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Article
MESSENGER
Vol. 1, 167–181, 2012
www.aspbs.com/messenger

Multivalent Benzene Polyphosphate Derivatives are Non-Ca^{2+}-Mobilizing $Ins(1,4,5)P_3$ Receptor Antagonists

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Inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$ 1] mobilizes intracellular Ca^{2+} through the $Ins(1,4,5)P_3$ receptor [$InsP_3R$]. Although some progress has been made in the design of synthetic $InsP_3R$ partial agonists and antagonists, there are still few examples of useful small molecule competitive antagonists. A "multivalent" approach is explored and new dimeric polyphosphorylated aromatic derivatives were designed, synthesized and biologically evaluated. The established weak $InsP_3R$ ligand benzene 1,2,4-trisphosphate [$Bz(1,2,4)P_3$ 2] is dimerized through its 5-position in two different ways, first directly as the biphenyl derivative biphenyl 2,2′,4,4′,5,5′-hexakisphosphate, [$BiPh(2,2',4,4',5,5')P_6$ 8] and with its regioisomeric biphenyl 3,3′,4,4′,5,5′-hexakisphosphate [$BiPh(3,3',4,4',5,5')P_6$ 11]. Secondly, a linker motif is introduced in a flexible ethylene-bridged dimer (9) with its corresponding 1,2-bisphosphate dimer (10), both loosely analogous to the very weak antagonist 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N*-tetraacetic acid (BAPTA 7). In permeabilized L15 fibroblasts overexpressing type 1 $InsP_3R$, $BiPh(2,2',4,4',5,5')P_6$ (8) inhibits $Ins(1,4,5)P_3$-induced Ca^{2+} release in a apparently competitive fashion [IC_{50} 187 nM] and the $Bz(1,2,4)P_3$ dimer (9) is only slightly weaker [IC_{50} 380 nM]. Compounds were also evaluated against type I $Ins(1,4,5)P_3$ 5-phosphatase. All compounds are resistant to dephosphorylation, with $BiPh(2,2',4,4',5,5')P_6$ (8), being the most effective inhibitor of any biphenyl derivative synthesized to date [IC_{50} 480 nM] and the $Bz(1,2,4)P_3$ ethylene dimer (9) weaker [IC_{50} 3.55 μM]. $BiPh(3,3',4,4',5,5')P_6$ (11) also inhibits 5-phosphatase [IC_{50} 730 nM] and exhibits unexpected Ca^{2+} releasing activity [EC_{50} 800 nM]. Thus, relocation of only a single mirrored phenyl phosphate group in (11) from that of antagonist (8) does not markedly change enzyme inhibitory activity, but elicits a dramatic switch in Ca^{2+}-releasing activity. Such new agents demonstrate the power of the multivalent approach and may be useful to investigate the chemical biology of signaling through $InsP_3R$ and as templates for further design.

Keywords: $Ins(1,4,5)P_3$ Receptor, Antagonist, Benzene Polyphosphate, Biphenyl Polyphosphate, Competitive Inhibition.

INTRODUCTION

$Ins(1,4,5)P_3$ receptors [$InsP_3Rs$] are intracellular ligand-gated Ca^{2+} release channels present in the endoplasmic reticulum, which is the major storage site for Ca^{2+} in the cell. Ca^{2+} is released when $Ins(1,4,5)P_3$ (**1**, Fig. 1) binds to its receptor, eliciting many intracellular cellular responses including regulation of gene expression, cell division, synaptic transmission etc. [Foskett, 2007]. In mammals three different isoforms of $InsP_3R$ are expressed with a high proportion of the type 1 receptor [$InsP_3R1$] found in the brain [Foskett, 2007]. The $InsP_3R$ is made up of four subunits and the *N*-terminal region (residues 1-604) contains the ligand binding (224–604) and suppressor (1–223) domains. The crystal structure of the ligand binding domain of $InsP_3R1$ (residues 224–604) was described [Bosanac, 2002] and more recently structures in the presence and absence of $Ins(1,4,5)P_3$ [Lin, 2011] and for the full *N*-terminal domain (residues 1–604) [Seo, 2012] have been reported. There are, however, no crystal structures containing partial agonists or antagonists which may provide further clues to the activation mechanism of the $InsP_3R$. Numerous groups have been engaged in synthesizing both natural and non-natural inositol polyphosphates and analogues since the discovery of $Ins(1,4,5)P_3$ [Potter, 1995].

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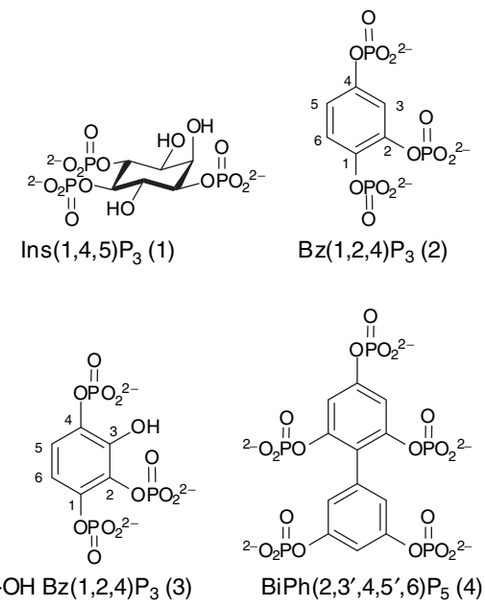

Figure 1. ***Myo*-inositol 1,4,5-trisphosphate and some known aromatic polyphosphates.**

After $Ins(1,4,5)P_3$ releases Ca^{2+} from intracellular stores it diffuses away from the $InsP_3R$ and is hydrolysed by type I $Ins(1,4,5)P_3$ 5-phosphatase [Verjans, 1994]. Type I 5-phosphatase enzyme is to a large extent membranous and ubiquitous [Storey, 1984; Erneux, 1986], one of a family of ten Mg^{2+}-dependent 5-phosphatase enzymes [Verjans, 1994] and is the only 5-phosphatase enzyme that hydrolyzes $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ [Erneux, 1989].

Heparin is a well-known $InsP_3$ antagonist [Bultynck, 2003]. There are a number of small molecule $Ins(1,4,5)P_3$ receptor antagonists currently used for biological studies, including 2-aminoethoxydiphenyl borate, xestospongins and caffeine, but none is competitive and specific for the $InsP_3R$ [Bootman, 2002; Bultynck, 2003] and results are often unreliable. *myo*-Inositol 1,3,4,5-tetrakisphosphate at high concentration is known to inhibit the $InsP_3R$ and several other phosphorylated derivatives can displace bound [^{3}H]$Ins(1,4,5)P_3$ at low to high micromolar concentration [Hermosura, 2000]. Inositol trisphosphorothioates, such as D-6-deoxy inositol 1,4,5-trisphosphorothioate [Safrany, 1993] and 1L-*chiro*-inositol 2,3,5-trisphosphorothioate [Safrany, 1993; Liu, 1994], *myo*-inositol 1,4,6-trisphosphorothioate and the related 1,3,6-trisphosphorothioate [Murphy, 2000] are competitive antagonists of the $InsP_3R$ in platelets, but are also very low efficacy partial agonists because they are still able to release a fraction of the Ca^{2+} store. Only one report of a small molecule full $InsP_3R$ competitive antagonist has been made, based upon the inositol core [Keddie, 2011], but the reported derivatives only very weakly inhibit the $InsP_3R$ at a millimolar level.

Aromatic polyphosphate derivatives have proved to be of potential in the phosphoinositide field. Benzene 1,2,4-trisphosphate [$Bz(1,2,4)P_3$ (**2**)] (Fig. 1) with phosphate groups arranged around a six-membered ring in a similar way to $Ins(1,4,5)P_3$ was synthesized and evaluated against three proteins that bind $Ins(1,4,5)P_3$ (**1**), the $InsP_3R$, $Ins(1,4,5)P_3$ type I 5-phosphatase and $Ins(1,4,5)P_3$ 3-kinase [Poitras, 1993]. $Bz(1,2,4)P_3$ is resistant both to dephosphorylation by 5-phosphatase and phosphorylation by $Ins(1,4,5)P_3$ 3-kinase. Introduction of a 3-hydroxyl motif into $Bz(1,2,4)P_3$ to give 3-hydroxybenzene 1,2,4-trisphosphate [3-OH-$Bz(1,2,4)P_3$ (**3**)] however, transforms the parent compound into a substrate for $Ins(1,4,5)P_3$ 5-phosphatase [Mills, 2006]. More recently, several benzene polyphosphates, including $Bz(1,2,4)P_3$ were also evaluated against $Ins(1,4,5)P_3$ 3-kinase but found to be ineffective at inhibiting the enzyme [Vandeput, 2007]. Interestingly, earlier data show that $Bz(1,2,4)P_3$ weakly interacts with the $InsP_3R$ [Poitras, 1993], and competitively blocks [^{3}H]$Ins(1,4,5)P_3$ binding 10,000-fold more weakly than $Ins(1,4,5)P_3$. Another study [Ward, 1995] showed that $Bz(1,2,4)P_3$ inhibits *in vitro* phosphatidylinositol 3-kinase activity. More recently, our biphenyl derivative $BiPh(2,3',4,5',6)P_5$ (**4**) (Fig. 1) was found to be a moderately potent $Ins(1,4,5)P_3$ receptor antagonist having an IC_{50} value in the low micromolar range [Vandeput, 2007]. Preliminary structure activity relationship (SAR) data indicate that the number and position of the phosphate groups might influence the recognition of benzene phosphate derivatives.

Our previous studies show that benzene polyphosphates are non-hydrolysable $Ins(1,4,5)P_3$ 5-phosphatase inhibitors [Mills, 2008] and biphenyl 2,3′,4,5′,6-pentakisphosphate [$BiPh(2,3',4,5',6)P_5$ (**4**)] [Vandeput, 2007], the first aromatic polyphosphorylated ligand with activity to possess more than one ring, is one of the most potent $Ins(1,4,5)P_3$ 5-phosphatase inhibitors that also inhibits $Ins(1,4,5)P_3$-induced Ca^{2+} release.

