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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 Bα

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Dedicated to Dr. Melanie N. Trusselle (1973–2008).

*Novel benzene polyphosphates were synthesised as inositol polyphosphate mimics and evaluated against type-I inositol 1,4,5-trisphosphate 5-phosphatase, which only binds soluble inositol polyphosphates, and against the PH domain of protein kinase Bα (PKBα), which can bind both soluble inositol polyphosphates and inositol phospholipids. The most potent trisphosphate 5-phosphatase inhibitor is benzene 1,2,4-trisphosphate (**2**, IC_{50} of 14 μM), a potential mimic of D-myo-inositol 1,4,5-trisphosphate, whereas the most potent tetrakisphosphate Ins(1,4,5)P_3 5-phosphatase inhibitor is benzene 1,2,4,5-tetrakisphosphate, with an IC_{50} of 4 μM. Biphenyl 2,3′,4,5′,6-pentakisphosphate (**4**) was the most potent inhibitor evaluated against type I Ins(1,4,5)P_3 5-phosphatase (IC_{50} of 1 μM). All new benzene polyphosphates are resistant to dephosphorylation by type I Ins(1,4,5)P_3 5-phosphatase. Unexpectedly, all benzene polyphosphates studied bind to the PH domain of PKBα with apparent higher affinity than to type I Ins(1,4,5)P_3 5-phosphatase. The most potent ligand for the PKBα PH domain, measured by inhibition of biotinylated diC_8-PtdIns(3,4)P_2 binding, is biphenyl 2,3′,4,5′,6-pentakisphosphate (**4**, $K_i = 27$ nM). The approximately 80-fold enhancement of binding relative to parent benzene trisphosphate is explained by the involvement of a cation–π interaction. These new molecular tools will be of potential use in structural and cell signalling studies.*

Introduction

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3, **1**] is a second messenger that releases Ca^{2+} ions from intracellular stores.[1,2] The crystal structure of Ins(1,4,5)P_3 bound in the ligand-binding domain of the type I Ins(1,4,5)P_3 receptor [Ins(1,4,5)P_3R] has been reported.[3] Many compounds have been synthesised[4] and evaluated at the Ins(1,4,5)P_3R and against the Ins(1,4,5)P_3 metabolising enzymes—Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 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 (for example, the Lowe syndrome)[6] and insulin signalling.[7] Type I Ins(1,4,5)P_3 5-phosphatase is probably the best characterised of the inositol 5-phosphatase enzymes, and we have a longstanding 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 might be useful tools for cell signalling studies. Benzene polyphosphates are compounds that accommodate phosphate groups around a six-membered planar aromatic ring: benzene 1,2,4-trisphosphate [Bz(1,2,4)P_3, **2**], for example, has the same regioisomeric arrangement of phosphate groups as in Ins(1,4,5)P_3 **1**. Such compounds are synthetically accessible, planar, achiral derivatives, in contrast with the ring-puckered conformations and optical activity of most of their inositol polyphosphate counterparts.

In particular, we have shown that such compounds can inhibit PI 3-kinase,[11] Ins(1,4,5)P_3 5-phosphatase[14] or the SH2 domain of inositol 5-phosphatase (SHIP2),[14] and one such derivative—Bz(1,2,3,4)P_4, **3**—binds to the PH domain of PKBα with relatively high affinity and has been co-crystallised with the protein and studied by X-ray crystallography, even though it is structurally very different from the natural lipid headgroup.[13]

Protein kinase B (PKB, also known as Akt) is an enzyme involved in inositol lipid signalling and is made up of three distinct, highly conserved enzymes: PKBα, PKBβ and PKBγ (also

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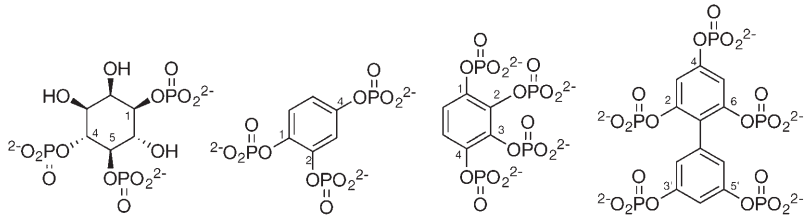


Ins(1,4,5) P_3 (**1**) Bz(1,2,4) P_3 (**2**) Bz(1,2,3,4) P_4 (**3**) BiPh(2,3',4,5',6)P_5 (**4**)

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)P_3], which is required for PKB phosphorylation and activation.[20] The kinase activity of PKB has received considerable attention as a drug target, since PKBα is constituently active in some cancerous cells and requires strict regulation. However, inhibition of PtdIns(3,4,5)P_3 binding to the PH domain by small molecules has also become a priority target for anticancer treatment.[21,22]

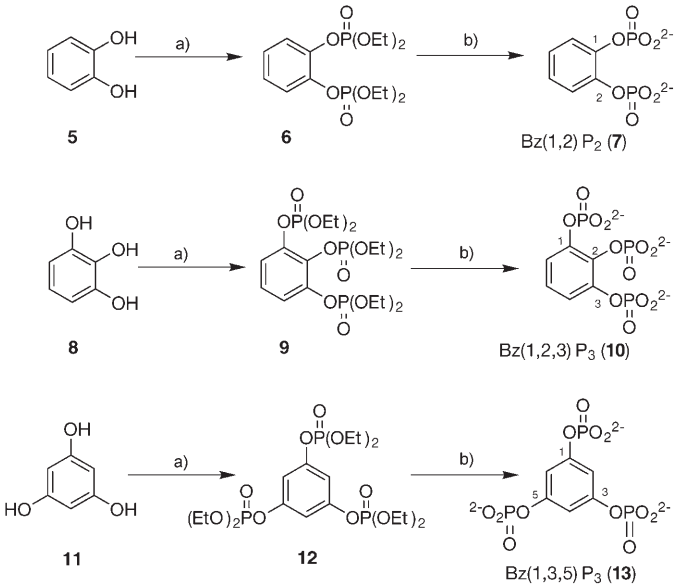
Here we report a structure–activity study of some novel regioisomeric benzene polyphosphates against type I Ins(1,4,5)P_3 5-phosphatase and PKBα PH domain and demonstrate further their potential as high-affinity ligands and emerging signalling tools.

