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Article

Both D- and L-Glucose Polyphosphates Mimic D-*myo*-Inositol 1,4,5-Trisphosphate: New Synthetic Agonists and Partial Agonists at the Ins(1,4,5)P_3 Receptor

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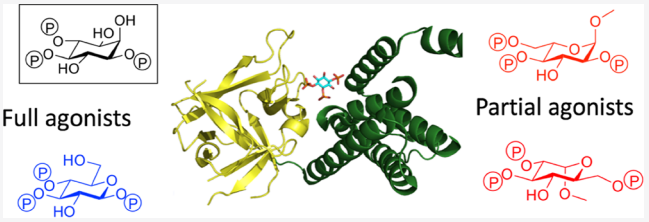
ABSTRACT: Chiral sugar derivatives are potential cyclitol surrogates of the Ca^{2+}-mobilizing intracellular messenger D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. Six novel polyphosphorylated analogues derived from both D- and L-glucose were synthesized. Binding to Ins(1,4,5)P_3 receptors [Ins(1,4,5)P_3R] and the ability to release Ca^{2+} from intracellular stores via type 1 Ins(1,4,5)P_3Rs were investigated. β-D-Glucopyranosyl 1,3,4-trisphosphate, with similar phosphate regiochemistry and stereochemistry to Ins(1,4,5)P_3, and α-D-glucopyranosyl 1,3,4-trisphosphate are full agonists, being equipotent and 23-fold less potent than Ins(1,4,5)P_3, respectively, in Ca^{2+}-release assays and similar to Ins(1,4,5)P_3 and 15-fold weaker in binding assays. They can be viewed as truncated analogues of adenophostin A and refine understanding of structure-activity relationships for this Ins(1,4,5)P_3R agonist. L-Glucose-derived ligands, methyl α-L-glucopyranoside 2,3,6-trisphosphate and methyl α-L-glucopyranoside 2,4,6-trisphosphate, are also active, while their corresponding D-enantiomers, methyl α-D-glucopyranoside 2,3,6-trisphosphate and methyl α-D-glucopyranoside 2,4,6-trisphosphate, are inactive. Interestingly, both L-glucose-derived ligands are partial agonists: they are among the least efficacious agonists of Ins(1,4,5)P_3R yet identified, providing new leads for antagonist development.



INTRODUCTION

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3, **1**] is a second messenger that binds to tetrameric D-*myo*-inositol 1,4,5-trisphosphate receptors [Ins(1,4,5)P_3Rs] on the endoplasmic reticulum. Ins(1,4,5)P_3Rs are Ca^{2+} channels that open to release Ca^{2+} to the cytosol.[1,2] The resulting local or global increases in cytosolic Ca^{2+} concentration regulate diverse cellular processes, including mitochondrial metabolism, cell proliferation, differentiation, smooth muscle contraction, secretion, exocytosis, and ion channel opening.[3]

Ins(1,4,5)P_3 (Figure 1a) binds to the Ins(1,4,5)P_3-binding core (IBC; residues 224−604) close to the N terminus of each of the four Ins(1,4,5)P_3R subunits (Figure 1b,c). The IBC consists of an α-helical domain and a β-trefoil domain between which there is a cleft rich in basic amino acid residues.[4] Ins(1,4,5)P_3 binding within the cleft allows phosphates at positions 1 and 5 to interact with the α-domain, while the 4-phosphate interacts with the β-domain (Figure 1c).[5] As Ins(1,4,5)P_3 interacts with both domains, it pulls the two sides of the clam-like IBC together.[6,7] The clam closure leads to channel opening, possibly by rearranging Ca^{2+}-binding sites such that Ca^{2+} can bind to the Ins(1,4,5)P_3R and trigger conformational changes that lead to opening of the Ca^{2+}-permeable pore.[1,6,7]

Modulators of Ins(1,4,5)P_3R activity are highly sought after, and many studies have examined structure−activity relationships (SARs) of ligands binding to Ins(1,4,5)P_3R, attempting to identify partial agonists or antagonists.[8−10] Synthetic analogues have played a key role in this process, including those of the potent glyconucleotides, the adenophostins[5,11] (Figure 1d), with a recent synthetic study reporting the effects of replacing the glucose moiety of adenophostin A (AdA) with a *chiro*-inositol core.[12] This also highlights developing interest in examining less explored isomers of inositol than the *myo*-form.[13]

The phosphates attached to the 4- and 5-positions of Ins(1,4,5)P_3 (Figure 1a) are thought to be essential to agonist activity as each interacts with a different domain of the IBC.[4,14] The 1-phosphate increases affinity, but it is not essential for receptor activation.[5] The hydroxyl group attached to the 6-position of Ins(1,4,5)P_3 appears to be important for optimal

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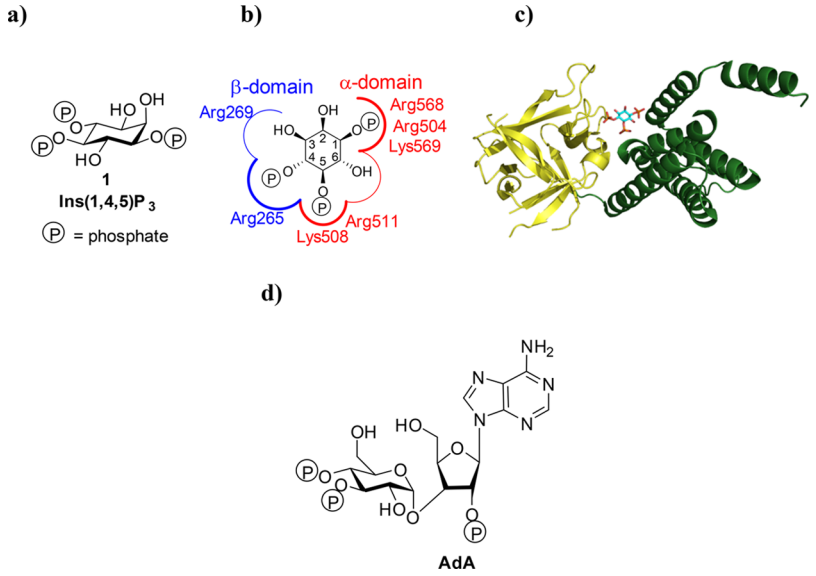

Figure 1. Structures of the Ins(1,4,5)$_3$R IBC and its agonists. (a) Structure of Ins(1,4,5)P_3 (**1**). (b) Binding mode of **1** to the IBC of Ins(1,4,5)P_3R with key amino acid residues involved in binding labeled. (c) Crystal structure of the IBC of type 1 InsP_3R with Ins(1,4,5)P_3 (**1**) bound (PDB: 1N4K, file available in the Associated Content)[4]. The α-domain is shown in green and the β-domain in yellow. (d) Structure of the Ins(1,4,5)P_3R agonist adenophostin A (AdA).

activity but it is not essential,[15] while hydroxyl groups attached to the 2- and 3-positions are less involved in ligand binding.[16]

A few Ins(1,4,5)P_3R antagonists have been identified, but these suffer major drawbacks including poor target selectivity, cell impermeability (heparin),[17] inconsistent effectiveness in assays (2-aminoethoxydiphenylborane, 2-APB),[17] low potency (caffeine),[17,18] and disputed activity (xestospongins).[17] For decades, analogues of Ins(1,4,5)P_3 have been synthesized and investigated in attempts to discover a selective antagonist for Ins(1,4,5)P_3R that could be made, at least temporarily, cell permeable with enzyme-cleavable or photolabile protecting groups.[19,20] Very recently, there has also been work carried out by Li et al. that describes the delivery of inositol phosphates into cells via lysosomes, rendering phosphate protecting groups unnecessary.[21] To date, however, only a small number of analogues of Ins(1,4,5)P_3 with minor structural modifications have been identified as partial agonists or antagonists at Ins(1,4,5)P_3R. Most of these compounds demonstrate that conservative modifications to the phosphates attached to positions 4 and 5 and the hydroxyl group attached to position 3 can lead to degrees of antagonist activity.[22−25] However, many analogues of Ins(1,4,5)P_3 with modifications to the same regions are inactive.[26] Attaching a bulky substituent to the axial 2-position hydroxyl can also lead to partial agonist activity,[27] and interestingly, even using a simple benzene ring as a surrogate for inositol in a benzene polyphosphate approach, as a dimer or biphenyl, can provide low-affinity antagonists.[28,29]

Stimulated in part by the discovery of the adenophostins,[5,10,30] there have been a number of studies to investigate polyphosphates of D-glucose and of other sugars[30−35] as inositol phosphate analogues. By using such carbohydrates, the need for optical resolution of protected cyclitol precursors or resulting phosphate regioisomers is bypassed as chiral starting materials are readily available. Also, the structural features of carbohydrates offer additional opportunities for synthetic versatility. In this study, we return to and expand upon the use of D-glucose to try to identify novel Ins(1,4,5)P_3R ligands. We also investigate, perhaps counterintuitively, the use of L-glucose as a starting material.

Although several studies have generated D-glucose-based ligands of Ins(1,4,5)P_3R,[30,31,34] no ligands based on the L-glucose enantiomer have been synthesized and nor is it known whether ligands with this scaffold would bind to Ins(1,4,5)P_3R. In a previous study,[5] Ins(4,5)P_2 [albeit a low-affinity Ins(1,4,5)P_3R ligand] was effectively mimicked by a D-Gluc(3,4)P_2 surrogate. We noted that both D- and L-glucose offer three hydroxyl groups of the requisite relative configuration that could, in principle, be used to mimic the 4,5,6-hydroxyl groups in *myo*-inositol. Thus, we anticipated that we could use this similarity (Figure 2, highlighted in red),

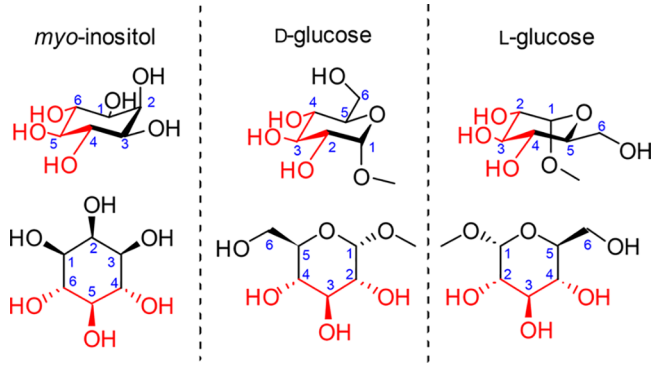

Figure 2. Structures of methyl α-D-glucopyranoside and methyl α-L-glucopyranoside relative to *myo*-inositol with the shared stereochemistry of the hydroxyl groups highlighted in red.

alongside intrinsic structural differences of L- and D-glucose, to prepare diverse chiral ligands with appropriately located phosphates. These would present different structural motifs to the Ins(1,4,5)P_3R and allow further investigation of the binding site and perhaps identify novel activity. With this in mind, we designed and synthesized six novel ligands based on

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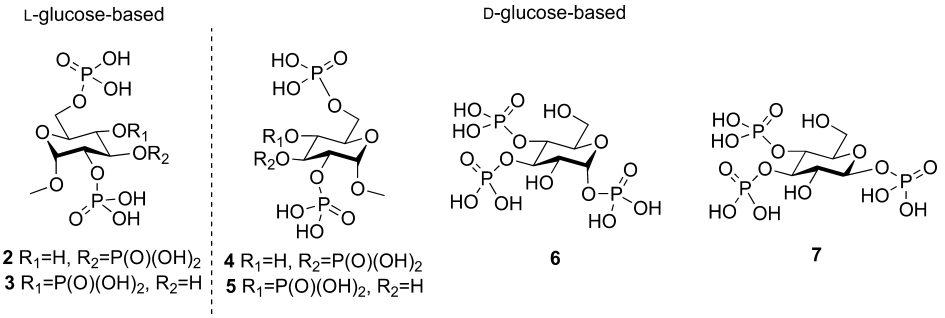
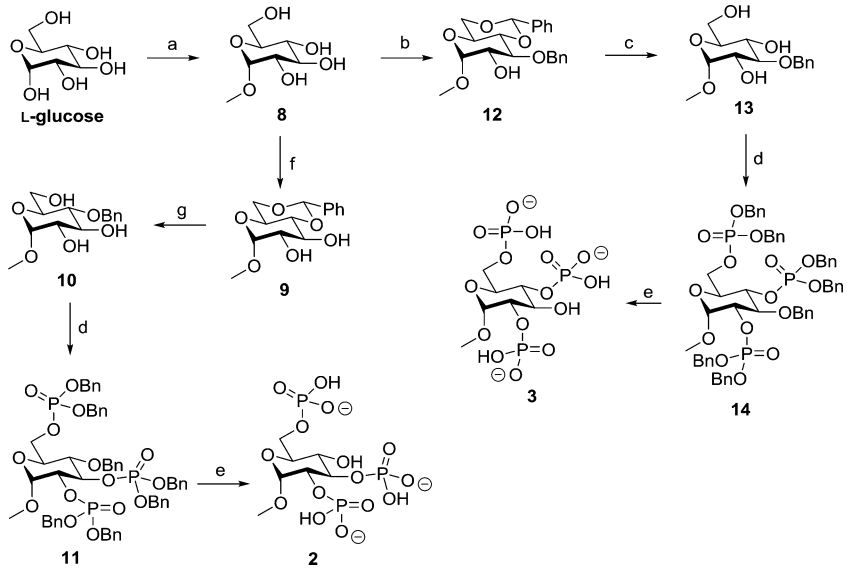


Figure 3. Structures of Ins(1,4,5)P_3 (**1**) analogues: methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) and methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**), methyl α-D-glucopyranoside 2,3,6-trisphosphate (**4**) and methyl α-D-glucopyranoside 2,4,6-trisphosphate (**5**), α-D-glucopyranosyl 1,3,4-trisphosphate (**6**), and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**).

