Synthesis of inositol phosphate-based competitive antagonists of inositol 1,4,5-trisphosphate receptors†

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Inositol 1,4,5-trisphosphate receptors (IP₃ Rs) are intracellular Ca²⁺ channels that are widely expressed in animal cells, where they mediate the release of Ca²⁺ from intracellular stores evoked by extracellular stimuli. A diverse array of synthetic agonists of IP₃ Rs has defined structure–activity relationships, but existing antagonists have severe limitations. We combined analyses of Ca²⁺ release with equilibrium competition binding to IP₃ R to show that (1,3,4,6)IP₄ is a full agonist of IP₃ R1 with lower affinity than (1,4,5)IP₃. Systematic manipulation of this meso-compound via a versatile synthetic scheme provided a family of dimeric analogs of 2-O-butyryl-(1,3,4,6)IP₄ and (1,3,4,5,6)IP₅ that compete with (1,4,5)IP₃ for binding to IP₃ R without evoking Ca²⁺ release. These novel analogs are the first inositol phosphate–based competitive antagonists of IP₃ Rs with affinities comparable to that of the only commonly used competitive antagonist, heparin, the utility of which is limited by off-target effects.

Introduction

Inositol 1,4,5-trisphosphate receptors (IP₃ Rs) are intracellular Ca²⁺ channels that are almost ubiquitously expressed in animal cells.¹,² IP₃ Rs are essential links between receptors in the plasma membrane that stimulate phospholipase C and release of Ca²⁺ from the endoplasmic reticulum (ER). The resulting cytosolic Ca²⁺ signals regulate many diverse cellular processes.³ The three subtypes of IP₃ Rs expressed in vertebrates (IP₃ R1-3) are closely related proteins and they are each regulated by both (1,4,5)IP₃ (1, Fig. 1) and Ca²⁺, but they differ in their sensitivity to other forms of regulation and in their subcellular and tissue distributions.⁴

Extensive structure–activity studies,⁴–⁸ reinforced by a high-resolution structure of (1,4,5)IP₃ bound to the IP₃-binding core of IP₃ R1 (Fig. 1A),⁹ established that the vicinal 4,5-bisphosphate moiety is essential for (1,4,5)IP₃ binding and the equatorial 6-hydroxyl and 1-phosphate confer high affinity (Fig. 1B). All high-affinity agonists of IP₃ R have structures equivalent to these substituents. The only endogenous inositol phosphate likely to bind to IP₃ Rs under physiological conditions is (1,4,5)IP₃, the immediate water-soluble product of phospholipase C-catalyzed hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate. However, synthetic ligands of IP₃ Rs, including many inositol phosphates⁵ and derivatives of adenosinephosphins,¹⁰–¹³ have provided insight into the structural determinants of IP₃ R activation. These ligands include analogs of (1,4,5)IP₃ that are resistant to degradation,¹³ fluorescent analogs,¹⁴ partial agonists,⁶ and synthetic derivatives of adenosinephosphins.¹⁰ There are, however, no ligands of IP₃ R that distinguish effectively between IP₃ R subtypes,⁵,¹⁵,¹⁶ and the only available antagonists have severe limitations.¹⁷ The commonly used antagonists are heparin, 2-aminoethyldiphenyl borate (2-APB), xestospongins and high concentrations of caffeine. The limitations of these antagonists include off-target effects, notably interactions with other Ca²⁺ channels, Ca²⁺ pumps, G proteins and other signalling pathways; membrane-impermeability (heparin) and, for xestospongins, an inconsistent history of effectiveness as discussed recently.¹⁷ This study was undertaken with the aim of developing more effective antagonists of IP₃ R.

(1,3,4,6)IP₄, which retains the essential pharmacophore of an IP₃ R agonist (Fig. 1B), stimulates Ca²⁺ release via IP₃ R, but its affinity is between 10 and 100-fold lower than that of
(1,4,5)IP₃, though not all, studies have suggested that (1,3,4,6)IP₄ may be a partial agonist, namely that it less effectively activates IP₃R than full agonists like (1,4,5)IP₃. It seems likely that the inverted position of the 2-OH in (1,3,4,6)IP₄ (equivalent to the 3-OH of (1,4,5)IP₃ when the structures are compared in orientations likely to reflect their interactions with IP₃R, Fig. 1B) is a major determinant of the reduced affinity.⁵,²⁰ Although (1,3,4,6)IP₄ is produced endogenously from (1,3,4)IP₃, it is unlikely to attain concentrations that regulate IP₃Rs.²⁴ Nevertheless, we chose (1,3,4,6)IP₄ to attempt development of novel antagonists of IP₃R because it and analogs in which its free hydroxyls are modified (3, 4) are meso compounds that make synthesis more straightforward, and we had initially supposed that (1,3,4,6)IP₄ might have reduced efficacy.¹⁹,²² We previously reported that dimers of inositol phosphates are high-affinity partial agonists of IP₃R.⁸ We have now developed a family of antagonists of IP₃Rs (5–12 in Fig. 1C) from the (1,3,4,6)IP₄ backbone by modification of its free hydroxyls and dimerization of the modified structures. Diesteric or dietheric linkages of various sizes (n = 1–3) were chosen for these 5-O-homodimers, which were synthesized by means of a diverse and versatile approach. The most useful of these ligands (8, 10 and 12) bind to IP₃R1 with an affinity comparable to that of the best available competitive antagonist of IP₃R, heparin, the utility of which is limited by its off-target effects.