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 by published procedures.

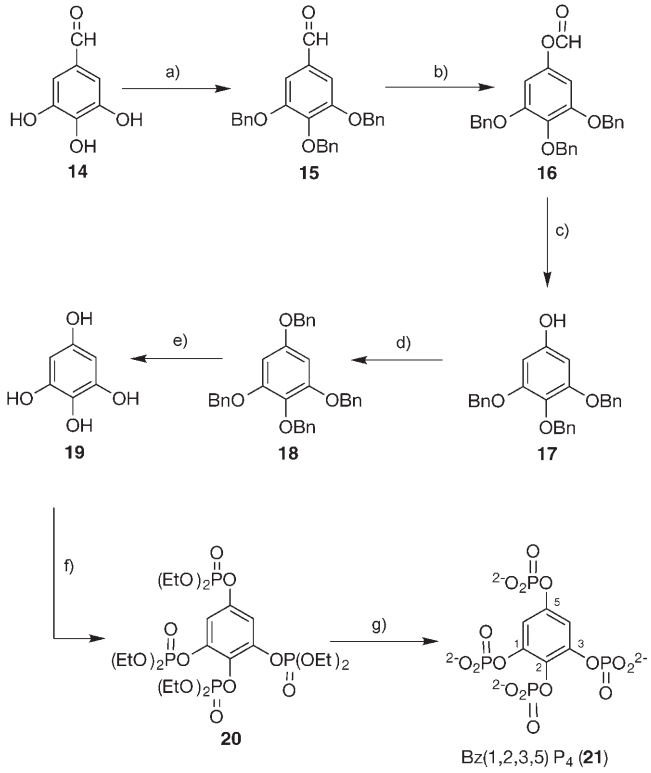
Chemistry

Benzene 1,2-bisphosphate (**7**), benzene 1,2,3-trisphosphate (**10**) and benzene 1,3,5-trisphosphate (**13**) were synthesised by phosphitylation of the appropriate polyphenols with diethyl chlorophosphite to give the corresponding bis- or trisphosphites (Scheme 1). The intermediates were oxidised with *m*-chloroperoxybenzoic acid (*m*CPBA) to afford the corresponding polyphosphorylated derivatives (**6**, **9** and **12**) in approximately 50% yield for each compound. The ethyl groups of the protected phosphates were removed by treatment with bromotrimethylsilane to give the corresponding silylphosphate derivatives. These reactions were monitored by ^{1}H NMR spectroscopy, since the ethyl groups from these compounds (**6**, **9** and **12**) were replaced by the corresponding trimethylsilyl groups; the corresponding ^{1}H NMR shifts moved upfield and the extent of the reaction was easily observed. ^{31}P NMR spectroscopy, however, was not always a reliable method for monitoring the upfield shifts 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 yields; final purification of each benzene polyphosphate was accomplished over a column of Q-Sepharose Fast Flow by using a gradient of triethylammonium bicarbonate (TEAB) buffer, and each compound was isolated as its glassy triethylammonium salt.

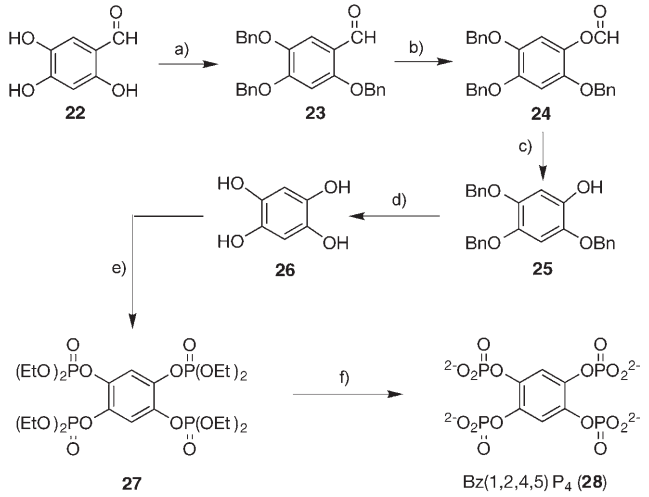


Scheme 1. Synthesis of benzene polyphosphates **7**, **10** and **13**. Reagents and conditions: a) $CDCl_3$, *N*,*N*-diisopropylethylamine $(EtO)_2PCl$, then addition to *m*CPBA in CH_2Cl_2 cooled in dry ice/acetone (**6** 52%, **9** 51%, **12** 47%); b) TMSBr, dry CH_2Cl_2, 20–21.5 h, then MeOH; purification over Q-Sepharose Fast Flow with TEAB (0→2.0 M) (**7** 89.5%, **10** 93%, **13** 57.5%).

3,4,5-Trihydroxybenzaldehyde monohydrate (**14**) was benzylated with benzyl bromide in the presence of caesium carbonate to provide **15** (Scheme 2). This 3,4,5-tribenzyloxybenzaldehyde (**15**) was oxidised to the formate ester **16** by treatment with *m*CPBA, the formate ester was removed by treatment with 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-tetrabenzyloxy derivative **18**. The benzyl groups were removed by $Pd(OH)_2$-catalysed hydrogenolysis 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 ex-



Scheme 2. Synthesis of benzene 1,2,3,5-tetrakisphosphate (**21**). Reagents and conditions: a) BnBr, Cs_2CO_3, DMF, 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% Pd(OH)$_2$ on carbon, H_2, THF, 71%; f) $CDCl_3$, *N*,*N*-diisopropylethylamine $(EtO)_2PCl$, then addition 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 with TEAB (0→2.0 M), 80%.