Multivalency is the use of two or more active groups to provide bioactivity that is more than additive. A new type of dimeric inositol polyphosphate derivative (**5**) (Fig. 2) was designed when two molecules of $Ins(1,4,5)P_3$ were synthetically linked via a polyethylene glycol spacer [Riley, 2002]. These dimers stimulate release of Ca^{2+} from permeabilized cells and are highly potent. A subsequent study [Riley, 2004] showed that an $Ins(1,4,5)P_3$ dimer linked via the 2-hydroxyl group with a short *N,N*-diethylurea spacer (**6**) (Fig. 2) has an EC_{50} value more than 12-fold lower than $Ins(1,4,5)P_3$ and is the most potent in the series. Further modifications of these ligands led to the discovery of their partial agonist properties [Rossi, 2009]. The multivalent approach thus offers attractive design potential and, with the established high potency of the multivalent short $Ins(1,4,5)P_3$ dimer (**6**), we therefore explored the possibility of further modification to the biphenyl core

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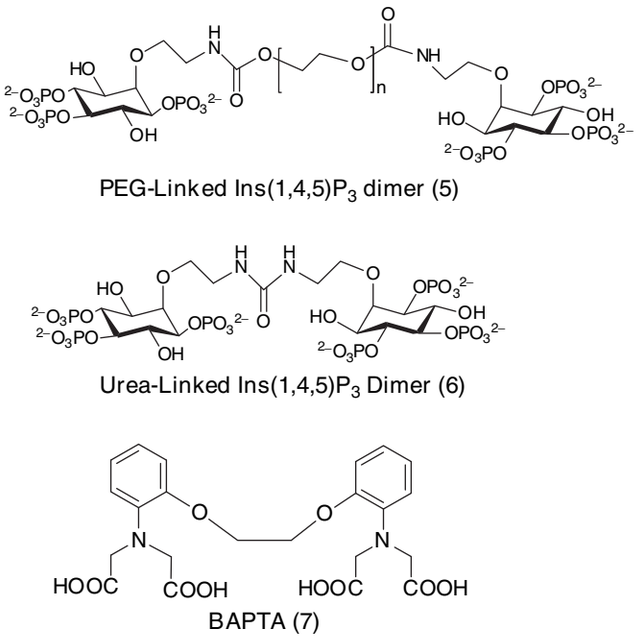

Figure 2. **Structures of $Ins(1,4,5)P_3$ dimers and BAPTA.**

lead structure by both changing the number and regiochemistry of the decorating phosphates and bridging the two phosphorylated aromatic rings using a linker.

In the search for small molecule $InsP_3R$ antagonist leads, our attention also focused on the Ca^{2+} indicator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA **7**, Fig. 2), a weak competitive antagonist at the $InsP_3R$ [Richardson, 1993; Morris, 1999]. The carboxylic acid groups and nitrogen heteroatoms are required to chelate Ca^{2+}, [Morris, 1999]. BAPTA competitively antagonizes $Ins(1,4,5)P_3$-stimulated Ca^{2+} mobilization (K_D = 1.8 mM). Although there is no speculation as to how molecules such as BAPTA are supposed to bind to the $InsP_3R$, its non-chelated carboxylic acids might obviously bind weakly to the extensive network of positively charged amino acids in the $Ins(1,4,5)P_3$ binding site. We hypothesized that such binding by BAPTA might be enhanced by replacing the carboxylic acids with other negatively charged groups.

We report here the synthesis of new dimeric benzene polyphosphates as both biphenyl derivatives and with short ethylene linkers akin to BAPTA (compounds **8–11**, Fig. 3). Thus, the weak ligand $Bz(1,2,4)P_3$ (**2**) was dimerized through its 5-position in two different ways, first directly as a biphenyl derivative and secondly by introducing a BAPTA-type linker motif. These compounds thus both develop further the earlier biphenyl template antagonist lead [Vandeput, 2007] and also exemplify a new, more flexible, related class. Compounds were evaluated for activity using permeablized L15 fibroblasts overexpressing $InsP_3R1$ and also on recombinantly expressed human type I $Ins(1,4,5)P_3$ 5-phosphatase.

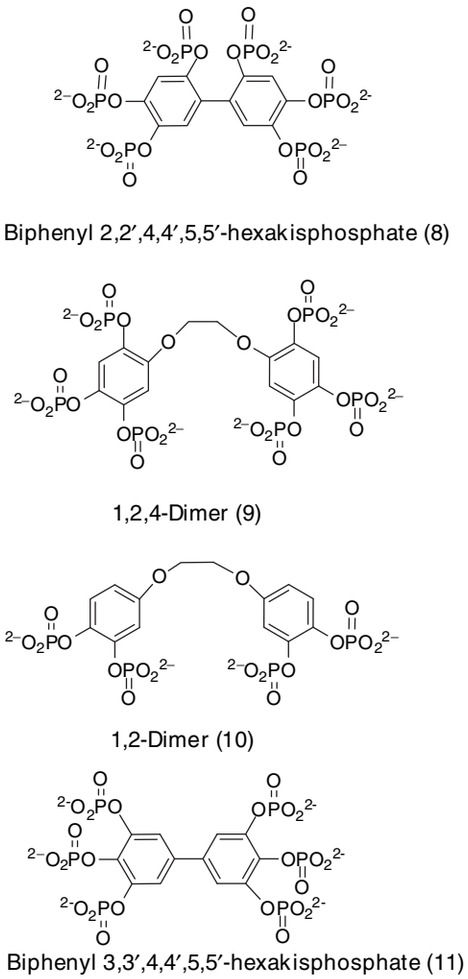

Figure 3. **Structures of dimeric aromatic polyphosphates.**

MATERIALS AND METHODS

Chemistry

General: Chemicals were purchased from Acros, Sigma-Aldrich, and Alfa Aesar. Thin-layer chromatography (TLC) was performed on pre-coated plates (Merck TLC aluminium sheets silica 60 F_{254}): products were visualized by dipping plates in an ethanolic solution of phosphomolybdic acid then heating at high temperature. Organic compounds were dried over $MgSO_4$. Flash chromatography was carried out on Fisher Scientific Silica 60A (particle size 35–70 micron). All final compounds were evaluated by standard spectroscopic methods and purified by ion exchange chromatography 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

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the Briggs test [Lampe, 1994]. NMR spectra (proton frequency 270 or 400 MHz) were referenced against $SiMe_4$, (HDO), [D_3-(CD_3CN)] or [D_6-(CD_3)$_2$SO], and ^{13}C NMR was carried out at 100 MHz. The ^{31}P NMR shifts (phosphorus frequency 162 MHz) were measured in ppm relative to external 85% phosphoric acid. Since the NMR spectra of all final compounds were recorded as their triethylammonium salts in D_2O, the pH was *ca*. 5. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler block or a Stanford Research Systems Optimelt automated melting point system. Microanalysis was carried out at the University of Bath. Mass spectra were recorded using electrospray (ES) with sodium formate as standard or Fast Atom Bombardment (FAB) where the standard was 3-nitrobenzyl alcohol (NOBA).

All final compounds were homogeneous, used as their triethylammonium salt after purification by ion exchange chromatography and were quantified by total phosphate assay [Lampe, 1994]. The latter procedure is more accurate than quantification by mass due to unknown amounts of residual water, inaccurate determination of the variable amount of triethylammonium counter ion and the inaccuracy in weighing out small quantities of material. All the final phosphorylated compounds are air stable and do not decompose when left in solution around pH 7, at least for a short time. 1.0 Micromole samples of compound in solution in an Eppendorf tube were carefully evaporated in a speedvac providing a pellet that was then used in biological studies.

2,2′,4,4′,5,5′-Hexamethoxybiphenyl (13)

$MoCl_5$ (8.19 g, 30 mmol) was added to a solution of 1,2,4-trimethoxybenzene (**12**) (2.52 g, 15 mmol) ($R_f = 0.24$, CH_2Cl_2) in anhydrous CH_2Cl_2 (50 mL) at room temperature under Argon. The solution turned dark green and the heterogeneous mixture was stirred for 30 min under nitrogen. MeOH (50 mL) was then added to the reaction mixture and the solvents were evaporated off to give an olive green residue and a red colouration. The remaining solid was partitioned between CH_2Cl_2 and water (100 mL of each), and the red colour was present in the aqueous layer and the green colour in the organic layer. The organic layer was filtered through filter paper and the solvent was evaporated to give a pale green solid. The crude material was suspended in MeOH and the resulting white solid was filtered off and washed with ether (100 mL). $R_f = 0.39$, (ether), $R_f = 0$, (CH_2Cl_2). (The compound is only soluble in cold CH_2Cl_2, not EtOAc). The compound was recrystallized from MeOH to give the title compound (**13**) as a white crystalline solid (2.24 g, 89%), (crystals were rod-like prisms) m.p. = 177–179 °C; Lit. [Waldvogel, 2002] 178 °C. ^1H NMR (400 MHz, $CDCl_3$) 3.74, 3.83, 3.92 (3 s, 18 H, 6 × ArO*Me*), 6.61, 6.81 (2 s, 4 H, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$) 56.06, 56.50, 56.85 (3 q, 6 × ArO*Me*), 98.35, 115.31 (2 d, *Ar*H), 118.91 (s, C_q *Ar-Ar*), 142.86, 148.77, 151.22 (3 s, C_q, *Ar*OMe); (HRMS, ESI^+) m/z Calcd for $C_{18}H_{23}O_6$ $[M+H]^+$ 335.1489. found 335.1488.

2,2′,4,4′,5,5′-Hexahydroxybiphenyl (14)

2,2′,4,4′,5,5′-hexamethoxybiphenyl (**13**) (1.003 g, 3.0 mmol) was partially dissolved in dry CH_2Cl_2 (10 mL) and the solution was cooled using a dry ice acetone mixture. A solution of BBr_3 in CH_2Cl_2 (1.0 M, 25 mL) was added over 5 min to the cooled solution to give a pale green-yellow solution, which was then allowed to warm to ambient temperature over a period of 19 h. An aqueous solution of 1 M HCl (50 mL) was added to the cooled mixture (dry ice-acetone) which resulted in a white precipitate. Water (100 mL) was then added and the layers separated. The aqueous layer was extracted with ethyl acetate (4 × 100 mL), dried ($MgSO_4$) and the solvent was evaporated to give a solid which had a violet colouration. The remaining solid was suspended in ether (40 mL) to dissolve any of the impurities and filtered to give the title compound as a violet (blue grey) coloured solid (**14**) (633 mg, 84%). The solid was not recrystallized but was pure enough for phosphorylation and pure by NMR. ^1H NMR (400 MHz, d_6-DMSO) 6.33, 6.50 (2 s, 4 H, Ar*H*), 8.21, 8.51, 8.72 (3 s, 6 H, D_2O exch, 6 × ArO*H*); ^{13}C NMR (100 MHz, d_6-DMSO) 104.28, 116.44 (2 d, 4 × *C*H, ArH), 117.78 (s, C_q, *Ar-Ar*), 138.165, 144.65, 146.09 (3 s, C_q, *Ar*OH); (HRMS, ESI^+) m/z Calcd for $C_{12}H_{11}O_6$ $[M+H]^+$ 251.0550. found 251.0549.