Results and discussion

Chemistry

Synthesis of IP₅₄ and IP₆₅. Phosphates 2–4 were all prepared from myo-inositol (13) (Scheme 1). Thus, tetradsodium (1,3,4,6)IP₄ (2) was synthesized from butanedione-derived acetal 1₄ in a previously published route. The pentasodium (1,2,3,4,6)IP₅ (4) was reached via pentol 1₅a and pentakis phosphate 1₅b. Modifications on the perphosphorylation and hydrogenolysis protocols of an inositol biscyclohexylidene acetal originated synthetic scheme, were applied in order to solely obtain the pentasodium salt. The preparation of butanoate 3 involved a novel approach. Thus, acetal 1₄ was initially selectively protected at the C-2 position as the PMB ether to yield 1₆a. Masking of the remaining C-5 hydroxyl as the benzyl ether gave the fully protected derivative 1₆b, which was very carefully deprotected upon treatment with aqueous DDQ to reach free alcohol 1₆c. Introduction of the required butyryl group was performed by esterification with butyric anhydride. The resulting ester (1₆d) was then exposed to aqueous TFA to cleave both acetals, and the corresponding tetraol (1₇a) was formed quantitatively. Perphosphorylation of crude 1₇a was accomplished using 1H-tetrazole solution in acetonitrile and dibenzyl N,N-diisopropylphosphoramidate at ambient temperature, followed by direct oxidation of the intermediate phosphate with m-chloroperbenzoic acid at low temperature. Finally, the obtained benzyl tetrakisphosphate 1₇b was subjected to hydrogenolysis...
in ethanol/water in the presence of Pd/C and sodium bicarbonate (exactly one equivalent per phosphate group) to yield quantitatively the desired tetrasodium salt 3.

**Synthesis of dimeric analogs of IP$_4$ and IP$_5$.** For the synthesis of dimers 5–12, we envisioned the retroynthetic analysis depicted in Scheme 2. Dimers 5–12 could be reached from the corresponding polyols 18 applying sequentially perphosphorylation and global deprotection protocols. The key to obtain all these compounds, differentially substituted on C-2, from a common intermediate (19) was to introduce orthogonal protective groups (PG and PG$'$) at an early stage of the synthesis. In this way, 19 could serve as the sole precursor for both series (2-O-butyrylated and 2-O-phosphorylated derivatives) by selective removal of PG$'$. Esters and ethers 19 could, in turn, be prepared by dimerization of the corresponding monomers 20 using the appropriate linkers. Since this process involved the relatively hindered secondary alcohols 20, we were keen to explore the feasibility of this approach. Finally, starting from myo-inositol (13) selective introduction of the required protective groups was expected to lead to monomers 20.

Monobenzyl ether 21 (Scheme 3) was recognized as a suitable derivative, appropriately functionalized to play the role of 20. Moreover 21 is easily accessible$^{25,29,30}$ from myo-inositol through butaneidine bisacetal 14. Direct dimerization of this compound was initially investigated using the Steglich esterification approach$^{31}$ and employing malonic ($n = 1$) and succinic acid ($n = 2$) as linkers (Scheme 3 and Table S1 in ESI†). However, these apparently simple couplings were found to be complicated, under various reaction conditions tested, by the formation of acetate 22 (in the first case) and N-acylureas 24a–c (in both cases). Thus, for malonic acid reactions, the presence of Pd/C and sodium bicarbonate (exactly one equivalent per phosphate group) to yield quantitatively the desired tetrasodium salt 3.

**Scheme 1** Synthesis of phosphates 2–4. Reagents and conditions: (a) ref. 25; (b) ref. 26; (c) ref. 27; (d) i. (BnO)$_2$PN(iPr)$_2$, 1H-tetrazole, CH$_3$CN, 25 °C, 48 h; ii. m-CPBA, CH$_2$Cl$_2$, −50 to 0 °C, 5 h, for 15b 73%; for 17b 63%; (e) Pd/C, H$_2$ (1 atm), NaHCO$_3$, EtOH/H$_2$O (1 : 1), 25 °C, 48–96 h, for 3 and 4 100%; (f) i. NaH, DMF, 0 °C, 1 h; ii. PMBCl 0 to 25 °C, 12 h, 67%; (g) i. NaH, DMF, 0 °C, 1 h; ii. BnBr, 0 to 25 °C, 12 h, 90%; (h) DDQ, CH$_2$Cl$_2$/H$_2$O (10 : 1), 25 °C, 24 h, 71%; (i) Bt$_2$O, Et$_3$N, DMAP, CH$_2$Cl$_2$, 25 °C, 12 h, 91%; (j) 90% aq. TFA, CH$_2$Cl$_2$, 25 °C, 2 h, 100%.

