$ as substrate.[23] Values are reported in Table 1 and all compounds except **29** were resistant to hydrolysis by type I 5-phosphatase. The general trend of activity for these compounds suggests that the number of phosphates and their position on the benzene ring dictate potency against type-I $Ins(1,4,5)P_3$ 5-phosphatase. Benzene 1,2-bisphosphate, $[Bz(1,2)P_2]$, **7**, possesses two adjacent phosphates on a benzene ring [like $Ins(4,5)P_2$], but is a poor inhibitor of 5-phosphatase (IC_{50} is $>200 \mu M$). Benzene 1,2,3-trisphosphate, $[Bz(1,2,3)P_3]$, **10** possesses three adjacent phosphates and is also a weak inhibitor, $IC_{50} = 86 \mu M$, providing a marginal improvement compared to **7**. However, benzene 1,3,5-trisphosphate, $[Bz(1,3,5)P_3]$, **13** ($IC_{50} = 16 \mu M$), which has three non-adjacent phosphates evenly spread around the ring, is more potent compared to both **7** and **10**. Compound **13** is similar in structure to $Ins(1,3,5)P_3$ ($K_i = 45 \mu M$ for 5-phosphatase);[24] however, $Ins(1,3,5)P_3$ is less potent than the aromatic counterpart **13**. We demonstrated earlier that the trisphosphorothioate analogue of $Ins(1,3,5)P_3$ had a K_i for this enzyme of 430 nM.[9] Thus, we would predict that the corresponding modification for **13** should provide an even better inhibitor.

Benzene 1,2,4-trisphosphate, $[Bz(1,2,4)P_3]$, **2**, 3-hydroxybenzene 1,2,4-trisphosphate $[3-HO-Bz(1,2,4)P_3]$ **29** and 3-benzyloxybenzene 1,2,4-trisphosphate, $[3-BnO-Bz(1,2,4)P_3]$ **30** (Scheme 5) were synthesised as described previously[11,12] and possess the same 1,2,4-trisphosphate pattern, albeit with different functional groups at position-3. **29** is the first example of a non-inositol polyphosphate substrate for 5-phosphatase, in which phosphate-2 can be hydrolysed to provide 2,3-dihydroxybenzene 1,4-bisphosphate.[12] The presence of the phenolic $-OH$ group at position-3 in **29** did not markedly alter the IC_{50} value towards type-I 5-phosphatase compared to **2**. [12] However, the presence of a benzyl group at position-3 of the related compound **30** increases the hydrophobic bulk, and makes it a weaker 5-phosphatase resistant inhibitor compared to **29** (Table 1). Thus, position-3 of compound **30** can most likely accommodate a small functional group such as OH. D-2,3,6-Trideoxy *myo*-inositol 1,2,4-trisphosphate ($K_i = 81.4 \mu M$) published some 15 years ago by Kozikowski[25] is similar in structure to $Bz(1,2,4)P_3$ **2** ($IC_{50} = 14 \mu M$), however the inositol 1,2,4-trisphosphate derivative is a substrate and much less potent than compound **2** against 5-phosphatase from human erythrocyte membranes, illustrating that these aromatic compounds are potentially useful $Ins(1,4,5)P_3$ 5-phosphatase-resistant inhibitors.

The addition of a fourth phosphate to $Bz(1,2,4)P_3$ **2** provides three possible compounds, benzene 1,2,3,4-tetrakisphosphate, $[Bz(1,2,3,4)P_4]$, **3**, benzene 1,2,3,5-tetrakisphosphate $[Bz(1,2,3,5)P_4]$ **21** and benzene 1,2,4,5-tetrakisphosphate $[Bz(1,2,4,5)P_4]$ **28**. Surprisingly, **21** which has the same phosphate pattern as $Ins(1,3,4,5)P_4$ (the most potent inhibitor of 5-phosphatase, as a substrate)[6,23] is a poor inhibitor of the enzyme ($IC_{50} = 78 \mu M$) and close to the value of $Bz(1,2,3)P_3$ **10** (IC_{50} value $86 \mu M$). The IC_{50} value of $Bz(1,2,3,5)P_4$ is approximately 5-fold higher than $Bz(1,3,5)P_3$ which has one less phosphate, suggesting that phosphate-2 of the three adjacent phosphate groups in $Bz(1,2,3,5)P_4$ is making an unfavourable interaction or displacing phosphates 1- and 3- by repulsion into an unfavourable environment. Thus, for type I $Ins(1,4,5)P_3$ 5-phosphatase, more phosphates on an aromatic ring does not necessarily mean more potent inhibition *per se*, only their number coupled also with their position on the aromatic ring. It would probably be naïve to assume that benzene polyphosphates which have ostensibly the same regioisomeric phosphate arrangement as their inositol polyphosphate counterparts will necessarily behave in a similar manner. $Bz(1,2,3,4)P_4$ **3** is a good example of this effect.[13] Therefore, each compound must be evaluated against an inositol phosphate binding protein individually. For benzene

polyphosphates, two adjacent phosphate groups is the optimum number for a good 5-phosphatase inhibitor, since three adjacent phosphate groups reduce the potency of the resulting compound, as is illustrated by the IC_{50} values of compounds **10** and **21**. Furthermore, compound **3** ($IC_{50} = 98 \mu M$) has four adjacent phosphate groups, indicating that the potency dramatically decreases when more than two adjacent phosphates are present on a single benzene ring. Finally, tetrakisphosphate Bz(1,2,4,5) P_4 **28** was found to be the most potent 5-phosphatase inhibitor in the tetrakisphosphate series of compounds ($IC_{50} = 4 \pm 2 \mu M$). **28** Has two pairs of adjacent phosphates, showing that benzene 1,2-bisphosphate **7** is successively made a more potent inhibitor of 5-phosphatase by the addition of another non-adjacent phosphate, first to give Bz(1,2,4) P_3 **2** ($IC_{50} = 14 \pm 2 \mu M$), and the same pattern of phosphates as Ins(1,4,5) P_3 , ($K_m = 15 \mu M$) and *scyllo*-Ins(1,2,4) P_3 ($K_m = 24.2 \mu M$) [26] then the addition of another phosphate group to Bz(1,2,4) P_3 **2**, to give Bz(1,2,4,5) P_4 **28**, ($IC_{50} = 4 \pm 2 \mu M$). **28** Nominally corresponds to the Ins(1,3,4,6) P_4 counterpart which has a K_i value of $7.7 \mu M$ [8] and also the known derivative, *scyllo*-inositol 1,2,4,5-tetrakisphosphate has a K_i value of $4.3 \mu M$ [26]. Both inositol polyphosphates are substrates with the same arrangement of phosphates as Bz(1,2,4,5) P_4 ; however, **28** is resistant to 5-phosphatase. Biphenyl 2,3',4,5',6-pentakisphosphate **4** was the most potent of all the compounds evaluated against type I Ins(1,4,5) P_3 5-phosphatase with an IC_{50} of $1 \mu M$. While there is no inositol polyphosphate equivalent of the biphenyl compound it is apparent that the two adjacent phosphorylated benzene rings increases the affinity for this enzyme. Further studies will optimise the number and position of phosphate groups on the biphenyl structure yielding tighter binding of these novel derivatives. These data demonstrate that while benzene polyphosphates are apparently better inhibitors of 5-phosphatase than their inositol polyphosphate counterparts, the effects are not substantial. However, unlike inositol polyphosphates benzene phosphates without free hydroxyl groups are as a rule not dephosphorylated by 5-phosphatase. Another important issue for compounds of this class is the interaction with the metabolic enzyme Ins(1,4,5) P_3 3-kinase and the Ins(1,4,5) P_3 receptor, an issue we have dealt with in a recent publication. [14] The benzene phosphates are rather poor inhibitors of Ins(1,4,5) P_3 3-kinase A, but biphenyl pentakisphosphate may well compete with Ins(1,4,5) P_3 at the level of the Ins(1,4,5) P_3 receptor.