2,2′,4,4′,5,5′-Hexakis(diethoxyphosphoryloxy)-biphenyl (15)

A mixture of diethyl chlorophosphite (1.33 mL, 7.8 mmol) and *N*,*N*-diisopropylethylamine (1.75 mL, 10.0 mmol) was stirred at room temperature in dry CH_2Cl_2 (10 mL) to give a yellow solution. 2,2′,4,4′,5,5′-Hexahydroxybiphenyl (**14**) (250 mg, 1 mmol) was added in small portions and the solid dissolved with the aid of ultrasound within 5–10 min with the solution remaining a yellow colour which was stirred for a further 30 min. The mixture was cooled using dry ice in acetone, then *m*CPBA (2.58 g, 15 mmol) in CH_2Cl_2 (25 mL) was added in one portion, the colour went a dark olive green and the solution was stirred for a further 30 min. Due to work up (sodium metabisulphite and $NaHCO_3$ wash) also destroying the compound, the mixture was purified directly by flash chromatography using EtOAc then EtOAc-EtOH (5:1) to give compound (**15**) as a syrup, (580 mg, 54 %), $R_f = 0.25$ (EtOAc-EtOH, 5:1). ^1H NMR (400 MHz, $CDCl_3$) 1.19–1.22 (12 H, t, $J = 7.0$ Hz, 2 × ArOP(O)(OCH$_2$C*H*$_3$)$_2$), 1.34–1.38 (24 H, 2 t, $J = 7.0$ Hz, 4 × ArOP(O)(OCH$_2$C*H*$_3$)$_2$), 3.91–4.01 (8 H, m, 2 × ArOP(O)(OC*H*$_2$CH$_3$)$_2$), 4.18–4.30 (16 H, m, 4 × ArOP(O)(OC*H*$_2$CH$_3$)$_2$), 7.33 (2 H, d, $J = 0.8$ Hz, C*H*, Ar), 7.53 (2 H, d, $J = 0.8$ Hz, C*H*, Ar);

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^{13}C NMR (100 MHz, $CDCl_3$) 15.82, 15.88, 16.00, 16.07 (4 q, 6 × ArOP(O)(OCH_2CH_3)$_2$), 64.76, 64.82, 64.85, 64.91, 64.97, 65.04 (6 t, 6 × ArOP(O)(OCH_2CH_3)$_2$), 113.27, 123.90 (2 d, 4 × *C*H, Ar), 124.55, 124.63 (C_q, 2 × *Ar-Ar*), 138.11, 138.16, 141.69, 144.79, 144.85 (C_q, 6 × *Ar*OP(O)(OCH_2CH_3)$_2$); ^{31}P NMR (162 MHz, $CDCl_3$) −6.43 (2 × ArO*P*(O)(OCH_2CH_3)$_2$), −6.70 (2 × ArO*P*(O)(OCH_2CH_3)$_2$), −7.17 (2 × ArO*P*(O)(OCH_2CH_3)$_2$). (HRMS, ESI^+) *m/z* Calcd for $C_{36}H_{65}O_{24}P_6$ $[M+H]^+$ 1067.2285. found 1067.2270, calcd for $C_{36}H_{64}O_{24}P_6$ C 40.53, H 6.05; found: C 40.3, H 5.73.

2,2′,4,4′,5,5′-Biphenylhexakisphosphate (8)

2,2′,4,4′,5,5′-Hexakis(diethoxyphosphoryloxy)biphenyl (**15**) (100 mg, 93.7 μmol), was dissolved in dry CH_2Cl_2 (5 mL). Bromotrimethylsilane (1.0 mL, 7.57) was added and the solution was stirred for 3 days, each day monitoring the disappearance of the ethyl groups from the compound. The solvents were evaporated and the remaining syrup was stirred in a mixed solvent of TEAB (1 mL) and water (2 mL) for 30 min. The title compound was purified over Q-Sepharose Fast Flow using a linear gradient of 0 → 2.0 M TEAB, eluting at 2.0 M buffer, and compound (**8**) was obtained as a glassy triethylammonium salt, (72.4 μmol, 77%). ^{1}H NMR (400 MHz, D_2O) 7.24, 7.28 (4 H, 2 s, Ar*H*); ^{13}C NMR (100 MHz, D_2O) 113.25, 124.63 (2 d, 4 × *C*H, Ar), 123.90, 123.98 (C_q, 2 × *Ar-Ar*), 139.20 (C_q, t, $J = 5.9$ Hz, *Ar*OPO_3^{2-}), 143.43 (C_q, t, $J = 5.9$ Hz, *Ar*OPO_3^{2-}), 145.76 (C_q, d, $J = 5.9$ Hz, *Ar*OPO_3^{2-}); ^{31}P NMR (162 MHz, D_2O) −2.28, −2.50, −3.17 (3 s, 6 × Ar*O*PO_3^{2-}), (HRMS, ESI^-) *m/z* Calcd for $C_{12}H_{15}O_{24}P_6$ $[M-H]^-$ 728.8384. found 728.8357.

2,4,5-Tribenzyloxyphenol (18)

*m*CPBA (2.9 g, 16.8 mmol) was added to a solution of 2,4,5-tribenzyloxybenzaldehyde (**16**) (4.245 g, 10 mmol) in dry CH_2Cl_2 (75 mL) and the mixture was stirred at room temperature for 18 h. The organic layer was washed with an aqueous solution of 10% sodium metabisulfite (2 × 100 mL) a saturated solution of sodium hydrogen carbonate (100 mL) and water (100 mL). The organic layer was filtered and the solvent was evaporated to give the crude formate ester derivative (**17**) ($R_f = 0.50$, CH_2Cl_2) which was purified by flash chromatography (CH_2Cl_2). The resulting solid was dissolved in a mixed solvent (CH_2Cl_2, 25 mL and MeOH, 25 mL) and 5 drops of concentrated hydrochloric acid were added. The reaction was stirred for 90 min and the solvents were evaporated to give the crude product. Purification of the title compound (**18**) was achieved using flash chromatography (CH_2Cl_2) to give the product as a solid, (2.73 g, 66%), ($R_f = 0.40$, CH_2Cl_2); m.p. 75–77 °C from ether-hexane. ^{1}H NMR (400 MHz, $CDCl_3$) 4.91, 5.00, 5.03 (6 H, 3 s, 3 × ArO*C*H_2Ph), 5.30 (1 H, s, ArO*H*, D_2O exch.), 6.60 (1 H, s, C*H*, Ar), 6.61 (1 H, s, C*H*, Ar), 7.24–7.40 (15 H, m, 3 × ArOCH_2*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$) 71.81, 71.97, 73.30 (3 t, ArO*C*H_2Ph), 103.48, 105.08 (2 d, *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: $(FAB)^+$ 91, 412.3; (HRMS, ESI^+) *m/z* Calcd for $C_{27}H_{25}O_4$ $[M+H]^+$ 413.1747. found 413.1739, calcd for $C_{27}H_{24}O_4$ C 78.62, H 5.86; found: C 78.8, H 5.88.

1,2-Bis(2,4,5-tris(benzyloxy)phenoxy)ethane (19)

A mixture of 2,4,5-tribenzyloxyphenol (**18**) (2.73 g, 6.61 mmol), ethylene glycol di-*p*-tosylate (1.22 g, 3.3 mmol) and K_2CO_3 (2.76 g, 20 mmol) was heated at 130 °C under N_2 in dry DMF (50 mL) for 22 h. The dark coloured DMF was evaporated and the remaining solid was partitioned between water and CH_2Cl_2 (200 mL of each) and the organic solvent was evaporated to give the crude product $R_f = 0.26$ (CH_2Cl_2) and lower R_f than the starting material $R_f = 0.40$ (CH_2Cl_2) on same TLC plate. The remaining solid was subject to flash chromatography (CH_2Cl_2) to give compound (**19**) as a pure crystalline white solid. Yield = (1.483 g, 53%) (EtOAc–petroleum ether 40-60 °C), m.p. = 128–129 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 4.21 (4 H, s, $(OBn)_3ArOCH_2CH_2OAr(OBn)_3$), 4.95, 4.99, 5.00 (12 H, 3 s, 6 × ArO*C*H_2Ph), 6.61, 6.67 (4 H, 2 s, Ar*H*), 7.25–7.38 (30 H, m, 6 × ArOCH_2*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$) 69.32 (t, $(OBn)_3ArOCH_2CH_2OAr(OBn)_3$), 72.49, 72.51, 72.60 (3 t, 6 × ArO*C*H_2Ph), 106.89, 107.28 (2 d, 4 × *Ar*H), 127.53, 127.60, 127.76, 127.79, 128.39, 128.40 (d, ArOCH_2*Ph*), 137.25, 137.29, 143.53, 143.64, 143.71, 143.76 (C_q, ArOCH_2*Ph*, *Ar*OCH_2Ph). (HRMS, ESI^+) *m/z* Calcd for $C_{56}H_{50}O_8Na$ $[M + Na]^+$ 873.3398. found 873.3406, calcd for $C_{56}H_{50}O_8$ C 79.04, H 5.92; found: C 78.9, H 5.87.

5,5′-(Ethane-1,2-diylbis(oxy))dibenzene-1,2,4-triol (20)

1,2-Bis(2,4,5-tris(benzyloxy)phenoxy)ethane (**19**) (1.254 g, 1.47 mmol) was dissolved in a mixed solvent of CH_2Cl_2 (50 mL) and MeOH (10 mL) and palladium on carbon (10%, 100 mg) was added. The air was expelled and the solution was stirred over an atmosphere of hydrogen for 18 h at room temperature. A further portion of MeOH (40 mL) was added and the mixture was hydrogenated for a further 4 h to ensure full deprotection. The colourless solution was filtered off and washed with more MeOH and the solvent was evaporated to yield a pale pink-grey-purple solid which was then washed with ether (439 mg, 1.415 mmol, 96%), compound (**20**) was not recrystallized. ^{1}H NMR (400 MHz, d_6-DMSO) 4.04

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(s, 4 H, $(OH)_3ArOCH_2CH_2OAr(OH)_3$), 6.29, 6.41 (4 H, 2 s, Ar*H*), 8.09 (4 H, s, D_2O exch. ArO*H*), 8.34 (2 H, s, D_2O exch. ArO*H*); ^{13}C NMR (100 MHz, d_6-DMSO) 68.89 (t, $(OH)_3ArOCH_2CH_2OAr(OH)_3$), 104.60, 105.59 (2 d, $4 \times ArH$), 136.97, 138.34, 139.56, 139.72 (4 s, C_q, $(OH)_3ArOCH_2CH_2OAr(OH)_3$); (HRMS, ESI^+) m/z Calcd for $C_{14}H_{15}O_8$ $[M+H]^+$ 311.0761. found 311.0754., calcd for $C_{14}H_{14}O_8$ C 54.20, H 4.55; found: C 53.6, H 4.70.