Scheme 3. Synthesis of benzene 1,2,4,5-tetrakisphosphate (**28**). Reagents and conditions: a) BnBr, Cs_2CO_3, DMF, 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, Pd(OH)$_2$, H_2, 94%; e) $(EtO)_2PCl$, *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%.

change 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 3). Aldehyde **23** was oxidised to give ester **24** by treatment with *m*CPBA, and acidic methanolysis gave the phenol **25** in 73% yield over two steps. Palladium hydroxide was added to a solution of 2,4,5-tribenzyloxyphenol dissolved in warm ethanol. The mixture was then stirred under 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 with the P^{III} reagent diethyl chlorophosphite. 1,2,4,5-Tetrahydroxybenzene (**26**) was added in small portions to the P^{III} solution, ultrasound-aided dissolution of the solid being accompanied by a number of colour changes during phosphitylation, and the P^{III} intermediate was oxidised with *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 by treatment with methanol, and final purification of Bz(1,2,4,5)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, measured by an inositol phosphatase assay with [^{3}H]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 positions on the benzene ring dictate their potencies 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 μM). Benzene 1,2,3-trisphosphate [Bz(1,2,3)P_3, **10**] possesses three adjacent phosphates and is also a weak inhibitor ($IC_{50} = 86$ μM), providing a marginal improvement relative to **7**. However, benzene 1,3,5-trisphosphate [Bz(1,3,5)P_3, **13**, $IC_{50} = 16$ μM], which has three non-adjacent phosphates evenly spread around the ring, is more potent than both **7** and **10**. Compound **13** is similar in structure to Ins(1,3,5)P_3 ($K_i = 45$ μM for 5-phosphatase);[24] however, Ins(1,3,5)P_3 is less potent than its aromatic counterpart **13**. We demonstrated earlier that the

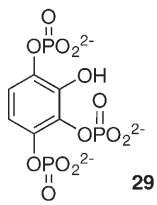
Table 1. Inhibition of Ins(1,4,5)P_3 5-phosphatase and PKBα PH domain by benzene polyphosphates.

Compound	Type I Ins(1,4,5)P_3 5-phosphatase IC_{50} [μM]	PKBα PH domain pIC_{50}	K_i [nM]
Bz(1,2)P_2 (**7**)	>200	n.d.	n.d.
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]	n.d.	n.d.
3-OBn Bz(1,2,4)P_3 (**30**)	68±16	n.d.	n.d.
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_8PtdIns(3,4,5)P_3	[c]	5.92±0.09	230
Ins(1,3,4,5)P_4	[d]	6.22±0.20	120

IC_{50} values were obtained for benzene polyphosphates evaluated against type I Ins(1,4,5)P_3 5-phosphatase in the presence of Ins(1,4,5)P_3 (1 μM) as substrate; data are from a minimum of three experiments. K_i values for inhibition at the PH domain of PKBα were obtained for a series of benzene polyphosphates measured by competition with biotinylated diC_8PtdIns(3,4)P_2. Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 are shown for comparison. [a] Compound **30** is the only benzene polyphosphate that is a substrate. [b] Data taken from ref. [13]. [c] See ref. [14] for data obtained under different conditions. [d] See ref. [6] for data obtained under different conditions. n.d. = not determined.

trisphosphorothioate analogue of Ins(1,3,5)P_3 had a K_i for this enzyme of 430 nM.[9] We would thus 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**] were synthesised as described previously[11,12] and possess the same 1,2,4-trisphosphate pattern, albeit with different functional groups at position 3. Compound **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 the 3-position in **29** did not markedly alter the IC_{50} value towards type-I 5-phosphatase relative to that of **2**.[12] However, the presence of a benzyl group at the 3-position of the related compound **30** increases the hydrophobic bulk, and makes it a weaker 5-phosphatase resistant inhibitor than **29** (Table 1). The 3-position in compound **30** can thus most probably accommodate a small functional group such as OH. D-2,3,6-Trideoxy *myo*-inositol 1,2,4-trisphosphate (K_i = 81.4 μM), reported some 15 years ago by Kozikowski,[25] is similar in structure to Bz(1,2,4)P_3 **2** (IC_{50} = 14 μM), but 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.



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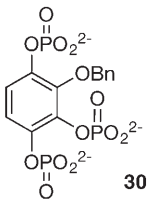

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The addition of a fourth phosphate to Bz(1,2,4)P_3 **2** provides three possible compounds: benzene 1,2,3,4-tetrakisphosphate [Bz(1,2,3,4)P_4 **3**], benzene 1,2,3,5-tetrakisphosphate [Bz-(1,2,3,5)P_4 **21**] and benzene 1,2,4,5-tetrakisphosphate [Bz-(1,2,4,5)P_4 **28**]. Surprisingly, compound **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 μM, close to the value for Bz(1,2,3)P_3 **10** (IC_{50} value 86 μM)]. The IC_{50} value of Bz(1,2,3,5)P_4 is approximately five times higher than that of Bz(1,3,5)P_3, which has one phosphate fewer, suggesting that phosphate-2 of the three adjacent phosphate groups in Bz(1,2,3,5)P_4 is making an unfavourable interaction or is displacing phosphates-1 and -3 into an unfavourable environment by repulsion. 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 together with their position on the aromatic ring. It would probably be naïve to assume that benzene polyphosphates ostensibly possessing the same regioisomeric phosphate arrangements 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 μM) 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±2 μM). Compound **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, firstly to give Bz(1,2,4)P_3 **2** (IC_{50} 14± 2 μM), and the same pattern of phosphates as in Ins(1,4,5)P_3 (K_m = 15 μM) and *scyllo*-Ins(1,2,4)P_3 (K_m = 24.2 μM),[26] and then, by 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±2 μM). Compound **28** nominally corresponds to the Ins(1,3,4,6)P_4 counterpart, which has a K_i value of 7.7 μM,[8] and the known derivative *scyllo*-inositol 1,2,4,5-tetrakisphosphate also has a K_i value of 4.3 μM.[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 μM. While there is no inositol polyphosphate equivalent of the biphenyl com-

pound, it is apparent that the two adjacent phosphorylated benzene rings increases the affinity for this enzyme. Further studies should optimise the number and positions 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 PKBα detected by FRET analysis: Table 1 shows the benzene polyphosphates that inhibit the binding of biotinylated diC_8-PtdIns(3,4)P_2 to the PH domain of PKBα.[13] Interestingly, the lipid analogue diC_8-PtdIns(3,4,5)P_3 and water-soluble headgroup Ins(1,3,4,5)P_4 show only 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 μM and 0.61 μM respectively); however Bz(1,3,5)P_3 **13** (K_i = 2.18 μM) is four times 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, consists of two phosphorylated benzene rings that, from modelling studies, appear to interact with more sites on PKBα PH domain than any other compound, resulting in a potent binding affinity (K_i = 27 nM), 8.5 times higher than that of diC_8PtdIns(3,4,5)P_3. The binding affinity for Bz(1,3,5)P_3 **13** (K_i = 2.18 μM) 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**). The latter motif thus appears to enhance the affinity of **13** some 80-fold. In an attempt to explain 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 PKBα.