Scheme 1. Synthesis of L-Glucose-Derived Ligands: Methyl α-L-Glucopyranoside 2,3,6-Trisphosphate (2) and Methyl α-L-Glucopyranoside 2,4,6-Trisphosphate (3)[a]



[a]Reagents and conditions: (a) MeOH, AcCl, reflux, 5 days; (b) (1) TMSCl, py, 22 h and (2) DCM, benzaldehyde, $FeCl_2 \cdot 6H_2O$, MeCN, triethylsilane, 0 °C to room temperature, 1.5 h; (c) MeOH, H_2O, 1 M $HCl_{(aq)}$, reflux, 3 h; (d) (1) DCM, 5-phenyl-1*H*-tetrazole, $(BnO)_2PN(^iPr)_2$, 20 h and (2) mCPBA, −78 °C to room temperature; (e) MeOH/H_2O (10:1 v/v), cat. $Pd(OH)_2$/C, H_2, 24 h; (f) MeCN, benzaldehyde dimethyl acetal, cat. CSA, 24 h; (g) BH_3/THF, $La(Tf)_3$, 7 days.

L-glucose and D-glucose (Figure 3) and evaluated their activity at Ins(1,4,5)P_3R.

We ensured that the designed ligands retained structures equivalent to the critical 4,5-bisphosphate motif of Ins(1,4,5)P_3 and had no major structural modifications in regions believed to be necessary for ligand binding; modifications in regions equivalent to the 2-*O*-position were permitted as these were expected to remain outside the binding pocket. We hypothesized that the L-glucose-based ligands [methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) and methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**)] might have sufficiently conservative structural changes relative to Ins(1,4,5)P_3 such that they could still bind to Ins(1,4,5)P_3R, possibly with novel activity. The D-glucose-based ligands [methyl α-D-glucopyranoside 2,3,6-trisphosphate (**4**) and methyl α-D-glucopyranoside 2,4,6-trisphosphate (**5**)] were not expected to adopt orientations that position the phosphate groups in appropriate regions of the IBC (see the Supporting Information SI-1, S1 for details of all possible predicted binding modes). Their bioassay was designed to enable confirmation of receptor enantioselectivity. From a practical standpoint, the synthesis of ligands **4** and **5** was optimized first with D-glucose before the commercially available, but considerably more costly, L-glucose was used to make the respective L-enantiomers, **2** and **3**. We also anticipated that the two ligands with phosphates at the anomeric carbon, α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**), would help to elucidate the basis for the high affinity of AdA.[5,12,36] We expected both novel truncated analogues to be agonists due to their structural similarity to Ins(1,4,5)P_3 but were interested to compare their activities with AdA and the previously analyzed truncated analogue Gluc(3,4)P_2.[5]

RESULTS

Chemistry. Methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) was synthesized in a five-step route from readily available L-glucose (Scheme 1). Refluxing L-glucose in an acidic methanol solution resulted in the protection of the anomeric hydroxyl with a methyl group. Multiple recrystallizations from ethanol afforded methyl α-L-glucopyranoside **8** in 45% yield.

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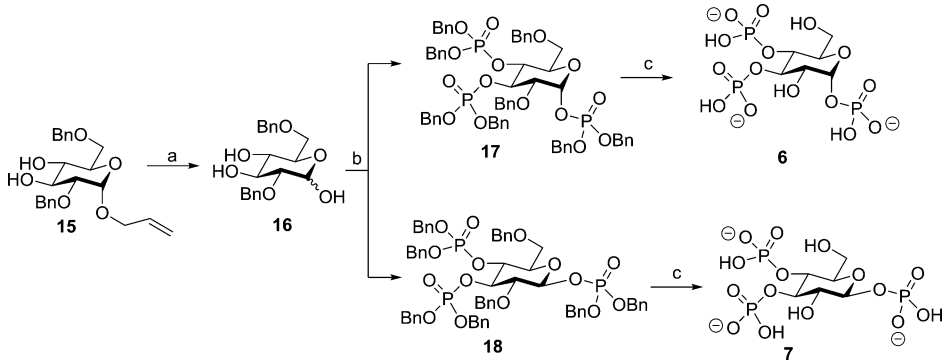
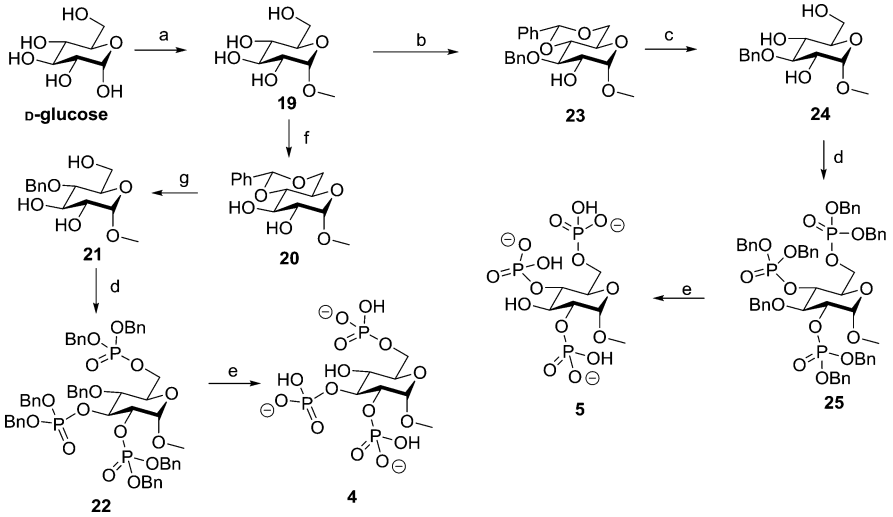
Scheme 2. Synthesis of Epimeric Ligands with a Phosphate Group at the Anomeric Carbon: α-D-Glucopyranosyl 1,3,4-Trisphosphate (6) and β-D-Glucopyranosyl 1,3,4-Trisphosphate (7)[a]



[a]Reagents and conditions: (a) MeOH, cat. $PdCl_2$, 6 h; (b) (1) DCM, 5-phenyl-1*H*-tetrazole, $(BnO)_2PN(^iPr)_2$, 20 h and (2) mCPBA, −78 °C to room temperature; (c) MeOH/H_2O (10:1 v/v), cat. $Pd(OH)_2/C$, H_2, $NaHCO_3$, 24 h.

Scheme 3. Synthesis of D-Glucose-Derived Ligands: Methyl α-D-Glucopyranoside 2,3,6-Trisphosphate (4) and Methyl α-D-Glucopyranoside 2,4,6-Trisphosphate (5)[a]



[a]Reagents and conditions: (a) MeOH, reflux, 5 days; (b) (1) TMSCl, py, 22 h and (2) DCM, benzaldehyde, $Fe(II)Cl_2·6H_2O$, MeCN, triethylsilane, 0 °C to room temperature, 1.5 h; (c) MeOH, H_2O, 1 M $HCl_{(aq)}$, reflux, 3 h; (d) (1) DCM, 5-phenyl-1*H*-tetrazole, $(BnO)_2PN(^iPr)_2$, 20 h and (2) mCPBA, −78 °C to room temperature; (e) MeOH/H_2O (10:1 v/v), cat. $Pd(OH)_2/C$, H_2, 24 h; (f) MeCN, benzaldehyde dimethyl acetal, cat. CSA, 24 h; (g) BH_3/THF, $La(Tf)_3$, 7 days.

The 4- and 6-position hydroxyls were protected with a benzylidene group to form **9** in 91% yield. This benzylidene group was reduced regioselectively, opening to form methyl 4-*O*-benzyl-α-D-glucopyranoside (**10**). The benzylidene reduction was first attempted with borane/THF and $AlCl_3$, but this was found to be insufficiently regioselective and produced inseparable regioisomers. The reaction was successfully carried out with borane/THF and $La(Tf)_3$ following the method of Shie et al.[37] to yield **10** in 31% yield following purification. The hydroxyl groups in triol **10** were then phosphitylated with dibenzyl diisopropylphosphoramidite, and subsequent oxidation with mCPBA formed **11** in 86% yield. The benzyl protecting groups on phosphates and *O*-4 were then removed by stirring a solution of **11** with Pearlman's catalyst under hydrogen overnight. After filtration to remove the catalyst and evaporation of the solvent, **2** was collected as the triethylammonium salt in 53% yield.

Methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**) was also synthesized in five steps from L-glucose, diverging from the synthesis of **2** after the initial methylation step. Methyl α-L-glucopyranoside (**8**) was protected in a regioselective one-pot reaction that involved persilylation followed by $FeCl_2$-catalyzed benzylidene protection as described by Bourdreux et al.[38] to yield **12** in 79% yield. The acid-labile benzylidene group was removed through reflux with $HCl_{(aq)}$ to form **13** in 93% yield. Phosphorylation using the standard phosphoramidite methodology[39] was then employed to give **14** in 40% yield. After debenzylation with hydrogen and Pearlman's catalyst and filtration and evaporation of the solvent, the final product, **3**, was collected as its triethylammonium salt in 90% yield.

Both α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**) were synthesized via a divergent route, starting with allyl 2,6-di-*O*-Bn-α-D-glucopyranoside (**15**) (Scheme 2).[34] Palladium chloride-catalyzed deallylation yielded **16**, although purification of this compound was found to be very difficult at this step and purification after phosphorylation proved to be much more effective. Thus, slightly impure **16** was phosphorylated to yield a pure, partially

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separable mixture of the epimeric phosphates **17** and **18** (56% yield total: 14% **17**, 19% **18**, and 23% mixed epimers). It was unclear how stable **17** and especially the phosphorylated β-epimer **18** would be as there are reports of compounds with phosphate groups at the anomeric carbon atom being unable to survive purification by silica column chromatography in some cases and not in others.[40−42]

We found in this case that both compounds survived silica gel chromatography, although **18** showed slight degradation by ^{31}P NMR over time (approx. 30% after 3 weeks at 4 °C). Catalytic hydrogenolysis of both compounds **18** and **19** was carried out in the presence of sodium bicarbonate to prevent acidic hydrolysis of the potentially labile C-1 phosphates. The final products **6** and **7** were purified by reversed-phase ion-pair chromatography and collected as their triethylammonium salts.

Methyl α-D-glucopyranoside 2,3,6-trisphosphate (**4**) and methyl α-D-glucopyranoside 2,4,6-trisphosphate (**5**) were synthesized using the same methods as described for their enantiomers (**2** and **3**), starting the route with D-glucose (Scheme 3).

The relative stabilities of α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**) were first investigated by allowing each compound (as triethylammonium salts) to remain in a solution of D_2O at room temperature at pH 7. Over the course of 2 months, neither isomer showed any sign of degradation. Following this, a mixture of the compounds in a known starting ratio was exposed to increasingly harsh conditions, and relative isomer degradation was monitored through ^{1}H NMR spectroscopy (see the Supporting Information, SI-1 Figure S5). From the results of the hydrolysis study, we determined that the β-epimer (**7**) degraded more readily than the corresponding α-epimer (**6**). Both compounds were, however, surprisingly durable and required strongly acidic conditions to be fully hydrolyzed (i.e., at pH 1 for 1 day), while strongly basic conditions (i.e., at pH 14 for 1 day) only produced limited degradation (and at pH 10 for a week, there was no change). We are, therefore, confident that both compounds remained intact during the near-neutral conditions of the biological assays. All final compounds were assessed by HPLC for purity (SI-1 Figure S6), and products from acid hydrolysis were examined by HPLC using a synthetic standard of D-glucose 3,4-bisphosphate[5] to confirm the expected hydrolysis product of both compounds (see the Supporting Information, SI-1 Figure S7).

Biology. Permeabilized HEK-Ins(1,4,5)P_3R1 cells were used to determine the ability of compounds **2**−**7** (with Ins(1,4,5)P_3 and AdA as controls) to evoke Ca^{2+} release from intracellular stores (Figure 4 and Table 1). Maximally effective concentrations of Ins(1,4,5)P_3, α-D-glucopyranosyl 1,3,4-trisphosphate (**6**), or β-D-glucopyranosyl 1,3,4-trisphosphate (**7**) released the same fraction (ca. 80%) of the intracellular Ca^{2+} stores, suggesting that these two epimeric compounds are both full agonists (Figure 4). Compound **7** was equipotent with Ins(1,4,5)P_3, and **6** was ca. 20 times less potent than Ins(1,4,5)P_3.

The L-glucose-based ligands methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) and methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**) were much less potent than Ins(1,4,5)P_3 (Figure 4), while their enantiomers methyl α-D-glucopyranoside 2,3,6-trisphosphate (**4**) and methyl α-D-glucopyranoside 2,4,6-trisphosphate (**5**) were, as predicted, inactive. The Ca^{2+} release evoked by maximally effective concentrations of **2** or **3** was only ca. 70% of that evoked by Ins(1,4,5)P_3, suggesting that **2** and **3** are partial agonists. Since partial agonists bind to Ins(1,4,5)P_3Rs but activate them less effectively than full agonists, a partial agonist must bind to more Ins(1,4,5)P_3Rs than a full agonist to evoke comparable Ca^{2+} release. We performed equilibrium competition binding assays using [^{3}H]-Ins(1,4,5)P_3 and the active ligands to examine relationships between ligand binding and functional responses. The affinities of **6** and **7** for Ins(1,4,5)P_3R aligned with their potencies in functional assays, with **7** having an affinity indistinguishable from that of Ins(1,4,5)P_3, while **6** had ca. 15-fold lower affinity (Figure 5 and Table 1). The EC_{50}/K_d values for Ins(1,4,5)P_3, AdA, **6**, and **7** were similar, consistent with each being a full agonist (Table 1). Comparison of the concentrations of **2** and **3** required to occupy 50% of binding sites (K_d) and to evoke release of 39% of the Ca^{2+} stores (EC_{39}, i.e., the Ca^{2+} release evoked by a half-maximally effective Ins(1,4,5)P_3 concentration) confirmed that **2** and **3** are weak partial agonists: their EC_{39}/K_d values (132 and 462, respectively) were much greater than that of Ins(1,4,5)P_3 (17). HPLC was used to confirm the purity of the compounds used in the biological assays (details in the Supporting Information SI-1, S6).