(Ethane-1,2-diylbis(oxy))bis (benzene-5,4,2,1-tetrayl)dodecaethyl-hexakisphosphate (21)

A mixture of diethyl chlorophosphite (1.33 mL, 7.8 mmol) and *N*,*N*-diisopropylethylamine (1.75 mL, 10.0 mmol) was stirred at room temperature in dry CH_2Cl_2 (10 mL) to give a yellow solution. 5,5′-(Ethane-1,2-diylbis(oxy))dibenzene-1,2,4-triol (**20**) (310 mg, 1 mmol) was added in small portions and the solid dissolved with the aid of ultrasound within 5–10 min with the solution remaining a yellow colour which was stirred for a further 30 min. The mixture was cooled using dry ice in acetone, then *m*CPBA (2.58 g, 15 mmol) in CH_2Cl_2 (25 mL) was added in one portion, the colour went a dark olive green and the solution was stirred for a further 30 min. The mixture was washed with 0.50 M aqueous phosphate buffer pH 7.4 (2×100 mL), dried, and was purified by flash chromatography using EtOAc then EtOAc-EtOH (5:1) to give the title compound (**21**) as a syrup, (809 mg, 72 %), $R_f = 0.19$ (EtOAc-EtOH, 5:1). ^1H NMR (400 MHz, $CDCl_3$) 1.28-1.39 (36 H, 2 t, $J = 7.0$ Hz, $4 \times ArOP(O)(OCH_2CH_3)_2$), 4.17–4.31 (24 H, m, $2 \times ArOP(O)(OCH_2CH_3)_2$), 4.35 (4 H, d, $J = 2.0$ Hz, -$ArOCH_2CH_2OAr$-), 7.13 (2 H, br m, Ar*H*), 7.39 (2 H, br m, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$) 15.81, 15.89, 15.97 (3 q, $6 \times ArOP(O)(OCH_2CH_3)_2$), 64.71, 64.77, 64.82, 64.86, 64.93 (5 t, $6 \times ArOP(O)(OCH_2CH_3)_2$), 67.80 (t, -$ArOCH_2CH_2OAr$-), 107.46, 114.88 (2 d, $4 \times ArH$), 134.63 (C_q, dd, $ArOP(O)(OCH_2CH_3)_2$), 136.35 (C_q, d, $J = 7.4$ Hz, $ArOP(O)(OCH_2CH_3)_2$), 138.43 (C_q, dd, $J = 6.6$ Hz, $ArOP(O)(OCH_2CH_3)_2$), 146.69, 146.75 (C_q, -$ArOCH_2CH_2OAr$-); ^{31}P NMR (162 MHz, $CDCl_3$) −7.73 ($2 \times ArOP(O)(OCH_2CH_3)_2$), −7.75 ($2 \times ArOP(O)(OCH_2CH_3)_2$), −8.01 ($2 \times ArOP(O)(OCH_2CH_3)_2$). (HRMS, ESI^+) m/z Calcd for $C_{38}H_{69}O_{26}P_6$ $[M+H]^+$ 1127.2497. found 1127.2511; $C_{38}H_{68}O_{26}P_6$ C 40.51, H 6.08; found: C 40.1, H 6.15.

Ethane-1,2-diylbis(oxy)bis (benzene-5,4,2,1-tetrayl) hexakisphosphate (9)

Compound (**21**) (113 mg, 100 μmol) was dissolved in dry CH_2Cl_2 (10 mL) and dry 2,4,6-collidine (0.5 mL, 3.84 mmol) was added and the solution was stirred over an atmosphere of nitrogen. Bromotrimethylsilane (1.0 mL, 7.57 mmol) was added and the solution was stirred for 3 days at room temperature (there was some precipitation of collidine derivative). The solvents were evaporated off and the reaction mixture was quenched using a mixed solvent of H_2O–(2 M) TEAB (3:1, 4 mL). Compound (**9**) was purified by ion exchange chromatography using Q-Sepharose Fast Flow and a gradient of triethylammonium bicarbonate, (TEAB) $0 \rightarrow 2.0$ M and the compound was identified by the Briggs test and eluted at 2.0 M TEAB buffer, (yield, 88 μmol, 88%). ^1H NMR (400 MHz, D_2O) 4.26 (4 H, s, -$ArOCH_2CH_2OAr$-), 7.00, 7.13 (4 H, 2 s, $4 \times ArH$); ^{13}C NMR (100 MHz, D_2O) 68.88 (t, -$ArOCH_2CH_2OAr$-), 109.85, 114.71 (2 d, $4 \times Ar$-H), 137.77 (dd, C_q, C-P, $J = 1.4$, 6.6 Hz, $ArOPO_3^{2-}$), 137.99 (d, C-P, $J = 6.6$ Hz, $ArOPO_3^{2-}$), 139.69 (dd, C-P, $J = 1.4$, 5.9 Hz), 145.41, 145.47 (2 s, $(OPO_3^{2-})_3ArOCH_2CH_2OAr(OPO_3^{2-})_3$); ^{31}P NMR (162 MHz, D_2O) −1.68 ($2 \times ArOPO_3^{2-}$), −1.77 ($2 \times ArOPO_3^{2-}$), −2.67 ($2 \times ArOPO_3^{2-}$); (HRMS, ESI^-) m/z Calcd for $C_{14}H_{19}O_{26}P_6$ $[M-H]^-$ 788.8596. found 788.8617.

3,4-Benzyloxyphenol (24)

3-Chloroperoxybenzoic acid (5.18 g, 30 mmol) was added to a solution of 3,4-dibenzyloxybenzaldehyde (**22**) (6.37 g, 20 mmol) in dry CH_2Cl_2 (100 mL) and the mixture was stirred for 22 h. The organic solution was washed with an aqueous solution of 10% sodium metabisulfite (2×100 mL) and a saturated aqueous solution of sodium hydrogen carbonate (2×100 mL). The organic solution was then evaporated to give an orange/yellow syrup, ($R_f = 0.54$, formate ester, **23**). The syrup was dissolved in a mixed solvent containing MeOH (25 mL) and CH_2Cl_2 (25 mL) and 5 drops of concentrated HCl were added and the solution was stirred for 90 mins. $NaHCO_3$ (5 g) was added and the solution turned orange/brown. The solvents were evaporated to give the crude product which was dissolved in CH_2Cl_2 (100 mL) and the grey solid was washed with 0.1 M HCl (2×100 mL) and water (100 mL). The organic solution was dried ($MgSO_4$) and the solvents were evaporated. The crude product was purified by flash chromatography (CH_2Cl_2), $R_f = 0.14$ (CH_2Cl_2) to give compound (**24**) as a white solid, (4.37 g, 71%) (m.p. = 109–110 °C) from EtOAc-hexane. ^1H NMR (400 MHz, CD_3CN) 4.99, 5.07 (4 H, 2 s, $2 \times ArOCH_2Ph$), 6.30 (1 H, dd, $J = 2.9$, 8.8 Hz, Ar*H*), 6.52 (1 H, d, $J = 2.9$ Hz, Ar*H*), 6.64 (1 H, s, D_2O exch, ArO*H*), 6.83 (1 H, d, $J = 8.8$ Hz, Ar*H*), 7.31-7.46 (10 H, m, $2 \times CH_2Ph$). ^{13}C NMR (100 MHz, CD_3CN) 71.35, 72.94 (2 t, $2 \times CH_2Ph$), 103.74, 107.44, 118.04, 118.30 (4 d, *C*H, Ar), 128.70, 128.73, 128.84, 128.87, 129.31, 129.44 (d, *C*H, $ArOCH_2Ph$), 138.33, 138.87, 142.88, 150.96, 152.91 (C_q, $ArOCH_2Ph$, $ArOCH_2Ph$, *Ar*OH). (MS, FAB^+) Calcd for

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$C_{20}H_{19}O_3$ $[M+H]^+$ 307.1334; found 307.1340. $C_{20}H_{18}O_3$ C 78.41, H 5.92; found: C 78.4, H 5.94.

1,2-bis(3,4-bis(benzyloxy)phenoxy)ethane (25)

A mixture of 3,4-benzyloxyphenol (**24**) (3.06 g, 10 mmol) ethylene glycol ditosylate (2.22 g, 6 mmol), K_2CO_3 (2.76 g, 20 mmol) was heated in dry DMF (100 mL) at 80 °C for 20 h. After this time the reaction was incomplete and a further 0.2 equivalents (0.74 g, 2 mmol) was added and heating continued at 80 °C for a further 17.5 h. The DMF was evaporated and the remaining solid was partitioned between water and CH_2Cl_2 (100 mL of each). The organic solvent was evaporated and the remaining solid was washed with ether-hexane (150 mL) mixture. The remaining solid was then subject to flash chromatography (CH_2Cl_2) to give pure compound (**25**) as a white solid. Yield (1.54 g, 48%), m.p. = 134–135 °C from (CH_2Cl_2-hexane). ^{1}H NMR (400 MHz, $CDCl_3$) 4.16 (4 H, s, $(OBn)_2ArOCH_2CH_2OAr(OBn)_2$), 5.09, 5.12 (4 H, 2 s, 2 × $ArOCH_2Ph$), 6.40 (2 H, dd, J = 2.7, 8.6 Hz, Ar*H*), 6.62 (2 H, d, J = 2.7 Hz, Ar*H*), 6.86 (2 H, d, J = 8.6 Hz, Ar*H*), 7.31–7.46 (20 H, m, 4 × CH_2Ph). ^{13}C NMR (100 MHz, $CDCl_3$) 66.87 (t, $(OBn)_2ArOCH_2CH_2OAr(OBn)_2$), 70.97, 72.48 (2 t, 2 × CH_2Ph), 103.59, 105.19, 116.86 (3 d, *C*H, Ar), 127.26, 127.49, 127.70, 128.80, 128.37, 128.47 (d, *C*H, $ArOCH_2Ph$), 136.96, 137.52, 143.22, 150.12, 153.80 (C_q, $(OBn)_2ArOCH_2CH_2OAr(OBn)_2$). (MS, FAB^+) Calcd for $C_{42}H_{38}O_6$ $[M]^+$ 638.2668; found 638.2735. $C_{42}H_{38}O_6$ C 78.97, H 6.00; found: C 79.0, H 5.98.

4,4′-(ethane-1,2-diylbis(oxy))bis(benzene-1,2-diol) (26)

1,2-bis(3,4-bis(benzyloxy)phenoxy)ethane (**25**) (1.47 g, 2.30 mmol) was dissolved in warm THF (100 mL). Palladium hydroxide (500 mg, 20%) on carbon was then added and the reaction mixture was stirred under an atmosphere of hydrogen for 17 h. TLC (EtOAc) revealed the disappearance of starting material and a new product R_f = 0.54 for the fully deprotected compound. The solution was filtered over a bed of celite and the solvents were evaporated to yield an off-white greyish solid. Purification of 4,4′-(ethane-1,2-diylbis(oxy))dibenzene-1,2-diol (**26**) was accomplished by recrystallization R_f = 0.54, EtOAc). Yield, (506 mg, 79 %), after recrystallization, m.p. = 179–181 °C (MeOH). ^{1}H NMR (400 MHz, d_6-DMSO) 4.06 (4 H, s, $(OH)_2ArOCH_2CH_2OAr(OH)_2$), 6.22 (2 H, dd, J = 2.7, 8.6 Hz, Ar*H*), 6.37 (2 H, d, J = 3.1 Hz, Ar*H*), 6.61 (2 H, d, J = 8.6 Hz, Ar*H*), 8.42, 8.91 (4 H, 2 s, $(OH)_2ArOCH_2CH_2OAr(OH)_2$). ^{13}C NMR (100 MHz, d_6-DMSO) 67.48 (t, $(OH)_2ArOCH_2CH_2OAr(OH)_2$), 104.01, 105.22, 116.49 (3 d, *C*H, Ar), 139.76, 146.29, 152.42 (C_q, $(OH)_2ArOCH_2CH_2OAr(OH)_2$). $C_{14}H_{14}O_6$ C 60.43, H 5.07; found: C 60.1, H 5.08.