Molecular modelling

Compound **4** was modelled into the binding site of the PKBα PH domain on the basis of 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 (Tables S1 and S2 in the Supporting Information; see above for numbering of **4**). One phosphate of the aryl 3′,5′-bisphosphate motif interacts with Arg25 and Arg23, whilst the other interacts only with Arg23 (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 noncovalent forces 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 the 3′,5′-bisphosphate motif of **4** could potentially account for the majority of the 80-fold increase in binding affinity (K_i = 27 nM) relative to Bz(1,3,5)P_3 **13** (K_i = 2.18 μM). Cation–π interactions are now increasingly recognised as important interactions and have been reviewed.[29–31]

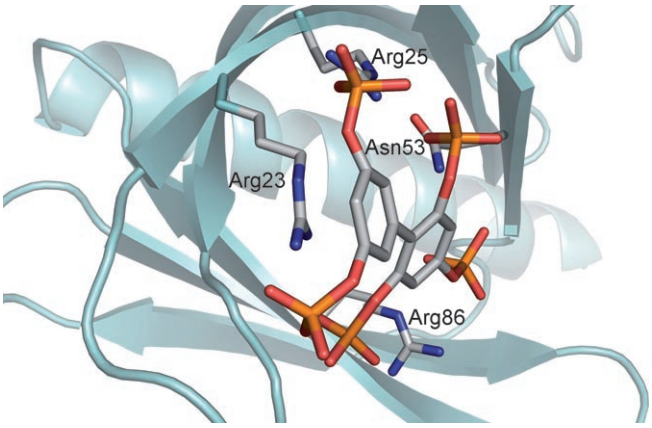

Figure 1. Minimised structure of biphenyl 2,3′,4,5′,6-pentakisphosphate (**4**) docked in the PH domain of PKBα. For purposes of clarity, not all amino acid residues that interact with **4** are shown.

Bz(1,2,3)P_3 **10** also has reasonable affinity for the PH domain of PKBα, but it is not obvious why three adjacent phosphates on a benzene ring give a more potent binder than the 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 (Table S1 and Figure 2). There seem, however, to be fewer obvious interactions of compound **10** than with Bz(1,2,4)P_3 **2** (Table S1 and 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)P_4 **21** (Figure S2) and Bz(1,2,3,4)P_4 **3** possessing three or four adjacent phosphates do not appear to enter into 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 PKBα and seems to be able to accommodate more phosphate groups in its binding site than for 5-phosphatase,

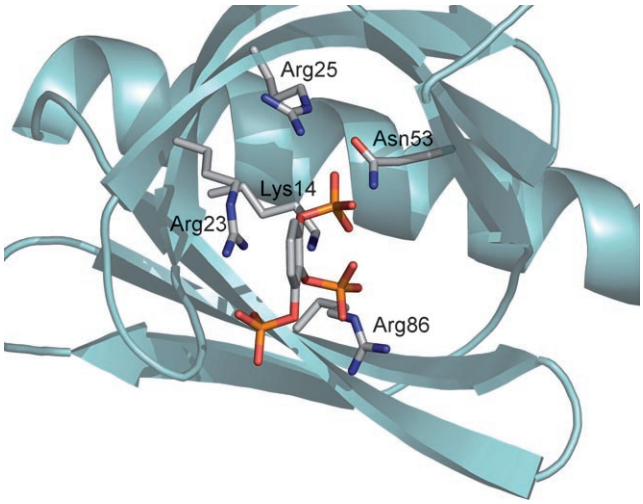

Figure 2. Minimised model of Bz(1,2,3)P_3 docked in the PH domain of PKBα. For purposes of clarity, not all amino acid residues of the PKBα are shown.

since three or four adjacent phosphates can be present on a single benzene ring with increasing potency, whereas Ins(1,4,5)P_3 5-phosphatase can only accommodate a maximum of two adjacent phosphates without binding being affected. Interestingly, only the same 1,2,4-trisphosphorylated motif is recognized in Bz(1,2,3,5)P_4 (1-, 2- and 5-phosphates) and Bz(1,2,3,4)P_4 (1-, 3- and 4-phosphates), demonstrating that this protein binds a specific pattern of phosphate groups present on a benzene ring. In Bz(1,2,3,4)P_4 this was demonstrated by a crystal structure analysis[13] that showed that H-bonding only occurred at phosphates-1, -3 and -4. Since the PKBα PH domain binds the phospholipids PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, 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 for Ins(1,3,4,5)P_4, the head group of PtdIns(3,4,5)P_3, have shown that phosphate-5 is solvent exposed, and only phosphates-1, -3 and -4 are important binding partners.[32] Recognition of **3** and **21** is thus most probably achieved through interaction of the protein with the surrogate 1,3,4-trisphosphate arrangement of the benzene trisphosphate.

Conclusions

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 for exploring membrane-permeable pro-drug forms in which the phosphate charge can be masked with suitable lipophilic groups and potentially provide more versatile tools for investigation of whole-cell inhibition of inositol polyphosphate and inositol lipid-binding proteins. Benzene 1,2,4-trisphosphate (**2**) is the most potent inhibitor of 5-phosphatase in the trisphosphate series, whereas benzene 1,2,4,5-tetrakisphosphate (**28**) is most potent in the tetrakisphosphate series. Inhibition of Ins(1,4,5)P_3 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 PKBα for molecules possessing three or more adjacent phosphate groups. Since there is no X-ray crystal structure of type I 5-phosphatase we are unable fully to interpret our results for this enzyme in a structural context at this stage as in the case of the PKBα PH domain. Synthesis of new benzene polyphosphates and exploration of improved 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 potential for introducing phenyl motifs in a number of structural contexts and possible examination of binding to inositol phospholipid binding proteins and enzymes. Exploration of the structural biology of such proteins in complex with benzene phosphates could be of particular interest. Their simple structures and lack of chirality mean that they could be easily tailored to provide ligands for this purpose and subsequently optimized for a given protein by 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 cocrystallisation of members of this ligand class with a range of signalling protein targets are underway.