**Scheme 2** Retrosynthetic analysis for target dimers 5–12.
of DMAP seemed to solely favor the decarboxylation process, regardless of the carbodiimide (DCC or DIC) and the solvent used.\textsuperscript{32} We could not securely determine whether this decarboxylation occurred prior to or after the first esterification. However, in other runs we isolated the \(N\)-acylureas \(24a\) and \(24b\), suggesting that acetate \(22\) is formed from malonic monoester. Although replacing DMAP with DIPEA eliminated this problem, the only product isolated was \(N\)-acylurea \(24b\), in very low yield, whereas starting material was quantitatively recovered when EDC was used. On the other hand, the reactions performed in the absence of base\textsuperscript{33} were productive, yielding the desired dimer (23a) along with the corresponding \(N\)-acylurea (24a or 24b). The best results were obtained in the case of the DCC-promoted coupling.\textsuperscript{34} Surprisingly, applying the same conditions (DCC in \(Et_2O\)) for the coupling of \(21\) with succinic acid was unsuccessful. In order to reach dimer 23b the presence of DMAP was a crucial factor using either DCC or EDC in \(CH_2Cl_2\).\textsuperscript{34} Again, the reaction with DCC furnished an inseparable mixture of dimer 23b and \(N\)-acylurea 24c, which was subsequently resolved upon hydrogenolysis. In contrast to

\begin{align*}
\text{Scheme 3} & \quad \text{Synthesis of dimers 23 and 25. Reagents and conditions: (a) ref. 29, 30; (b) CH}_3\text{COOH, DCC or DIC, DMAP, CH}_2\text{Cl}_2, 25 ^\circ\text{C, 24 h, 37–41%; (c) CH}_2\text{NH}_2\text{COOH, DCC, Et}_2\text{O, 25 }^\circ\text{C, 24 h, 62% of 23a and 12% of 24b; (d) HOCO(CH}_2)_2\text{COOH, DCC, DMAP, CH}_2\text{Cl}_2, 25 }^\circ\text{C, 96 h, 56% of 23b and 12% of 24c; (e) TsOCH}_2\text{(CH}_2)_n\text{CH}_2\text{OTs (26 or 27), KOH, BnH/DMSO (4:1), 55 }^\circ\text{C, 120 h, for 25a 45%, for 25b 62%.}
\end{align*}

\begin{align*}
\text{Scheme 4} & \quad \text{Synthesis of dimeric phosphates 5–12. Reagents and conditions: (a) Pd/C, H}_2 (1 \text{ atm}), \text{MeOH, 25 }^\circ\text{C, 24 h, 93–100%; (b) Bt}_2\text{O, Et}_3\text{N, DMAP, CH}_2\text{Cl}_2, 25 }^\circ\text{C, 24 h, 79–97%; (c) 90% aq. TFA, CH}_2\text{Cl}_2, 25 }^\circ\text{C, 2 h, 98–100%; (d) i. (BnO)_2PN(iPr)_2, 1H-tetrazole, CH}_2\text{CN, 25 }^\circ\text{C, 48 h; ii. m-CPBA, CH}_2\text{Cl}_2, -50 \text{ to } 0 ^\circ\text{C, 5 h, 60–76%; (e) Pd/C, H}_2 (1 \text{ atm}), \text{NaHCO}_3, \text{EtOH/H}_2\text{O (1:1), 25 }^\circ\text{C, 48–72 h, 95–100%.}}
\end{align*}
esters 23,24,25,26,27 the synthesis of dimeric ethers 25,26,27 was accomplished in a more facile way. Williamson etherifications, through the in situ formed (NaH) sodium alkoxide of 21, were initially attempted in DMF using the required diido- or dibromo-alkanes, but with poor results. Replacing halo-electrophiles with the more reactive ditosylates 26,27 and applying a protocol28 which involved KOH as base and a more polar solvent (DMSO) furnished the desired dimers (25,26,27) in a clean way and in good yields.29

With the key intermediate dimers in our hands, we proceeded to the next steps, which involved installation of the butyryl and phosphate groups. Pd-catalyzed hydrogencylation of 23,24,25,26,27 led to the corresponding diols 28, which were esterified upon exposure to butyric anhydride to give 29 in very good yields (Scheme 4).

Both 28 and 29 were then used to reach the final targets. Thus, careful treatment of these dimers (especially in the case of 29) with aqueous TFA furnished octaols and decaols 30, in nearly quantitative yields (Scheme 4). Perphosphorylation of these crude polyols was accomplished as described for 17b (Scheme 1) to obtain the protected polyphosphates 31. The latter were debenzyolated upon hydrogencylation in the presence of sodium bicarbonate to yield the octakis and decakis phosphate salts 5–12.30

**Biology**

(1,3,4,6)IP₄ is a full agonist of IP₃R. Both (1,4,5)IP₃ (1) and (1,3,4,6)IP₄ (2) stimulated a concentration-dependent release of Ca²⁺ from the intracellular stores of permeabilized DT40-IP₃R1 cells (Fig. 2A and B). The maximal Ca²⁺ release evoked by each ligand was similar, but (1,3,4,6)IP₄ was 21 ± 3-fold less potent that (1,4,5)IP₃ (Table S2 in ESI†). Membranes from Sf9 cells expressing rat IP₃R1 (Sf9-IP₃R1 cells) were used for equilibrium competition binding studies with [³H-(1,4,5)IP₃, because these membranes express full-length IP₃R1 at ~20-fold higher levels than cerebellar membranes, the richest source of endogenous IP₃R1 (Fig. 2C, inset). In these binding analyses, the equilibrium dissociation constants (KD) for (1,4,5)IP₃ and (1,3,4,6)IP₄ differed by 46 ± 19-fold (Fig. 2C and Table S2 in ESI†).

