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Synthesis and Biological Activity of Metabolically Stabilized Cyclopentyl Trisphosphate Analogues of D-*myo*-Ins(1,4,5)P_3

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*We describe the synthesis of four novel metabolically stabilized analogues of Ins(1,4,5)P_3 based on the known cyclopentane pentaol tris(phosphate) **2**: tris(phosphorothioate) **3**, tris(methylenephosphate) **4**, tris(sulfonamide) **5**, and tris(sulfate) **6**. Of these analogues, only the tris(phosphorothioate) **3** and parent tris(phosphate) **2** bound to the type I InsP_3R construct. In addition, both the tris(phosphorothioate) **3** and parent tris(phosphate) **2** elicited calcium release in MDA MB-435 breast cancer cells. The Ins-(1,4,5)P_3 agonist activities of these two compounds can be rationalized on the basis of computational docking of the ligands to the binding domain of the type I InsP_3R.*

Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3, **1**) is a second messenger produced primarily by phospholipase C metabolism of phosphatidylinositol-4,5-bisphosphate (PIP_2) in response to the stimulation of G-protein-coupled receptors or receptor tyrosine kinases.[1] Ins(1,4,5)P_3 rapidly releases Ca^{2+} from intracellular Ca^{2+} pools within the endoplasmic reticulum and other cellular membranes by binding to the Ins(1,4,5)P_3 receptors (InsP_3Rs). InsP_3Rs are involved in many signaling pathways in the control of cellular and physiological processes as diverse as cell division, cell proliferation, apoptosis, fertilization, development, behavior, memory, and learning. To date, none of the inositol-based analogues of Ins(1,4,5)P_3 has proved to be more potent than Ins(1,4,5)P_3 itself, with the exception of the dimeric Ins-(1,4,5)P_3 analogues.[2,3] A search for InsP_3R agonists revealed the highly potent naturally occurring adenophostins,[4] for which an extensive set of synthetic analogues and corresponding data of structure–activity relationships has been accumulated.[5] We sought to prepare stabilized, cell-permeant, high-affinity small molecules that could act as Ins(1,4,5)P_3 agonists or antagonists. To this end, we turned to the cyclopentane-based InsP_3R ligands, in particular trisphosphate **2** (Figure 1), which had only a 2–4-fold lower affinity than Ins(1,4,5)P_3.[6] The cyclopentane-based scaffold was capable of mobilizing cellular Ca^{2+} stores and offered easier synthetic access to examine several phosphomimetic functionalities.[7] We describe herein the preparation of four analogues of the parent trisphosphate **2**. We also present evaluation of ligand binding to the InsP_3R using a fluorescence resonance energy transfer method, and we use computational algorithms to dock the synthetic analogues into the type I InsP_3R.

Phosphate mimics have been used extensively to enhance stability of naturally occurring phosphates. We have previously employed phosphorothioate and methylenephosphonates to prepare phosphatase-resistant analogues of phosphoinositides[8–10] and lysophosphatidic acid.[11] In each case, the analogues were less susceptible to acidic or enzymatic cleavage

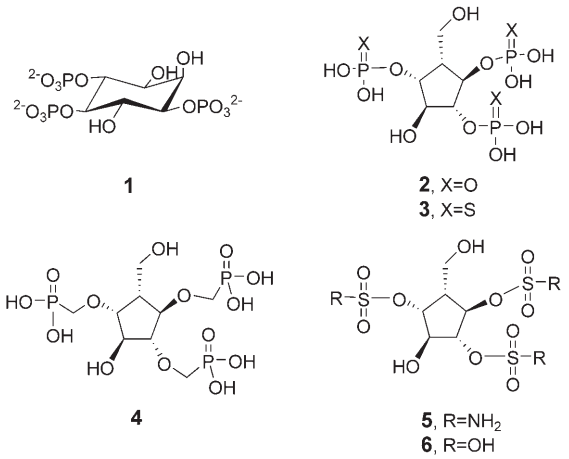

Figure 1. Ins(1,4,5)P_3 and stabilized analogues.

and some bound to cognate binding proteins with reduced affinity. On the basis of these data, we designed two analogues of **2**, trisphosphorothioate **3** and tris(methylenephosphonate) **4**. We also designed two novel isosteric analogues of phosphate, tris(sulfonamide) **5** and tris(sulfate) **6**. These former two functionalities have been previously investigated for Ins-(1,4,5)P_3 analogues[12,13] and lysophosphatidic acid ana-

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logues.[14] Herein, we report the asymmetric total synthesis of the four compounds **3**, **4**, **5**, **6**, and the original compound **2**. As the X-ray crystal structure of type 1 $InsP_3R$ binding with Ins(1,4,5)P_3 has been published recently,[15] the computational docking of the ligands was performed to rationalize the binding mode of cyclopentane-based ligands to $InsP_3R$.