Experimental Section

Chemistry: Chemicals were purchased from Acros, Aldrich, Alfa Aesar and Fluka. Thin-layer chromatography was performed on precoated plates (Merck TLC aluminium sheets silica 60 F_{254}): products were visualised by dipping in an ethanolic solution of phosphomolybdic acid, followed by heating at high temperature. Organic compounds were dried over $MgSO_4$. Flash chromatography was carried out on Fisher Scientific Silica 60 A (particle size 35–70 micron). All final compounds were judged by standard spectroscopic methods, purified by ion-exchange chromatography and used in all biological evaluations as their triethylammonium salts. Ion-exchange chromatography was performed on an LKB-Pharmacia Medium Pressure Ion Exchange Chromatograph on Q-Sepharose Fast Flow with gradients of triethylammonium bicarbonate (TEAB, 0→2.0 M) as eluent. Column fractions containing benzene polyphosphates were identified by UV 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 $SiMe_4$ (HDO), $(CD_3)_2CO$ or CD_3CN. The ^{31}P NMR shifts were measured in ppm relative to external 85% phosphoric acid. Melting points (uncorrected) were determined with a Reichert–Jung Thermo Galen Kofler block. Microanalysis was carried out at the University of Bath. Mass spectra were recorded by electron impact (EI), electrospray (ES) and positive and negative Fast Atom Bombardment (FAB) with 3-nitrobenzyl alcohol (NBA) as the matrix, and the standard for electrospray was sodium formate. It was

assumed that *m*CPBA used in oxidation reactions was 100% for calculation purposes only, and it was always used in excess.

Enzyme methods

Expression and purification of recombinant type I 5-phosphatase: Expression and purification of human brain Type I $InsP_3$ 5-phosphatase in pTrcHis was performed as previously described.[33]

Ins(1,4,5)P_3 5-phosphatase assay: The assay was carried out according to the literature procedure,[14] with Ins(1,4,5)P_3 (1.0 μM) as substrate to calculate the IC_{50} values.

Malachite green phosphatase assay: This assay was only used to evaluate an analogue in terms of inorganic phosphate release at 100 μM, and thus its potential to function as a substrate. Phosphatase activity was measured by a phosphate release assay with an acidic malachite green dye as reported.[23] The benzene polyphosphates (100 μM) were diluted in assay buffer (30 μL) consisting of Hepes (50 mM, pH 7.4), $MgCl_2$ (2 mM), bovine serum albumin (1 mg mL^{-1}) 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 EDTA (0.1 M, 15 μL). Malachite green reagent [ammonium molybdate (0.5% *w/v*), Tween 20 (0.057% *v/v*) and malachite green oxalate (0.34 mM), 75 μL] was added to the reaction solution (50 μL). Samples were left for 10 min for colour development, after which absorbance was measured at 650 nm. Inorganic phosphate release was quantified by comparison with a standard curve of KH_2PO_4 in MilliQ water.

Determination of IC_{50}: All analogues were evaluated over the 1–100 μM range by the published procedure by Erneux et al.,[23] and data were adjusted by nonlinear regression with use of GraphPad prism4 (http://www.graphpad.com).

FRET binding experiments at the PH domain of PKBα: Quantitative time-resolved-fluorescence resonance energy transfer (TR-FRET) analysis of binding was performed with 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 BamH1 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 (pH 6.8) consisting of HEPES (50 mM), NaCl (150 mM), $MgCl_2$ (5 mM), DTT (5 mM), CHAPS (0.5%) and EDTA (1 mM).

Determination of K_i values: To determine K_i values for biotinylated diC_8-PtdIns(3,4)P_2, increasing amounts (0–100 nM) of biotinylated diC_8-PtdIns(3,4)P_2 (Cell Signals) were incubated with GST-tagged PKBα PH domain (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 diC_8-PtdIns(3,4)P_2 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 diC_8-PtdIns(3,4)P_2 and increasing amounts of competing agent. IC_{50} values were determined, from which K_i values were calculated.[34] All curve fitting was performed with Prism (GraphPad).

Molecular modelling methods: Biphenyl 2,3′,4,5′,6-pentakisphosphate (**4**) was built with the program SYBYL (v7.1) and optimized to convergence with the MMFFs force field with MMFF charges. Angles between the carbon atoms were constrained by use of 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 experimentally determined 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)P_4 were removed. Hydrogen atoms were added, and their positions were optimized to convergence with 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 by use of 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)P_3.

*1,2-Bis(diethoxyphosphoryloxy)benzene (**6**)*: Dry *N*,*N*-diisopropylethylamine (2.1 mL, 12 mmol) was stirred in dry $CDCl_3$ (5 mL) at room temperature. Benzene-1,2-diol (**5**, 330 mg, 3 mmol) was added to the solution, which produced a grey/green suspension. The solution was cooled with dry ice, diethyl chlorophosphite (1.22 mL, 7 mmol) was added, and the mixture was stirred for a further 10 min to give a yellow solution. At this stage ^{31}P NMR showed $\delta_P = +134$ ppm, which indicated phosphitylation of the phenolic groups. The solution was cooled with dry ice in acetone, *m*CPBA (1.72 g, 10 mmol) in CH_2Cl_2 (5 mL) was then added, and the mixture was stirred for 30 min. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was diluted with CH_2Cl_2 (50 mL) and washed with aqueous sodium metabisulfite (10%, 2×50 mL), aqueous 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%). ^{1}H NMR (270 MHz, $CDCl_3$): $\delta = 1.28$–1.33 (m, 12H; 2ArOP(O)(OCH$_2$C*H*$_3$)$_2$), 4.15–4.26 (m, 8H; 2ArOP(O)-(OC*H*$_2$CH$_3$)$_2$), 7.06–7.12 (m, 2H; C*H*, Ar), 7.33–7.38 ppm (m 2H; C*H*, Ar,); ^{31}P NMR (109 MHz, $CDCl_3$): $\delta = -5.81$ ppm (s, 2P; 2ArO*P*(O)-(OCH$_2$CH$_3$)$_2$); MS (FAB$^+$): *m/z*: 383.0, 355.0, 270.9, 252.9, 190.0, 173.0, 98.9; HRMS (FAB$^+$): *m/z* calcd for $C_{14}H_{25}O_8P_2$: 383.1024 [*M*+H]$^+$; found: 383.1011.