Because both agonists (1 and 2) released the same amount of Ca²⁺ at maximally effective concentrations, a comparison of EC₅₀ and Kd values allows the effectiveness with which each promotes opening of the IP₃R Ca²⁺ channel to be determined. A partial agonist needs to occupy more receptors to elicit the same response, which is then reflected in a higher EC₅₀/Kd ratio (and a lower value for pEC₅₀-pKd, where p denotes the negative log).6 (1,3,4,6)IP₄ and (1,4,5)IP₃ did not differ significantly in their pEC₅₀-pKd values (Table S2 in ESI†) suggesting that (1,4,5)IP₃ and (1,3,4,6)IP₄ have similar efficacies. We conclude that (1,3,4,6)IP₄ is a full agonist with lower affinity than (1,4,5)IP₃, in agreement with a previous report,21 but inconsistent with suggestions that it is a partial agonist.19,22

2-O-Butyryl-(1,3,4,6)IP₄ is a partial agonist and (1,2,3,4,6)IP₅ is an antagonist of IP₃R. We synthesized and assessed the biological activity of two analogs with modifications at the 2-position of (1,3,4,6)IP₄, 2-O-butyryl-(1,3,4,6)IP₄ (3) and (1,2,3,4,6)IP₅...
(4) (Fig. 1B). The analogs retained both the essential pharmacophore (Fig. 1B, blue), and the 5-hydroxyl and 6-phosphate groups [equivalent to the 6-hydroxyl and 1-phosphate of (1,4,5)IP3] that increase binding affinity (Fig. 1B, green).

A maximally effective concentration of 2-O-butyryl-(1,3,4,6)IP4 released a smaller fraction of the intracellular Ca2+ stores than did (1,4,5)IP3 (Fig. 2B) and it bound to the IP3R1 with 50 ± 22-fold lower affinity than (1,4,5)IP3 (Fig. 2C). The lesser maximal Ca2+ release evoked by 2-O-butyryl-(1,3,4,6)IP4, suggests that it is less efficacious than (1,4,5)IP3. Although 2-O-butyryl-(1,3,4,6)IP4 and (1,3,4,6)IP4 differed in their ability to evoke Ca2+ release, they bound to IP3R with similar affinities (Fig. 2C and Table S2 in ESI†). Hence, addition of a butyryl moiety to the 2-position of (1,3,4,6)IP4 decreased efficacy without affecting affinity. 2-O-Butyryl-(1,3,4,6)IP4 (3) thus replaced (1,3,4,6)IP4 as a lead compound from which we attempted to develop ligands that bind to IP3R without activating it (i.e. competitive antagonists).

Even a very high concentration (100 µM) of (1,2,3,4,6)IP5 (4) failed to release Ca2+ (Fig. 2B), but it bound to IP3R1 with a 2-position moiety to the 2-position of (1,3,4,6)IP4 decreased efficacy without affecting affinity. 2-O-Butyryl-(1,3,4,6)IP4 (3) thus replaced (1,3,4,6)IP4 as a lead compound from which we attempted to develop ligands that bind to IP3R without activating it (i.e. competitive antagonists).

Pre-equilibration of permeabilized DT40-IP3R1 cells with (1,2,3,4,6)IP5 (100 µM, 2 min), shifted the sensitivity of the Ca2+ release evoked by (1,4,5)IP3 by 2.4 ± 0.2-fold, without affecting either the maximal Ca2+ release or Hill coefficient (Fig. 3 and Table S3, ESI†). From the dose ratios (see Experimental section), this functional analysis suggests that (1,2,3,4,6)IP5 binds to the (1,4,5)IP3-binding site of IP3R1 and has 230 ± 100-fold lower affinity (Fig. 2C, and Table S2, ESI†). (1,2,3,4,6)IP5 retains the essential pharmacophore and moieties known to be crucial for high-affinity binding (Fig. 1B), but it has an axial phosphate at the 2-position [equivalent to the 3-position of (1,4,5)IP3]. Others have reported that an axial phosphate at the 3-position of (1,4,5)IP3 reduced affinity. The important observation is that addition of an axial 2-phosphate to (1,3,4,6)IP4, to give (1,2,3,4,6)IP5, abolishes residual efficacy, albeit with some (5.2 ± 0.5-fold) loss of affinity.

Fig. 3 (1,2,3,4,6)IP5 (4) and a dimeric analog (6) are competitive antagonists of IP3R1. (A) Typical experiment showing the Ca2+ content of the ER after addition of Mg-ATP to permeabilized DT40-IP3R1 cells, followed by addition of 4 or 6 (100 µM, antagonist) and then cyclopiazonic acid with (1,4,5)IP3 (100 nM the three upper lighter lines, or 100 µM darker lines). Results show fluorescence as means from 4 repeats within one experiment. (B) Summary shows the concentration-dependent effects of (1,4,5)IP3 on Ca2+ release alone or after preincubation with 4 or 6 (100 µM). Results are means ± s.e.m., n = 3. Summary results in ESI in Table S3.†

Fig. 4 Dimers of 2-O-butyryl-(1,3,4,6)IP4 (5, 7, 9, 11) or (1,2,3,4,6)IP5 (6, 8, 10, 12) are competitive antagonists of IP3R1. (A, B) Experiments similar to those shown in Fig. 3 were used to assess the effects of the indicated concentrations of (1,4,5)IP3 on Ca2+ release from permeabilized DT40-IP3R1 cells after preincubation (2 min) with the indicated dimers (5–12, 100 µM). Results are means ± s.e.m., n = 3. Summary results in Table 1.
O-butyryl-(1,3,4,6)IP_4, the loss of e
(1,3,4,6)IP_4 or (1,2,3,4,6)IP_5 might improve a
K_a(1,4,5)IP_3 without a
(1,4,5)IP_3 relative to (1,4,5)IP_3. Hence, dimerization of 2-O-butyryl-(1,3,4,6)IP_5, to give 5, 7, 9 and 11, successfully reduced efficacy, but without improving affinity (Table 1).