Results and Discussion

Molecular docking of Ins(1,4,5)P_3 and cyclopentyl analogues

To obtain insight on interactions of the cyclopentane-based analogues with $InsP_3R$, we studied the possible binding mode of compound **3** with $InsP_3R1$ by AutoDock3.0.5,[16–18] based on the crystal structure of type I $InsP_3R$ complexed with Ins(1,4,5)P_3[15,19] and compared with the docking results of Ins(1,4,5)P_3 **1** and the parent compound **2** (as shown in Figure 2). Three phosphonate (or phosphorothioate) groups formed good hydrogen-bonding networks within the base-rich binding pocket of $InsP_3R$ binding pocket, in which Arg and Lys residues dominate the interactions. Figure 2A shows successful steric simulation in the geometry of **2** and **3**; the phosphonate groups dock into nearly identical binding positions relative to the position of Ins(1,4,5)P_3. In particular, the phosphonate at the 4-position of **3** which appears to mimic the P1 of Ins(1,4,5)P_3, can form hydrogen bonds with Arg 568 and Lys 569 of $InsP_3R$. The bis(methylene)phosphonates at the 1,2-position of **3** mimic the bisphosphates at the P4,5-position of Ins(1,4,5)P_3, forming hydrogen bonds with Arg 265 and Arg 269 of $InsP_3R$ (Figure 2B). These interactions located the cyclopentane rings at the center of the pocket occupied normally by the inositol ring of Ins(1,4,5)P_3. The docking studies revealed the properties of the $InsP_3R$ active site, including multiple basic residues (especially Arg 265, Gly 268, Arg 269, Lys 508, Arg 511, Arg 568, and Lys 569), and presented the possible binding mode of cyclopentane-based compounds with $InsP_3Rs$. In contrast, the binding energies of tris(SU) **6** and tris(SA) **5** were considerably lower (data not shown), confirming earlier observations that these functionalities abrogated the biological activity of Ins(1,4,5)P_3.[13] The model and method of $InsP_3R$ structural modeling provide a perspective on future design of Ins(1,4,5)P_3 analogues as $InsP_3R$ ligands.

Synthesis

The synthesis of key intermediate **12**, followed the published synthetic strategy[6] with modifications as shown in Scheme 1. Compound **7** was prepared from α-D-glucopyranoside,[6] but benzylation with **7** with NaH and *p*-methoxybenzyl chloride in DMF proceeded in low yield. We found that the use of Bu_4NI as a phase transfer catalyst in THF as solvent for the *p*-methoxybenzylation gave **8** in 96% yield. Next, **9** was obtained from **8** in three steps:[6] selective reduction of the 4-PMB group with $LiAlH_4$ and $AlCl_3$, followed Swern oxidation and SmI_2-mediated ring contraction. Benzylation of **9** gave a mixture of **11** and its isomer, 4-benzylated isomer, in a ratio of 2:3 as found previously.[6] Thus, we developed a new regioselective method, using selective reduction of acetal **10** with the nonhygroscopic reductive solid reagent $BH_3{\cdot}Me_2NH$ and $BF_3{\cdot}OEt_2$, which proceeds with no detectable 4-benzyl by-product.[20] Thus, diol **9** was heated with benzaldehyde dimethyl acetal and TsOH in DMF to give the acetal **10** in 94% yield; the reductive opening with $BH_3{\cdot}Me_2NH/BF_3{\cdot}OEt_2$ in CH_3CN was carried out at room temperature to give **11** in 92% yield. Hydrolysis of **11** in 1 M HCl/EtOH gave the key triol intermediate **12** in 83% yield.