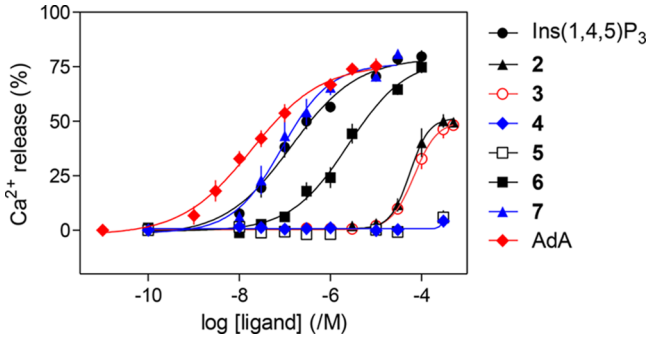

Figure 4. Concentration-dependent effects of Ins(1,4,5)P_3 and related ligands on Ca^{2+} release from intracellular stores of permeabilized HEK-Ins(1,4,5)P_3R1 cells. Results are means ± SEM from 5 to 11 independent experiments, each with duplicate determinations.

DISCUSSION

Of the glucose polyphosphates considered in this study, the two that bound to Ins(1,4,5)P_3R with the highest affinity were α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**). Both compounds, which can be considered as truncated analogues of adenophostin A (AdA, Figure 6),[44] were found to be full agonists of Ins(1,4,5)P_3R, and the β-epimer (**7**) was equipotent with Ins(1,4,5)P_3.

Compounds containing a phosphate group attached to the anomeric carbon atom, as featured in **6** and **7**, have not been previously investigated as Ins(1,4,5)P_3R ligands, presumably due to concerns over their stability, at least in the case of the β-epimer.[31] Nevertheless, we found that both α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**) were surprisingly durable as their triethylammonium salts and neither compound showed signs of degradation after 2 months in neutral aqueous solution at room temperature. Both **6** and **7** were eventually degraded under strongly acidic conditions, and HPLC traces were taken to confirm their hydrolysis to glucose 3,4-bisphosphate (details in the Supporting Information SI-1, S7).

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Table 1. Ins(1,4,5)P_3R Binding and Ca^{2+} Release Mediated by Ins(1,4,5)P_3, AdA, and Compounds 2−7[a]

	Ca^{2+} release			binding			
ligand	pEC_{50}; EC_{50}	release (%)	h	pK_d; K_d (nM)	h	EC_{50}/K_d	EC_{39}/K_d[b]
Ins(1,4,5)P_3	6.90 ± 0.12; 126 nM	78.8 ± 1.3	0.7 ± 0.1	8.06 ± 0.03; 8.7	1.1 ± 0.2	14 (5−46)	17 (5−63)
2	4.06 ± 0.09[c]; 87.7 μM	56.2 ± 2.6[c]	1.4 ± 0.2	5.91 ± 0.03[c]; 1230	0.9 ± 0.1	71 (34−148)	132 (49−355)
3	3.98 ± 0.04[c]; 104 μM	53.1 ± 5.0[c]	1.3 ± 0.2	6.26 ± 0.07[c]; 549	0.9 ± 0.1	191[c] (123−295)	462[c] (128−1667)
4	ND	4.3 ± 2.1[d]	ND	48 ± 12[e]	ND	ND	
5	ND	5.9 ± 2.1[d]	ND	53 ± 3[e]	ND	ND	
6	5.53 ± 0.20[c]; 2.96 μM	78.8 ± 3.0	0.9 ± 0.2	6.89 ± 0.09[c]; 129	0.7 ± 0.1	23 (5−105)	
7	7.09 ± 0.18; 80 nM	75.2 ± 1.4	1.0 ± 0.1	7.95 ± 0.05; 11.2	1.1 ± 0.2	7 (2−29)	
AdA	7.62 ± 0.12[c]; 24 nM	77.8 ± 4.5	0.8 ± 0.1	8.86 ± 0.14[c]; 1.4	1.2 ± 0.2	17 (5−51)	

[a]Effects of ligands on Ca^{2+} release from the intracellular stores of permeabilized HEK-Ins(1,4,5)P_3R1 cells and on [^{3}H]-Ins(1,4,5)P_3 binding to cerebellar membranes are summarized. Results from functional assays are means ± SEM (pEC_{50} (−log of the half-maximally effective concentration), Ca^{2+} release (%), and Hill coefficient (h)) and means (EC_{50}) from 5 to 11 independent experiments, each performed in duplicate. Results from binding experiments are means ± SEM (pK_d (−log of the equilibrium dissociation constant) and h) and means (K_d) from three independent experiments. The pK_d values for Ins(1,4,5)P_3 and AdA have been published (Mills et al.)[43] and are reproduced with permission. Final columns show EC_{50}/K_d or (for partial agonists and Ins(1,4,5)P_3) EC_{39}/K_d (mean and 95% CI). ND, not determined. [b]EC_{39} reports the concentration of ligand required to evoke the same Ca^{2+} release (39% of the intracellular stores) as evoked by a half-maximally effective concentration of Ins(1,4,5)P_3. [c]$P < 0.05$ relative to Ins(1,4,5)P_3. [d]Ca^{2+} release evoked by 300 μM ligand. [e]Specific binding of [^{3}H]-Ins(1,4,5)P_3 in the presence of 30 μM competing ligand.

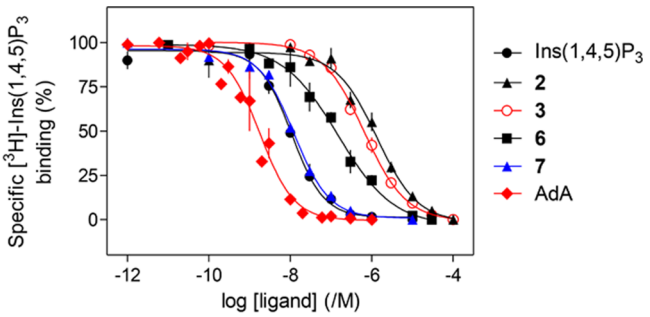

Figure 5. Equilibrium competition binding to cerebellar membranes using [^{3}H]-Ins(1,4,5)P_3 (1.5 nM) and the indicated concentrations of competing ligands. Results are means ± SEM from three independent experiments. The results for Ins(1,4,5)P_3 and AdA have been published (Mills et al.).[43]

Previous studies using synthetic analogues of AdA[30−34,45] demonstrated that the adenine moiety significantly increases potency of the agonist, the vicinal phosphates are crucial to activity, and minor adjustments to the placement of the auxiliary phosphate can be tolerated.[5,34] The general consensus for AdA binding is that the ligand interacts with the binding site of Ins(1,4,5)P_3R with the 3″-, 4″-, and 2′-phosphates mimicking the 5, 4, and 1 phosphates of Ins(1,4,5)P_3, respectively.[27,46] However, these previous studies employed analogues that differed from Ins(1,4,5)P_3 in several ways, and it has, therefore, been difficult to isolate the specific impact of replacing the *myo*-inositol ring with D-glucopyranose.

This has implications for the possible mode of action of AdA and related compounds. Indeed, a cryo-EM study[47] of tetrameric Ins(1,4,5)P_3R1 has recently proposed that AdA interacts with the IBC in a completely different way from Ins(1,4,5)P_3, with the two domains of the IBC being pulled together by the 3″- and 4″-phosphate groups of AdA interacting with one domain and the adenine moiety interacting with the other.[47] In this model of AdA binding to Ins(1,4,5)P_3R, the glucose bisphosphate structure of AdA only coincidentally resembles the *myo*-inositol 4,5-bisphosphate of Ins(1,4,5)P_3, and there is no structural correspondence between the glucose ring of AdA and the inositol ring of Ins(1,4,5)P_3. However, this conclusion does not support the observed activities of the compounds in this study and other AdA analogues; it should, therefore, be viewed with caution.[12,47]

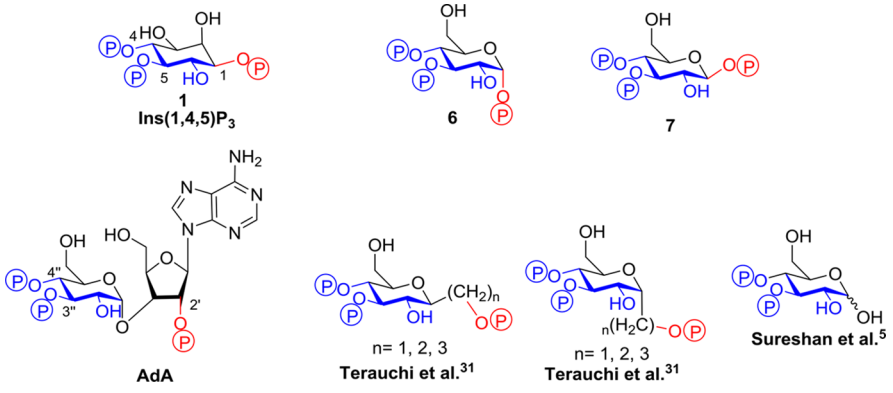

Figure 6. Structural comparison of Ins(1,4,5)P_3 (**1**), AdA, and some of its truncated analogues including α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**). The conserved regions of the structures involved in binding are drawn in blue, while the differing auxiliary phosphate is shown in red.

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In the present study, we found that the closest possible glucose-containing analogue of Ins(1,4,5)P_3, namely, compound **7**, is effectively indistinguishable from Ins(1,4,5)P_3 in our assays of Ins(1,4,5)P_3R binding and Ca^{2+} release. This is entirely consistent with the idea that, in the Ins(1,4,5)P_3 binding site, the glucopyranoside ring of **7** is closely analogous to the *myo*-inositol ring of Ins(1,4,5)P_3. In turn, this establishes that the flexible Ins(1,4,5)P_3 binding site can accommodate the equatorial glucopyranoside hydroxymethyl (CH_2OH) group and pyranoside ring oxygen in place of the *myo*-inositol 3-OH group and C-2, respectively, with no impact on activity. The α-epimer **6** is approximately 27-fold less potent than β-epimer **7** in Ca^{2+} release. This shows that the axial phosphate group in **6** can still contribute to binding [Gluc(3,4)P_2 is much less potent] and is also consistent with an earlier report that D-*chiro*-Ins(1,3,4)P_3, the C-1 epimer of Ins(1,4,5)P_3 having an axial 1-phosphate group, had 25-fold lower potency than Ins(1,4,5)P_3.[48]

Thus, the effects of trisphosphates **6** and **7** provide strong support for the argument that compounds containing the D-glucopyranosyl 3,4-bisphosphate structure mimic Ins(1,4,5)P_3 due to the direct structural analogy between glucopyranosyl and *myo*-inositol rings depicted in Figures 2 and 6. In such compounds, it is highly likely that the glucose 3,4-bisphosphate structure simply pulls together the two domains of the IBC in the manner proposed for the inositol 4,5-bisphosphate motif of Ins(1,4,5)P_3. Ligands **6** and **7** were docked into the IBC to explore potential binding site interactions (see Supporting Information SI-1 Figures S2 A and B, respectively, molecular docking files of **6** and **7** in 1N4K available in the Associated Content). We recently reported studies in which the glucose ring of AdA[12] and ribophostin[43] was replaced by D-*chiro*-inositol, leading to both modest and significant increases in biological activity. It therefore remains to be conclusively established whether the additional components present in the AdA molecule can, counterintuitively, induce a completely unrelated role for the glucose bisphosphate component of AdA itself, as suggested in the cryo-EM study.[47]

Previous studies of C-glycosidic truncated analogues of AdA with different chain lengths tethering the third, auxiliary phosphate group (Figure 6) have demonstrated that positioning of this phosphate has a significant effect on the affinity of the agonist.[30−34] It has been observed that the Ca^{2+}-releasing potency of these analogues at Ins(1,4,5)P_3R decreases as the length and flexibility of the linkage to the auxiliary phosphate increases.[34] The orientation of the linkage also plays a role, with axial linkages usually resulting in more potent compounds. However, even the most potent of these compounds (Figure 6, Terauchi et al.,[31] axial linkage, n = 2) is still weaker than Ins(1,4,5)P_3 and compound **7**.

Methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) was found to be a partial agonist of Ins(1,4,5)P_3R1, while its D-glucose-based enantiomer, compound **4**, was inactive. This supports the structural alignment of trisphosphate **2** with Ins(1,4,5)P_3 shown in Figure 7 and in our molecular modeling in SI-1 Figure S3 (molecular docking file of **2** in 1N4K available in the Associated Content). No such alignment is possible for compound **4** because it does not possess a vicinal bisphosphate motif whose stereochemistry matches that of Ins(1,4,5)P_3.

In the predicted binding conformation of methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) (SI-1 Figure S3 molecular docking file of **2** in 1N4K available in the Associated Content), the axial methyl group is positioned in a region of the binding site normally occupied by the 3-hydroxyl of Ins(1,4,5)P_3. In the design of **2**, we further anticipated that the phosphate group at C-6 of L-glucose would mimic the auxiliary 1-phosphate of Ins(1,4,5)P_3 to some extent as there is evidence from previous studies showing that the Ins(1,4,5)P_3R can accommodate more sterically demanding groups in this region of the binding site.[5,49−51] A very recent example of this is that the replacement of the Ins(1,4,5)P_3 1-phosphate by a pyrophosphate, which increases both charge and steric bulk, does not affect activity.[49] In addition, trisphosphate **2** contains an hydroxyl group appropriately placed to mimic the important 6-OH group of Ins(1,4,5)P_3.