The antagonist 12 is one of three dimers of (1,2,3,4,6)IP_5 (4) with equally high affinity, and it shifted the EC_{50} for (1,4,5)IP_3 by 19.4 ± 6.5-fold, suggesting an apparent K_a of ~7 µM (Fig. 4B). Given the similar affinities of the dimers 8, 10 and 12 (K_a 7-8 µM) in functional assays (Table 1), we examined only 12 in equilibrium competition binding experiments. The K_a value for 12 determined in these experiments (7.7 µM) concurs with the results from functional analyses (Table 1).

These results establish that 8, 10 and 12 are competitive antagonists of IP_3R with low-micromolar affinity. Although modifications of the 6-hydroxyl of (1,4,5)IP_3 reduced affinity, dimerization through the analogous 5-hydroxyls of 3 and 4 caused more modest decreases or increases in affinity, respectively (Table 1). That pattern is similar across the four different linkers used (Fig. 1C). For each linker, dimers of 4 had 2 to 3-fold greater affinity than dimers of 3, even though monomeric 4 has significantly lower affinity than monomeric 3 (Fig. 4, Tables 1 and S2 in ESI†). For dimers of both 3 and 4, the shortest linker (n = 1, Fig. 1C) less effectively increased affinity than did the longer linkers (n = 2–3) (Table 1).

**Conclusions**

There is a need for selective antagonists of IP_3R. Aiming to discover new lead-compounds of this type, a series of novel (1,3,4,6)IP_4 and (1,2,3,4,6)IP_5 homodimers were synthesized following a practical synthetic strategy. Among these homodimers, ligands 8, 10 and 12 were the antagonists with highest affinity for IP_3R (K_a 7-8 µM). 5-Carboxymethyl-(1,4)IP_3 was reported to partially inhibit IP_3-evoked Ca^{2+} release, but only at an extremely high concentration (5 mM). Recently, (1,3,4,5,6)IP_5 is the only other inositol phosphate previously shown to be a competitive antagonist, but it bound to IP_3R1 with lower affinity.

| Table 1 Dimers of 2-O-butyryl-(1,3,4,6)IP_4 and (1,2,3,4,6)IP_5 are competitive antagonists of IP_3R^a |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Ca^{2+} release |                  |                  | Binding         |                  |
|                  | pEC_{50} (µM)  | ΔpEC_{50} (µM)  | EC_{50} (nM)    | Maximal release (%) | n_H  | K_d (µM)  | pK_d (µM)  | K_d (µM)  |
| (1,4,5)IP_3      | 7.03 ± 0.02     | —               | 94              | 70 ± 1           | 1.31 ± 0.18     | —               | 6.90 ± 0.19 | 0.13       |
| +5              | 6.51 ± 0.06*    | 0.51 ± 0.03     | 306             | 65 ± 6           | 0.86 ± 0.17     | 44              |
| +6              | 6.03 ± 0.06*    | 1.00 ± 0.07     | 931             | 58 ± 4           | 1.28 ± 0.08     | 11              |
| +7              | 6.14 ± 0.08*    | 0.89 ± 0.08     | 719             | 59 ± 4           | 1.67 ± 0.57     | 15              |
| +8              | 5.89 ± 0.11*    | 1.14 ± 0.09     | 1300            | 60 ± 5           | 1.49 ± 0.39     | 8               |
| +9              | 6.24 ± 0.07*    | 0.79 ± 0.09     | 571             | 64 ± 4           | 1.63 ± 0.42     | 20              |
| +10             | 5.86 ± 0.06*    | 1.17 ± 0.05     | 1393            | 64 ± 3           | 1.58 ± 0.17     | 7               |
| +11             | 6.27 ± 0.13*    | 0.76 ± 0.15     | 537             | 59 ± 4           | 1.08 ± 0.17     | 21              |
| +12             | 5.84 ± 0.16*    | 1.19 ± 0.18     | 1449            | 60 ± 5           | 2.13 ± 0.55     | 7               |
| +14             | 5.89 ± 0.11*    | 0.76 ± 0.09     | 719             | 64 ± 4           | 1.63 ± 0.42     | 20              |
| +15             | 6.24 ± 0.07*    | 0.79 ± 0.09     | 571             | 64 ± 4           | 1.63 ± 0.42     | 20              |
| +16             | 5.86 ± 0.06*    | 1.17 ± 0.05     | 1393            | 64 ± 3           | 1.58 ± 0.17     | 7               |
| +17             | 6.27 ± 0.13*    | 0.76 ± 0.15     | 537             | 59 ± 4           | 1.08 ± 0.17     | 21              |
| +18             | 5.84 ± 0.16*    | 1.19 ± 0.18     | 1449            | 60 ± 5           | 2.13 ± 0.55     | 7               |