To compare the biological activity of the four new compounds with the parent trisphosphate **2**, we phosphorylated **12** with (benzyloxy)(*N*,*N*-diisopropylamino)phosphine in the presence of 1*H*-tetrazole, followed by oxidation to the protected phosphates with *m*-CPBA and water to give **13** (Scheme 2). The benzyl protecting groups of **13** were removed simultaneously by catalytic hydrogenolysis with $Pd(OH)_2/C$ in aqueous

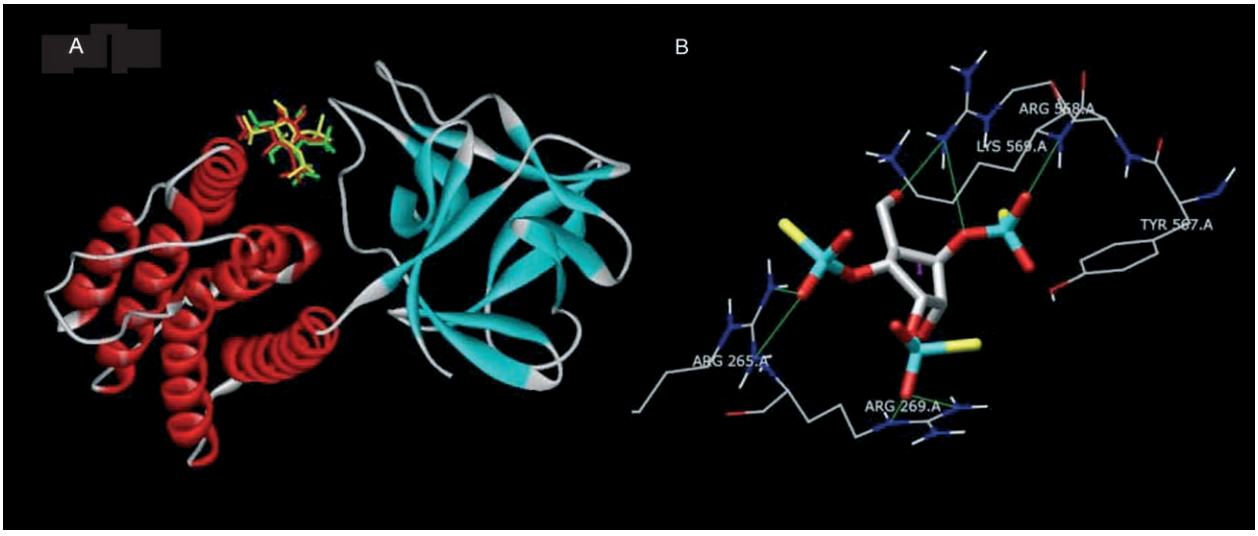
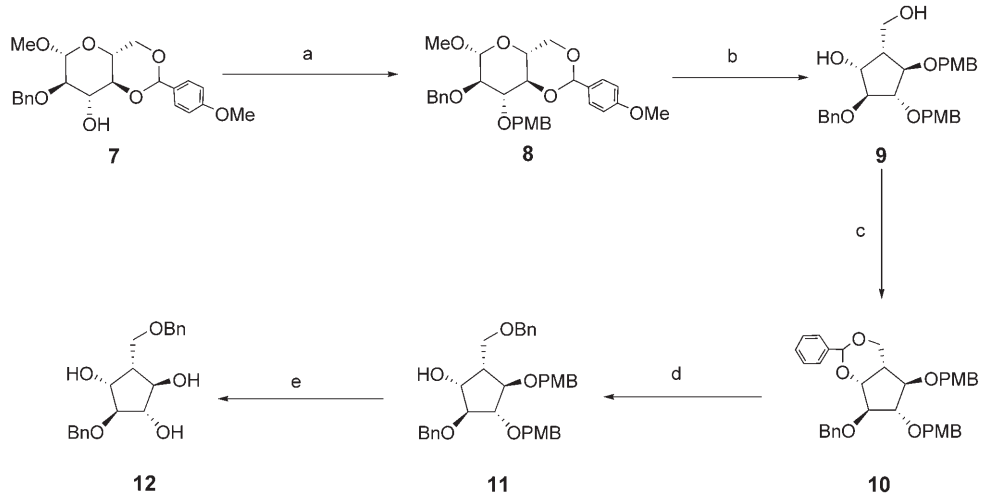
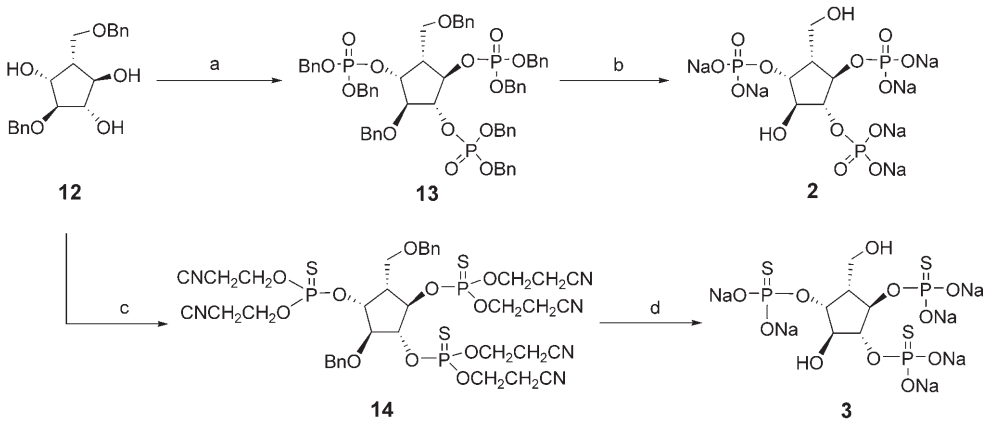