Octabenzyl((ethane-1,2-diylbis(oxy)bis (benzene-4,2,1-triyl))tetrakisphosphate (27)

A mixture of carbon tetrachloride, (1.93 mL, 20 mmol), *N*,*N*-diisopropylethylamine (1.46 mL, 8.4 mmol), *N*,*N*-dimethylaminopyridine (49 mg, 0.4 mmol) and 4,4′-(ethane-1,2-diylbis(oxy))dibenzene-1,2-diol (**26**) (278 mg, 1 mmol) was stirred for 15 min at −10 °C in acetonitrile (25 mL) and the suspension turned pale green in colour. Dibenzylphosphite (2.66 mL, 12.0 mmol) was then added dropwise over 5 min at −10 °C (dry ice alone) and the mixture was stirred for a further 1 h under N_2 with the aid of ultrasound once the phosphorylating reagent was added to give a clear yellow solution. The solvents were evaporated and the remaining yellow syrup was dissolved in dichloromethane (50 mL), washed with water (50 mL), dried, and the title compound was purified by flash chromatography R_f = 0.32 (EtOAc-Petroleum ether (40–60 °C), 2:1), to give the product (**27**) as a colourless syrup (300 mg, 23 %). ^{1}H NMR (400 MHz, $CDCl_3$) 4.35 (4 H, s, -$ArOCH_2CH_2OAr$-), 5.14–5.18 (16 H, m, 4 × $ArOP(O)(OCH_2Ph)_2$), 6.73 (2 H, dd, J = 2.7, 9.0 Hz, 2 × Ar*H*), 7.02 (2 H, d, J = 3.0 Hz, 2 × Ar*H*), 7.30–7.35 (42 H, m, 4 × $ArOP(O)(OCH_2Ph)_2$, 2 × Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$) 66.66 (t, -$ArOCH_2CH_2OAr$-), 69.84, 69.90, 69.95, 70.01 (4 t, 4 × $ArOP(O)(OCH_2Ph)_2$), 107.78, 107.81, 111.68 (3 d, 6 × *C*H, ArH), 121.95, 121.97 (d, *C*H, ArH), 127.79, 127.84, 128.36, 128.38, 128.43 (d, *C*H, $ArOP(O)(OCH_2Ph)_2$), 135.11, 135.19, 135.21, 135.25, 135.28, 141.64, 155.72 (s, C_q, $(^{2-}O_3PO)_2ArOCH_2CH_2OAr(OPO_3^{2-})_2$); ^{31}P NMR (162 MHz, $CDCl_3$) −5.69 (2 × $ArOP(O)(OCH_2Ph)_2$), −6.44 (2 × $ArOP(O)(OCH_2Ph)_2$), (MS, ES^+) Calcd for $C_{70}H_{67}O_{18}P_4$ $[M+H]^+$ 1319.3272; found 1319.3277. calcd for $C_{70}H_{66}O_{18}P_4$ C 63.73, H 5.04; found: C 63.5, H 4.92.

Ethane-1,2-diylbis(oxy)bis (benzene-4,2,1-triyl)-tetrakisphosphate (10)

The octabenzylphosphate (**27**) (138 mg, 105 μmol) was dissolved in dry CH_2Cl_2 (15 mL) and dry 2,4,6-collidine (1.17 mL, 9 mmol) was added and the solution was stirred over an atmosphere of nitrogen. Bromotrimethylsilane (1.0 mL, 7.57 mmol) was added and the solution was stirred for 24 h at room temperature and then a further 2 days since it was difficult to monitor (there was some precipitation of collidine derivative). The solvents were evaporated off and the reaction mixture was quenched using a mixed solvent of H_2O–(2 M) TEAB (3:1, 4 mL). Compound (**10**) was purified by ion exchange chromatography using Q-Sepharose Fast Flow and a gradient of triethylammonium bicarbonate, (TEAB) 0 → 2.0 M and the compound was identified by the Briggs test and eluted at 1.50–2.0 M TEAB buffer. An impurity due

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to the incomplete deprotection of the phosphate triester was present. To complete the deprotection the phosphate derivative was dissolved in water and 10% Pd/C (100 mg) was added to the mixture which was stirred over hydrogen for 20 h, yield, (14 μmol, 13%).

^{1}H NMR (400 MHz, D_2O) 4.25 (4 H, s, -ArOCH_2 CH_2OAr-), 6.66 (2 H, dd, $J = 3.1$, 9.0 Hz, ArH), 6.92 (2 H, d, $J = 2.7$ Hz, ArH), 7.14 (2 H, d, $J = 9.0$ Hz, ArH), ^{13}C NMR (100 MHz, D_2O) 67.41 (t, -ArOCH_2CH_2OAr-), 108.87, 110.64, 122.63 (3 d, $6 \times CH$ ArH), 138.13 (dd, C_q, C-P, $J = 5.9$, 6.6 Hz, ArOPO$_3^{2-}$), 144.29 (d, C-P, $J = 5.1$, 6.6 Hz, ArOPO$_3^{2-}$), 154.42 (2 s, (OPO$_3^{2-}$)$_3Ar$OCH$_2$CH$_2$OAr(OPO$_3^{2-}$)$_3$); ^{31}P NMR (162 MHz, D_2O) -1.90 ($2 \times$ ArOPO_3^{2-}), -2.31 ($2 \times$ ArOPO_3^{2-}). (HRMS, ESI$^-$) m/z Calcd for $C_{14}H_{17}O_{18}P_4$ $[M–H]^-$ 596.9371. found 596.9383.

Biphenyl 3,3′,4,4′,5,5′-hexakisphosphate

Outline spectroscopic data for the final compound are given in the footnote[1]. Full data will be described elsewhere.

Biological Materials and Methods

Unidirectional $^{45}Ca^{2+}$ fluxes. $InsP_3R1$-overexpressing L15 cells [Miyawaki, 1990] were seeded in twelve-well clusters (Costar, MA) at a density of 6×10^4 cells per well. Experiments were carried out on confluent cell monolayers, between the 6th and 8th day after seeding. $^{45}Ca^{2+}$ unidirectional flux experiments were performed at 30 °C on saponin-permeabilized cells, essentially as previously described [Missiaen, 1992; Parys, 1993]. Permeabilization was for 10 min in a solution containing 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA and 40 μg ml^{-1} saponin. The non-mitochondrial Ca^{2+} stores were subsequently loaded for 45 min in 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3 and 150 nM free $^{45}Ca^{2+}$ (28 μCi ml^{-1}). Efflux was initiated by incubation in efflux medium (120 mM KCl, 1 mM EGTA, 30 mM imidazole-HCl pH 6.8) containing thapsigargin (10 μM) and Ca^{2+} release from the stores was subsequently assessed in efflux medium every 2 min. After 10 min, $Ins(1,4,5)P_3$ or Ca^{2+} ionophore A23187 (10 μM) were added to the efflux medium for 2 min. Compounds (**4**, **8**, **9** and **10**) were added for 2 min before the addition of $Ins(1,4,5)P_3$ and remained present until 2 min after the $Ins(1,4,5)P_3$ addition. As compound (**11**) induced Ca^{2+} release by itself, it was applied in the absence of $Ins(1,4,5)P_3$. At the end of the experiment, all $^{45}Ca^{2+}$ remaining in the stores was released by incubation with 1 ml of a 2% (w/v) sodium dodecyl sulfate solution for 30 min. Concentration-response curves were fitted using Origin 8.0 (Northampton, MA) software using the Hill equation.

$Ins(1,4,5)P_3$ Type I 5-Phosphatase Assay

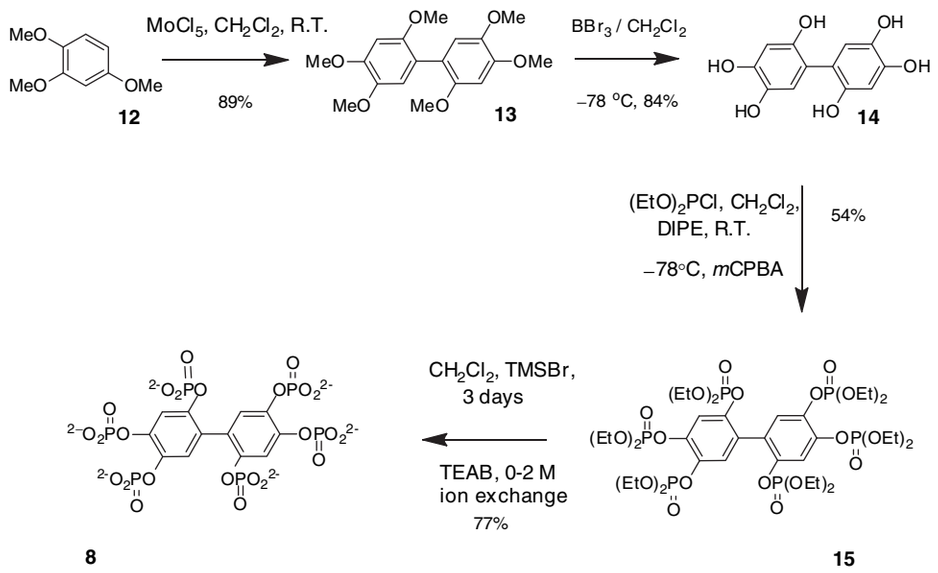
Substrate properties of (**4**, **8**, **9**, **10** and **11**) against 5-phosphatase were investigated by use of the malachite green phosphate assay and inhibition of enzyme activity was also evaluated as reported earlier [Vandeput, 2007], but using 1.0 μmol $Ins(1,4,5)P_3$ as substrate.