Inhibition at the PH domain of PKB α detected by FRET analysis—Table 1 shows the benzene polyphosphates which inhibit the binding of biotinylated diC₈-PtdIns(3,4) P_2 to the PH domain of PKB α . [13] Interestingly, the lipid analogue diC₈-PtdIns(3,4,5) P_3 and water-soluble headgroup Ins(1,3,4,5) P_4 only show a two-fold difference in K_i values, demonstrating that the 1-position has available space for the hydrophobic lipid motif. Bz(1,2,3) P_3 **10** and Bz(1,2,4) P_3 **2** also have similar K_i values ($0.59 \mu M$ and $0.61 \mu M$ respectively); however Bz(1,3,5) P_3 **13** ($K_i = 2.18 \mu M$) is 4-fold weaker. The 1,3,5-trisphosphate motif exhibits the weakest binding of the three possible arrangements of the three phosphate groups, and these inhibition data do not follow the pattern observed for 5-phosphatase.

Biphenyl 2,3',4,5',6-pentakisphosphate **4**, also a molecule of the benzene phosphate class, is composed of two phosphorylated benzene rings which from modelling studies appears to interact with more sites on PKB α PH domain than any other compound, resulting in potent binding affinity ($K_i = 27 nM$), with 8.5-fold higher affinity than diC₈-PtdIns(3,4,5) P_3 . The binding affinity for Bz(1,3,5) P_3 **13** ($K_i = 2.18 \mu M$) is interesting in the context of **4**, since one ring is functionalised with the same benzene 1,3,5-trisphosphate motif (2,4,6-trisphosphate of **4**) and the adjacent ring has a benzene 1,3-bisphosphate motif (3',5'-bisphosphate of **4**). Thus, the latter motif appears to enhance the affinity of **13**, by some 80-fold. In an attempt to rationalize this we undertook molecular modelling of biphenyl 2,3',4,5',6-pentakisphosphate **3** using the known X-ray crystal structure of the PH domain of PKB α .

Molecular Modelling

4 Was modelled into the binding site of PKB α PH domain based upon the published X-ray crystal structure for this protein[13] as described in the experimental section (Figure 1). The aryl 2,4,6-trisphosphate motif of **4** seems to dock to the same amino acid residues as the simpler compound **13** although **4** binds with an additional H-bond between phosphate-6 and Asn53 (Supplementary Tables S1 & S2), (see Scheme 1 for numbering of **4**). One phosphate of the aryl 3',5'-bisphosphate motif interacts with Arg25 and Arg23 whilst the other interacts with Arg23 only (Figure 1). Interestingly, the aromatic core of the 3',5'-bisphosphorylated aryl motif is also close to and is nearly parallel with the guanidinium cation of Arg23, initiating a potential cation- π interaction, an interaction obviously not possible for inositol polyphosphate derivatives. However, GOLD does not recognize cation- π interactions, so an X-ray crystal structure would be the only definitive approach to identify this interaction. Cation- π interactions are non-covalent forces which are important in small molecule recognition with some amino acids.[27] Energetically, they are similar to and possibly stronger than hydrogen bonds. Cation- π interactions are also recognized in highly conserved arginine residues among known Src Homology 2 (SH2) domain-phosphotyrosine-peptide complexes,[28] and our model illustrates this type of interaction. The cation- π interaction and the favourable location of 3,5-bisphosphate motif of **4** could potentially account for the majority of the 80-fold increase in binding affinity ($K_i = 27$ nM) compared to Bz(1,3,5)P₃ **13** ($K_i = 2.18$ μ M). Cation- π interactions are now increasingly recognised as important interactions and have been reviewed.[29-31]

Bz(1,2,3)P₃ **10** also has reasonable affinity for the PH domain of PKB α , but it is not obvious why three adjacent phosphates on a benzene ring give a more potent inhibitor compared to other phosphate arrangements. When **10** was minimized in the presumed binding site of the PH domain only potential interactions with phosphate-1 (Arg23) and phosphate-3 and a single H-bond of phosphate-2 with Lys14 were revealed (Supplementary Table S1 & Figure 2). There seem however, fewer obvious interactions of compound **10** than with Bz(1,2,4)P₃ **2** (Supplementary Table S1 & Figure S1). The model of compound **10** also suggests a potential cation- π interaction between Arg23 and the aromatic ring (Figure 2). Compounds, such as Bz(1,2,3,5)P₄ **21** (Supplementary Figure S2) and Bz(1,2,3,4)P₄ **3** which have 3- or 4- adjacent phosphates do not appear to make such a cation- π interaction, since Arg23 and the aromatic ring are not parallel or close to each other. However, **3** is nevertheless a potent binder at the PH domain of PKB α and seems to be able to accommodate more phosphate groups in its binding site than for 5-phosphatase since 3-4 adjacent phosphates can be present on a single benzene ring with increasing potency, whereas Ins(1,4,5)P₃ 5-phosphatase can only accommodate a maximum of two adjacent phosphates without affecting binding. Interestingly, only the same 1,2,4-trisphosphorylated motif is recognized in Bz(1,2,3,5)P₄ (1-, 2-, and 5-phosphates) and Bz(1,2,3,4)P₄ (1-, 3- and 4-phosphates) respectively, demonstrating that this protein binds a specific pattern of phosphate groups present on a benzene ring. In Bz(1,2,3,4)P₄ this was demonstrated by the crystal structure analysis[13] that showed H-bonding only occurred at phosphates 1, 3 and 4. Since PKB α PH domain binds the phospholipids PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, this means that the common pharmacophore is the two phosphates at the 3, and 4 positions of the inositol ring together with the diacylglycerol phosphodiester link at the 1-position. X-ray crystal structure data has shown that for Ins(1,3,4,5)P₄, the head group of PtdIns(3,4,5)P₃, phosphate-5 is solvent exposed, and only -1, -3 and -4 are important binding partners.[32] Thus, recognition of **3** and **21** is most likely achieved by interaction of the protein with the surrogate 1,3,4-trisphosphate arrangement of the benzene trisphosphate.