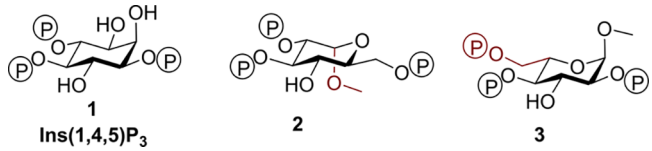

Figure 7. Ins(1,4,5)P_3 (**1**) and analogues methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) and methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**) with their structural differences contribute to their Ins(1,4,5)P_3R partial agonist activity in dark red.

In studies of Ins(1,4,5)P_3 analogues as partial agonists, it has been shown that perturbations in the equivalent of the 3-hydroxyl of Ins(1,4,5)P_3 can result in partial agonist activity,[52] especially when this disruption (often by means of stereochemical inversion) occurs in conjunction with a modification to the vicinal phosphate pair or other region of the ligand.[18,22,25,52] It has been observed in multiple studies that limited, equatorial extension of substituents from the 3-position equivalent can be tolerated,[53−55] but larger groups hinder binding[56,57] and inversion of the 3-hydroxyl to axial results in a slight decrease in ligand activity.[22,58−60] Figure 7 therefore suggests that the axial *O*-methyl group is the most likely component of **2** that causes it to display partial agonist activity, perhaps by interfering with the ligand binding to the β-domain of the IBC or by reducing the extent of domain closure.

Methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**) was designed to bind to the Ins(1,4,5)P_3R in a manner that would potentially satisfy the essential binding requirements by positioning the pyranoside ring oxygen in place of the nonessential 3-OH group of Ins(1,4,5)P_3, while the axial 1-methoxy group occupied the place of the unimportant 2-OH group of Ins(1,4,5)P_3 (Figure 7 and SI-1 Figure S4, molecular docking file of **3** in 1N4K available in the Associated Content). In this docked binding mode, the L-glucose 6-phosphate group would enter the region of the binding site usually occupied by the 4-phosphate of Ins(1,4,5)P_3.

In previous studies, it has been shown that conservative modifications to the phosphates attached to the 4 and 5 equivalent positions (and sometimes in conjunction with a modification to the 3-position equivalent) can produce partial agonists and even low-affinity antagonists.[22−25,52,56,61] Bello et al.[26] hypothesized that if a ligand could bind to only one side of the IBC (through disruption of the interactions of either the 4 or 5 equivalent phosphates), it would be unable to pull the clam-like structure of the binding site closed and would therefore be unable to activate the receptor. Thus, a suitable modification to the 4-phosphate of Ins(1,4,5)P_3 might weaken the important interaction with the β-domain of the clam shell

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structure and induce suboptimal activation of Ins(1,4,5)P_3R. However, previous studies attempting to generate partial agonists with modifications solely to the 4-phosphate have failed to identify any active Ins(1,4,5)P_3 analogues.[26]

Pleasingly, our assays show that L-glucose trisphosphate **3** also behaves as a partial agonist of Ins(1,4,5)P_3R1, with improved binding affinity and a higher EC_{50}/K_d ratio than partial agonist **2**. The fact that the D-enantiomer **5** is inactive supports the structural alignment of **3** with Ins(1,4,5)P_3 depicted in Figure 7. The "extended" 4-phosphate group equivalent in **3** may thus disrupt the interaction of the ligand with the β-domain of the IBC as theorized.[26] It is likely that the absence in **3** of an equivalent to the 3-OH group in Ins(1,4,5)P_3 also contributes to a decreased interaction between the β-domain of the IBC and the ligand. Indeed, activity for 3-deoxy-Ins(1,4,5)P_3 at Ins(1,4,5)P_3R has been reported to drop up to 40-fold.[10]

The two partial agonists, α-L-glucopyranoside 2,3,6-trisphosphate (**2**) and α-L-glucopyranoside 2,4,6-trisphosphate (**3**), indicate that perturbations on the ring structure of the ligand are sufficient to induce partial agonism. Both ligands suffer from low affinity and, as a result, structurally related compounds are currently being developed that will incorporate similar structural differences to Ins(1,4,5)P_3 and hopefully maintain the desired decreased efficacy while increasing affinity. The most promising avenue seems to be adapting the structure of **3** by generating other ligands with extended 4-position phosphate equivalents. This could hypothetically be continued with L-glucose, but inositol could also prove to be a useful starting material as ligands could be synthesized with a similar extension of the 4-position hydroxyl without the loss of an equivalent hydroxyl to position 3 in Ins(1,4,5)P_3, perhaps thereby improving ligand affinity while retaining partial agonist activity. Such work is in progress.

CONCLUSIONS

We have synthesized four novel active ligands for the Ins(1,4,5)P_3R based on both D-glucose and L-glucose templates as inositol surrogates. The two ligands based on L-glucose, namely, methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) and methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**), are low-affinity, low-efficacy partial agonists of Ins(1,4,5)P_3R, while their respective D-glucose-based enantiomers **4** and **5** are inactive. Two further synthetic D-glucose-based trisphosphates, α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) and β-D-glucopyranosyl 1,3,4-trisphosphate (**7**), can be regarded as close analogues of Ins(1,4,5)P_3, but they are also related structurally to the naturally occurring glyconucleotide Ins(1,4,5)P_3R agonist adenophostin A (AdA). They can, therefore, further our understanding of how AdA binds to Ins(1,4,5)P_3R. Both **6** and **7** were found to be full agonists of Ins(1,4,5)P_3R, with the perhaps surprisingly stable β-epimer **7** being equipotent to Ins(1,4,5)P_3 itself and potentially useful as a chemical biology tool under physiological conditions (with degradation induced only under extremes of pH). The potency of **7** demonstrates that the structural differences between *myo*-inositol and D-glucose need not result in any decrease in ligand activity. This is consistent with the D-glucopyranosyl 3,4-bisphosphate moiety of AdA directly mimicking the D-*myo*-inositol 4,5-bisphosphate structure of Ins(1,4,5)P_3 at the binding site of Ins(1,4,5)P_3Rs. All four active ligands **2**, **3**, **6**, and **7** were docked into the IBC to explore potential binding site interactions.

Partial agonists **2** and **3** are the first L-glucose-derived ligands that have been synthesized for Ins(1,4,5)P_3R. Both compounds provide evidence for the viability of generating partial agonists and potential antagonists of Ins(1,4,5)P_3R by deliberately disrupting the crucial moieties involved in binding to the IBC clam shell and pulling the domains together upon ligand binding. We hypothesize that the axial *O*-methyl group of compound **2** and the extended phosphate in the equivalent of the 4-position phosphate in Ins(1,4,5)P_3 of compound **3** cause the partial agonist activity of these compounds either by disrupting the interactions of the ligand with the β-domain of the IBC or by preventing complete closure of the IBC upon binding. These partial agonists could prove to be interesting starting points to generate structurally similar compounds with even lower efficacy and higher affinity that could result in the generation of improved partial agonists or antagonists.

EXPERIMENTAL SECTION

General Synthesis. Chemicals were purchased from Sigma-Aldrich, Acros, or Alfa Aesar. Anhydrous solvents were purchased from Sigma-Aldrich. TLC was performed on precoated plates (Merck aluminum sheets, silica 60 F254, art no. 5554). Chromatograms were visualized under UV light and by dipping plates into phosphomolybdic acid in EtOH followed by heating. Flash column chromatography was performed using RediSep Rf disposable flash columns on an ISCO CombiFlash Rf automated flash chromatography machine. Reversed-phase chromatography was performed on LiChroprep RP-18 (25−40 μm, Merck) using a BioLogic LP system (BioRad), eluting at 5 mL min^{-1} with a gradient of 0−10% MeCN in 0.05 M triethylammonium bicarbonate (TEAB) buffer, collecting 7 mL fractions. Fractions containing the target polyphosphate were identified using a modification of the Briggs phosphate assay.[62] The purity of all of the final compounds used in biological assays was assessed by HPLC and found to be >95% pure (vide infra and HPLC data in the Supporting Information). Proton ^{1}H NMR and COSY spectra were recorded on a Bruker Avance III (400 MHz) spectrometer. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.0 ppm) or with the solvent reference relative to TMS employed as the internal standard ($CDCl_3$, δ 7.26 ppm; CD_3OD, δ 3.31 ppm). The following abbreviations are used to describe the multiplicity of the chemical shifts: br, broad; s, singlet; d, doublet; dd, double doublet; q, quartet; m, multiplet; and t, triplet. ^{13}C and HSQC spectra were recorded on a Bruker Avance III (100 MHz) spectrometer with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, δ 77.0 ppm, CD_3OD, δ 49.0 ppm). ^{31}P NMR spectra were recorded on a Bruker Avance III (162 MHz) spectrometer with complete proton decoupling. Phosphorus chemical shifts are reported in ppm (δ) relative to an 85% H_3PO_4 external standard (H_3PO_4, δ 0.0 ppm). All NMR data were collected at 25 °C. Optical rotations were measured at ambient temperature using an Optical Activity Ltd. AA-10 polarimeter in a cell volume of 5 cm^3, and specific rotation is given in $degmLg^{-1}dm^{-1}$. Melting points were determined using a Stanford Research Systems Optimelt MPA100 automated melting point system and are uncorrected. Mass spectra were recorded on a Thermo Orbitrap Exactive mass spectrometer. All reactions were carried out under an argon atmosphere employing oven-dried glassware unless stated otherwise.

Methyl α-L-Glucopyranoside (8). In a modified version of the Li et al.[63] procedure, L-glucose (850 mg, 4.7 mmol) was dissolved in anhydrous MeOH (6.5 mL). A solution of hydrogen chloride was prepared by adding acetyl chloride (0.25 mL) to anhydrous MeOH (1.5 mL) at 0 °C, and this solution was added dropwise to the glucose reaction solution. The reaction was refluxed for 5 days while under nitrogen before the MeOH was evaporated to yield the crude product. The product was recrystallized from EtOH as a white crystalline solid, which contained approximately 5% of the β-anomer. The product was

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recrystallized from EtOH again to yield the pure α-anomer of the product as white crystals (414 mg, 2.13 mmol, 45% yield). mp (EtOH) 167.2−168.1 °C (lit.[64] mp (EtOH) 161−163 °C). $[\alpha]_D^{23}$ −168.4 (c = 1.00, MeOH) [lit.[64] $[\alpha]_D$ −161 (c = 1.0, MeOH)]. ^{1}H NMR (CD_3OD, 400 MHz): δ 4.67 (d, J = 3.8 Hz, 1H, H-1), 3.81 (dd, J = 11.8, 2.4 Hz, 1H, H-6), 3.67 (dd, J = 11.8, 5.8 Hz, 1H, H-6), 3.61 (t, J = 9.2 Hz, 1H, H-3), 3.55−3.50 (m, 1H, H-5), 3.41 (s, 3H, OMe), 3.38 (dd, J = 9.7, 3.8 Hz, 1H, H-2), 3.27 (dd, J = 10.0, 9.0 Hz, 1H, H-4). ^{13}C NMR (CD_3OD, 100 MHz): δ 101.2 (C-1), 75.1 (C-3), 73.54 (C-5), 73.53 (C-2), 71.8 (C-4), 62.7 (C-6), 55.5 (OMe).

Methyl 3-*O*-Benzyl-4,6-*O*-benzylidene-α-L-glucopyranoside (12). Methyl α-L-glucopyranoside (**8**) (93 mg, 0.480 mmol) was dissolved in dry pyridine (1 mL) and put under argon. To this solution, trimethylsilyl chloride (0.30 mL, 2.39 mmol, 5 equiv) was added dropwise. The solution was allowed to stir for 22 h at room temperature. The reaction was then diluted with EtOAc and washed with water (2 × 30 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to yield the persilylated glucopyranoside crude product. The persilylated product was not purified, but ^{1}H NMR was used to confirm that the reaction had proceeded to completion. The persilylated product was dissolved and co-evaporated twice with toluene before being dissolved in dry DCM (0.7 mL) and put under argon. To this solution, benzaldehyde (0.15 mL, 1.42 mmol, 3 equiv) was added, and the reaction was cooled in an ice bath. To this chilled solution, iron(III) chloride hexahydrate (3.4 mg, 0.013 mg, 0.026 equiv) dissolved in MeCN (0.12 mL) was added dropwise. Triethylsilane (0.08 mL, 0.528 mmol, 1.1 equiv) was then added dropwise, and the reaction was allowed to warm to room temperature and stir for 1.5 h. After this time, the solution was diluted with EtOAc (50 mL), washed with sat. $NaHCO_3$ solution (50 mL), and extracted from the aqueous phase twice more with EtOAc (2 × 30 mL). The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo to yield the crude product. The product was purified with flash chromatography (petroleum ether/EtOAc, 0−100%) to yield the pure product as a white solid (140.7 mg, 0.378 mmol, 79% yield). mp 184.0−185.3 °C. $[\alpha]_D^{21}$ −87.5 (c = 1.00, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51−7.27 (m, 10H, Ar), 5.57 (s, 1H, H-7), 4.97 (d, J = 11.6 Hz, 1H, CH_2Ph), 4.82 (d, J = 3.9 Hz, 1H, H-1), 4.79 (d, J = 11.6 Hz, 1H, CH_2Ph), 4.30 (dd, J = 4.3, 9.8 Hz, 1H, H-6), 3.87−3.71 (m, 4H, H-2, H-3, H-5, H-6), 3.65 (t, J = 9.2 Hz, 1H, H-4), 3.45 (s, 3H, OMe), 2.29 (d, J = 7.2 Hz, 1H, OH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138. 6 (Ar), 137.5 (Ar), 129.1 (Ar), 128.6 (Ar), 128.4 (Ar), 128.2 (Ar), 127.9 (Ar), 126.2 (Ar), 101.4 (C-7), 100.0 (C-1), 82.1 (C-4), 79.0 (C-2 or 3 or 5), 75.0 (CH_2Ph), 72.6 (C-2 or 3 or 5), 69.2 (C-6), 62.7 (C-2 or 3 or 5), 55.6 (OMe). HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{21}H_{24}O_6$, 395.14651; found, 395.14658.