^a Summary results from Fig. 4 show the effects of (1,4,5)IP_3 alone or in the presence of 100 µM of each analog. Results show pEC_{50}, ΔpEC_{50} (pEC_{50} - pEC_{50, antagonist}) and Hill coefficients (n_H) (means ± s.e.m.) and EC_{50} for (1,4,5)IP_3-evoked Ca^{2+} release (n = 3). K_d is shown calculated from functional assays and from equilibrium competition binding experiments (n = 3). Statistical differences were determined by one-way ANOVA and Tukey’s post hoc test, and refer to the results with (1,4,5)IP_3 alone, *P < 0.05.
(K_d ~ 40 µM)⁴⁴ than (1,2,3,4,6)IP_5 (4, K_d ~ 23 µM) and with substantially lower affinity than the dimers of 4. These comparisons are consistent with our observation that 10 µM (1,2,4,5,6)IP_5 had no detectable effect on (1,4,5)IP_3-evoked Ca^{2+} release,⁶ whereas the same concentration of 12 caused a 2.8-fold decrease in (1,4,5)IP_3-sensitivity (not shown). A dimeric benzene with six attached phosphate groups (biphenyl 2,2′,4,4′,5,5′-hexakisphosphate) was recently reported to be a rather high-affinity (K_d ~ 200 nM) antagonist of IP_3R, but it inhibited IP_3-5-phosphatase with very similar potency.⁴⁶ Compounds 8, 10 and 12 are the most potent inositol phosphate-based antagonists of IP_3R so far reported. The affinity of these antagonists for IP_3R1 (K_d ~ 7–8 µM) is comparable to that of heparin (K_d ~ 4 µM),¹⁷ but the new dimeric antagonists are smaller than heparin (M_r ~1200 and ~5000, respectively), and less likely to interact with as many additional intracellular targets. None of these antagonists is membrane-permeant, but based on the versatility of our synthetic approach, it may be feasible to esterify the phosphate groups of the dimeric antagonists to allow them to cross the plasma membrane and then be de-esterified by endogenous intracellular esterases.⁴⁶

Experimental

Chemistry

Materials and methods. All commercially available reagent-grade chemicals and solvents were used without further purification. Dry solvents were prepared by literature methods and stored over molecular sieves. Whenever possible, reactions were monitored using commercially available precoated TLC plates (layer thickness 0.25 mm) of Kieselgel 60F 254. Compounds were visualized by use of a UV lamp and/or phosphomolybdic acid (PMA) or Seebach’s stains upon warming. Column chromatography was performed in the usual way using Merck 60 (40–60 mm) silica gel using as eluents the solvents indicated in each case. Yields are reported for isolated compounds with >96% purity, as established by NMR spectroscopy. FTIR spectra were obtained in a Nicolet 6700 spectrometer. NMR spectra were recorded with a 300 MHz Bruker Avancei spectrometer (¹H: 300 MHz, ¹³C: 75 MHz, ³¹P: 121 MHz) or an Agilent 500/54 spectrometer (¹H: 500 MHz, ¹³C: 126 MHz, ³¹P: 202 MHz) using the deuterated solvent indicated. Chemical shifts are given in parts per million and J values in Hertz using solvent or TMS as an internal reference.

Assignments of protons were confirmed based on 2D NMR experiments (¹H–¹H COSY, HSQC, and HMBC, recorded using a standard pulse-program library). High resolution mass spectra (HRMS) were recorded on micrOTOF GC-MS QP 5050 Shimadzu single-quadrupole mass spectrometer. For each known compound ¹H and/or ¹³C NMR spectra along with HRMS spectra were used to establish identity.

Esterification of malonic acid with 21. Malonic acid (280 mg, 2.69 mmol), and DCC (4.43 g, 21.5 mmol) were successively added to a solution of alcohol 21 (2.69 g, 5.39 mmol) in dry Et_2O (50 mL). The resulting slurry was vigorously stirred under an Ar atmosphere at room temperature for 24 h, while the reaction progress was monitored by TLC. Upon completion, the solvent was removed in vacuo. The residue was triturated with Et_2O and filtered. The solid was further washed with Et_2O (25 mL) and the filtrates were concentrated in vacuo and the residue was purified with flash column chromatography (hexanes/EtOAc 5 : 1 to 2 : 1) to give 1.78 g (62%) of diester 23a and 510 mg (12%) of ureido derivative 24b.

Esterification of succinic acid with 21. Succinic acid (160 mg, 1.36 mmol), DMAP (133 mg, 1.1 mmol), and DCC (1.69 g, 8.2 mmol) were successively added to a solution of alcohol 21 (1.36 g, 2.72 mmol) in dry CH_2Cl_2 (25 mL). The resulting slurry was vigorously stirred under an Ar atmosphere at room temperature for 96 h, while the reaction progress was monitored by TLC. Upon completion, the reaction mixture was washed with H_2O (25 mL) and saturated brine (25 mL). The combined aqueous phases were back-extracted with CH_2Cl_2 (4 × 50 mL), the combined organic phases were dried over Na_2SO_4, and the solvents were removed in vacuo. The residue was purified with flash column chromatography (hexanes/EtOAc 3 : 1 to 1 : 1) to give 820 mg (56%) of diester 23b and 260 mg (12%) of ureido derivative 24c.

General procedure A: preparation of 5,5′-ethers 25a,b. Alcohol 21 (1 mmol) was dissolved in a 4 : 1 mixture of toluene and DMSO (2.5 mL), powdered KOH (140 mg, 2.5 mmol) was added and the mixture was warmed up to 55 ºC. Then, 26 or 27 (0.5 mmol) was added in one portion and the resulting slurry was heated at the same temperature for 120 h, while the progress of the reaction was monitored by TLC. Upon completion, the mixture was neutralized with the addition of a saturated aqueous NH_4Cl solution. Then, water was added to dissolve all solids and the clear solution was extracted with toluene (50 mL) and CH_2Cl_2 (2 × 50 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified with flash column chromatography (hexanes/EtOAc 5 : 1 to 1 : 1) to give ethers 25a,b.