Recently, we have shown that benzene polyphosphates most of which appear to be 5-phosphatase-resistant, can substitute for inositol polyphosphates and their lipid counterparts

in a number of structural[13] and in vitro cell signalling contexts.[12,14] Benzene polyphosphates are also good candidates to explore membrane-permeable pro-drug forms in which the phosphate charge can be masked with suitable lipophilic groups and potentially provide more versatile tools to investigate whole cell inhibition of inositol polyphosphate and inositol lipid binding proteins. Benzene 1,2,4-trisphosphate **2** is the most potent inhibitor in the trisphosphate series and benzene 1,2,4,5-tetrakisphosphate **28** is most potent in the tetrakisphosphate series against 5-phosphatase. Inhibition of Ins(1,4,5)P₃ 5-phosphatase is considerably reduced when three or more adjacent phosphate groups are present on a single benzene ring leaving a maximum number of adjacent phosphates as two per benzene ring for 5-phosphatase. Contrastingly, we observed a small increase in potency at the PH domain of PKB α , for molecules having three or more adjacent phosphate groups. Since there is no X-ray crystal structure of type I 5-phosphatase we are unable to fully interpret our results for this enzyme in a structural context at this stage as for PKB α PH domain. Synthesis of new benzene polyphosphates and exploration of more optimised biphenyl derivatives with new functionality could provide new tools to investigate the phosphoinositide signalling pathways. The identification here of potential cation- π interactions provides interesting prospects to introduce phenyl motifs in a number of structural contexts to possibly examine binding to inositol phospholipid binding proteins and enzymes. Of particular interest could be the exploration of the structural biology of such proteins in complex with benzene phosphates. Their simple structure and lack of chirality means that they could be easily tailored to provide ligands for this purpose and subsequently optimized for a given protein using structure-based design. Our recent report[13] paves the way for this exploration. We have thus demonstrated that benzene polyphosphates can bind to and inhibit proteins, both an inositol polyphosphatase and one that also bind phospholipids and in one case with high affinity. Studies to explore co-crystallisation of members of this ligand class with a range of signalling protein targets are underway.

Experimental Section

Materials and Methods

Chemistry—Chemicals were purchased from Acros, Aldrich, Alfa Aesar and Fluka. Thin-layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminium sheets silica 60 F₂₅₄): products were visualised by dipping in an ethanolic solution phosphomolybdic acid, then heated at high temperature. Organic compounds were dried over MgSO₄. Flash chromatography was carried out on Fisher Scientific Silica 60A (particle size 35–70 micron). All final compounds were judged by standard spectroscopic methods, purified by ion exchange chromatography, and were used in all biological evaluations as their triethylammonium salts. Ion exchange chromatography was performed on an LKB-Pharmacia Medium Pressure Ion Exchange Chromatograph using Q-Sepharose Fast Flow with gradients of triethylammonium bicarbonate (TEAB, 0→2.0 M) as eluent. Column fractions containing benzene polyphosphates were identified by U.V. spectroscopy at 254 nm and were quantified for total phosphate by a modification of the Briggs test.[9] NMR spectra (proton frequency 270 or 400 MHz) were referenced against SiMe₄, (HDO), [D₆-(CD₃)₂CO] or [D₃-(CD₃CN)]. The ³¹P NMR shifts were measured in ppm relative to external 85% phosphoric acid. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler block. Microanalysis was carried out at the University of Bath. Mass spectra were recorded using electron impact (EI), electrospray (ES) and positive and negative Fast Atom Bombardment (FAB) with 3-nitrobenzyl alcohol (NBA) as the matrix and for electrospray the standard was sodium formate. It is assumed that *m*CPBA used in oxidation reactions is 100% for calculation purposes only and was always used in excess.

Enzyme Methods

Expression and Purification of Recombinant Type I 5-Phosphatase—Expression and purification of human brain Type I InsP₃ 5-phosphatase in pTrcHis was performed as previously described.[33]

Ins(1,4,5)P₃ 5-Phosphatase Assay—The assay was carried out according to the literature procedure,[14] using Ins(1,4,5)P₃ (1.0 μM) as substrate to calculate the IC₅₀ values.

Malachite Green Phosphatase Assay—This assay was only used to evaluate the analogue in terms of inorganic phosphate release at 100 μM and thus its potential to be a substrate or inhibitor. Phosphatase activity was measured with a phosphate release assay using an acidic malachite green dye as reported.[23] The benzene polyphosphates (100 μM) were diluted in assay buffer (30 μL), comprising of Hepes (50 mM, pH 7.4), MgCl₂ (2 mM) bovine serum albumin (1 mg/mL) and DTT (5 mM). The phosphatase reaction was initiated by adding the enzyme diluted in assay buffer (15 μL) to the substrate. Samples were incubated at 37 °C. After 10 min, reactions were stopped by the addition of 0.1 M EDTA (15 μL). Malachite green reagent (75 μL) (ammonium molybdate 0.5% [w/v], Tween-20 (0.057%) [v/v] and malachite green oxalate 0.34 mM) were added to the reaction solution (50 μL). Samples were left for 10 min for colour development before measuring absorbance at 650 nm. Inorganic phosphate release was quantified by comparison to a standard curve of KH₂PO₄ in MilliQ water.

Determination of IC₅₀—All analogues were evaluated in the 1–100 μM range according to the published procedure by Erneux et al[23] and data were adjusted by non linear regression using GraphPad prism4 (www.graphpad.com).

FRET Binding Experiments at the PH domain of PKBα—The quantitative time-resolved-fluorescence resonance energy transfer (TR-FRET) analysis of binding was performed using a BMG Labtech PHERAstar at the following settings: excitation 337 nm filter, emission 665 and 620 nm filters, 300 flashes per well 10-μs flashes, read for 400 μs following a 50 μs delay. The PH domain of PKBα (corresponding to amino acids 1–123) was subcloned into the BamHI restriction site of the *Escherichia coli* expression vector pGEX2T. The resulting construct encodes for the bacterial expression of the PH domain of PKBα with an N-terminal glutathione-S-transferase (GST) tag, as previously described.[32] Assays were performed in a buffer comprising: HEPES, (50 mM), pH 6.8; NaCl, (150 mM); MgCl₂, (5 mM); DTT, (5 mM); CHAPS, (0.5%); EDTA, (1 mM).

Determination of K_i Values—To determine K_i values for biotinylated diC₈-PtdIns(3,4)P₂, increasing amounts (0–100 nM) of biotinylated diC₈-PtdIns(3,4)P₂ (Cell Signals) were incubated with GST tagged PH domain of PKBα (20 nM) in the presence of excess europium-labelled goat anti-GST antibody and streptavidin-conjugated allophycocyanin (APC). The binding of the biotinylated lipid to the protein allowed FRET to occur between europium (donor) and APC (acceptor). Fluorescence was monitored at 665 and 620 nm; the ratio of these signals allows the determination of the relative amount of binding. The pK_D for biotinylated diC₈-PtdIns(3,4)P₂ was 8.23 ± 0.09 (mean ± s.e.m., n=3), giving a K_D of 5.9 nM. Competition assays were performed with a fixed amount (25 nM) of biotinylated diC₈-PtdIns(3,4)P₂ and increasing amounts of competing agent. IC₅₀ values were determined, from which K_i values were calculated.[34] All curve fitting was performed using Prism (GraphPad).

Molecular Modelling Methods

Biphenyl 2,3',4,5',6-pentakisphosphate **4** was built using the program SYBYL (v7.1) and optimized to convergence using the MMFFs force field with MMFF charges. Angles between the carbon atoms were constrained using a force constant of 200 kcal mol⁻¹ during the optimization procedure, and this induced a twisted conformation of the biphenyl moiety; the angle between the planes of the rings post-optimization was 109.6°.