Methyl 3-*O*-Benzyl-α-L-glucopyranoside (13). Methyl 3-*O*-benzyl-4,6-*O*-benzylidene-α-L-glucopyranoside (**12**) (135.7 mg, 0.364 mmol) was dissolved in MeOH (3 mL). To this solution, water (0.15 mL) and 1 M $HCl_{(aq)}$ (0.3 mL) were added. The reaction was heated to reflux for 3 h. After this time, the reaction was quenched with the addition of $NaHCO_{3(aq)}$ (25.2 mg in 5 mL of water). The solution was concentrated in vacuo, and the residue was dissolved and co-evaporated with toluene twice to yield the crude product. The product was purified with flash chromatography (petroleum ether/EtOAc, 0−100%) to yield the pure product as a white solid (96.3 mg, 0.339 mmol, 93% yield). mp 91.2−93.8 °C. $[\alpha]_D^{22}$ −89.5 (c = 0.93, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.39−7.29 (m, 5H, Ar), 5.03 (d, J = 11.5 Hz, 1H, CH_2Ph), 4.76 (d, J = 3.9 Hz, 1H, CH_2Ph), 4.73 (d, J = 11.5 Hz, 1H, H-1), 3.88−3.75 (m, 2H, H-6 ×2), 3.70−3.52 (m, 4H, H-2, H-3, H-4, H-5), 3.44 (s, 3H, OMe), 2.30 (d, J = 2.4 Hz, 1H, OH), 2.14 (d, J = 9.2 Hz, 1H, OH), 1.93 (dd, J = 5.7, 7.2 Hz, 1H, OH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.6 (Ar), 128.8 (Ar), 128.1 (Ar), 99.7 (C-1), 82.8 (C-3), 75.1 (CH_2Ph), 72.9 (C-2), 71.1 (C-5), 70.2 (C-4), 62.5 (C-6), 55.5 (OMe). HRMS (ESI) m/z: $[M + Na]^+$ calcd for $C_{14}H_{20}O_6$, 307.11521; found, 307.11515.

Methyl 3-*O*-Benzyl-α-L-glucopyranoside 2,4,6-Tris(dibenzyl phosphate) (14). Methyl 3-*O*-benzyl-α-L-glucopyranoside (**13**) (96.3 mg, 0.339 mmol) was dissolved in dry DCM (4 mL), and the solution was put under argon. 5-Phenyl-1*H*-tetrazole (297 mg, 2.03 mmol, 6 equiv) was added to the solution followed by dibenzyl diisopropylphosphoramidite (0.55 mL, 1.52 mmol, 4.5 equiv). The reaction was allowed to stir at room temperature overnight. The next day, after the confirmation of successful phosphitylation with ^{31}P NMR, the reaction flask was cooled to −78 °C, and mCPBA (502 mg, 70% purity, 2.03 mmol, 6 equiv) was added. The reaction was allowed to stir at room temperature for 10 min before the solution was diluted with EtOAc (50 mL), washed with 10% Na_2SO_3 solution (2 × 30 mL), dried over $MgSO_4$, and concentrated to yield the crude product. The crude product was purified with flash chromatography (petroleum ether/EtOAc, 0−100%) to yield the pure product as a colorless oil (144.2 mg, 0.135 mmol, 40% yield). $[\alpha]_D^{21}$ −38.8 (c = 1.01, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.34−7.08 (m, 35H, Ar), 5.03 (s, 2H, CH_2Ph ×2), 5.01 (s, 2H, CH_2Ph ×2), 4.98 (d, J = 3.2 Hz, 1H, CH_2Ph), 4.96 (d, J = 3.2 Hz, 1H, CH_2Ph), 4.94−4.85 (m, 6H, H-1, CH_2Ph ×5), 4.80−4.75 (m, 3H, CH_2Ph ×3), 4.42−4.18 (m, 4H, H-2, H-3, H-6 ×2), 3.98 (t, J = 9.4 Hz, 1H, H-4), 3.86 (dd, J = 10.0, 5.2 Hz, 1H, H-5), 3.28 (s, 3H, OMe). ^{31}P NMR ($CDCl_3$, 162 MHz): δ −0.99, −1.74, −1.86. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.1 (Ar), 136.0 (Ar), 135.9 (Ar), 135.8−135.6 (m, Ar), 128.7−128.5 (m, Ar), 128.3 (Ar), 128.0 (m, Ar), 127.9 (Ar), 127.7 (Ar), 127.5 (Ar), 97.6 (C-1), 78.4−78.3 (m, C-4), 76.8−76.7 (m, C-2 or 3), 75.1 (C-2 or 3, CH_2Ph), 69.8−69.4 (m, CH_2Ph), 69.0−68.9 (m, C-5), 66.1 (d, J = 5.1 Hz, C-6), 55.7 (OMe). HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{56}H_{59}O_{15}P_3$, 1065.31396; found, 1065.31280.

Methyl α-L-Glucopyranoside 2,4,6-Trisphosphate Triethylammonium Salt (3). Methyl 3-*O*-benzyl-α-L-glucopyranoside 2,4,6-tris(dibenzyl phosphate) (**14**) (141.9 mg, 0.133 mmol) was dissolved in MeOH (7.1 mL). Ultrapure water (0.71 mL) was added dropwise to the solution, ensuring that the precipitate formed upon addition was able to dissolve back into solution. $Pd(OH)_2$/C (20%, ≥50% wet, 71.0 mg) was added to the solution, and the reaction flask was flushed with hydrogen. The reaction was allowed to stir at room temperature for 24 h after which the catalyst was filtered off and the collected filtrate was evaporated to yield the product as a free acid. No purification steps were deemed to be necessary, but triethylamine was added to sharpen the ^{31}P NMR signals and to convert the product from the free acid into the triethylammonium salt. The product was concentrated in vacuo, lyophilized, and collected as a colorless glass (96.1 mg, 0.120 mmol, 90% yield). $[\alpha]_D^{22}$ −46.5 (c = 0.88, MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 4.90 (d, J = 3.0 Hz, 1H, H-1), 4.29−4.18 (m, 2H, H-3, H-6), 4.05−3.97 (m, 3H, H-2, H-4, H-6), 3.68 (d, J = 10.0 Hz, 1H, H-5), 3.38 (s, 3H, OMe), 3.14 (q, J = 7.3 Hz, approx. 18H, TEA CH_2CH$_3$), 1.30 (t, 7.3 Hz, approx. 27H, TEA CH$_2$CH_3). ^{31}P NMR (CD_3OD, 162 MHz): δ 1.90, 1.71, 1.28. ^{13}C NMR (CD_3OD, 100 MHz): δ 100.2 (d, J = 4.0 Hz, C-1), 76.3 (d, J = 5.1 Hz, C-2 or 4), 74.6 (d, J = 4.0 Hz, C-2 or 4), 73.5 (d, J = 4.9 Hz, C-3), 71.0−71.2 (m, C-5), 63.9 (d, J = 4.7 Hz, C-6), 55.6 (OMe), 46.9 (TEA CH_2CH_3), 9.4 (TEA CH_2CH_3). HRMS (ESI) m/z: $[M - H]^-$ for $C_7H_{17}O_{15}P_3$, 432.97075; found, 432.97065.

Methyl 4,6-*O*-Benzylidene-α-L-glucopyranoside (9). In a version of the Tseberlidis et al.[65] method, methyl α-L-glucopyranoside (**8**) (100 mg, 0.514 mmol) was suspended in dry MeCN (1.7 mL) and put under a nitrogen atmosphere. To this suspension, benzaldehyde dimethyl acetal (0.24 mL, 1.55 mmol, 3 equiv) and catalytic camphor-10-sulfonic acid (1.6 mg, 0.0068 mmol) were added, and the reaction was allowed to stir at room temperature overnight. After 24 h, the reaction was neutralized with a few drops of triethylamine and evaporated to yield the crude product as a white crystalline solid. The crude product was purified through flash chromatography (petroleum ether/EtOAc, 0−100%), and the pure product was collected as a white solid (132.6 mg, 0.470 mmol, 91% yield). mp 163.1−164.4 °C (lit.[64] mp 161−162 °C). $[\alpha]_D^{21}$ −110.5 (c = 0.69, $CDCl_3$) [lit.[64] $[\alpha]_D$ −95 (c = 1.0, MeOH)]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51−7.48 (m, 2H, Ar), 7.40−7.34 (m, 3H, Ar), 5.53 (s, 1H, H-7), 4.79 (d, J = 4.0 Hz, 1H, H-1), 4.29 (dd, J = 9.6, 4.3 Hz, 1H, H-6), 3.93 (td, J = 9.3, 2.2 Hz, 1H, H-3), 3.84−3.78 (m, 1H, H-5), 3.75 (q, J = 10.3 Hz, 1H, H-6), 3.63 (td, J = 9.3, 3.9 Hz, 1H, H-2), 3.49 (t, J = 9.3 Hz, 1H, H-4), 3.46 (s, 3H, OMe), 2.76 (d, J = 2.2 Hz, 1H, OH), 2.30 (d, J = 9.5 Hz, 1H, OH). ^{13}C NMR ($CDCl_3$, 100

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MHz): δ 137.2 (Ar), 129.4 (Ar), 128.5 (Ar), 126.4 (Ar), 102.1 (C-7), 99.9 (C-1), 81.1 (C-4), 73.0 (C-2), 72.0 (C-3), 69.1 (C-6), 62.5 (C-5), 55.7 (OMe).

Methyl 4-*O*-Benzyl-α-L-glucopyranoside (10). Methyl 4-*O*-benzyl-α-L-glucopyranoside was made as described for methyl 4-*O*-benzyl-α-D-glucopyranoside (**21**) using both method A to generate large amounts of impure product and method B to generate smaller amounts of pure product (26 mg, 37% yield). mp 128.5−132.0 (lit.[66] mp 125−127 °C). $[\alpha]_D^{25}$ −142.5 (*c* = 0.3, MeOH) [lit.[66] $[\alpha]_D^{25}$ −144.2 (*c* = 1.2, MeOH)]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.37−7.27 (m, 5H, Ar), 4.87 (d, *J* = 11.5 Hz, 1H, CH_2Ph), 4.75 (d, *J* = 3.9 Hz, 1H, H-1), 4.72 (d, *J* = 11.4 Hz, 1H, CH_2Ph), 3.86 (t, *J* = 9.2 Hz, 1H, H-3), 3.83 (dd, *J* = 11.8, 2.6 Hz, 1H, H-6), 3.75 (dd, *J* = 11.8, 3.8 Hz, H-6), 3.67−3.62 (m, 1H, H-5), 3.50 (dd, *J* = 3.9, 9.4 Hz, 1H, H-2), 3.45 (t, *J* = 9.4 Hz, 1H, H-4), 3.40 (s, 3H, OMe). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.3 (Ar), 128.7 (Ar), 128.2 (Ar), 127.9 (Ar), 99.2 (C-1), 77.3 (C-4), 75.3 (C-3), 74.8 (CH_2Ph), 72.9 (C-2), 70.9 (C-5), 62.1 (C-6), 55.5 (OMe).

Methyl 4-*O*-Benzyl-α-L-glucopyranoside 2,3,6-Tris(dibenzyl phosphate) (11). Methyl 4-*O*-benzyl-α-L-glucopyranoside (**10**) (65.5 mg, 0.230 mmol) was dissolved in dry DCM (2 mL) and put under argon. 5-Phenyl-1*H*-tetrazole (202 mg, 1.38 mmol, 6 equiv) was added to the solution followed by dibenzyl diisopropylphosphoramidite (0.36 mL, 1.04 mmol, 4.5 equiv). The reaction was allowed to stir at room temperature overnight. The following day, the reaction was cooled to −78 °C, and mCPBA (70% pure, 342 mg, 1.38 mmol, 6 equiv) was added. The reaction was allowed to stir for 10 min at room temperature before it was diluted with EtOAc (50 mL) and washed twice with 10% Na_2SO_3 solution (2 × 30 mL). The organic layer was dried over $MgSO_4$ and concentrated to yield the crude product. The residue was purified with flash chromatography (petroleum ether/EtOAc, 0−100%) to yield the pure product as a colorless oil (210.5 mg, 0.198 mmol, 86% yield). $[\alpha]_D^{21}$ −42.3 (*c* = 1.06, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.33−7.09 (m, 35H, Ar), 5.05−4.81 (m, 16H, H-1, H-3, H-6, CH_2Ph ×13), 4.46 (d, *J* = 10.7 Hz, 1H, CH_2Ph), 4.25 (ddd, *J* = 13.3, 6.2, 3.6 Hz, 1H, H-2), 4.19−4.16 (m, 1H, H-6), 3.75 (dq, *J* = 10.0, 2.3 Hz, 1H, H-5), 3.50 (t, *J* = 9.5 Hz, 1H, H-4), 3.24 (s, 3H, OMe). ^{31}P NMR ($CDCl_3$, 162 MHz): δ −0.76, −1.32, −2.01. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 137.6 (Ar), 136.1−135.7 (m, Ar), 128.7−127.8 (m, Ar), 97.6 (C-1), 78.4 (dd, *J* = 8.7, 6.5 Hz, C-3), 76.2 (C-4), 75.3 (dd, *J* = 4.7, 3.2 Hz, C-2), 74.6 (CH_2Ph), 69.8 (d, *J* = 5.6 Hz, CH_2Ph), 69.6−69.4 (m, CH_2Ph), 69.1 (d, *J* = 8.3 Hz, C-5), 65.8 (d, *J* = 5.6 Hz, C-6), 55.5 (OMe). HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{56}H_{59}O_{15}P_3$, 1065.31396; found, 1065.31297.

