Development of inositol-based antagonists for the D-*myo*-inositol 1,4,5-trisphosphate receptor†‡

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The syntheses of four D-*myo*-inositol 1,4,5-trisphosphate ($Ins P_3$) derivatives, incorporating phosphate bioisosteres at the 5-position, are reported. The methyl phosphate ester and sulfate derivatives retain $Ins P_3$ receptor ($Ins P_3R$) agonist activity; the compounds that possess a methylphosphonate or a carboxymethyl moiety are $Ins P_3R$ antagonists.

D-*myo*-Inositol 1,4,5-trisphosphate ($Ins P_3$, **1**, Fig. 1) is a ubiquitous intracellular second messenger that mediates a plethora of cellular functions *via* interaction with defined receptors ($Ins P_3Rs$).[1,2] These receptors are ligand-gated Ca^{2+}-releasing channels that are located predominantly on the endo- or sarcoplasmic reticulum (ER/SR). The central signalling role of $Ins P_3$ has promoted many chemical syntheses of the natural compound and numerous unnatural derivatives.[3,4] With only a few exceptions,[5–8] these compounds have all displayed full agonist activity at $Ins P_3Rs$. However, to enable chemical dissection of the $Ins P_3$ signalling pathway, the development of selective $Ins P_3R$ antagonists is an essential aim. Although some antagonists of this receptor do exist, these compounds are not selective for the $Ins P_3Rs$ over other Ca^{2+} channels and pumps. For example, 2-aminoethoxydiphenyl borate (2-APB), the most commonly used $Ins P_3R$ antagonist,[9] displays a number of biological actions, including inhibition of SERCA pumps[10] and inhibition of store operated Ca^{2+} entry.[11,12]

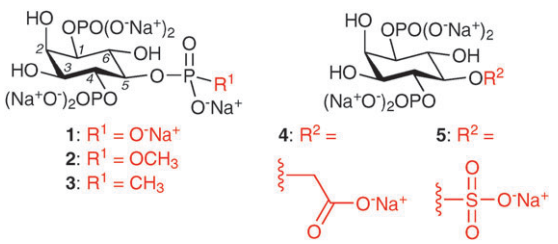

Fig. 1 The structures of $Ins P_3$ (**1**) and its four 5-position derivatives, **2–5**.

Most existing $Ins P_3R$ antagonists are not structurally related to $Ins P_3$; indeed, only one $Ins P_3$ derivative (**3**) has been reported to act as an $Ins P_3R$ antagonist and no biological data were provided for this compound.[7,8] However, it seems probable that $Ins P_3$-based antagonists are most likely to be selective for $Ins P_3Rs$ over other cellular targets. The elucidation of the X-ray crystal structure of the mouse type 1 $Ins P_3R$ in complex with $Ins P_3$ enables the rational design of $Ins P_3R$ ligands.[13] This structure shows that $Ins P_3$ binds between two flexible lobes, the α-domain and the β-domain, leading to proposals of a "clam-shell-like" mode of receptor activation.[14] The 1- and 5-position phosphates of $Ins P_3$ bind to the α-domain, whereas the 4-position phosphate binds predominantly to the β-domain. The structure confirms the findings of previous structure–activity studies that the 4- and 5-position phosphate groups are the most important for $Ins P_3$-receptor interactions.[3] We previously hypothesised that reduction of charge at the 4-position, by introducing a phosphate isostere, would lead to compounds that can bind to the $Ins P_3Rs$. The reduced charge of these compounds would lead to them being unable to evoke the conformational change required for receptor activation, hence they would acts as competitive antagonists.[15] None of the 4-position-modified compounds synthesised exhibited agonist or antagonist activity at $Ins P_3Rs$, leading to the conclusion that the 4-position phosphate group is essential for $Ins P_3R$ binding.[15] Furthermore, we hypothesised that the conformational change undergone by $Ins P_3Rs$ upon $Ins P_3$ binding is modest, and mediated by interaction of the 5-position phosphate of $Ins P_3$ with its receptor. To test this hypothesis, our attention turned to the development of $Ins P_3$ derivatives that contain phosphate bioisosteres at the 5-position (Fig. 1). Again, phosphate isosteres that possess reduced charge, compared to a phosphate group, were utilised. However, more polar isosteres than used previously have been employed, to maximise the likelihood of these compounds binding to the polar $Ins P_3$ binding pocket of the $Ins P_3Rs$.