The experimental structure of the PKB PH domain crystallized with benzene 1,2,3,4,-tetrakisphosphate[13] was used for the docking study (residue 17 modelled as Glu). Waters of crystallization, the triethylammonium bicarbonate counter ions and Bz(1,2,3,4)P₄ were removed. Hydrogen atoms were added and their positions optimized to convergence using the TRIPOS force field and Gasteiger-Hückel charges within the SYBYL program (v7.1). Biphenyl 2,3',4,5',6-pentakisphosphate **4** was docked a total of 40 times to the PKB PH domain using the GOLD program[35] (v3.0.1) with the GOLDScore fitness function. The terminal phosphate oxygen atoms of the ligand were specified as type O.co2. The binding site of the PH domain was defined as a 10 Å sphere around atom NZ of Lys14. A similar method was used to model Bz(1,2,3)P₃.

1,2-Bis(diethoxyphosphoryloxy)benzene (6)—Dry *N,N*-diisopropylethylamine (2.1 mL, 12 mmol) was stirred in dry CDCl₃ (5 mL) at room temperature. Benzene 1,2-diol **5** (330 mg, 3 mmol) was added to the solution which formed a grey/green suspension. The solution was cooled using dry ice and diethyl chlorophosphite (1.22 mL, 7 mmol) was added and stirred for a further 10 min to give a yellow solution. At this stage ($\delta_P = +134$ ppm), which indicated phosphorylation of the phenolic groups. The solution was cooled using dry ice in acetone then *m*CPBA (1.72 g, 10 mmol) was added in CH₂Cl₂ (5 mL) and the mixture was stirred for 30 min. The reaction was warmed to room temperature and stirred overnight. The mixture was diluted with CH₂Cl₂ (50 mL) and washed with an aqueous solution of 10% sodium metabisulphite (2 × 50 mL), an aqueous solution of sodium hydrogen carbonate (2 × 50 mL) and water (50 mL). The compound was purified by flash chromatography (EtOAc, *R_f* = 0.27) to give compound **6** as an oil (598 mg, 52%). ¹H NMR (270 MHz, CDCl₃) 1.28–1.33 (12 H, m, 2 × ArOP(O)(OCH₂CH₃)₂), 4.15–4.26 (8 H, m, 2 × ArOP(O)(OCH₂CH₃)₂), 7.06–7.12 (2 H, m, CH, Ar), 7.33–7.38 (2 H, m, CH, Ar); ³¹P NMR (109 MHz, CDCl₃) –5.81 (2 P, s, 2 × ArOP(O)(OCH₂CH₃)₂); (MS, FAB⁺) 383.0, 355.0, 270.9, 252.9, 190.0, 173.0, 98.9; (HRMS, FAB⁺) *m/z* calcd for C₁₄H₂₅O₈P₂ [M + H]⁺ 383.1024, found 383.1011.

Benzene 1,2-bisphosphate (7)—A mixture of compound **6** (76 mg, 200 μmoles) and bromotrimethylsilane (1.0 mL, 7.58 mmol), in dry CH₂Cl₂ (5 mL) was stirred for 20 h under an atmosphere of nitrogen. The solvents were evaporated and the residue was dissolved in MeOH (5 mL) and the solution was stirred for 5 min. MeOH was evaporated and TEAB (2 M, 1 mL) was added to form the salt. Final purification was achieved over Q-Sepharose Fast Flow using a gradient of TEAB (0 → 2.0 M) to give compound **7** as a glass, (179 μmoles, 89.5%). ¹H NMR (270 MHz, D₂O) 7.00–7.09 (2 H, m, CH, 2 × ArOPO₃²⁻), 7.24–7.27 (2 H, m, CH, 2 × ArOPO₃²⁻); ³¹P NMR (109 MHz, D₂O) –2.83 (2 P, s, 2 × ArOPO₃²⁻); (MS, FAB⁻): 539.0, 349.0, 269.0, 189.0, 171.0, 78.9; (HRMS, FAB⁻) *m/z* calcd for C₆H₇O₈P₂ [M – H]⁻ 268.9616, found 268.9616.

1,2,3-Tris(diethoxyphosphoryloxy)benzene (9)—Dry *N,N*-diisopropylethylamine (2.1 mL, 12 mmol) was stirred in dry CDCl₃ (5 mL) at room temperature. The mixture was cooled using dry ice alone and diethyl chlorophosphite (1.74 mL, 10 mmol) was added to the mixture. Pyrogallol **8** (378 mg, 3 mmol) was added in small quantities and ultrasound aided dissolution of the solid which was stirred for a further 30 min. *m*CPBA (2.58 g, 15

mmol) in dry CH_2Cl_2 (10 mL) was added to the intermediate under cooling conditions using dry ice and acetone, and the mixture was stirred overnight. The solution was diluted with CH_2Cl_2 (50 mL) and washed with a 10% aqueous solution of sodium metabisulphite (2×50 mL), a saturated aqueous solution of sodium hydrogen carbonate (2×50 mL) and water (50 mL). The solvent was evaporated and the residue was purified by flash chromatography (EtOAc then EtOAc/EtOH, 5:1) to give compound **9**, (814 mg, 51%) as an oil (EtOAc/EtOH 5:1, $R_f = 0.35$). ^1H NMR (400 MHz, CDCl_3) 1.33–1.40 (18 H, m, $3 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 4.23–4.34 (12 H, m, $3 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 7.13 (1 H, dt, $J = 1.2, 9.1$ Hz, CH, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 7.31 (2 H, d, $J = 8.5$ Hz, CH, $2 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); ^{13}C NMR (100 MHz, CDCl_3) 15.71, 15.77 (2 q, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 64.56, 64.61, 64.67 (3 t, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 116.41, 124.82 (2 d, CH, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 143.42, 143.47, 143.51 (C_q , $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); ^{31}P NMR (162 MHz, CDCl_3) –6.39 (1 P, s, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), –7.01 (2 P, s, $2 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); (MS, FAB^+): 535.1, 507.0, 268.9; (HRMS, FAB^+) m/z Calcd for $\text{C}_{18}\text{H}_{34}\text{O}_{12}\text{P}_3$ $[\text{M} + \text{H}]^+$ 535.1263, found 535.1242; calcd for $\text{C}_{18}\text{H}_{33}\text{O}_{12}\text{P}_3$: C 40.46, H 6.22; found: C 40.3, H 6.37.