Figure 2. Panel A: Docking conformations of Ins(1,4,5)P_3 (small molecule in red), **2** (small molecule in green) and **3** (small molecule in yellow) in Type I IP_3R (macromolecule) binding pocket using AutoDock3.0. Panel B: Binding interaction of **3** with relevant residues of IP_3R active site (hydrogen bonds are shown as green lines).



Scheme 1. Synthesis of key intermediate for cyclopentyl analogues. a) PMBCl, NaH, Bu_4NI, THF, reflux, 96%; b) 3 steps; c) $PhCH(OMe)_2$, TsOH, DMF, 50 °C, 94%; d) BH_3, Me_2NH, BF_3, Et_2O, CH_3CN, RT, 92%; e) 1 M HCl, EtOH, reflux, 83%.



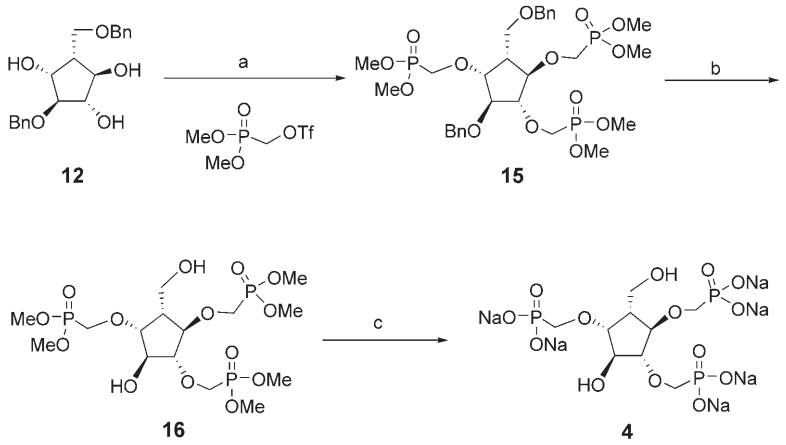
Scheme 2. Synthesis of tris(PO) **2** and tris(PT) **3**. a) 1) *i*-$Pr_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, RT, 2) *m*-CPBA, -78 °C, H_2O, 57%; b) H_2, $Pd(OH)_2/C$, CH_2Cl_2, CH_3OH, H_2O, RT; c) 1) *i*-$Pr_2NP(OCH_2CH_2CN)_2$, 1*H*-tetrazole, CH_2Cl_2, RT, 2) S, CS_2, Py, RT, 96%; d) Na/NH_3, -78 °C, 59%.

$MeOH/CH_2Cl_2$.[21] Debenzylation at a high pressure of hydrogen was required to overcome the steric hindrance around the cyclopentane scaffold. The sodium salt of tris(PO) **2** was prepared by ion exchange.