General procedure B: preparation of diols 28. 10% Pd/C (200 mg) was added to a solution of dibenzyl ether 23 or 25 (1 mmol) in MeOH (60 mL). This mixture was vigorously stirred under H_2 (1 atm) at room temperature for 24 h. Then, it was filtered through a pad of Celite®, which was further washed with MeOH (20 mL), CH_2Cl_2 (20 mL), and MeOH (20 mL). Diols 28 were found to be sufficiently pure and used in the next steps without any further purification.

General procedure C: preparation of butyrates 29. Dry Et_2N (0.56 mL, 4 mmol) and DMAP (50 mg, 0.4 mmol) were added to a solution of diol 28 (1 mmol) in dry CH_2Cl_2 (10 mL) under an Ar atmosphere at room temperature. Butyric anhydride (0.50 mL, 3 mmol) was added and the mixture was stirred at room temperature until the full consumption of starting material (TLC monitoring, about 24 h). The reaction mixture was diluted with CH_2Cl_2 (20 mL) and successively washed with saturated aqueous sodium bicarbonate solution (3 × 10 mL) and saturated brine (10 mL). The aqueous phase was back-extracted with CH_2Cl_2 (10 mL) and the combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. The
residue was purified with flash column chromatography (hexanes/EtOAc 7:1 to 1:1) to give butyrates 29.

**General procedure D: removal of acetal protecting groups.** A 90% aqueous solution of TFA (10 mL) was added dropwise to a solution of starting acetal (16d or 28 or 29, 1 mmol) in CH2Cl2 (10 mL) at room temperature. The resulting mixture was stirred at the same temperature for 2 h. Then, the volatiles were removed under reduced pressure (40 °C). The residue was successively treated with toluene (10 mL) and absolute EtOH (3 × 10 mL) and each time the solvent was removed under reduced pressure. The resulting polyl was found to be sufficiently pure by NMR and used in the next step without any further purification.

**General procedure E: phosphorylation of polyols.** A 0.45 M solution of 1H-tetrazole in CH3CN (3 equiv. per OH) was added to a flask containing neat starting polyl (15a or 17a or 30, 1 mmol) under an Ar atmosphere at room temperature. Then, dienyl benzyl N,N-diisopropylphosphoramidite (1.6 equiv. per OH) was added dropwise over a period of 30 min. The resulting mixture was stirred for 24 h at room temperature, and an additional amount of the phosphorylating agent was added (0.3 equiv. per OH). After 24 h the reaction mixture was diluted with CH3Cl2 (10 mL) and cooled to −50 °C. A solution of 70% m-CPBA (2.4 equiv. per OH) in CH2Cl2 (1.6 mL per mmol m-CPBA) was added dropwise and the mixture was left to vigorously stir for 5 h at 0 °C. The reaction mixture was further diluted with CH3Cl2 (120 mL) and successively washed with a 10% aqueous solution of sodium sulfite (2 × 120 mL), a 10% aqueous solution of sodium sulfite (2 × 120 mL), and H2O (120 mL). The combined aqueous phases were back-extracted with CH2Cl2 (120 mL) and successively washed with CH2Cl2 (120 mL) and dried over Na2SO4. The volatiles were removed under reduced pressure. The resulting polyol was found to be efficiently pure by NMR and used in the next step without any further purification.

**General procedure F: final deprotection.** The starting benzyl phosphate (15b or 17b or 31, 1 mmol) was dissolved in EtOH (50–70 mL). Deionized H2O (50–70 mL) and NaHCO3 (1 equiv. per phosphate group) were added. Then, 10% Pd/C (1 g) was added to the resulting emulsion and the mixture was vigorously stirred under H2 (1 atm) at room temperature for the indicated period of time. The reaction progress was monitored by 1H NMR. Upon completion the catalyst was removed by filtration through an LCR/PTFE hydrophilic membrane (0.5 mm); the membrane was washed with a 1:1 mixture of EtOH and deionized H2O (3 × 30 mL). The combined filtrates were evaporated under reduced pressure (55 °C), and the resulting residue was dried under high vacuum for 24 h to yield the desired phosphate salt.

**Analysis.** For each individual experiment, concentration-effect relationships were fitted to a Hill equation using non-linear curve-fitting (GraphPad Prism, version 5). From each experiment, pEC50 or pIC50 [−log of the half-maximally effective (EC50) or inhibitory (IC50) concentration in M], Hill coefficient (nH), and the maximal response were obtained and then used for statistical analyses. All reported comparisons of ligand potencies rely on comparisons within experiments because EC50 values for (1,4,5)IP3-evoked Ca2+ release can vary between passages of cells. For convenience, figures illustrating concentration-effect relations show average results from several experiments, but the values (pEC50, etc.) determined from fitting curves to individual experiments were used for statistical analyses. Most statistical comparisons were paired, and used Student’s t-test or one-way ANOVA with Tukey’s post hoc test as appropriate. P < 0.05 is considered significant.