Herein, we report the synthesis of four 5-position-modified $Ins P_3$ derivatives (Fig. 1). It is shown that, while compounds with a methyl phosphate ester (**2**) or a sulfate group (**5**) at the 5-position retain $Ins P_3R$ agonist activity, compounds with a methylphosphonate (**3**) and a carboxymethyl (**4**) derivative act as $Ins P_3R$ antagonists.

The synthesis of compounds **3–5** commenced from *myo*-inositol, and employed a well-established sequence of

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‡ Electronic supplementary information (ESI) available: Additional biological data, compound characterisation data and NMR spectra for compounds **2–5**. See DOI: 10.1039/c0cc03003a

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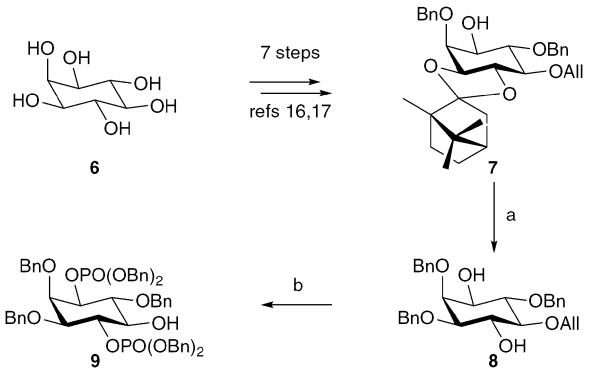
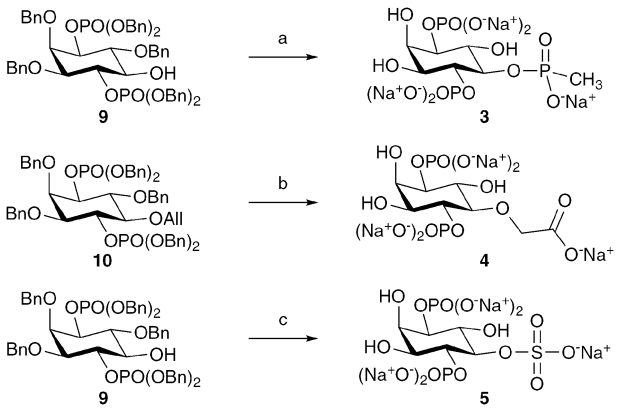


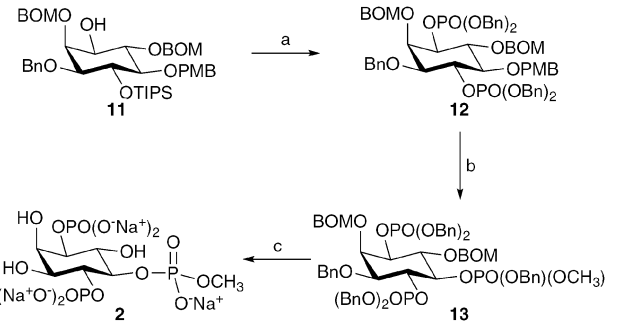
Scheme 1 The synthesis of the key intermediate **9**. *Reagents and conditions:* a. i. AcCl, MeOH, CH_2Cl_2, 83%; ii. Bu_2SnO, BnBr, TBABr, 3 Å molecular sieves, MeCN, 79%; b. i. 1. $(BnO)_2PN^iPr_2$, 1*H*-tetrazole, CH_2Cl_2; 2. *m*CPBA, CH_2Cl_2, 81%; ii. $PdCl_2$, MeOH, 80–84%.

transformations to afford the chiral, non-racemic, camphor derivative **7** (Scheme 1).[16,17] Removal of the chiral auxiliary under acidic conditions followed by treatment with dibutyltin oxide and benzyl bromide furnished the desired 3-*O*-benzyl regioisomer (**8**) in high yield. Phosphitylation and oxidation of the 1- and 4-position hydroxyl groups gave the protected bisphosphate. The allyl group was removed by treatment with $PdCl_2$ in methanol, yielding the key intermediate **9**. The methyl phosphonate was installed using conditions reported by Dreef *et al.* (Scheme 2).[7,8] In our hands, this reaction was capricious and the greatest success was achieved when using freshly prepared reagents and rigorously dry conditions. Hydrogenolysis in the presence of sodium bicarbonate afforded the methyl phosphonate **3** as its presumed pentakis sodium salt. The carboxymethyl derivative **4** was synthesised by cleavage and oxidation of the allyl group,[18,19] followed by hydrogenolysis as before. The sulfate **5** was afforded by reaction of **9** with sulfur trioxide in pyridine, followed by hydrogenolysis.