*Benzene 1,2-bisphosphate (**7**)*: A mixture of compound **6** (76 mg, 200 μmol) and bromotrimethylsilane (1.0 mL, 7.58 mmol) in dry CH_2Cl_2 (5 mL) was stirred for 20 h under nitrogen. The solvents were evaporated, 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 with a gradient of TEAB (0→2.0 M) to give compound **7** as a glass (179 μmol, 89.5%). ^{1}H NMR (270 MHz, D_2O): $\delta = 7.00$–7.09 (m, 2H; C*H*, 2*Ar*OPO$_3^{2-}$), 7.24–7.27 ppm (m, 2H; C*H*, 2*Ar*OPO$_3^{2-}$); ^{31}P NMR (109 MHz, D_2O): $\delta = -2.83$ (s, 2P; 2ArO*P*O$_3^{2-}$); MS (FAB$^-$): *m/z*: 539.0, 349.0, 269.0, 189.0, 171.0, 78.9; HRMS (FAB$^-$): *m/z* calcd for $C_6H_7O_8P_2$: 268.9616 [*M*−H]$^-$; found: 268.9616.

*1,2,3-Tris(diethoxyphosphoryloxy)benzene (**9**)*: Dry *N*,*N*-diisopropylethylamine (2.1 mL, 12 mmol) was stirred in dry $CDCl_3$ (5 mL) at room temperature. The mixture was cooled with 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, dissolution of the solid was aided with ultrasound, and the mixture 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 with cooling (dry ice and acetone), and the mixture was stirred overnight. The solution was diluted with CH_2Cl_2 (50 mL) and washed with aqueous sodium metabisulfite (10%, 2×50 mL), saturated aqueous 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). $R_f = 0.35$; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 1.33–1.40 (m, 18H; 3 ArOP(O)(OCH_2CH_3)$_2$), 4.23–4.34 (m, 12H; 3 ArOP(O)(OCH_2CH_3)$_2$), 7.13 (dt, $J = 1.2$, 9.1 Hz, 1H; C*H*, *Ar*OP(O)(OCH_2CH_3)$_2$), 7.31 ppm (d, $J = 8.5$ Hz, 2H; C*H*, 2 *Ar*OP(O)(OCH_2CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 15.71$, 15.77 (2q; ArOP(O)(OCH_2CH_3)$_2$), 64.56, 64.61, 64.67 (3t; ArOP(O)(OCH_2CH_3)$_2$), 116.41, 124.82 (2d; *C*H, *Ar*OP(O)(OCH_2CH_3)$_2$), 143.42, 143.47, 143.51 ppm (C_q; *Ar*OP(O)(OCH_2CH_3)$_2$); ^{31}P NMR (162 MHz, $CDCl_3$): $\delta = -6.39$ (s, 1P; ArO*P*(O)(OCH_2CH_3)$_2$), −7.01 ppm (s, 2P; 2 ArO*P*(O)(OCH_2CH_3)$_2$); MS (FAB$^+$): *m/z*: 535.1, 507.0, 268.9; HRMS (FAB$^+$): *m/z* calcd for $C_{18}H_{34}O_{12}P_3$: 535.1263 $[M+H]^+$; found: 535.1242; elemental analysis calcd (%) for $C_{18}H_{33}O_{12}P_3$: 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 the mixture was 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 by ion-exchange chromatography over Q-Sepharose Fast Flow with elution with a linear gradient of triethylammonium bicarbonate buffer (0→2.0 M). Compound **10** eluted between 1.3 and 1.7 M buffer and was isolated as a glassy triethylammonium salt (186 μmol, 93%). ^{1}H NMR (270 MHz, D_2O): $\delta = 7.01$ (m, 1H; C*H*, *Ar*OPO_3^{2-}), 7.05 ppm (br m, 2H; C*H*, 2 *Ar*OPO_3^{2-}); ^{31}P NMR (109 MHz, D_2O): $\delta = -2.17$ (s, 2P; 2 Ar*O*PO_3^{2-}), −2.33 ppm (s, 1P; Ar*O*PO_3^{2-}); MS (FAB$^-$): *m/z*: 365.0, 335.1, 303.1, 285.0, 264.0, 200.1, 121.1; HRMS (FAB$^-$): *m/z* calcd for $C_6H_8O_{12}P_3$: 364.9228 $[M-H]^-$; 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 with 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 with dry ice in acetone, *m*CPBA (2.58 g, 15 mmol) in CH_2Cl_2 (10 mL) was then added, and the mixture was stirred for a further 30 min. The solution was diluted with CH_2Cl_2 (50 mL) and washed with aqueous sodium metabisulfite (10%, 2×50 mL), saturated aqueous 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). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 1.33–1.39 (m, 18H; 3 ArOP(O)(OCH_2CH_3)$_2$), 4.21–4.25 (m, 12H; 3 ArOP(O)(OCH_2CH_3)$_2$), 6.99 ppm (s, 3H; C*H*, *Ar*OP(O)(OCH_2CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 16.03$, 16.11 (2q; ArOP(O)(OCH_2CH_3)$_2$), 64.95, 65.01 (2t; ArOP(O)(OCH_2CH_3)$_2$), 108.96, 109.01, 109.07 (3d; *C*H, *Ar*OP(O)(OCH_2CH_3)$_2$), 151.62, 151.69 ppm (C_q; *Ar*OP(O)(OCH_2CH_3)$_2$); ^{31}P NMR (162 MHz, $CDCl_3$): $\delta = -7.12$ ppm (s, 3P; 3 ArO*P*(O)(OCH_2CH_3)$_2$); MS (FAB$^+$): *m/z*: 1076.1, 877.1, 724.1, 643.2, 535.2, 507.1, 366.9; HRMS (FAB$^+$): *m/z* calcd for $C_{18}H_{34}O_{12}P_3$: 535.1263 $[M+H]^+$; found: 535.1245; elemental analysis calcd (%) for $C_{18}H_{33}O_{12}P_3$: 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 by ion-exchange chromatography over Q-Sepharose Fast Flow with a gradient of triethylammonium bicarbonate buffer (0→2.0 M). Compound **13** eluted between 1.3 and 1.7 M buffer and was isolated as a glassy triethylammonium salt (115 μmol, 57.5%). ^{1}H NMR (270 MHz, D_2O): $\delta = 6.67$ ppm (s, 3H; C*H*, 3 *Ar*OPO_3^{2-}); ^{31}P NMR (109 MHz, D_2O): $\delta = -3.03$ ppm (s, 3P; 3 ArO*P*O_3^{2-}); HRMS (FAB$^-$): *m/z* calcd for $C_6H_8O_{12}P_3$: 364.9228 $[M-H]^-$; 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 and 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%), m.p. 103–105 °C from CH_2Cl_2/hexane, $R_f = 0.42$ ($CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.14$ (s, 6H; 3 ArOC*H*$_2$Ph), 7.15 (s, 2H; 2 C*H*, Ar), 7.21–7.41 (m, 15H; 3 ArOCH$_2$*Ph*), 9.76 (s, 1H; ArC*H*O); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 71.18$, 75.16 (t; ArO*C*H$_2$Ph), 108.70 (d; *C*H, Ar), 127.30, 127.89, 127.97, 128.35, 128.45 (d; ArOCH$_2$*Ph*), 131.59, 136.19, 137.04, 143.50, 152.99 (s; C_q, ArOCH$_2$*Ph*), 190.69 ppm (Ar*C*HO); MS (FAB$^+$): *m/z*: 425.1, 333.1, 91.0; HRMS (FAB$^+$): *m/z* calcd for $C_{28}H_{25}O_4$: 425.1752 $[M+H]^+$; found: 425.1745; elemental analysis calcd (%) for $C_{28}H_{24}O_4$: 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 aqueous sodium metabisulfite (10%, 2×100 mL), saturated 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 that 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 with CH_2Cl_2 as eluent (3.50 g, 85%); m.p. 110–111 °C, from CH_2Cl_2/hexane. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.99$ (s, 4H; 2 ArOC*H*$_2$Ph), 5.02 (s, 2H; ArOC*H*$_2$Ph), 5.42 (s, D_2O exch, 1H; ArO*H*), 6.12 (s, 2H; 2 *C*H, Ar), 7.26–7.46 ppm (m, 15H; 3 ArOCH$_2$*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 70.92$ (t; 2 ArO*C*H$_2$Ph), 75.60 (t; ArO*C*H$_2$Ph), 95.36 (d; 2 *C*H, Ar), 127.41, 127.90, 128.20, 128.53, 128.80 (d; ArOCH$_2$*Ph*), 131.80, 136.93, 137.75, 152.27, 153.23 ppm (C_q, ArOCH$_2$*Ph*, *Ar*OH, *Ar*OBn); MS (FAB$^+$): *m/z*: 412.2, 321.1, 181.1, 71.0, 55.0; elemental analysis calcd (%) for $C_{27}H_{24}O_4$: C 78.62, H 5.86; found: C 78.3, H 5.88.