**Euripidase expression in X. laevis oocytes.** For each experiment, 10 oocytes were loaded with Fura-2 AM (25 µM), incubated for 1 h, and then immediately superfused with an external Ringer’s solution containing (in mM): 125 NaCl, 2.5 KCl, 1.5 MgCl2, 1 CaCl2, 10 HEPES, 5 D-glucose, and 0.25% bovine serum albumin, pH 7.5. After 10 min of superfusion, oocytes were superfused with a 10 mM solution of CPA (100 µM, R&D Systems Europe, Oxford, UK) to inhibit Ca2+ release. CPA was added 2 min before (1,4,5)IP3. Reactions were terminated after 5 min by centrifugation (20,000g, 5 min, 4 °C). The pellet was washed with 700 µL of CLM, resuspended in 200 µL of CLM, and radioactivity was determined by liquid scintillation counting. Culture of Sf9 cells, infection with baculovirus encoding rat IP3R1, and preparation of membranes were as described previously.49 Quantification of IP3R1 expression by Western blotting, using an anti-peptide antiserum to IP3R1 was performed as described.50

**Equilibrium binding of 3H-(1,4,5)IP3 and competing ligands to IP3R1.** These assays were performed at 4 °C in 500 µL of CLM containing 1.5 mM MgATP, membranes (−20 µg protein) prepared from Sf9 cells expressing rat IP3R1 (S9-IP3R1 cells), 3H-(1,4,5)IP3 (1.5 nM, 19.3 Ci per mmol, Perkin Elmer, Waltham, MA, USA) and appropriate concentrations of competing ligand. Non-specific binding was determined by addition of 10 µM (1,4,5)IP3 (Enzo Life Sciences, Exeter, UK). Reactions were terminated after 5 min by centrifugation (20,000g, 5 min, 4 °C). The pellet was washed with 700 µL of CLM, resuspended in 200 µL of CLM, and radioactivity was determined by liquid scintillation counting. Culture of Sf9 cells, infection with baculovirus encoding rat IP3R1, and preparation of membranes were as described previously.49 Quantification of IP3R1 expression by Western blotting, using an anti-peptide antiserum to IP3R1 was performed as described.50

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antagonists from functional assays:

\[
K_d = \frac{[\text{Antagonist}]}{(\text{DR} - 1)}
\]

From equilibrium competition binding experiments, the \(K_d\) of competing ligands was calculated from the concentration (IC\(50\)) required to cause 50% displacement of the specifically bound \(^3\text{H}-(1,4,5)\text{IP}_3\):

\[
K_d = \frac{\text{IC}_{50}}{1 + \left[\frac{[\text{H}-(1,4,5)\text{IP}_3]}{K_d}\right]^{1,4,5}\text{IP}_3}
\]

The \(^3\text{H}-(1,4,5)\text{IP}_3\) was 1.5 nM, and \(K_d^{1,4,5}\text{IP}_3^{127}\text{nM}\) (ESI Table S2). \(pK_d\) values were then used for statistical analyses.\(^{51}\)

For comparisons of differences between \(pEC_{50}\) and \(pK_d\) values (\(pEC_{50} - pK_d\)), the standard deviation of the difference (\(\sigma_{pEC_{50}-pK_d}\)) was calculated from the individual variances (\(\sigma_{pEC_{50}}\) and \(\sigma_{pK_d}\)).\(^{52}\)

\[
\sigma_{pEC_{50}-pK_d} = \sqrt{\sigma_{pEC_{50}}^2 - \sigma_{pK_d}^2}
\]

**Author contributions**

V. K. and J. G. S. contributed equally. A. E. K. initiated the study and supervised the chemistry. C. W. T. designed, interpreted and supervised the biological analyses. V. K. designed, performed and analyzed the biological experiments. J. G. S. and E. D. S. designed the chemical part, performed the synthesis of dimers and interpreted all spectral data. N.-A. T. I. and N. V. P. E. D. S. designed the chemical part, performed the synthesis of monomers. K. C. F. contributed to formulating the initial rationale. All authors have contributed to the manuscript and approved the final version.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Bt</td>
<td>Butyryl</td>
</tr>
<tr>
<td>CLM</td>
<td>Cytosol-like medium</td>
</tr>
<tr>
<td>m-CPBA</td>
<td>m-Chloro-perbenzoic acid</td>
</tr>
<tr>
<td>DCC</td>
<td>(N,N')-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DDQ</td>
<td>2,3-Dichloro-5,6-dicyano-1,4-benzoquinone</td>
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<tr>
<td>DIC</td>
<td>(N,N')-Diisopropylcarbodiimide</td>
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<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>EC(50)</td>
<td>Half-maximal effective (inhibitory) concentration</td>
</tr>
<tr>
<td>IC(50)</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>IP(_2)</td>
<td>Inositol trisphosphate</td>
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<td>IP(_3)R</td>
<td>IP(_3) receptor</td>
</tr>
<tr>
<td>IP(_4)</td>
<td>Inositol tetrakisphosphate</td>
</tr>
<tr>
<td>IP(_5)</td>
<td>Inositol pentakisphosphate (structures of the analogs and their codes are shown in Fig. 1)</td>
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</tbody>
</table>

**PMB** | \(p\)-Methoxybenzyl |
| TFA | Trifluoroacetic acid |

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**Notes and references**

The reaction was closely monitored in order to avoid the oxidative removal of the benzyl group.

We have practically recovered the rest of unreacted starting material (21).

For large scale runs we have found it more convenient to subject the mixture of 23 and 24 to hydrogenolysis (see Scheme 4), since the debensylated derivatives were more easily separable.

Only traces of the corresponding 5-O-butyryl- and 5-O-pentyl-4-aryl-derivatives (elimination products) were detected in the reaction mixture.

For compounds 5 and 6 a rapid H-D exchange of malonic protons in the NMR solvent (D2O) occurs. Therefore, these protons disappear in the 1H NMR spectrum, whereas a quintet is observed in the 13C NMR spectrum for the CD2 group (around 40 ppm). The complete insolubility of these compounds in non-protic solvents did not allow us to run other NMR experiments. The provided data (NMR and HRMS) are consistent with the given structures.