The methyl phosphate ester **2** was synthesised from intermediate **11**, which was prepared as described previously.[20] Deprotection of



Scheme 2 The synthesis of the 5-position-modified InsP_3 derivatives **3–5**. *Reagents and conditions:* a. i. 1. Bis[6-(trifluoromethyl)benzotriazol-1-yl] methylphosphonate, pyridine; 2. BnOH, 36–60%; ii. Pd black, H_2, ^{t}BuOH, H_2O, $NaHCO_3$, 93%. b. i. $RuCl_3{\cdot}H_2O$, $NaIO_4$, CCl_4, MeCN, H_2O, 44%; ii. Pd black, H_2, ^{t}BuOH, H_2O, $NaHCO_3$, 98%. c. i. SO_3, pyridine, 52%; ii. ^{t}BuOH, H_2O, $NaHCO_3$, 94%.



Scheme 3 The synthesis of the 5-position-modified InsP_3 derivative **2**. *Reagents and conditions:* a. i. TBAF, THF, 89%; ii. 1. $(BnO)_2PN^iPr_2$, 1*H*-tetrazole, CH_2Cl_2; 2. *m*CPBA, CH_2Cl_2, 89%; b. i. CAN, MeCN, H_2O, 75%; ii. 1. $(BnO)(OCH_3)PN^iPr_2$, 1*H*-tetrazole, CH_2Cl_2; 2. *m*CPBA, CH_2Cl_2, 92%; c. Pd black, H_2, ^{t}BuOH, H_2O, $NaHCO_3$, 51%.

the TIPS group, using TBAF, furnished a 1,4-diol. This compound was phosphitylated and oxidised to give the protected bisphosphate **12** (Scheme 3). Oxidative cleavage of the PMB ether, followed by phosphitylation and oxidation, gave the fully protected methyl phosphate ester **13**. Hydrogenolysis, as above, furnished **2** as the presumed pentakis sodium salt.

The ability of **2–5** to mobilise Ca^{2+} or inhibit InsP_3-induced Ca^{2+} release (IICR) was investigated using either a unidirectional $^{45}Ca^{2+}$ flux assay in permeabilised cells or a Ca^{2+} fluorescence assay in sea urchin egg homogenate. In the $^{45}Ca^{2+}$ assay, either MEF cells or L15 cells were employed.¶ Using the $^{45}Ca^{2+}$ assay in MEF cells, the methyl phosphate ester (**2**) acts as an InsP_3R agonist, with an EC_{50} = 9.7 μM (InsP_3 EC_{50} = 5.2 μM in the same system, see ESI‡). The sulfate **5** also acts as a weak InsP_3R agonist, evoking InsP_3R-mediated Ca^{2+} release when applied at 100 and 300 μM in permeabilised L15 cells (see ESI‡). The methylphosphonate **3** showed little activity when applied to permeabilised L15 cells alone. However, application of **3** (300 μM) before and during application of InsP_3 (0.25 μM) caused approximately 40% reduction in the amount of IICR (Fig. 2).

The biological activity of **3** and **4** was also investigated using the sea urchin egg homogenate-based assay.[21–23]‖ In this assay both **3** and **4** behaved as InsP_3R antagonists (Fig. 3). The methylphosphonate (**3**) gave 37% inhibition of IICR when applied at a concentration of 1.67 mM. The carboxymethyl derivative **4** demonstrated 58% inhibition of IICR when applied at a concentration of 5.00 mM. In both cases, the compounds evoked no Ca^{2+} release when applied in the absence of InsP_3. Additionally, preliminary data (not shown) indicate that **4** has no significant effect on cyclic adenosine diphosphate ribose-induced Ca^{2+} release, which occurs *via* ryanodine receptors. These data indicate that **4** is acting at InsP_3Rs and not non-selectively at other Ca^{2+} channels or pumps. Taken together, our data show that **3** and **4** are acting as InsP_3R antagonists. These data represent the first conclusive proof that InsP_3 derivatives can act as antagonists at InsP_3Rs.