Benzene 1,2,3-trisphosphate (10)—1,2,3-Tris(diethoxyphosphoryloxy)benzene **9** (107 mg, 200 μmol) was dissolved in dry dichloromethane (5 mL). Bromotrimethylsilane (1.0 mL, 7.58 mmol) was added to the solution and stirred for 20 h at room temperature. The volatile solvents were evaporated and the residue was dissolved in methanol (5 mL). Final purification of compound **10** was achieved using ion exchange chromatography over Q-Sepharose Fast Flow and eluted with a linear gradient of triethylammonium bicarbonate buffer (0 $\rightarrow$ 2.0 M). Compound **10** eluted between 1.3–1.7 M buffer and was isolated as a glassy triethylammonium salt, (186 μmoles , 93%). ^1H NMR (270 MHz, D_2O) 7.01 (1 H, m, CH, ArOPO_3^{2-}), 7.05 (2 H, br m, CH, $2 \times \text{ArOPO}_3^{2-}$); ^{31}P NMR (109 MHz, D_2O) –2.17 (2 P, s, $2 \times \text{ArOPO}_3^{2-}$), –2.33 (1 P, s, ArOPO_3^{2-}); (MS, FAB^-) 365.0, 335.1, 303.1, 285.0, 264.0, 200.1, 121.1; (HRMS, FAB^-) m/z calcd for $\text{C}_6\text{H}_8\text{O}_{12}\text{P}_3$ $[\text{M} - \text{H}]^-$ 364.9228, found 364.9239.

1,3,5-Tris(diethoxyphosphoryloxy)benzene (12)—A mixture of dry CDCl_3 (5 mL) and dry *N,N*-diisopropylethylamine (2.1 mL, 12 mmol) was cooled using dry ice. Diethyl chlorophosphite (1.74 mL, 10 mmol) was added and the solution turned yellow. Benzene 1,3,5-triol **11** (378 mg, 3 mmol) was added and the solution was stirred for 30 min. The mixture was cooled using dry ice in acetone, then *m*CPBA (2.58 g, 15 mmol) in CH_2Cl_2 (10 mL) was added and stirred for a further 30 min. The solution was diluted with CH_2Cl_2 (50 mL) and washed with 10% aqueous solution of sodium metabisulphite (2×50 mL), a saturated aqueous solution of sodium hydrogen carbonate (2×50 mL) and water (50 mL). The organic solvent was evaporated to give an oil and purified by flash chromatography (EtOAc then EtOAc/EtOH, 5:1) to give compound **12** (760 mg, 47%) as an oil, $R_f = 0.56$ (CHCl_3 –acetone, 1 : 1). ^1H NMR (400 MHz, CDCl_3) 1.33–1.39 (18 H, m, $3 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 4.21–4.25 (12 H, m, $3 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 6.99 (3 H, s, CH, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); ^{13}C NMR (100 MHz, CDCl_3) 16.03, 16.11 (2 q, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 64.95, 65.01 (2 t, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 108.96, 109.01, 109.07 (3 d, CH, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 151.62, 151.69 (C_q , $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); ^{31}P NMR (162 MHz, CDCl_3) –7.12 (3 P, s, $3 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); (MS, FAB^+): 1076.1, 877.1, 724.1, 643.2, 535.2, 507.1, 366.9; (HRMS, FAB^+) m/z calcd for $\text{C}_{18}\text{H}_{34}\text{O}_{12}\text{P}_3$ $[\text{M} + \text{H}]^+$ 535.1263, found 535.1245; calcd for $\text{C}_{18}\text{H}_{33}\text{O}_{12}\text{P}_3$: C 40.46, H 6.22; found: C 40.2, H 6.26.

Benzene 1,3,5-trisphosphate (13)—1,3,5-Tris(diethoxyphosphoryloxy)benzene **12** (107 mg, 200 μmol) was dissolved in dry dichloromethane (5 mL). Bromotrimethylsilane (1.0 mL, 7.58 mmol) was added to the solution which was stirred for 21.5 h at room temperature. The volatile solvents were evaporated and the residue was dissolved in

methanol (5 mL). Final purification of compound **13** was achieved using ion exchange chromatography over Q-Sepharose Fast Flow using a gradient of triethylammonium bicarbonate buffer (0→2.0 M). Compound **13** eluted between 1.3–1.7 M buffer and was isolated as a glassy triethylammonium salt, (115 μ moles, 57.5%). ^1H NMR (270 MHz, D_2O) 6.67 (3 H, s, CH , $3 \times \text{ArOPO}_3^{2-}$); ^{31}P NMR (109 MHz, D_2O) –3.03 (3 P, s, $3 \times \text{ArOPO}_3^{2-}$); MS: (HRMS, FAB^-) m/z calcd for $\text{C}_6\text{H}_8\text{O}_{12}\text{P}_3$ $[\text{M} - \text{H}]^-$ 364.9228, found 364.9234.

3,4,5-Tribenzyloxybenzaldehyde (15)—A mixture of 3,4,5-trihydroxybenzaldehyde monohydrate **14** (3.08 g, 17.89 mmol), caesium carbonate (32.58 g, 100 mmol) and benzyl bromide (11.9 mL, 100 mmol) in dry DMF (100 mL) was stirred at 80 °C for 17 h. The solution was filtered over a bed of Celite, washed with acetone and the solvents were evaporated to give the crude product. The residue was dissolved in CH_2Cl_2 (200 mL) and washed with water (200 mL), the organic layer was dried and the solvent was evaporated. The crude product was purified by flash chromatography (CH_2Cl_2) to give compound **15** as a solid, (6.62 g, 87%), from CH_2Cl_2 –hexane, mp 103–105 °C, R_f = 0.42 (CHCl_3). ^1H NMR (400 MHz, CDCl_3) 5.14 (6 H, s, $3 \times \text{ArOCH}_2\text{Ph}$), 7.15 (2 H, s, $2 \times \text{CH}$, Ar), 7.21–7.41 (15 H, m, $3 \times \text{ArOCH}_2\text{Ph}$), 9.76 (1 H, s, ArCHO); ^{13}C NMR (100 MHz, CDCl_3) 71.18, 75.16 (t, ArOCH_2Ph), 108.70 (d, CH , Ar), 127.30, 127.89, 127.97, 128.35, 128.45 (d, ArOCH_2Ph), 131.59, 136.19, 137.04, 143.50, 152.99 (s, C_q , ArOCH_2Ph), 190.69 (ArCHO); (MS, FAB^+) 425.1, 333.1, 91.0; (HRMS, FAB^+) m/z calcd for $\text{C}_{28}\text{H}_{25}\text{O}_4$ $[\text{M} + \text{H}]^+$ 425.1752, found 425.1745; calcd for $\text{C}_{28}\text{H}_{24}\text{O}_4$ C 79.23, H 5.70; found: C 79.0, H 5.77.