*1,2,3,5-Tetrabenzyloxybenzene (**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%), m.p. 113–114 °C (from hexane), $R_f = 0.22$ (CH_2Cl_2/hexane 2:1). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.90$ (s, 2H; ArOCH_2Ph), 4.97 (s, 2H; ArOCH_2Ph), 5.04 (s, 4H; 2ArOCH_2Ph), 6.27 (s, 2H; 2C*H*, Ar), 7.21–7.40 ppm (m, 20H; 4ArOCH$_2$*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 70.24$ (t; ArOCH$_2$Ph), 71.05 (t; 2ArOCH$_2$Ph), 75.21 (t; ArOCH$_2$Ph), 95.15 (d; 2CH, Ar), 127.07, 127.28, 127.40, 127.51, 127.72, 127.79, 128.14, 128.27, 128.29 (d; ArOCH$_2$*Ph*), 136.45, 136.70, 137.61, 152.82, 154.80 (C_q; ArOCH$_2$*Ph*, *Ar*OCH$_2$Ph); MS (FAB$^+$): *m/z*: 503.0, 411.0, 91.0; HRMS (FAB$^+$): *m/z* calcd for $C_{34}H_{31}O_4$: 503.2222 $[M+H]^+$; found: 503.2204; elemental analysis calcd (%) for $C_{34}H_{30}O_4$: 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-tetrabenzyloxybenzene (**18**, 2.29 g, 4.56 mmol), dissolved in THF (100 mL). The air was expelled from the vessel, and the mixture was stirred under 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 a brown solid (461 mg, 71%). ^{1}H NMR (400 MHz, CD_3CN): $\delta = 5.89$ (s, 2H; 2C*H*, Ar), 6.24–6.71 ppm (br, 4H; 4Ar*OH*); ^{13}C NMR (100 MHz, CD_3CN): $\delta = 94.69$ (d; *C*H, Ar), 124.94, 143.74, 146.28, 150.52 (C_q; *Ar*OH); HRMS (EI$^+$): *m/z* calcd for $C_6H_6O_4$: 142.0266 $[M]^+$; 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 redissolved in dichloromethane and applied to the column, where the product was purified directly by elution with ethyl acetate and 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 UV at 254 nm). ^{1}H NMR (270 MHz, $CDCl_3$): $\delta = 1.29$–1.36 (m, 24H; 4ArOP(O)(OCH$_2$CH_3)$_2$), 4.13–4.29 (m, 16H; 4ArOP(O)(OCH_2CH$_3$)$_2$), 7.20 ppm (s, 2H; C*H*, Ar); ^{31}P NMR (109 MHz, $CDCl_3$): $\delta = -5.43$ (s, 1P; ArO*P*(O)(OCH$_2$CH$_3$)$_2$), -6.28 (s, 1P; ArO*P*(O)(OCH$_2$CH$_3$)$_2$), -6.46 ppm (s, 2P; ArO*P*(O)(OCH$_2$CH$_3$)$_2$); MS (ES$^+$): *m/z* calcd for $C_{22}H_{43}O_{16}P_4$: 687.1496 $[M+H]^+$; found: 687.1493.