The structural similarity of the methyl phosphate ester **2** and the methylphosphonate **3**, which differ by one oxygen atom yet possess opposing activity at InsP_3Rs, potentially provides unique insight into the mechanism of InsP_3R activation and inhibition. Our current hypothesis is that all compounds bind

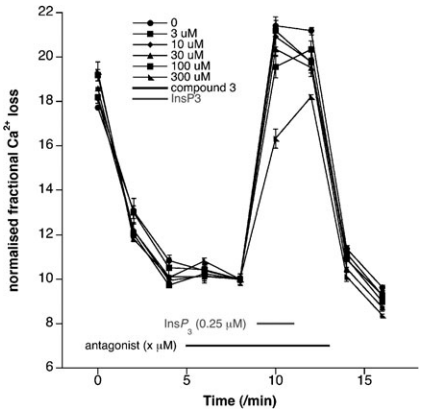


Fig. 2 Fractional loss plots of $^{45}Ca^{2+}$ in L15 cells when treated with the methylphosphonate analogue **3**. The cells were permeabilised, leading to an initial loss of $^{45}Ca^{2+}$ (0–3 min). Application of InsP_3 evokes a large loss of $^{45}Ca^{2+}$ as a result of InsP_3R activation. This loss of $^{45}Ca^{2+}$ is reduced by ~40% in the presence of the methylphosphonate **3** (300 μM), indicating that this compound is behaving as an InsP_3R antagonist.

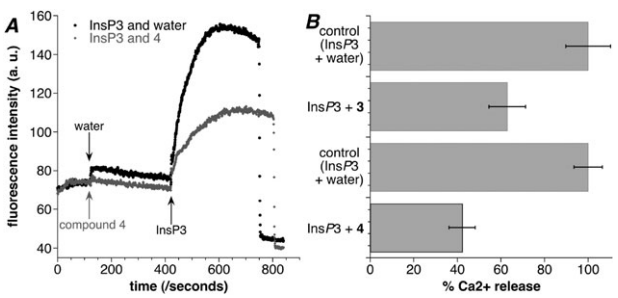


Fig. 3 Compounds **3** and **4** inhibit IICR. (A) A representative trace obtained from sea urchin egg homogenate loaded with the fluorescent Ca^{2+} dye Fluo-3. Application of InsP_3 (400 nM) evokes an increase in fluorescence due to Ca^{2+} release from InsP_3Rs (black trace). Application of compound **4** (5.00 mM) alone does not cause Ca^{2+} release, however, application of InsP_3 (400 nM) in the presence of **4** evokes significantly reduced Ca^{2+} release, demonstrating that **4** is an InsP_3R antagonist (grey trace). (B) A comparison of the Ca^{2+} release evoked by InsP_3 alone and in the presence of compound **3** or compound **4**. The Ca^{2+} release caused by InsP_3 (400 nM) alone is taken as 100% (top bar). When InsP_3 (400 nM) and compound **3** (1.67 mM) were applied to the sea urchin egg homogenate, a smaller increase in fluorescence (63%, $n = 4$) was observed (middle bar), consistent with reduced Ca^{2+} release from InsP_3Rs. When InsP_3 and compound **4** (5.00 mM) were applied a similarly reduced increase in fluorescence was seen (42%, $n = 4$, bottom bar). These data show that both **3** and **4** are acting as InsP_3R antagonists. Error bars show SEM.

to the InsP_3R ligand-binding domain in a similar orientation to InsP_3. The methyl phosphate ester (**2**) and the sulfate (**5**) derivatives have the same number of atoms as a phosphate group (such as in InsP_3) from which to make polar contacts. However, the methylphosphonate (**3**) and the carboxylate (**4**) have one less polar atom at the 5-position. It is possible that this reduced interaction with the receptor renders the compounds unable to evoke the conformation change required for InsP_3R activation, and hence **3** and **4** act as competitive antagonists at InsP_3Rs.

In conclusion, four InsP_3 derivatives with phosphate bioisosteres at the 5-position have been synthesised. Biological evaluation of these compounds has revealed the methyl phosphate ester (**2**) and sulfate (**5**) derivatives retain InsP_3R agonist activity. However, the methylphosphonate (**3**) and carboxymethyl (**4**) derivatives behave as InsP_3R antagonists. These studies provide the first proof that InsP_3 derivatives can act as antagonists at InsP_3Rs. The compounds developed herein will likely prove useful tools for the study of InsP_3Rs and provide new insight into the mechanism of InsP_3R activation and inhibition.

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Notes and references

¶ MEF cells are immortalised wild-type mouse embryonic fibroblast cells expressing normal levels of InsP_3R1 and InsP_3R3. L15 cells are mouse L-fibroblast cells stably over-expressing InsP_3R1 by ~8–10 fold.
‖ Sea urchin eggs possess only one subtype of InsP_3R.

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