RESULTS

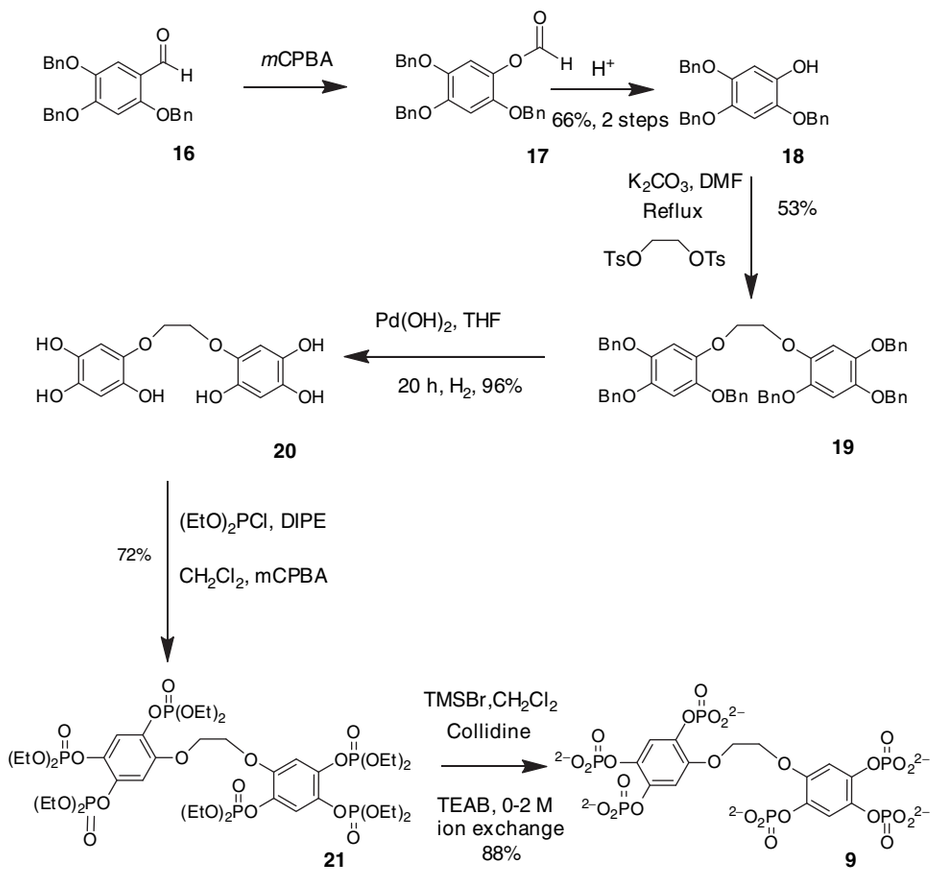
Chemistry

The critical step for the synthesis of compound (**8**), the more structurally rigid dimer, was the formation of the aryl-aryl bond using the fully protected compound (**13**). The oxidative coupling reaction was achieved in high yield using molybdenum pentachloride [Waldvogel, 2002] under an atmosphere of argon to give a clean product (**13**) in high yield. Removal of the methyl groups to expose the six phenolic groups was achieved using excess boron tribromide in dichloromethane. The resulting 2,2′,4,4′,5,5′-hexakisbiphenol (**14**) is soluble in ethyl acetate and the impurities from the reaction dissolve in the aqueous layer leaving a clean solution of the product. Phosphorylation of six phenolic groups was achieved using P(III) reagent diethoxychlorophosphine followed by oxidation with *m*-chloroperoxybenzoic acid using a cooling mixture of dry ice and acetone to give the P(V) derivative. This method for introducing the phosphate groups is more efficient than using dibenzyl phosphite, as for the 1,2-phosphorylated dimer as yields are higher and by-products derived from the deprotection of the ethyl groups using bromotrimethylsilane are easy to remove. However, the benzyl protected octabenzylphosphate derivative (**27**) is easier to identify and purify than the ethylpolyphosphate derivative (**15**) due to its higher U.V. activity. The critical step for the synthesis of both the ethylene dimers was the coupling of the two aromatic groups via a phenolate to ethylene glycol di-*p*-tosylate. The potassium phenolate (potassium salt of compounds **18** and **24**) doubly displaced both tosyl groups from ethylene glycol di-*p*-tosylate to provide two aromatic rings linked via an ethylene group (**19** and **25** respectively). Since the yields of the displacement were only 50% there was probably elimination of the tosylate competing with the substitution reaction. The full synthesis of compound (**11**) will be published elsewhere. Compounds (**8**, **9** and **10**) were synthesised according to Schemes (**1**, **2** and **3**).

[1]Compound **11** preliminary data: ^{31}P NMR -5.82 (s, 6 P, $6 \times$ ArOPO_3^{2-}). (HRMS, ESI$^-$) m/z Calcd for $C_{12}H_{14}O_{24}P_6Na$ $[M+Na–H]^-$ 750.8204. found 750.8215.



Scheme 1. Synthesis of biphenyl 2,2′,4,4′,5,5′-hexakisphosphate 8.



Scheme 2. Synthesis of the ethylene-linked benzene 1,2,4-trisphosphate dimer 9.

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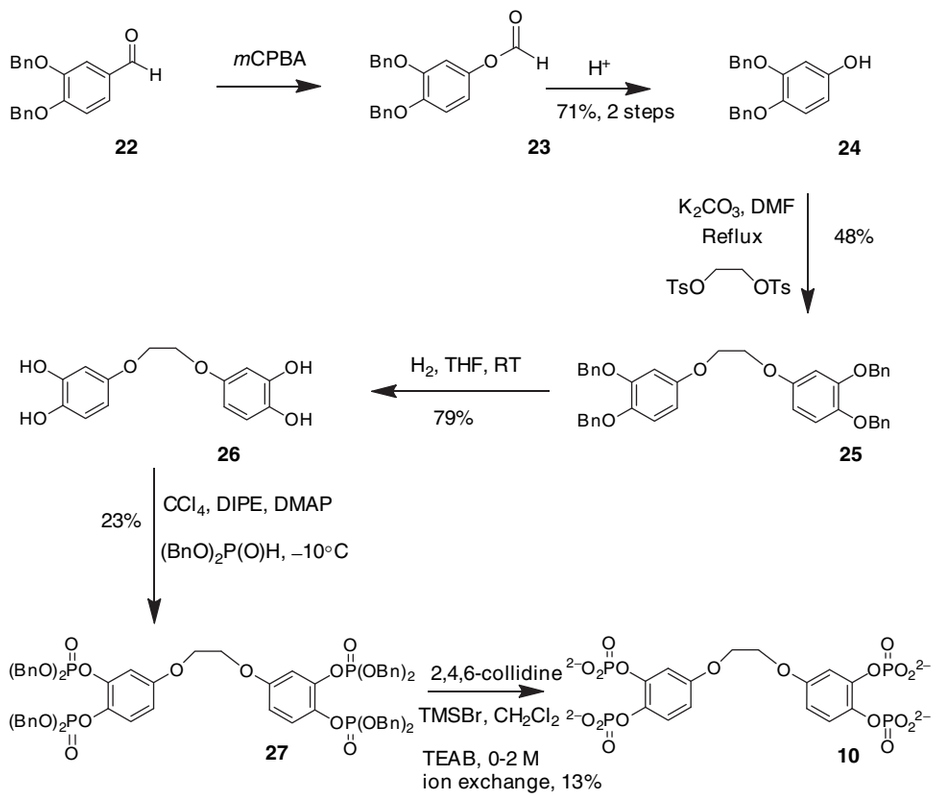

Scheme 3. Synthesis of ethylene-linked benzene 1,2-bisphosphate dimer 10.

Biology

Effects on $InsP_3R$ and on Ca^{2+} Release

Adherent cells form after permeabilization a particularly suitable model system for the investigating the properties of the $InsP_3R$. In this way they allow the loading of the intracellular Ca^{2+} stores until steady state with $^{45}Ca^{2+}$ and the accurate measurement of its unidirectional release under various conditions [Missiaen, 1992; Parys, 1993a]. This system was used previously for investigating the effects of various pharmacological compounds on $Ins(1,4,5)P_3$-induced Ca^{2+} release; for example the sulfhydryl reagent thimerosal [Parys et al., 1993b] methylxanthines [Missiaen, 1994] or KN-93 and related compounds [Smyth, 2002].

As the $InsP_3R1$ is the most studied isoform, most studies have made use of cell lines that express predominantly that the latter isoform endogenously or heterologously express high amounts of it, such as L15 fibroblasts [Miyawaki, 1990]. Saponin-permeabilized L15 fibroblasts have recently been used in a previous study, investigating potential antagonists of the $InsP_3R$ [Keddie, 2011]. Figure 4 demonstrates in saponin-permeabilized L15 fibroblasts the effect of low (0.1 μM) and high (3 μM) concentrations of $Ins(1,4,5)P_3$ on unidirectional $^{45}Ca^{2+}$ release from the non-mitochondrial Ca^{2+} stores, whereby the additional inclusion of compound (**8**) (200 nM) strongly inhibited $Ins(1,4,5)P_3$-induced Ca^{2+} release at low but not at high concentrations of $Ins(1,4,5)P_3$.

The inhibition observed for compound (**8**) was further analyzed (Fig. 5(B)) and compared to that obtained by compound (**4**) (Fig. 5(A)), which was previously investigated [Vandeput, 2007], as well as the effect of the new ligands, (**9**) (Fig. 5(C)) and (**10**) (Fig. 5(D)). The various compounds inhibited $Ins(1,4,5)P_3$-induced Ca^{2+} release in the following rank-order: (**8**) (IC_{50} 187 ± 21 nM) > (**9**) (IC_{50} 380 ± 89 nM) $\approx$ (**4**) (IC_{50} 417 ± 39 nM) $\ggg$ (**10**) (IC_{50} >5 μM). These data are collected in Table I. It was further investigated how the most potent compound (**8**) acts on the $InsP_3R$ by investigating the effect of a fixed concentration (200 nM, its approximate IC_{50} value) at various $Ins(1,4,5)P_3$ concentrations (Fig. 6). From the Lineweaver-Burk plot (Figure 6, inset), (**8**) appears to act as a competitive inhibitor shifting the apparent K_d for $Ins(1,4,5)P_3$ at the receptor from 400 nM to 900 nM.

Finally, it is interesting to observe that the related compound (Fig. 7) (**11**), with slightly different phosphate regiochemistry in contrast to the other compounds evaluated, has an effect on the Ca^{2+} store content in the absence of $Ins(1,4,5)P_3$, inducing Ca^{2+} release with an EC_{50} of 800 nM.