3,4,5-Tribenzyloxyphenol (17)—*m*CPBA (2.9 g, 16.8 mmol) was added to a solution of 3,4,5-tribenzyloxybenzaldehyde **15** (4.245 g, 10 mmol) in dry CH_2Cl_2 (100 mL) and the mixture was stirred for 20 h at room temperature. The yellow solution was washed with an aqueous solution of 10% sodium metabisulphite (2×100 mL), a saturated solution of sodium hydrogen carbonate (2×100 mL) and water (100 mL). The organic layer was dried and the solvent was evaporated to give the crude formate ester (R_f = 0.60, CH_2Cl_2) as an orange residue which was purified by flash chromatography to give **16** as a yellow solid. Compound **16** was dissolved in a mixed solvent (CH_2Cl_2 –MeOH 1:1, 100 mL) and stirred for 4 h in the presence of Amberlyst 15 ion exchange resin (1.0 g). TLC indicated a new compound with a lower R_f = 0.32 (CH_2Cl_2). The Amberlyst was filtered off and the organic solution was concentrated. Compound **17** was purified by flash chromatography using CH_2Cl_2 as eluent (3.50 g, 85%); mp 110–111 °C, from CH_2Cl_2 –hexane. ^1H NMR (400 MHz, CDCl_3) 4.99 (4 H, s, $2 \times \text{ArOCH}_2\text{Ph}$), 5.02 (2 H, s, ArOCH_2Ph), 5.42 (1 H, s, D_2O exch, ArOH), 6.12 (2 H, s, $2 \times \text{CH}$, Ar), 7.26–7.46 (15 H, m, $3 \times \text{ArOCH}_2\text{Ph}$); ^{13}C NMR (100 MHz, CDCl_3) 70.92 (t, $2 \times \text{ArOCH}_2\text{Ph}$), 75.60 (t, ArOCH_2Ph), 95.36 (d, $2 \times \text{CH}$, Ar), 127.41, 127.90, 128.20, 128.53, 128.80 (d, ArOCH_2Ph), 131.80, 136.93, 137.75, 152.27, 153.23 (C_q , ArOCH_2Ph , ArOH , ArOBn); m/z (MS, FAB^+) 412.2, 321.1, 181.1, 71.0, 55.0; calcd for $\text{C}_{27}\text{H}_{24}\text{O}_4$ C 78.62, H 5.86; found C 78.3, H 5.88.

1,2,3,5-Tetraphenylbenzene (18)—A mixture of 3,4,5-tribenzyloxyphenol **17** (2.17 g, 5.26 mmol), caesium carbonate (3.26 g, 10 mmol) and benzyl bromide (1.19 mL, 10 mmol) in dry DMF (50 mL) was stirred for 18 h at 80 °C. The solvent was evaporated and the residue was partitioned between CH_2Cl_2 and water (100 mL of each). The crude product was dried, the solvent was evaporated and the residue was purified by flash chromatography (CH_2Cl_2 –hexane 2:1, then CH_2Cl_2), to give the title compound **18** as a white crystalline solid, (2.37 g, 89%), mp 113–114 °C (from hexane), R_f = 0.22 (CH_2Cl_2 –hexane, 2:1). ^1H NMR (400 MHz, CDCl_3) 4.90 (2 H, s, ArOCH_2Ph), 4.97 (2 H, s, ArOCH_2Ph), 5.04 (4 H, s, $2 \times \text{ArOCH}_2\text{Ph}$), 6.27 (2 H, s, $2 \times \text{CH}$, Ar), 7.21–7.40 (20 H, m, $4 \times \text{ArOCH}_2\text{Ph}$); ^{13}C NMR (100 MHz, CDCl_3) 70.24 (t, ArOCH_2Ph), 71.05 (t, $2 \times \text{ArOCH}_2\text{Ph}$), 75.21 (t, ArOCH_2Ph),

95.15 (d, $2 \times \text{CH}$, Ar), 127.07, 127.28, 127.40, 127.51, 127.72, 127.79, 128.14, 128.27, 128.29 (d, ArOCH_2Ph), 136.45, 136.70, 137.61, 152.82, 154.80 (C_q , ArOCH_2Ph , ArOCH_2Ph); m/z (MS, FAB^+) 503.0, 411.0, 91.0; (HRMS, FAB^+) calcd for $\text{C}_{34}\text{H}_{31}\text{O}_4$ $[\text{M} + \text{H}]^+$ 503.2222, found 503.2204; calcd for $\text{C}_{34}\text{H}_{30}\text{O}_4$ C 81.25, H 6.02; found C 81.4, H 5.94.

1,2,3,5-Tetrahydroxybenzene (19)—Palladium hydroxide on carbon (20%, 500 mg) was added to 1,2,3,5-tetrabenzoyloxybenzene **18** (2.29 g, 4.56 mmol), dissolved in THF (100 mL). The air was expelled from the vessel and the mixture was stirred over an atmosphere of hydrogen for 17 h at room temperature. The resulting yellow solution was filtered over a bed of Celite and washed with more THF which was then evaporated to give compound **19** as brown solid (461 mg, 71%). ^1H NMR (400 MHz, CD_3CN) 5.89 (2 H, s, $2 \times \text{CH}$, Ar), 6.24–6.71 (4 H, br, $4 \times \text{ArOH}$); ^{13}C NMR (100 MHz, CD_3CN) 94.69 (d, CH , Ar), 124.94, 143.74, 146.28, 150.52 (C_q , ArOH); (HRMS, EI^+) m/z Calcd for $\text{C}_6\text{H}_6\text{O}_4$ $[\text{M}]^+$ 142.0266, found 142.0264.

1,2,3,5-Tetrakis(diethoxyphosphoryloxy)benzene (20)—A mixture of diethyl chlorophosphite (0.77 mL), *N,N*-diisopropylethylamine (1.05 mL, 6.0 mmol), and CDCl_3 (5 mL), was stirred at room temperature. 1,2,3,5-Tetrahydroxybenzene **19** (142 mg, 1 mmol) was added to the solution in small amounts and ultrasound was used to solubilise the solid which was stirred for 15 min, becoming a deeper yellow/orange colour as it dissolved. In a second flask, *m*CPBA (1.38 g, 8.0 mmol) in CH_2Cl_2 (25 mL) was stirred in a dry ice/acetone bath (some *m*CPBA precipitated out). The phosphitylated 1,2,3,5-tetrahydroxybenzene derivative was added to the cooled solution of *m*CPBA in dichloromethane, which was stirred for a further 30 min. The solvents were evaporated and the residue was re-dissolved in dichloromethane and applied to the column where the product was purified directly using ethyl acetate then ethyl acetate–ethanol (5:1), to give the product as a pale yellow syrup, (346 mg, 51%) R_f = 0.37 (EtOAc–EtOH, 5:1, only visualised at high concentration, by U.V. at 254 nm). ^1H NMR (270 MHz, CDCl_3) 1.29–1.36 (24 H, m, $4 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 4.13–4.29 (16 H, m, $4 \times \text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), 7.20 (2 H, s, CH , Ar); ^{31}P NMR (109 MHz, CDCl_3) –5.43 (1 P, s, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), –6.28 (1 P, s, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$), –6.46 (2 P, s, $\text{ArOP}(\text{O})(\text{OCH}_2\text{CH}_3)_2$); (MS, ES^+) calcd for $\text{C}_{22}\text{H}_{43}\text{O}_{16}\text{P}_4$ $[\text{M} + \text{H}]^+$ 687.1496, found 687.1493.

Benzene 1,2,3,5-tetrakisphosphate (21)—A mixture of compound **20** (171 mg, 249 μmoles) and bromotrimethylsilane (2.0 mL, 15.16 mmol) was stirred in dry CH_2Cl_2 for 17 h. The solvents were evaporated, MeOH (5 mL) was added and the mixture was stirred for a further 5 min. TEAB (2 mL) was added to the residue and the solvents were evaporated. The compound was purified by ion exchange chromatography over Q-Sepharose Fast Flow using a gradient of TEAB (0 $\rightarrow$ 2.0 M) in which compound **21** eluted at 2.0 M buffer to give a glassy triethylammonium salt, (220 mg, 200 μmoles , 80%). ^1H NMR (270 MHz, D_2O) 6.85 (2 H, s, CH , Ar); ^{31}P NMR (109 MHz, D_2O) 0.60 (1 P, $\text{ArOP}(\text{O}_3^{2-})$), 0.66 (2 P, $2 \times \text{ArOP}(\text{O}_3^{2-})$), 1.25 (1 P, $\text{ArOP}(\text{O}_3^{2-})$); (MS, FAB^-) 923.0, 760.5, 562.3, 461.1, 381.1, 283.1, 159.1, (HRMS, FAB^-) calcd for $\text{C}_6\text{H}_9\text{O}_{16}\text{P}_4$ $[\text{M} - \text{H}]^-$ 460.8841, found 460.8836.