*Benzene 1,2,3,5-tetrakisphosphate (**21**)*: A mixture of compound **20** (171 mg, 249 μmol) 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 with a gradient of TEAB (0→2.0 M), in which compound **21** eluted at 2.0 M buffer to give a glassy triethylammonium salt (220 mg, 200 μmol, 80%). ^{1}H NMR (270 MHz, D_2O): $\delta = 6.85$ ppm (s, 2H; C*H*, Ar); ^{31}P NMR (109 MHz, D_2O): $\delta = 0.60$ (s, 1P; ArOPO_3^{2-}), 0.66 (s, 2P; 2ArOPO_3^{2-}), 1.25 ppm (s, 1P; ArOPO_3^{2-}); MS (FAB$^-$): *m/z*: 923.0, 760.5, 562.3, 461.1, 381.1, 283.1, 159.1; HRMS (FAB$^-$): *m/z* calcd for $C_6H_9O_{16}P_4$: 460.8841 $[M-H]^-$; found: 460.8836.

*2,4,5-Tribenzyloxybenzaldehyde (**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 UV light at 254 nm. The solution was cooled and was 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). The organic solvent was evaporated to give the crude product. Crude **23** was purified by flash chromatography (CH_2Cl_2) to give the title compound as a white crystalline solid (7.58 g, 89%), from EtOAc/hexane, $R_f = 0.40$, m.p. 136–137 °C. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.02$, 5.08, 5.14 (3s, 6H; 3ArOCH_2Ph), 6.54 (s, 1H; C*H*, Ar), 7.28–7.42 (m, 16H; C*H*, Ar, 3ArOCH$_2$*Ph*), 10.30 ppm (s, 1H; ArC*H*O); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 70.84$, 71.13, 71.34 (3t; ArOCH$_2$Ph), 100.05, 112.31 (2d; *C*H, 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; ArOCH$_2$*Ph*), 135.70, 136.46 (C_q; ArOCH$_2$*Ph*), 143.00, 155.06, 157.25 (s; C_q, Ar), 187.45 ppm (d; ArCHO); MS: (FAB)$^+$: *m/z*: 91, 425.1; HRMS (FAB$^+$): *m/z* calcd for $C_{28}H_{25}O_4$: 425.1752 $[M+H]^+$; found: 425.1738; elemental analysis calcd (%) for $C_{28}H_{24}O_4$: 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 CH_2Cl_2 (150 mL), and the mixture was stirred at room temperature for 22 h. The organic layer was washed with aqueous sodium metabisulfite (10%, 2×100 mL), saturated 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** ($R_f = 0.40$, $CHCl_3$). The crude mixture was dissolved in a mixed solvent (CH_2Cl_2, 25 mL and MeOH, 25 mL), and concentrated hydrochloric acid (5 drops) was added. The reaction mixture was stirred for 90 min and then neutralised by the addition of solid $NaHCO_3$ (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 by flash chromatography (CH_2Cl_2) to give the product as a solid (3.0 g, 73%); $R_f = 0.50$ (CH_2Cl_2); m.p. 75–77 °C from ether/hexane. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.91$, 5.00, 5.03 (3s, 6H; 3ArOCH_2Ph), 5.30 (s, D_2O exch, 1H; ArO*H*), 6.60 (s, 1H; C*H*, Ar), 6.61 (s, 1H; C*H*, Ar), 7.24–7.40 ppm (m, 15H; 3ArOCH$_2$*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 71.81$, 71.97, 73.30 (3t; ArOCH$_2$Ph), 103.48, 105.08 (2d; *C*H, Ar), 127.15, 127.45, 127.49, 127.50, 127.54, 128.05, 128.08, 128.13, 128.37 (d; ArOCH$_2$*Ph*), 136.08, 136.92, 137.18, 138.96, 140.66, 141.31, 144.19 (C_q; ArOCH$_2$*Ph*, *Ar*OH, *Ar*OBn); MS (FAB)$^+$: *m/z*: 91, 412.3; HRMS (ESI$^+$): *m/z* calcd for $C_{27}H_{25}O_4$: 413.1747 $[M+H]^+$; found: 413.1739; elemental analysis calcd (%) for $C_{27}H_{24}O_4$: 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 under 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%); ^{1}H NMR (270 MHz, CD_3CN): $\delta = 6.09$ (brs, 4H; 4Ar*OH*), 6.35 ppm (s, 2H; 2C*H*, Ar); MS (EI$^+$): *m/z*: 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 $C_6H_6O_4$: 142.0266 $[M]^+$; 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 CH_2Cl_2 (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 and then orange and was stirred for a further 30 min. The clear solution was cooled with dry ice and acetone, and *m*CPBA (1.035 g, 6 mmol) dissolved in dry CH_2Cl_2 (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 workup 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, $CDCl_3$): $\delta = 1.28$–1.34 (m, 24H; 4 ArOP(O)(OCH_2CH_3)$_2$), 4.14–4.26 (m, 16H; 4 ArOP(O)(OCH_2CH_3)$_2$), 7.45 ppm (s, 2H; 2 *CH*, *Ar*OP(O)(OCH_2CH_3)$_2$); ^{31}P NMR (109 MHz, $CDCl_3$): $\delta = -5.96$ ppm (s, 4P; 4 Ar*O*P(O)(OCH_2CH_3)$_2$); MS (FAB^+): *m/z*: calcd for $C_{22}H_{43}O_{16}P_4$: 687.1501 $[M+H]^+$; found: 687.1521; elemental analysis calcd (%) for $C_{22}H_{42}O_{16}P_4$: 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 CH_2Cl_2 (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 with a linear gradient of TEAB buffer (0→2.0 M) in which the compound eluted between 1.3 and 1.5 M buffer (379 μmol (86%); ^1H NMR (270 MHz, D_2O): $\delta = 7.18$ ppm (s, 2H; 2 *CH*, *Ar*OPO_3^{2-}); ^{31}P NMR (109 MHz, D_2O): $\delta = +2.57$ ppm (s, 4P; 4 Ar*O*PO_3^{2-}); MS (FAB^-): *m/z*: calcd for $C_6H_9O_{16}P_4$: 460.8841 $[M-H]^-$; found: 460.8838.

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