The ligands evaluated against $InsP_3R$ were also evaluated against type I 5-phosphatase. Table I shows three biphenyl compounds (**4**, **8** and **11**) containing five or

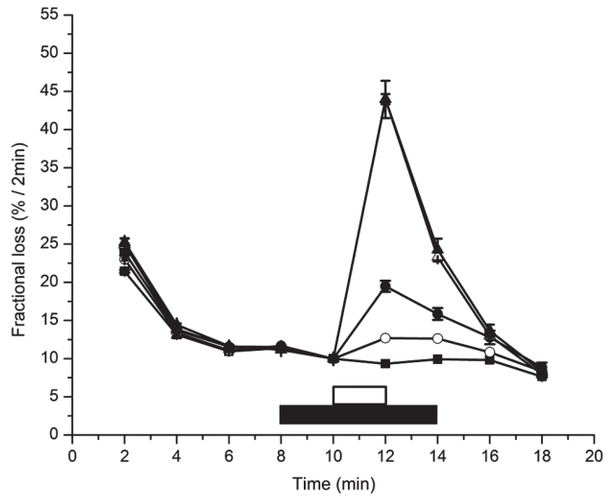

Figure 4. Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilized L15 cells. Fractional Ca^{2+} loss from non-mitochondrial Ca^{2+} stores was measured in saponin-permeabilized L15 fibroblasts in the absence of both $Ins(1,4,5)P_3$ and compound (**8**) (closed squares) or in the presence of 0.1 μM (circles) or 3 μM $Ins(1,4,5)P_3$ (triangles) in the absence (closed symbols) or presence of 200 nM compound (**8**) (open symbols). EGTA (1 mM) was present throughout the efflux. Fractional loss is defined as the amount of Ca^{2+} released in 2 min divided by the total store Ca^{2+} content at that time. $Ins(1,4,5)P_3$ was added for 2 min, as indicated by the white bar. When present, (**8**) was added to the medium for 6 min, as indicated by the black bar. Results are representative for 3 experiments, each performed in duplicate. The S.D. is indicated, unless it is smaller than the symbol.

six phosphate groups spread over two aromatic rings and two dimers (**9** and **10**) containing six or four phosphate groups respectively, spread over two rings and linked via an ethylene group. In general, the most potent inhibitors are derived from the rigid biphenyl derivatives and the two compounds (**8** and **11**) containing six phosphates are the most potent inhibitors with IC_{50} values of less than 1 μM for each. The more flexible dimers are less potent inhibitors of 5-phosphatase, however, the more phosphate groups a molecule contains, the more potent the inhibition of 5-phosphatase in the series.

DISCUSSION

Chemistry

(**4**) Was previously synthesised and evaluated [Vandeput, 2007]. Compounds (**4**, **8**, **9**, **10** and **11**) were evaluated at the $InsP_3R$ and type I inositol $Ins(1,4,5)P_3$ 5-phosphatase. All compounds are resistant to dephosphorylation by 5-phosphatase, as previously demonstrated for (**4**) and other compounds [Vandeput, 2007].

$Bz(1,2,4)P_3$(**2**) can displace [^{3}H]-$Ins(1,4,5)P_3$ from bovine adrenal cortex microsomes, but is *ca* 10,000-fold weaker than $Ins(1,4,5)P_3$ itself [Poitras, 1993]. Although our previous study also shows that $Bz(1,2,4)P_3$ is nearly completely ineffective [Vandeput, 2007], the introduction of another phosphate into the $Bz(1,2,4)P_3$ structure to give benzene 1,2,4,5-tetrakisphosphate nevertheless generates a weak antagonist (IC_{50} *ca* 10 μM), the most potent benzene tetrakisphosphate to inhibit $Ins(1,4,5)P_3$ binding to the $InsP_3R$. This provided a clue that increasing the number of phosphate groups around a core could enhance antagonism. Our new synthetic approach to explore this idea towards more potent $InsP_3R$ antagonists is to link two aromatic rings and decorate them with phosphate groups. The extended framework of negative charges over a biphenyl motif, for example, could interact with more of the positively charged amino acids in the $InsP_3R$ ligand binding site. $BiPh(2,3',4,5',6)P_5$ (**4**) was the first biphenyl polyphosphate derivative that is an $InsP_3R$ antagonist and 5-phosphatase inhibitor [Vandeput, 2007] and is used here as a control and compared with the newly synthesized ligands (**8**) and (**11**). Derivative (**8**) comprises a biphenyl core with a relative arrangement of phosphates on each aromatic ring the same as $Ins(1,4,5)P_3$. This core has little flexibility, only rotational mobility and the phosphate groups are more restricted than for $Ins(1,4,5)P_3$ (**1**). $BiPh(3,3',4,4',5,5')P_6$ (**11**) is a regioisomer of (**8**) with one phosphate group on each benzene ring relocated by one carbon atom to form a 1,2,3-trisphosphate motif on both aromatic rings.

The second synthetic approach is to loosely base the core structure of new compounds (**9**) and (**10**) on the Ca^{2+} chelator BAPTA (**7**), a known weak antagonist of the $InsP_3R$ [Morris, 1999]. Ligands were designed in which the two *N*-methylene carboxylic acids of BAPTA (**7**) are replaced with two phosphate groups flanking adjacent carbon atoms on the aromatic ring to give compound (**10**) and introduction of a $Bz(1,2,4)P_3$ (**2**) motif to give (**9**) distributes the phosphate groups around the aromatic core in a similar way to those of $Ins(1,4,5)P_3$ (**1**). Compound (**9**), containing two copies of (**2**) joined *via* a bridged ethylene linker, may also show more potent antagonism at the $InsP_3R$ than $Bz(1,2,4)P_3$ since, like biphenyl polyphosphate (**8**), it possesses a wider spread of phosphate groups than (**2**) to interact with the positively charged $InsP_3R$ ligand binding site.

Biology

Compounds (**4** and **8-11**) were evaluated in L15 cells overexpressing $InsP_3R1$. Compounds (**4**, **8** and **9**) showed similar shaped concentration-inhibition curves in the presence of 250 nM $Ins(1,4,5)P_3$, where low concentrations of compound (**4**, **8** and **9** at less than 100 nM for each) have little effect on the amount of Ca^{2+} released. As the concentration of competing ligand increases in the range from 100 nM to 1000 nM the amount of Ca^{2+} release decreases to nearly zero, demonstrating antagonism of the $Ins(1,4,5)P_3$-induced Ca^{2+}-release. $BiPh(2,2',4,4',5,5')P_6$ (**8**) with IC_{50}

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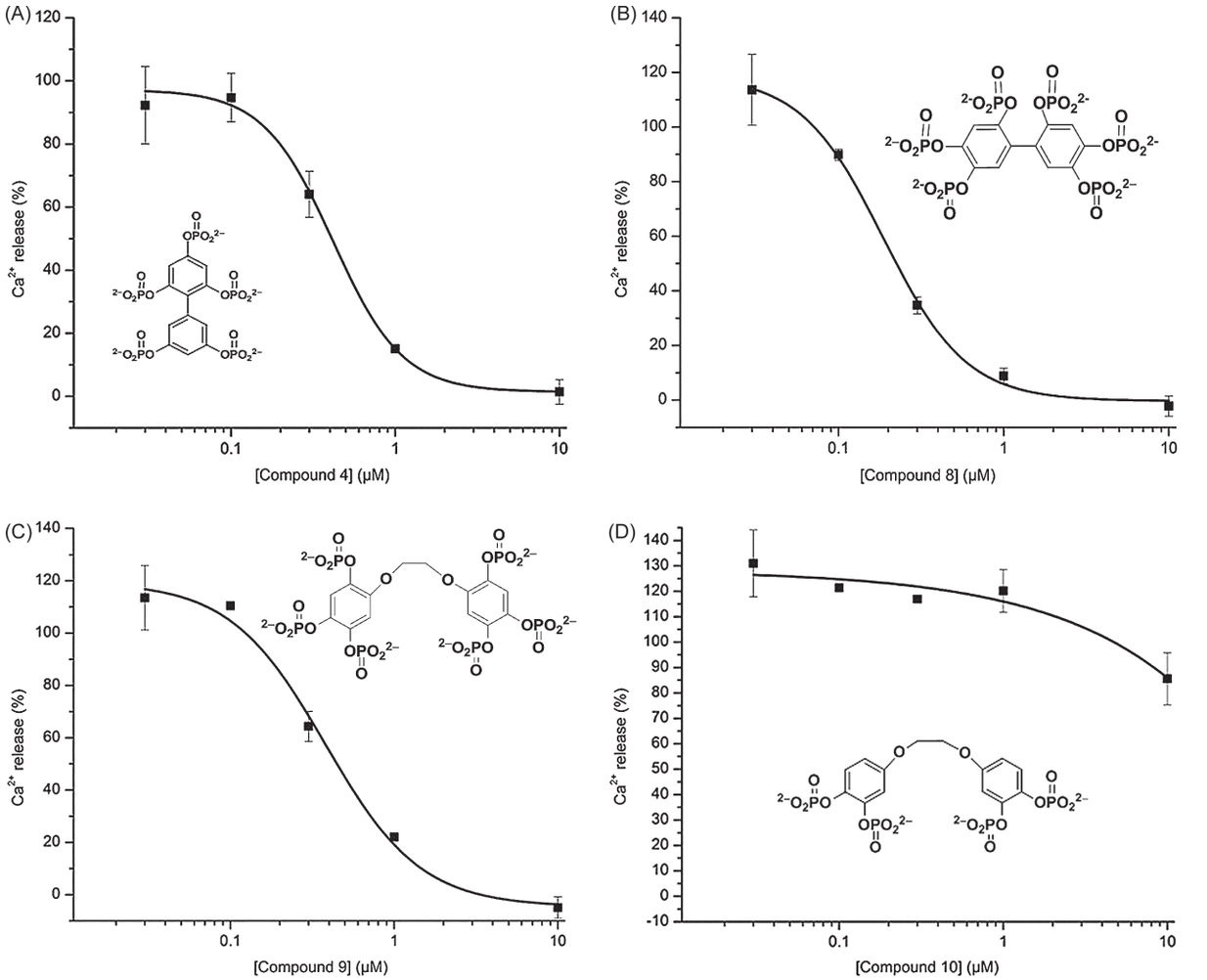

Figure 5. Effect of compounds 4, 8, 9, and 10 on $Ins(1,4,5)P_3$-induced Ca^{2+} release. The concentration-response curves for compounds (**4**) (panel A), (**8**) (panel B), (**9**) (panel C) and (**10**) (panel D) are shown; Ca^{2+} release was induced by 250 nM $Ins(1,4,5)P_3$. Measurement of $Ins(1,4,5)P_3$-induced Ca^{2+} release, incubation with the compounds, and the composition of the efflux medium was exactly as in Figure 4. $Ins(1,4,5)P_3$-induced Ca^{2+} release was defined as the increase in fractional loss over the basal leak observed after 2 min of incubation with $Ins(1,4,5)P_3$. The $Ins(1,4,5)P_3$-induced Ca^{2+} release measured in the absence of the compounds was taken as 100%. Each value represents the mean ± S.E. of 3 or 4 experiments, each performed in duplicate.

187 ± 21 nM is the most potent inhibitor of Ca^{2+} release evaluated, two-fold more potent than either the $Bz(1,2,4)P_3$ dimer (**9**) IC_{50} 380 ± 89 nM or $BiPh(2,3',4,5',6)P_5$ (**4**), IC_{50} 417 ± 39 nM. The benzene 1,2-bisphosphate dimer (**10**) is ineffective at inhibiting Ca^{2+} release up to 10 μM demonstrating, perhaps unsurprisingly, that structurally, two 1,2-bisphosphorylated aromatic rings are insufficient for activity. Dimer (**9**) is more flexible and slightly longer than biphenyl derivative (**8**), but the more rigid polyphosphorylated derivative is more potent. The only other compound known with similar inhibition data is a recently reported 5-modified $Ins(1,4,5)P_3$ analogue, where one of the oxygen atoms is replaced with a methyl group [Keddie, 2011] and reported as one of the first examples of an inositol derivative with full antagonistic behavior at the $InsP_3R$. Under similar experimental conditions application of this analogue at high concentration (300 μM) caused a 40% reduction in the amount of $Ins(1,4,5)P_3$-induced Ca^{2+} release. The inhibitory effect of $BiPh(2,2',4,4',5,5')P_6$ (**8**) on the $InsP_3R$ is thus much greater than that of this analogue and (**8**) appears to be a competitive inhibitor of $Ins(1,4,5)P_3$-induced Ca^{2+}-release and shifts the apparent K_d for $Ins(1,4,5)P_3$ from 400 nM to 900 nM. While the data for (**8**) appear to fit the classical definition of competitive antagonism, more work, e.g. Schild analysis and competition-binding experiments with labeled $Ins(1,4,5)P_3$ is required for a totally unambiguous classification.