2,4,5-Tribenzoyloxybenzaldehyde (23)—A mixture of 2,4,5-trihydroxybenzaldehyde **22** (3.08 g, 20 mmol), caesium carbonate (32.58 g, 100 mmol) and benzyl bromide (11.9 mL, 100 mmol) in dry DMF (100 mL) was stirred for 17 h at 70 °C. TLC indicated one major product which appeared fluorescent on the TLC plate under U.V. light at 254 nm. The solution was cooled then filtered over a bed of Celite and washed with a further portion of acetone until it remained colourless. The solvents were evaporated to give a solid which was partitioned between CH_2Cl_2 and water (200 mL of each) and the organic solvent was evaporated to give the crude product. Crude **23** was purified by flash chromatography

(CH₂Cl₂) to give the title compound as a white crystalline solid, (7.58 g, 89%), from EtOAc–hexane, R_f = 0.40, mp 136–137 °C. ¹H NMR (400 MHz, CDCl₃) 5.02, 5.08, 5.14 (6 H, 3 s, 3 × ArOCH₂Ph), 6.54 (1 H, s, CH, Ar), 7.28–7.42 (16 H, m, CH, Ar, 3 × ArOCH₂Ph), 10.30 (1 H, ArCHO); ¹³C NMR (100 MHz, CDCl₃) 70.84, 71.13, 71.34 (3 t, ArOCH₂Ph), 100.05, 112.31 (2 d, CH, Ar), 118.05 (s, C_q, Ar), 126.75, 126.99, 127.14, 127.59, 127.86, 127.97, 128.15, 128.37, 128.40 (d, ArOCH₂Ph), 135.70, 136.46 (C_q, ArOCH₂Ph), 143.00, 155.06, 157.25 (s, C_q, Ar), 187.45 (d, ArCHO); MS: (FAB)⁺ 91, 425.1; (HRMS, FAB⁺) m/z Calcd for C₂₈H₂₅O₄ [M + H]⁺ 425.1752. found 425.1738, calcd for C₂₈H₂₄O₄ C 79.23, H 5.70; found: C, 79.3, H, 5.74.

2,4,5-Tribenzyloxyphenol (25)—*m*CPBA (2.9 g, 16.8 mmol) was added to a solution of 2,4,5-tribenzyloxybenzaldehyde **23** (4.245 g, 10 mmol) in dry CH₂Cl₂ (150 mL) and the mixture was stirred at room temperature for 22 h. The organic layer was washed with an aqueous solution of 10% sodium metabisulphite (2 × 100 mL) a saturated solution of sodium hydrogen carbonate (2 × 100 mL) and water (100 mL). The organic layer was dried and the solvent was evaporated to give the crude formate ester derivative **24** (R_f = 0.40, CHCl₃). The crude mixture was dissolved in a mixed solvent (CH₂Cl₂, 25 mL and MeOH, 25 mL) and 5 drops of concentrated hydrochloric acid were added. The reaction was stirred for 90 min then neutralised by the addition of solid NaHCO₃ (5 g). The solid was filtered off and the solvents were evaporated to give the crude product. Purification of the title compound **25** was achieved using flash chromatography (CH₂Cl₂) to give the product as a solid, (3.0 g, 73%), (R_f = 0.50, CH₂Cl₂); mp 75–77 °C from ether–hexane. ¹H NMR (400 MHz, CDCl₃) 4.91, 5.00, 5.03 (6 H, 3 s, 3 × ArOCH₂Ph), 5.30 (1 H, s, ArOH, D₂O exch), 6.60 (1 H, s, CH, Ar), 6.61 (1 H, s, CH, Ar), 7.24–7.40 (15 H, m, 3 × ArOCH₂Ph); ¹³C NMR (100 MHz, CDCl₃) 71.81, 71.97, 73.30 (3 t, ArOCH₂Ph), 103.48, 105.08 (2 d, CH, Ar), 127.15, 127.45, 127.49, 127.50, 127.54, 128.05, 128.08, 128.13, 128.37 (d, ArOCH₂Ph), 136.08, 136.92, 137.18, 138.96, 140.66, 141.31, 144.19 (C_q, ArOCH₂Ph, ArOH, ArOBn); MS: (FAB)⁺ 91, 412.3; (HRMS, ESI⁺) m/z Calcd for C₂₇H₂₅O₄ [M + H]⁺ 413.1747. found 413.1739, calcd for C₂₇H₂₄O₄ C 78.62, H 5.86; found: C 78.8, H 5.88.

1,2,4,5-Tetrahydroxybenzene (26)—2,4,5-Tribenzyloxyphenol **25** (2.20 g, 5.33 mmol) was dissolved in warm EtOH (100 mL) and palladium hydroxide (20%, 500 mg) was added. The air was expelled and the solution was stirred over an atmosphere of hydrogen for 20 h at room temperature. The yellow solution was filtered over a bed of Celite and the solvent was evaporated to yield a dark coloured solid, (708 mg, 94 %); ¹H NMR (270 MHz, CD₃CN) 6.09 (br s, 4 H, 4 × ArOH), 6.35 (s, 2 H, 2 × CH, Ar); (MS, EI⁺) 142.0, 129.1, 113.0, 96.0, 84.0, 73.0, 69.0, 60.0, 55.0, 43.0; (HRMS, EI⁺) m/z Calcd for C₆H₆O₄ [M]⁺ 142.0266, found 142.0266.

1,2,4,5-Tetrakis(diethoxyphosphoryloxy)benzene (27)—A mixture of diethyl chlorophosphite (0.77 mL, 4.5 mmol) and *N,N*-diisopropylethylamine (1.05 mL, 6.0 mmol) was stirred at room temperature in dry CH₂Cl₂ (10 mL). 1,2,4,5-Tetrahydroxybenzene **26** (142 mg, 1 mmol) was added in small portions and ultrasound was used to dissolve the solid. The solution turned yellow then orange and was stirred for a further 30 min. The clear solution was cooled using dry ice and acetone and *m*CPBA (1.035 g, 6 mmol) dissolved in dry CH₂Cl₂ (5.0 mL) was added in one portion to the tetrakisphosphite. The solution was stirred for a further 30 min and became a dark orange colour. The mixture was purified by flash chromatography (EtOAc–EtOH, 5:1) without work up to give the title compound as a dark yellow oil, (450 mg, 66%), R_f = 0.30 (EtOAc–EtOH, 5:1); ¹H NMR (270 MHz, CDCl₃) 1.28–1.34 (24 H, m, 4 × ArOP(O)(OCH₂CH₃)₂), 4.14–4.26 (16 H, m, 4 × ArOP(O)(OCH₂CH₃)₂), 7.45 (2 H, s, 2 × CH, ArOP(O)(OCH₂CH₃)₂); ³¹P NMR (109 MHz, CDCl₃)

−5.96 (4 P, s, 4 × ArOPO(O)(OCH₂CH₃)₂); (MS, FAB⁺) Calcd for C₂₂H₄₃O₁₆P₄ [M + H]⁺ 687.1501; found 687.1521; calcd for C₂₂H₄₂O₁₆P₄ C 38.49, H 6.17; found C 38.0, H 5.97.