Methyl α-L-Glucopyranoside 2,3,6-Trisphosphate Triethylammonium Salt (2). Methyl 4-*O*-benzyl-α-L-glucopyranoside 2,3,6-tris(dibenzyl phosphate) (**11**) (60 mg, 0.056 mmol) was dissolved in MeOH (3 mL). To this solution, ultrapure water (0.3 mL) was added dropwise, ensuring that the white precipitate that formed returned to solution. $Pd(OH)_2$/C (20%, ≥50% wet, 30 mg) was added to the solution, and the flask was flushed with hydrogen. The reaction was left to stir under hydrogen at room temperature overnight. The palladium catalyst was filtered off with a PTFE filter, and the solution was concentrated to yield the product as a free acid. No purification was deemed necessary. The free acid was converted to the triethylammonium salt through the addition of triethylamine to the free acid followed by concentration in vacuo. The product was lyophilized and collected as a colorless glass (44.5 mg, 0.056 mmol, 100% yield). $[\alpha]_D^{21}$ −37.6 (*c* = 1.00, MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 4.92 (d, *J* = 3.6 Hz, 1H, H-1), 4.35 (q, *J* = 8.7 Hz, 1H, H-3), 4.17 (ddd, *J* = 11.1, 5.2, 2.0 Hz, 1H, H-6), 4.07−3.98 (m, 2H, H-2, H-6), 3.73−3.68 (m, 1H, H-5), 3.55 (dd, *J* = 9.8, 8.7 Hz, 1H, H-4), 3.39 (s, 3H, OMe), 3.09 (q, *J* = 7.3 Hz, approx. 18H, TEA CH_2CH$_3$), 1.27 (t, *J* = 7.3 Hz, approx. 27H, TEA CH$_2$CH_3). ^{31}P NMR (CD_3OD, 162 MHz): δ 2.50, 1.53, 1.15. ^{13}C NMR (CD_3OD, 100 MHz): δ 100.5 (C-1), 78.5 (C-3), 75.5 (C-2), 72.4 (d, *J* = 8.7 Hz, C-5), 72.0 (C-4), 65.7 (d, *J* = 4.9 Hz, C-6), 55.4 (OMe), 47.0 (TEA CH_2CH$_3$), 9.4 (TEA CH$_2$CH_3). HRMS (ESI) *m/z*: $[M - H]^-$ calcd for $C_7H_{17}O_{15}P_3$, 432.97075; found, 432.97067.

2,6-Di-*O*-benzyl-D-glucopyranose (16). Allyl 2,6-di-*O*-benzyl-D-glucopyranoside (**15**) (60 mg, 0.133 mmol) as synthesized in the method outlined by Jenkins and Potter[30] was dissolved in dry MeOH (1.54 mL). To this solution, $PdCl_2$ (6.2 mg, 0.03 mmol, 0.25 equiv) was added, and the reaction was allowed to stir at room temperature for 6 h with a drying tube affixed to the flask. After this time, the reaction was quenched with the addition of excess $NaHCO_3$ and allowed to stir for 5 min before being filtered through Celite and concentrated to yield the crude product. The product of this reaction could not be successfully purified; although following phosphorylation (see below), the products could be successfully isolated.

2,6-Di-*O*-benzyl-α-D-glucopyranosyl 1,3,4-Tris(dibenzyl phosphate) (17) and 2,6-Di-*O*-benzyl-β-D-glucopyranosyl 1,3,4-Tris(dibenzyl phosphate) (18). 2,6-Di-*O*-benzyl-D-glucopyranose (**16**) (154.7 mg, 0.429 mmol) was added to dry DCM (4.5 mL). To this suspension, 5-phenyl-1*H*-tetrazole (376 mg, 2.58 mmol, 6 equiv) was added, followed by dibenzyl diisopropylphosphoramidite (0.67 mL, 1.93 mmol, 4.5 equiv). The reaction was allowed to stir under argon at room temperature overnight after which it was cooled to −78 °C and mCPBA (70% purity, 636 mg, 2.58 mmol, 6 equiv) was added. The reaction was then diluted with EtOAc (100 mL) and washed twice with 10% Na_2SO_3 solution (2 × 30 mL). The organic phase was dried over $MgSO_4$ and concentrated to yield the crude product. The product was purified using flash chromatography (petroleum ether/EtOAc, 0−100%). The stereoisomers of the product were partially isolated and collected as colorless oils (total: 276.6 mg, 0.242 mmol, 56% yield; α-epimer: 70.4 mg, 0.062 mmol, 14% yield; β-epimer: 92.4 mg, 0.081 mmol, 19% yield; remaining unseparated mix of epimers: 113.8 mg, 0.100 mmol, 23% yield).

2,6-Di-*O*-benzyl-α-D-glucopyranosyl 1,3,4-Tris(dibenzyl phosphate) (17). R_f (EtOAc), 0.63. $[\alpha]_D^{20}$ +35.7 (*c* = 0.71, chloroform). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.35−7.09 (m, 40H, Ar), 5.91 (dd, *J* = 3.3, 7.0 Hz, 1H, H-1), 5.07−4.81 (m, 13H, H-3, CH_2Ph ×12), 4.72−4.61 (m, 3H, H-4, CH_2Ph ×2), 4.44 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.31 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 3.96 (dq, *J* = 10.0, 1.6 Hz, 1H, H-5), 3.70 (dd, *J* = 11.2, 3.9 Hz, 1H, H-6), 3.65 (dt, *J* = 9.7, 3.1 Hz, 1H, H-2), 3.54 (dd, *J* = 11.2, 1.8 Hz, 1H, H-6). ^{31}P NMR ($CDCl_3$, 162 MHz): δ −1.66, −2.31, −2.50. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.0 (Ar), 137.0 (Ar), 136.2−135.6 (m, Ar), 128.6−127.6 (m, Ar), 94.8 (d, *J* = 5.6 Hz, C-1), 77.9−77.8 (m, C-3), 77.0 (m, C-2), 73.7−73.6 (m, C-4), 73.3 (CH_2Ph), 73.0 (CH_2Ph), 71.6 (C-5), 70.0−69.9 (m, CH_2Ph), 69.7−69.6 (m, CH_2Ph), 69.5−69.4 (m, CH_2Ph), 67.5 (C-6). HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{62}H_{63}O_{15}P_3$, 1163.32720; found, 1163.32495.

2,6-Di-*O*-benzyl-β-D-glucopyranosyl 1,3,4-Tris(dibenzyl phosphate) (18). R_f (EtOAc), 0.57. $[\alpha]_D^{21}$ +2.68 (*c* = 1.01, chloroform). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.31−7.03 (m, 40H, Ar), 5.31 (t, *J* = 7.2 Hz, 1H, H-1), 5.04−4.87 (m, 11H, CH_2Ph ×11), 4.81−4.60 (m, 5H, H-3, H-4, CH_2Ph ×3), 4.42 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.31 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 3.77−3.55 (m , 4H, H-2, H-5, H-6 ×2). ^{31}P NMR ($CDCl_3$, 162 MHz): δ −1.90, −2.22, −2.59. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.0 (Ar), 137.7 (Ar), 136.1−135.4 (m, Ar), 128.6−127.6 (m, Ar), 98.6 (C-1), 80.1 (m, C-3 or 4), 79.7−79.6 (m, C-2 or 5), 74.5 (d, *J* = 3.5 Hz, C-3 or 4), 74.1 (CH_2Ph), 74.0−73.9 (m, C-2 or 5), 73.3 (CH_2Ph), 70.0 (d, *J* = 5.8 Hz, CH_2Ph), 69.8−69.7 (m, CH_2Ph), 69.6−69.5 (m, CH_2Ph), 68.1 (C-6). HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{62}H_{63}O_{15}P_3$, 1163.32720; found, 1163.32505.

α-D-Glucopyranosyl 1,3,4-Trisphosphate Triethylammonium Salt (6). α-D-Glucopyranosyl 1,3,4-trisphosphate was prepared with 2,6-di-*O*-benzyl-α-D-glucopyranosyl 1,3,4-tris(dibenzyl phosphate) (**17**) (31.9 mg, 0.028 mmol) using the same hydrogenation method as described for the synthesis of β-D-glucopyranosyl 1,3,4-trisphosphate (**7**). The crude sodium salt of the product was purified by ion-pair column chromatography on RP-18 and lyophilized to yield the triethylamine salt of the product as a colorless glass (8.0 mg, 0.011 mmol, 39% yield). $[\alpha]_D^{20}$ +33.9 (*c* = 0.97, methanol). ^{1}H NMR (CD_3OD, 400 MHz): δ 5.58 (dd, *J* = 7.0, 3.6 Hz, 1H, H-1), 4.45 (q, *J* = 9.0 Hz, 1H, H-3), 4.12 (q, *J* = 9.8 Hz, 1H, H-4), 3.98−3.91 (m, 2H, H-5, H-6), 3.76−3.72 (m, 1H, H-6), 3.61 (ddd, *J* = 9.5, 3.5, 2.4 Hz,

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1H, H-2), 3.16 (q, J = 7.3 Hz, approx. 18H, TEA CH_2CH$_3$), 1.31 (t, J = 7.3 Hz, approx. 27H, TEA CH$_2$CH_3). ^{31}P NMR (CD$_3$OD, 162 MHz): δ 2.06, 2.06, −0.62. ^{13}C NMR (CD$_3$OD, 100 MHz): δ 96.3 (d, J = 5.4 Hz, C-1), 79.2−79.1 (m, C-3), 73.8−73.6 (m, C-2, C-4, C-5), 62.1 (C-6), 47.3 (TEA CH_2CH$_3$), 9.2 (TEA CH$_2$CH$_3$). HRMS (ESI) m/z: [M − H]$^-$ calcd for $C_6H_{15}O_{15}P_3$, 418.9551; found, 418.95422.

β-D-Glucopyranosyl 1,3,4-Trisphosphate Triethylammonium Salt (7). 2,6-Di-*O*-benzyl-β-D-glucopyranosyl 1,3,4-tris(dibenzyl phosphate) (**18**) (23.3 mg, 0.020 mmol) was dissolved in MeOH (1.5 mL). To this solution, ultrapure water (0.15 mL) was added dropwise, ensuring that the precipitate that formed upon addition returned to solution. NaHCO$_3$ (5.15 mg, 0.061 mmol, 3 equiv) was then added, followed by Pd(OH)$_2$/C (20%, ≥50% wet, 11.7 mg). The reaction flask was flushed with hydrogen and left to stir at room temperature for 24 h. The catalyst was then filtered off, and the collected filtrate was evaporated to yield the crude product as a sodium salt. The product was purified by ion-pair column chromatography on RP-18 and lyophilized to yield the triethylamine salt of the product as a colorless glass (6.7 mg, 0.008 mmol, 40% yield). $[\alpha]_D^{21}$ +3.76 (c = 0.61, methanol). ^{1}H NMR (CD$_3$OD, 400 MHz): δ 4.98 (t, J = 7.6 Hz, 1H, H-1), 4.23 (q, J = 8.8 Hz, 1H, H-3), 4.08 (q, J = 9.8 Hz, 1H, H-4), 3.89 (dd, J = 12.7, 4.4 Hz, 1H, H-6), 3.84 (dd, J = 12.7, 2.1 Hz, 1H, H-6), 3.43−3.38 (m, 2H, H-2, H-5), 3.13 (q, J = 7.0 Hz, approx. 18H, TEA CH_2CH$_3$), 1.29 (t, J = 7.2 Hz, approx. 27H, TEA CH$_2$CH_3). ^{31}P NMR (CD$_3$OD, 162 MHz): δ 1.38, 1.06, −0.77. ^{13}C NMR (CD$_3$OD, 125 MHz): δ 99.3 (C-1), 81.4 (C-3), 77.9 (C-2 or 5), 76.2 (C-2 or 5), 73.8 (C-4), 62.4 (C-6), 47.2 (TEA CH_2CH$_3$), 9.3 (TEA CH$_2$CH$_3$). HRMS (ESI) m/z:[M − H]$^-$ calcd for $C_6H_{15}O_{15}P_3$, 418.9551; found, 418.95425.

Methyl 4-*O*-Benzyl-α-D-glucopyranoside (21). *Method A.* In a version of the Daragics et al.[67] method, methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (100 mg, 0.354 mmol) was dissolved in dry DCM (5.3 mL) and put under argon. The solution was cooled in an ice bath, and borane/THF (1 M, 1.8 mL, 1.77 mmol, 5 equiv) was added, followed by a solution of AlCl$_3$ (94.4 mg, 0.708 mmol, 2 equiv) in dry diethyl ether (0.9 mL). The solution was allowed to gradually warm to room temperature and then stir for 24 h. After this time, the reaction was quenched with the addition of triethylamine (0.2 mL) followed by MeOH (0.9 mL). The reaction solution was concentrated in vacuo to form a solid residue. This residue was dissolved in DCM (50 mL) and washed with 1 M HCl$_{(aq)}$, sat. NaHCO$_{3(aq)}$, and water. The combined aqueous washes were also extracted three times with EtOAc, and the organic layers were combined, dried over MgSO$_4$, and concentrated to yield the crude product. The crude product was purified through silica column chromatography using petroleum ether and EtOAc. It should be noted that this product was not entirely pure as a very small amount of methyl 6-*O*-benzyl-α-D-glucopyranoside was generated as well. This regioisomer could not be separated from the desired product (although separation of the regioisomers post-phosphorylation was achievable).