Unlike the inositol ring, the rigid benzene ring cannot alter its shape and phosphate groups attached similarly to a benzene core will have different relative degrees of freedom. The binding domain of the $InsP_3R$ is thought to work in a "clam-like" fashion [Rossi, 2009] and $Ins(1,4,5)P_3$, in

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Table I. Inhibition of recombinant type I inositol 1,4,5-trisphosphate 5-phosphatase and $Ins(1,4,5)P_3$—induced Ca^{2+} release by biphenyl polyphosphates and dimeric benzene polyphosphates. 1.0 μM $Ins(1,4,5)P_3$ was used as substrate in the 5-phosphatase assay.

Compound	5-Phosphatase inhibition IC_{50} (nM)	$InsP_3R$ antagonism IC_{50} (nM)
$BiPh(2,3',4,5',6)P_5$ **(4)**	1850	417
$BiPh(2,2',4,4',5,5')P_6$ **(8)**	480	187
1,2,4-Dimer **(9)**	3550	380
1,2-Dimer **(10)**	>10,000	>5000
$BiPh(3,3',4,4',5,5')P_6$ **(11)**	730	N/A

principle, can also undergo conformational changes. However, phosphorylated biphenyl compounds have in comparison just rotational mobility but little manoeuvrability and flexibility. These effects may also play some part in the antagonistic behavior of these compounds.

The symmetrical 1,2,4-trisphosphate arrangement gives the most potent inhibitor of 5-phosphatase (Table I) with $BiPh(2,2',4,4',5,5')P_6$ (**8**) having an IC_{50} of 480 nM. It is interesting to note for 5-phosphatase inhibition that even though the 1,2,4-trisphosphate arrangement is the same for compounds (**8**) (IC_{50} 480 nM) and (**9**) (IC_{50} 3.55 μM) the more rigid and shorter biphenyl polyphosphate (**8**) is the superior inhibitor.

For 5-phosphatase inhibition, (**8**) is also *ca* 4 times better than the original biphenyl polyphosphate synthesized

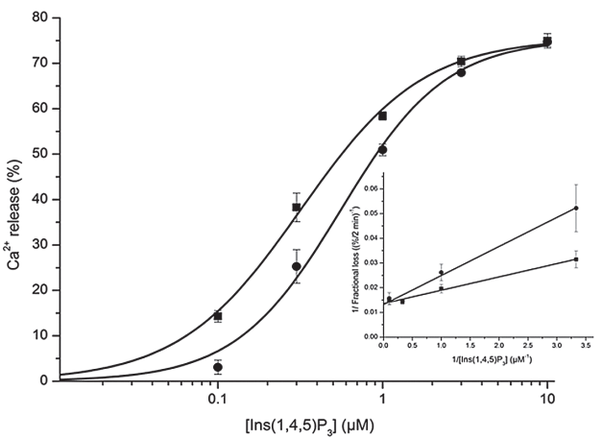

Figure 6. Compound 8 is a competitive inhibitor of the $Ins(1,4,5)P_3$ receptor. $Ins(1,4,5)P_3$-induced Ca^{2+} release was measured at various concentrations of $Ins(1,4,5)P_3$ in the absence (squares) or presence (circles) of 200 nM (**8**). The maximal Ca^{2+} release, i.e., the release induced by 10 μM of the Ca^{2+} ionophore A23187, was taken as 100%. The inset shows a Lineweaver-Burk representation of the same data. Fitting was performed by linear regression. (**8**) Appears to be a competitive inhibitor of the $InsP_3R$, and the apparent K_d for $Ins(1,4,5)P_3$ changes from 400 to 900 nM after inclusion of (**8**)**.** Each data point represents the mean of 3 or 4 independent experiments, each performed in duplicate. S.E. is indicated, unless smaller than the symbol.

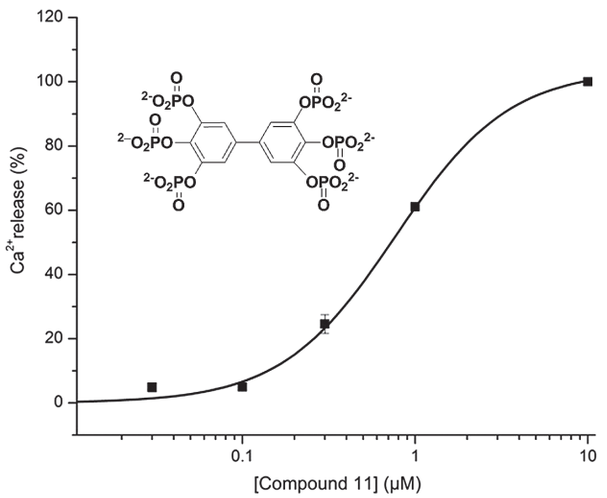

Figure 7. Compound 11 induces Ca^{2+} release. Fractional Ca^{2+} loss from the non-mitochondrial Ca^{2+} stores was measured in saponin-permeabilized L15 fibroblasts in the presence of various concentrations of compound (**11**), EGTA (1 mM) was present throughout the efflux. The Ca^{2+} release induced by 10 μM (**11**) was taken as 100%. The experiment was performed in duplicate and S.D. is indicated, unless smaller than the symbol.

(**4**) with 5 phosphate groups and regioisomer (**11**) is intermediate. It is obviously difficult to draw firm SAR conclusions with such a limited compound set but the trend is that potency of the phosphorylated ligands increases (Table I) with the number of phosphate groups on the aromatic core. Four phosphate groups in a 1,2-bisphosphate arrangement spread over two rings (**10**) exhibit weaker 5-phosphatase inhibition, whereas (**8**) is a minimum of 20-fold more potent. For the small set of compounds evaluated the 1,2,4-trisphosphate arrangement around a biphenyl core delivers the most potent ligand.

Regioisomeric $BiPh(3,3',4,4',5,5')P_6$ (**11**), with a different arrangement of phosphate groups on the aromatic ring compared to (**8**), was also evaluated. (**11**) Releases Ca^{2+} from intracellular stores in a concentration-dependent manner and in the absence of $Ins(1,4,5)P_3$. This is not due to an inhibitory effect on the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps as we measure unidirectional efflux of Ca^{2+} in the presence of thapsigargin, a specific SERCA inhibitor, and EGTA. Therefore, we suppose that (**11**) might interact with one of the proteins involved in the Ca^{2+} leak pathway. This pathway is responsible for a continuous leakage of Ca^{2+} from the stores. The protein(s) responsible for it is(are) however not known with certainty [Sammels, 2010] and possibilities include the translocon complex, polycystin-2, presenilins, Bax-inhibitor-1 and pannexins. Alternatively, it cannot yet be excluded (**11**) is acting on another type of intracellular Ca^{2+} release channel such as the cyclic ADP ribose or nicotinic acid-adenine dinucleotide phosphate (NAADP)-sensitive channel [Fliegert, 2007]. This

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dramatically contrasting effect, elicited by the relocation of only a single phosphate on each phenyl group, is very striking and is worthy of further investigation.

Duchenne muscular dystrophy is an X-chromosome linked muscle wasting disease caused by a defective gene encoding the 427-kDa cytoskeletal protein dystrophin present in muscle [Hoffman, 1987]. A dysfunctional calcium pathway via $InsP_3Rs$ releases too much Ca^{2+} into the skeletal muscle. Interestingly, if the $InsP_3R$ is blocked using the non-specific and non-competitive inhibitor 2-aminoethoxydiphenyl borate [Mondin, 2009], there is an improvement of survival of dystrophin-deficient myotubules. The authors suggest targeting the $Ins(1,4,5)P_3/Ca^{2+}$ pathway as a new approach to alleviate symptoms. Understanding the machinery of the $InsP_3R$ is thus a priority at both a mechanistic and wider level and intervention may afford leads to target muscular dystrophy and other diseases. Unfortunately, there are few $InsP_3R$ antagonists available as leads for such exploitation. Thus, although our new compounds are unlikely to be membrane permeant, we have developed new multivalent ligands with improved antagonist potency and diverse preliminary SAR features. One has been demonstrated to be a competitive antagonist in a functional context and all may be useful tools to investigate the chemical biology of signaling through $InsP_3R$ and particularly as templates for further design. Moreover, while a "multivalent" approach has been pioneered for inositol phosphate based ligand design [Riley, 2002; 2004; Rossi, 2009], importantly it now seems clear that this is also applicable for non-inositol polyphosphate ligands, since present data demonstrate a significant enhancement of antagonistic activity for dimers of the very weak $Bz(1,2,4)P_3$ compound achieved in two different ways.

CONCLUSIONS

We demonstrate that two new types of dimeric benzene phosphate derivative based on $Bz(1,2,4)P_3$ act as potent sub-micromolar antagonists of Ca^{2+} release through the $InsP_3R$. Exploration of an earlier structural template based upon a biphenyl core through multivalent considerations has generated an improved antagonist (**8**) with preliminary SAR considerations and support for a competitive mode of action. Surprisingly, the regioisomeric relative (**11**) releases Ca^{2+}, and may be interacting with one or more of the proteins connected with the Ca^{2+} leak pathway. A more flexible multivalent benzene phosphate derivative (**9**) based loosely around the known weak $InsP_3R$ antagonist BAPTA is also more potent than the parent $Bz(1,2,4)P_3$ fragment. The more rigid (**8**) is the most potent antagonist of Ca^{2+} release and is also the most potent inhibitor of type I $Ins(1,4,5)P_3$5-phosphatase, with regioisomer (**11**) being only slightly weaker. The more rigid structure with the same phosphate arrangement is also a superior 5-phosphatase inhibitor, with flexible derivative (**9**) being 7-fold weaker than (**8**). Such templates offer new leads for antagonist design and pharmacological intervention in the polyphosphoinositide pathway of cell signaling.

Conflict of Interest

The Authors disclose no conflict of interest.

Acknowledgments: We thank the Wellcome Trust (grant number 082837) for Programme Grant support to BVLP. We acknowledge K. Mikoshiba for the generous gift of L15 fibroblasts. This work was supported by Interuniversity Attraction Poles Program P6/28 (Belgian Science Policy) to J. B. Parys and C. Erneux.

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Received: 21 May 2012. Accepted: 20 June 2012.

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