Benzene 1,2,4,5-Tetrakisphosphate (28)—1,2,4,5-Tetrakis(diethoxyphosphoryloxy)-benzene **27** (303 mg, 441 μmol) was dissolved in dry CH₂Cl₂ (10 mL).

Bromotrimethylsilane (2 mL, 15.16 mmol) was added to the solution which was then stirred for 20 h. The solvents were evaporated and the residue was stirred in MeOH (5 mL). The title compound was purified on a column of Q-Sepharose Fast Flow using a linear gradient of TEAB buffer (0→2.0 M) in which the compound eluted between 1.3–1.5 M buffer, (379 μmoles (86%); ¹H NMR (270 MHz, D₂O) 7.18 (2 H, s, 2 × CH, ArOPO₃^{2−}); ³¹P NMR (109 MHz, D₂O) +2.57 (4 P, ArOPO₃^{2−}); (MS, FAB[−]) calcd for C₆H₉O₁₆P₄ [M − H][−] 460.8841; found 460.8838.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Ins(1,4,5)P₃ **1**, compared to Bz(1,2,4)P₃ **2**, Bz(1,2,3,4)P₄ **3** and biphenyl 2,3',4,5',6-pentakisphosphate **4**.

Scheme 2.

Synthesis of benzene polyphosphates **7**, **10**, **13**; *Reagents and Conditions*: (a) CDCl₃, *N,N*-diisopropylethylamine, (EtO)₂PCl, then add to *m*CPBA in CH₂Cl₂ cooled in dry ice–acetone, [(**6**) 52%, (**9**) 51%, (**12**) 47%]; (b) TMSBr, dry CH₂Cl₂, 20–21.5 h, then MeOH; purification over Q-Sepharose Fast Flow using TEAB (0→2.0 M), [(**7**) 89.5%, (**10**) 93%, (**13**) 57.5%].

Scheme 3.

Synthesis of benzene 1,2,3,5-tetrakisphosphate **21**. *Reagents and Conditions:* (a) Cs_2CO_3 , DMF, BnBr, 80 °C, 17 h, 87%; (b) *m*CPBA, CH_2Cl_2 ; (c) CH_2Cl_2 –MeOH (1:1, 100 mL), 1% Amberlyst, 85%; (d) BnBr, Cs_2CO_3 , DMF, 18 h, 80 °C, 89%; (e) 20% $\text{Pd}(\text{OH})_2$ on carbon, H_2 , THF, 71%; (f) CDCl_3 , *N,N*-diisopropylethylamine, $(\text{EtO})_2\text{P}(\text{Cl})$, then add to *m*CPBA in CH_2Cl_2 cooled in dry ice–acetone, 51%; (g) TMSBr, CH_2Cl_2 , 17 h, then MeOH; purification over Q-Sepharose Fast Flow using TEAB (0→2.0 M), 80%.

Scheme 4.

Synthesis of benzene 1,2,4,5-tetrakisphosphate **28**. *Reagents and Conditions:* **(a)** Cs_2CO_3 , DMF, BnBr, 70 °C, 17 h, 89%; **(b)** *m*CPBA, CH_2Cl_2 ; **(c)** CH_2Cl_2 –MeOH (1:1, 100 mL), 5 drops Conc. HCl, 73%; **(d)** EtOH, $\text{Pd}(\text{OH})_2$, H_2 , 94%; **(e)** $(\text{EtO})_2\text{PCl}$, *N,N*-diisopropylethylamine, CH_2Cl_2 , 30 min, then *m*CPBA in CH_2Cl_2 , dry ice–acetone, 66%; **(f)** Bromotrimethylsilane, CH_2Cl_2 , 20 h, then MeOH; purification Q-Sepharose Fast Flow, gradient of TEAB buffer (0→2.0 M), 86%.

Scheme 5.

Structures of 3-hydroxybenzene 1,2,4-trisphosphate **29** and 3-benzyloxybenzene 1,2,4-trisphosphate **30** both of which have the Bz(1,2,4) P_3 core.

Figure 1. Minimised structure of biphenyl 2,3',4,5',6-pentakisphosphate **4** docked in the PH domain of PKB α . Not all amino acid residues which interact with **4** are shown for the sake of clarity.

Figure 2.
Minimized model of Bz(1,2,3)P₃ docked in the PH domain of PKBα. Not all amino acid residues of the PKBα are shown for the sake of clarity.

Table 1Inhibition of Ins(1,4,5)P₃ 5-phosphatase and PKB α PH domain by benzene polyphosphates.

Compound	Type I Ins(1,4,5)P ₃ 5-Phosphatase (IC ₅₀ /μM)	PKB α PH-Domain (pIC ₅₀)	PKB α PH-Domain (K _i /nM)
Bz(1,2)P ₂ (7)	> 200	N/A	N/A
Bz(1,2,3)P ₃ (10)	86 ± 28	5.51 ± 0.06	590
Bz(1,3,5)P ₃ (13)	16 ± 9	4.95 ± 0.21	2180
Bz(1,2,4)P ₃ (2)	14 ± 9	5.50 ± 0.20	610
3-OH Bz(1,2,4)P ₃ (29)	21 ± 7 ^[a]	ND	ND
3-OBn Bz(1,2,4)P ₃ (30)	68 ± 16	ND	ND
Bz(1,2,3,4)P ₄ (3)	98 ± 16	6.36 ± 0.15	80 ^[b]
Bz(1,2,3,5)P ₄ (21)	78 ± 50	5.95 ± 0.29	220
Bz(1,2,4,5)P ₄ (28)	4 ± 2	6.23 ± 0.59	110
Biphenyl(2,3',4,5',6)P ₅ (4)	1 ± 2	6.85 ± 0.09	27
diC ₈ PtdIns(3,4,5)P ₃	[c]	5.92 ± 0.09	230
Ins(1,3,4,5)P ₄	[d]	6.22 ± 0.20	120

IC₅₀ values were obtained for benzene polyphosphates evaluated against type I 5-phosphatase in the presence of 1 μM Ins(1,4,5)P₃ as substrate; data are from a minimum of three experiments. K_i Values for inhibition at the PH-domain of PKB α were obtained for a series of benzene polyphosphates measured using competition with biotinylated diC₈PtdIns(3,4)P₂. Ins(1,3,4,5)P₄ and PtdIns(3,4,5)P₃ are shown for comparison and data are derived from a minimum of three experiments.

Key:

ND = not determined.

^[a]Compound 30 is the only benzene polyphosphate that is a substrate.^[b]Data taken from reference 13.^[c]See reference 14 for data obtained under different conditions.^[d]See reference 6 for data obtained under different conditions.