Method B. Using a version of the Shie et al.[37] procedure, methyl 4,6-*O*-benzylidene α-D-glucopyranoside (100 mg, 0.355 mmol) was added to borane/THF (1 M, 1.8 mL, 1.8 mmol, 5 equiv), and the reaction was put under argon. The solution was allowed to stir for 10 min before lanthanum triflate (31.2 mg, 0.053 mmol, 0.15 equiv) was added, and the reaction was allowed to stir at room temperature for a week. The reaction was then cooled to 0 °C, and the reaction was quenched with triethylamine (0.5 mL, 1 equiv) followed by MeOH (0.7 mL). The reaction was concentrated in vacuo and co-evaporated with MeOH twice before the crude product was isolated as a white solid. The product was purified with flash chromatography ((1) petroleum ether/EtOAc, 0−100% and (2) DCM/EtOAc, 0−100%). As impurities were still present, an aqueous workup was carried out. The product was dissolved in EtOAc and washed with 1 M HCl$_{(aq)}$, sat. NaHCO$_3$, and water. The aqueous washes were extracted with EtOAc again, and the organic phases were combined, dried over MgSO$_4$, and concentrated to yield the pure product as a white solid (30.8 mg, 0.108 mmol, 31% yield). mp 123.9−129.0 °C (lit.[68] mp 126−127 °C). $[\alpha]_D^{21}$ +116.2 (c = 1.47, CHCl$_3$) [lit.[68] $[\alpha]_D$ +154.1 (c = 1, CHCl$_3$)]. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.36−7.28 (m, 5H, Ar), 4.86 (d, J = 11.4 Hz, 1H, CH$_2$Ph), 4.75 (d, J = 3.9 Hz, 1H, H-1), 4.72 (d, J = 11.4 Hz, 1H, CH$_2$Ph), 3.86 (t, J = 9.2 Hz, 1H, H-3), 3.83 (dd, J = 11.6, 2.6 Hz, 1H, H-6), 3.75 (dd, J = 11.9, 3.6 Hz, H-6), 3.63 (dt, J = 9.8, 3.3 Hz, 1H, H-5), 3.51 (brs, 1H, H-2), 3.45 (t, J = 9.4 Hz, 1H, H-4), 3.39 (s, 3H, OMe). ^{13}C NMR (CDCl$_3$, 100 MHz): δ 138.3 (Ar), 128.7 (Ar), 128.2 (Ar), 128.1 (Ar), 99.2 (C-1), 77.2 (C-4), 75.1 (C-3), 74.8 (CH$_2$Ph), 72.8 (C-2), 70.9 (C-5), 62.0 (C-6), 55.5 (OMe).

Methyl 4-*O*-Benzyl-α-D-glucopyranoside 2,3,6-Tris(dibenzyl phosphate) (22). Methyl 4-*O*-benzyl-α-D-glucopyranoside (**21**) (50 mg, 0.176 mmol) was dissolved in dry DCM (2 mL), and the solution was put under argon. 5-Phenyl-1*H*-tetrazole (154 mg, 1.06 mmol, 6 equiv) was added to the solution, followed by dibenzyl diisopropylphosphoramidite (0.27 mL, 0.792 mmol, 4.5 equiv). The reaction was allowed to stir at room temperature overnight. The following day, the reaction was cooled to −78 °C, and mCPBA (70% pure, 261 mg, 1.06 mmol, 6 equiv) was added. The reaction was allowed to stir for 10 min at room temperature before it was diluted with EtOAc (50 mL) and washed twice with 10% Na$_2$SO$_3$ solution (2 × 30 mL). The organic layer was dried over MgSO$_4$ and concentrated to yield the crude product. The product was purified with flash chromatography ((1) petroleum ether/EtOAc, 0−100% and (2) DCM/EtOAc, 0−100%). The pure product was collected as a colorless oil (81.9 mg, 0.077 mmol, 44% yield). $[\alpha]_D^{22}$ +35.7 (c = 1.00, CHCl$_3$). ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.33−7.09 (m, 35H, Ar), 5.05−4.82 (m, 16H, H-1, H-3, H-6, CH_2Ph ×13), 4.47 (d, J = 10.7 Hz, 1H, CH_2Ph), 4.26 (ddd, J = 9.6, 6.2, 3.6 Hz, 1H, H-2), 4.19−4.16 (m, 1H, H-6), 3.76 (dq, J = 9.6, 2.7 Hz, 1H, H-5), 3.50 (t, J = 9.4 Hz, 1H, H-4), 3.25 (s, 3H, OMe). ^{31}P NMR (CDCl$_3$, 162 MHz): δ −0.76, −1.32, −2.01. ^{13}C NMR (CDCl$_3$, 100 MHz): δ 137.6 (Ar), 136.1−135.7 (m, Ar), 128.7−128.0 (m, Ar), 97.6 (C-1), 78.5−78.4 (m, C-3), 76.2 (C-4), 75.4−75.3 (m, C-5), 74.6 (CH$_2$Ph), 69.8 (d, J = 5.6 Hz, CH$_2$Ph), 69.6−69.4 (m, CH$_2$Ph), 69.1 (d, J = 8.1 Hz, C-5), 65.8 (d, J = 5.5 Hz, C-6), 55.5 (OMe); HRMS (ESI) m/z: [M + H]$^+$ calcd for $C_{56}H_{59}O_{15}P_3$, 1065.31396; found, 1065.31364.

Methyl α-D-Glucopyranoside 2,3,6-Trisphosphate Triethylammonium Salt (4). Methyl 4-*O*-benzyl-α-D-glucopyranoside 2,3,6-tris(dibenzyl phosphate) (**22**) (55.5 mg, 0.052 mmol) was dissolved in MeOH (2.8 mL). To this solution, ultrapure water (0.28 mL) was added dropwise, ensuring that the white precipitate that formed returned to solution. Pd(OH)$_2$/C (20%, ≥50% wet, 27.8 mg) was added to the solution, and the flask was flushed with hydrogen. The reaction was left to stir under hydrogen at room temperature overnight. The palladium catalyst was filtered off with a PTFE filter, and the solution was concentrated to yield the product as a free acid. No purification was deemed necessary. The free acid was converted to the triethylammonium salt through the addition of triethylamine to the free acid followed by concentration in vacuo. The product was lyophilized and collected as a colorless glass (24.8 mg, 0.034 mmol, 65% yield). $[\alpha]_D^{22}$ +38.2 (c = 1.00, MeOH). ^{1}H NMR (CD$_3$OD, 400 MHz): δ 4.91 (d, J = 3.6 Hz, 1H, H-1), 4.35 (q, J = 8.7 Hz, 1H, H-3), 4.17 (ddd, J = 11.0, 5.2, 2.0 Hz, 1H, H-6), 4.07−3.98 (m, 2H, H-2, H-6), 3.72−3.68 (m, 1H, H-5), 3.54 (dd, J = 9.8, 8.8 Hz, 1H, H-4), 3.39 (s, 3H, OMe), 3.11 (q, J = 7.3 Hz, approx. 18H, TEA CH_2CH$_3$), 1.28 (t, J = 7.3 Hz, approx. 27H, TEA CH$_2$CH_3). ^{31}P NMR (CD$_3$OD, 162 MHz): δ 2.21, 1.25, 0.94. ^{13}C NMR (CD$_3$OD, 100 MHz): δ 100.2 (C-1), 78.5 (t, J = 5.7 Hz, C-3), 75.3 (t, J = 4.9 Hz, C-2), 72.2 (d, J = 8.3 Hz, C-5), 71.7 (C-4), 65.6 (d, J = 5.2 Hz, C-6), 55.5 (OMe), 47.2 (TEA, CH_2CH$_3$), 9.1 (TEA, CH$_2$CH$_3$). HRMS (ESI) m/z: [M − H]$^-$ calcd for $C_7H_{17}O_{15}P_3$, 432.97075; found, 432.97076.

Methyl 3-*O*-Benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (23). As described by Bourdreux et al.,[38] **23** was synthesized using methyl α-D-glucopyranoside (100 mg, 0.515 mmol). The crude product was purified with flash chromatography (petroleum ether/EtOAc, 0−100%), and the pure product was collected as a white solid (143 mg, 0.384 mmol, 74% yield). mp 178.4−181.0 °C (lit.[69] mp 184 °C). $[\alpha]_D^{22}$ +87.7 (c = 0.70, CHCl$_3$) [lit.[69] $[\alpha]_D^{20}$ +77 (c = 0.91, CHCl$_3$)]. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.52−7.27 (m, 10H, Ar), 5.57 (s, 1H, H-7), 4.97 (d, J = 11.6 Hz, 1H, CH_2Ph), 4.82 (d, J = 3.8

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Hz, 1H, H-1), 4.79 (d, J = 11.6 Hz, 1H, CH_2Ph), 4.30 (dd, J = 4.3, 9.8 Hz, 1H, H-6), 3.87−3.71 (m, 4H, H-2, H-3, H-5, H-6), 3.65 (t, 1H, J = 9.1 Hz, H-4), 3.45 (s, 3H, OMe), 2.29 (d, 1H, J = 7.4 Hz, OH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.6 (Ar), 137.4 (Ar), 129.1 (Ar), 128.5 (Ar), 128.4 (Ar), 128.1 (Ar), 127.9 (Ar), 126.1 (Ar), 101.4 (H-7), 100.0 (C-1), 82.1 (C-4), 79.0 (C-2 or 3 or 5), 74.9 (CH_2Ph), 72.5 (C-2 or 3 or 5), 69.1 (C-6), 62.7 (C-2 or 3 or 5), 55.5 (OMe).

Methyl 3-*O*-Benzyl-α-D-glucopyranoside (24). As described by Boettcher et al.,[70] **24** was synthesized using methyl 4,6-*O*-benzylidene-3-*O*-benzyl-α-D-glucopyranoside (**23**) (120 mg, 0.322 mmol). The product was purified with flash chromatography (petroleum ether/EtOAc, 0−100%) to yield the pure product as a white solid (75.7 mg, 0.266 mmol, 83% yield). mp 92.1−93.9 °C (lit.[71] mp 85−86 °C). $[\alpha]_D^{23}$ +89.9 (c = 1.05, $CHCl_3$) [lit.[71] $[\alpha]_D^{23}$ +95.1 (c = 1.16, $CHCl_3$)]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.39−7.27 (m, 5H, Ar), 4.99 (d, J = 11.6, 1H, CH_2Ph), 4.74 (d, J = 11.6 Hz, 1H, CH_2Ph), 4.73 (d, J = 3.7 Hz, 1H, H-1), 3.82−3.74 (m, 2H, H-6 ×2), 3.67−3.52 (m, 4H, H-2, H-3, H-4, H-5), 3.42 (s, 3H, OMe), 2.74 (d, J = 2.5 Hz, 1H, OH), 2.32 (d, J = 8.7 Hz, 1H, OH), 2.27 (t, J = 6.4 Hz, 1H, OH). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.6 (Ar), 128.7 (Ar), 128.10 (Ar), 128.07 (Ar), 99.7 (C-1), 82.8 (C-3), 75.1 (CH_2Ph), 72.9 (C-2), 71.2 (C-5), 70.1 (C-4), 62.3 (C-6), 55.5 (OMe).

Methyl 3-*O*-Benzyl-α-D-glucopyranoside 2,4,6-Tris(dibenzyl phosphate) (25). Methyl 3-*O*-benzyl-α-D-glucopyranoside (**24**) (51.9 mg, 0.183 mmol) was dissolved in dry DCM (2 mL), and the solution was put under argon. 5-Phenyl-1*H*-tetrazole (160 mg, 1.10 mmol, 6 equiv) was added to the solution, followed by dibenzyl diisopropylphosphoramidite (0.30 mL, 0.82 mmol, 4.5 equiv). The reaction was allowed to stir at room temperature overnight. The next day, after the confirmation of successful phosphitylation with ^{31}P NMR, the reaction flask was cooled to −78 °C, and mCPBA (270.7 mg, 70% purity, 1.10 mmol, 6 equiv) was added. The reaction was allowed to stir at room temperature for 10 min before the solution was diluted with EtOAc (50 mL), washed with 10% Na_2SO_3 solution (2 × 30 mL), dried over $MgSO_4$, and concentrated to yield the crude product. The product was purified with flash chromatography (petroleum ether/EtOAc, 0−100%) to yield the pure product as a colorless oil (138.1 mg, 0.130 mmol, 71% yield). $[\alpha]_D^{21}$ +41.4 (c = 0.61, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.35−7.08 (m, 35H, Ar), 5.03 (s, 2H, CH_2Ph ×2), 5.01 (s, 2H, CH_2Ph ×2), 4.98 (d, J = 3.2 Hz, 1H, CH_2Ph), 4.97 (d, J = 3.2 Hz, 1H, CH_2Ph), 4.94−4.85 (m, 6H, H-1, CH_2Ph ×5), 4.81−4.75 (m, 3H, CH_2Ph ×3), 4.43−4.18 (m, 4H, H-2, H-3, H-6 ×2), 3.98 (t, J = 9.3 Hz, 1H, H-4), 3.86 (dd, J = 10.0, 5.2 Hz, 1H, H-5), 3.28 (s, 3H, OMe). ^{31}P NMR ($CDCl_3$, 162 MHz): δ −0.98, −1.73, −1.85. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.1 (Ar), 136.0 (Ar), 135.9 (Ar), 135.8−135.6 (m, Ar), 128.7−128.5 (m, Ar), 128.3 (Ar), 128.0 (m, Ar), 127.8 (Ar), 127.7 (Ar), 127.5 (Ar), 97.6 (C-1), 78.3 (m, C-4), 76.7 (m, C-2 or 3), 75.0 (C-2 or 3, CH_2Ph), 69.6 (m, CH_2Ph), 69.4 (m, CH_2Ph), 68.9 (m, C-5), 66.1 (m, C-6), 55.7 (OMe). HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{56}H_{59}O_{15}P_3$, 1065.31396; found, 1065.31336.

Methyl α-D-Glucopyranoside 2,4,6-Trisphosphate Triethylammonium Salt (5). Methyl 3-*O*-benzyl-α-D-glucopyranoside 2,4,6-tris(dibenzyl phosphate) (**25**) (132.4 mg, 0.124 mmol) was dissolved in MeOH (6.6 mL). Ultrapure water (0.66 mL) was added dropwise to the solution, ensuring that the precipitate formed upon addition was able to dissolve back into solution. $Pd(OH)_2$/C (20%, ≥50% wet, 66.2 mg) was added to the solution, and the reaction flask was flushed with hydrogen. The reaction was allowed to stir at room temperature for 24 h after which the catalyst was filtered off and the collected filtrate was evaporated to yield the product as a free acid. No purification steps were deemed to be necessary, but triethylamine was added to sharpen the ^{31}P NMR signals and to convert the product from the free acid into the triethylammonium salt. The product was concentrated in vacuo before being lyophilized and collected as a colorless glass (86 mg, 0.10 mmol, 94% yield). $[\alpha]_D^{22}$ +39.3 (c = 0.43, MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 4.91 (d, J = 2.8 Hz, 1H, H-1), 4.26−4.16 (m, 2H, H-3, H-6), 4.05−3.98 (m, 3H, H-2, H-4, H-6), 3.70 (brd, J = 9.8 Hz, 1H, H-5), 3.39 (s, 3H, OMe), 3.12 (q, J = 7.5 Hz, approx. 18H, TEA CH_2CH$_3$), 1.29 (t, J = 7.5 Hz, approx. 27H, TEA CH$_2$CH_3). ^{31}P NMR (CD_3OD, 162 MHz): δ 1.99, 1.75, 1.32. ^{13}C NMR (CD_3OD, 100 MHz): δ 100.2 (C-1), 76.3 (d, 5.6 Hz, C-2 or 4), 74.6 (d, 3.4 Hz, C-2 or 4), 73.5 (d, 5.5 Hz, C-3), 71.1 (m, C-5), 63.9 (C-6), 55.6 (OMe), 47.0 (TEA CH_2CH_3), (TEA, CH_2CH_3) 9.3. HRMS (ESI) m/z: $[M - H]^-$ calcd for $C_7H_{17}O_{15}P_3$, 432.97075; found, 432.97063.

HPLC. For analysis and stability experiments, the sugar phosphates were resolved by anion-exchange HPLC on a 3 × 250 mm CarboPac PA200 column (Dionex) fitted with 3 × 50 mm guard cartridge of the same material. Compounds were eluted with a gradient derived from buffer reservoirs containing water (A) and 0.6 M methanesulfonic acid (B) delivered at a flow rate of 0.4 mL min^{-1} according to the following schedule: time (min), % B; 0, 0; 20, 80; 21, 0; and 31, 0. Compounds were detected with the phosphate detection reagent of Phillippy and Bland.[72] For this purpose, the column eluate was mixed in a mixing Tee with a solution of 0.1% (w/v) ferric nitrate nonahydrate in 2% (w/v) perchloric acid delivered at a flow rate of 0.2 mL min^{-1} and passed through a 0.192 mL internal volume knitted reaction coil before being transferred to a UV detector set at 290 nm. Typically, samples of 40 μL of 500 μM solutions in water were injected. Data were exported from the ChromNav2 software as x,y data files and redrawn in GraFit.v7.[73]

Biology Methods. *Materials.* HEK-293 cells with all three Ins(1,4,5)P_3R subtypes disrupted using CRISPR/Cas9 technology (HEK-3KO)[74] were from Kerafast (Boston, USA). Dulbecco's modified Eagle's medium/nutrient mixture F-12 with GlutaMAX (DMEM/F-12 GlutaMAX) and Mag-Fluo-4 AM were from Thermo Fisher. TransIT-LT1 transfection reagent was from GENEFLOW (Elmhurst, Lichfield, UK). Most chemicals and fetal bovine serum (FBS) were from Sigma-Aldrich (Gillingham, UK). Cyclopiazonic acid (CPA) was from Tocris (Bristol, UK). G418 was from Formedium (Norfolk, UK). Protease inhibitor cocktail tablets were from Roche. Half-area 96-well black-walled plates were from Greiner. Ins(1,4,5)P_3 was from Enzo (Exeter, UK). [^{3}H]-Ins(1,4,5)P_3 was from PerkinElmer.

Cell Culture and Transfection. HEK cells were cultured in DMEM/F-12 GlutaMAX medium supplemented with 10% FBS (37 °C in 95% air and 5% CO_2). Cells were either passaged or used for experiments when they reached confluence. HEK cells expressing only Ins(1,4,5)P_3R1 (HEK-Ins(1,4,5)P_3R1) were generated by transfecting HEK-3KO cells with the gene encoding rat Ins(1,4,5)P_3R1 (lacking the S1 splice site)[27] cloned into pcDNA3.1(−)/Myc-His B plasmid[75] using the TransIT-LT1 reagent following the manufacturer's instructions. To generate stable cell lines, cells were passaged 48 h after transfection in medium with G418 (1 mg mL^{-1}). Selection was maintained for 2 weeks, and the medium was changed every 3 days. Monoclonal cell lines were selected by plating cells (~1 cell well^{-1}) into 96-well plates in medium containing G418 (1 mg mL^{-1}). After 4 days, wells with only one cell were identified, and the cells were allowed to reach confluence. These cell lines were then expanded, and their expression of Ins(1,4,5)P_3R1 was confirmed by western blot using an anti-Ins(1,4,5)P_3R1 antibody.[27]

Ca^{2+}-Release Assays. The free [Ca^{2+}] within the lumen of the endoplasmic reticulum (ER) was measured using the low-affinity Ca^{2+} indicator Mag-Fluo-4.[76,77] The ER of HEK-Ins(1,4,5)P_3R1 cells was loaded with the Ca^{2+} indicator by incubating cells (2.4 × 10^7 cells mL^{-1}, 1 h, 22 °C) in HEPES-buffered saline (HBS; 135 mM NaCl, 5.9 mM KCl, 11.6 mM HEPES, 1.5 mM $CaCl_2$, 11.5 mM glucose, 1.2 mM $MgCl_2$, pH 7.3) supplemented with BSA (1 mg mL^{-1}), Pluronic F127 (0.4 mg mL^{-1}), and Mag-Fluo-4 AM (20 μM). Cells were then suspended in the Ca^{2+}-free cytosol-like medium (CLM: 20 mM NaCl, 140 mM KCl, 1 mM EGTA, 20 mM Pipes, 2 mM $MgCl_2$, pH 7.0) and permeabilized with saponin (10 μg mL^{-1}, 3 min, 37 °C). Permeabilized cells were centrifuged (650 × g, 3 min) and incubated in CLM (7 min, 37 °C) to allow the Ca^{2+} stores to empty. Cells were then centrifuged (650 × g, 3 min) and resuspended in CLM without Mg^{2+} but supplemented with 375 μM $CaCl_2$ to give a final free [Ca^{2+}] of 220 nM after the addition of 1.5 mM MgATP. Cells (~4 × 10^5 well^{-1}) were added to poly-L-lysine-coated half-area 96-well black-

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walled plates. Fluorescence was recorded at 20 °C at intervals of 1.44 s using a FlexStation III plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively. MgATP (1.5 mM) was added to initiate Ca^{2+} uptake, and when the ER had loaded to the steady state (~2.5 min), cyclopiazonic acid (CPA, 10 μM) was added to inhibit the ER Ca^{2+} pump. Ins(1,4,5)P_3 or other ligands were added after a further 60 s. The amount of Ca^{2+} release was calculated as a percentage of the fluorescence signal from fully loaded stores (F_{full}) minus the signal from nonloaded stores ($F_{full} - F_{empty}$). Results are presented as means ± SEM from 5 to 11 independent experiments, each run in duplicate.

[^{3}H]-Ins(1,4,5)P_3 Binding to Cerebellar Membranes. Cerebellar membranes, which are rich in Ins(1,4,5)P_3R1, were prepared from the cerebella of adult Wistar rats. Frozen cerebella were homogenized at 4 °C in the homogenization medium (HM: 1 mM EDTA, 50 mM Tris, protease inhibitors, pH 8.3) supplemented with 100 mM NaCl. After centrifugation (130,000 × *g*, 1 h, 4 °C), the membranes were resuspended in HM (~6 mg protein mL^{-1}) and stored at −80 °C. Equilibrium competition binding assays were performed at 4 °C in a final volume of 500 μL of Tris/EDTA medium (TEM: 50 mM Tris, 1 mM EDTA, pH 8.3) with [^{3}H]-Ins(1,4,5)P_3 (19.3 Ci $mmol^{-1}$, 1.5 nM), competing ligands, and 25 μL of membranes.[27] After 5 min, during which equilibrium was attained, bound and free ligands were separated by centrifugation (20,000 × *g*, 5 min, 4 °C), and the pellet was then rinsed and resuspended in TEM (200 μL) before liquid scintillation counting (1 mL, Ecoscint A). Nonspecific binding, determined by the addition of 10 μM Ins(1,4,5)P_3, was always <10% of total binding, and <10% of the added [^{3}H]-Ins(1,4,5)P_3 was bound. Results are presented as means ± SEM from three independent experiments without replicates.

Data Analysis. Equilibrium binding results and concentration−effect relationships were fitted to Hill equations (GraphPad Prism, version 5) from which −log IC_{50} (pIC_{50}) and −log EC_{50} (pEC_{50}) values were obtained. For equilibrium competition binding assays pK_d values were calculated using the Cheng and Prusoff equation.[78] Because pEC_{50} and pK_d values are normally distributed, these results are presented as means ± SEM from *n* independent experiments. For comparisons of the ratios between mean values (EC_{50}/K_d), statistical analyses compared the differences between their log values (pEC_{50} and pK_d),[79] with the SEM calculated as follows, assuming that the population variances are the same (confirmed using an F test)[80]

$$\mathrm{SEM} = s_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where s_p is the estimate of the population variance

$$s_p = \sqrt{\frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2}{n_1 - n_2 - 2}}$$

where s_1 and s_2 are the sample standard deviations, and n_1 and n_2 are the sample sizes. Although all analyses were performed using log values, for greater clarity, we present ratios as the antilogs of the means and the 95% confidence interval.

Statistical analysis used ANOVA followed by Bonferroni's multiple comparison test (GraphPad Prism, version 5). $P < 0.05$ was considered significant.

EC39.4% release/K_d was calculated because some ligands did not fully release the Ins(1,4,5)P_3-sensitive stores. The ratio was calculated using the concentration of each ligand that caused a release of 39.4% of the total content of the stores (which is the % released by Ins(1,4,5)P_3 at its EC_{50}).

Molecular Docking. The X-ray crystal structure of the N-terminal IBC of the Type 1 Ins(1,4,5)P_3R in complex with Ins(1,4,5)P_3 (PDB: 1N4K)[4] was used for the molecular docking experiments of ligands (**1**−**7**). Compounds **1**−**7** were built and minimized using Chem3D version 15.1 and Mercury version 3.10. Docking methods were optimized by docking Ins(1,4,5)P_3 into the IBC structure with GOLD[81] version 5.6.1 to reproduce the bound Ins(1,4,5)P_3 conformation. The most successful docking runs were achieved with two water molecules in the binding site (1139 and 1198) being allowed to toggle and spin while the remaining water molecules were removed.[82] The lysine residues in the binding site (K412, K508, and K569) were permitted constrained movement and internal H-bonds were allowed. Compounds **2**−**7** were docked 100 times and scored using the GoldScore scoring function. The highest scoring solutions for **2**, **3**, **6**, and **7** were exported, and figures were prepared using PyMOL (DeLano Scientific LLC). More details are given in Supporting Information SI-1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00215.

Overlays, molecular docking, and ligand interaction diagrams for **2**, **3**, **6**, and **7**; HPLC data for compounds **2**−**7**; stability studies of compounds **6** and **7** in SI-1 (PDF)

NMR spectral data for all compounds in SI-2 (PDF)

Molecular formula strings for all compounds (CSV)

Data for 1N4K (PDB)

Molecular docking file for methyl α-L-glucopyranoside 2,3,6-trisphosphate (**2**) (PDB)

Molecular docking file for methyl α-L-glucopyranoside 2,4,6-trisphosphate (**3**) (PDB)

Molecular docking file for α-D-glucopyranosyl 1,3,4-trisphosphate (**6**) (PDB)

Molecular docking file for β-D-glucopyranosyl 1,3,4-trisphosphate (**7**) (PDB)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AdA, adenophostin A; ATP, adenosine triphosphate; COSY, correlation spectroscopy; CPA, cyclopiazonic acid; mCPBA, *meta*-chloroperoxybenzoic acid; cryo-EM, cryogenic electron microscopy; CSA, camphorsulfonic acid; DCM, dichloromethane; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FBS, fetal bovine serum; Gluc(3,4)P_2, D-glucose 3,4-bisphosphate; HBS, HEPES-buffered saline; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSQC, heteronuclear single quantum coherence; IBC, Ins(1,4,5)P_3-binding core; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,4,5)P_3R, D-*myo*-inositol 1,4,5-trisphosphate receptor; NMR, nuclear magnetic resonance; SAR, structure−activity relationship; THF, tetrahydrofuran; TMS, tetramethylsilane

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