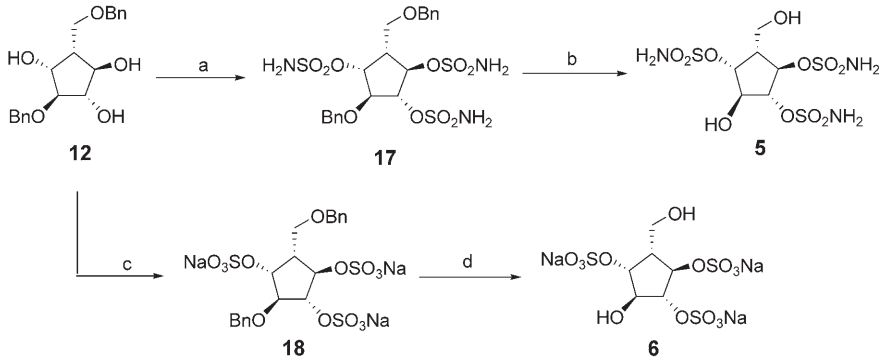
The synthesis of tristhiophosphate **3** is outlined in Scheme 2.[22] Phosphorylation of the triol **12** with a mixture of bis(2-cyanoethyl)*N*,*N*-diisopropylphosphoramidite and 1*H*-tetrazole in anhydrous CH_2Cl_2, followed by oxidation with elemental sulfur in a solution of pyridine and CS_2 to yield the corresponding cyanoethyl-protected thiophosphate **14**. Subsequent treatment of **14** with Na/NH_3 removed the benzyl groups and cyanoethyl group simultaneously to give trisphosphorothioate, tris(PT) **3**. This analogue was purified by ion-exchange chromatography on DEAE-Sephadex, eluting with a gradient of triethylammonium bicarbonate buffer, and assayed with Ellman's reagent. The sodium salt of **3** was prepared by ion exchange.

For the synthesis of trismethylenephosphate **4** (see Scheme 3), (dimethoxyphosphinyl)methyl triflate was prepared as described,[23] and was treated with alkoxide salt of **12** in THF to give **15** in modest yield. Debenzylation of **15** by hydrogenolysis gave **16**, and the methyl esters were removed with trimethylbromosilane (TMBS) using an in situ protection-deprotection strategy. First, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added to a solution of **16** in CH_2Cl_2 to protect the hydroxy groups, and then TMBS was added to deprotect the phosphate methyl esters. After concentration in vacuo, 95% methanol was added to hydrolyze the silyl phosphate esters to give tris(MP) **4** as a sodium salt, after passage through an ion-exchange resin.

The protected tris(sulfonamide) derivative **17** was prepared using Okada's method.[24] First, sulfamoyl chloride was prepared using formic acid reduction of chlorosulfonyl isocyanate. A solution of **12** in *N*,*N*-dimethylacetamide (DMA) was added to the solution of sulfamoyl chloride in CH_3CN to afford **17** in good yield. DMA worked as a moderate base and a solvent to accelerate the sulfamoylation. Finally, the benzyl group of **17** was removed using high pressure hydrogenation to afford the target compound tris(SA) **5**. Sulfation of **12** was first attempted without success using either sulfur trioxide pyridine complex or sulfur trioxide triethylamine complex. The desired intermedi-



Scheme 3. Synthesis of tris(MP) **4**. a) 0 °C, NaH, THF, 37%; b) H_2, $Pd(OH)_2$/C, CH_2Cl_2, CH_3OH, H_2O, RT, 86%; c) 1) BSTFA, TMBS, CH_2Cl_2, RT, 2) Na^+ Resin, 94%.



Scheme 4. Synthesis of tris(SA) **5** and tris(SU) **6**. a) $ClSO_2NH_2$, DMA, 0 °C, 53%; b) $Pd(OH)_2$/C, H_2, 50 atm, CH_3OH/CH_2Cl_2/H_2O, RT, 73%; c) $ClSO_3H$, Py, 0 °C, 91%; d) $Pd(OH)_2$/C, H_2, 50 atm, CH_3OH/CH_2Cl_2/H_2O, RT, 90%.

ate **18** was ultimately obtained using chlorosulfuric acid as the sulfating reagent, and then debenzylated as before to afford the target compound, tris(SU) **6** (see Scheme 4).

Binding studies

The binding ability of these compounds was examined with a FRET-based Ins(1,4,5)P_3 biosensor LIBRA[25] and its negative control LIBRA-N.[26] Figure 3 shows the effects of tris(PT) **3**, tris(PO) **2**, and Ins(1,4,5)P_3 **1** on permeabilized LIBRA-expressing cells and LIBRA-*N*-expressing COS-7 cells. Ins(1,4,5)P_3 increased the emission ratio (480/535 nm) of LIBRA in a concentration-dependent manner (Figure 3A), but had no effect on LIBRA-N (Figure 3B). Sequential application of 10 μM and 100 μM tris(PT) **3** elicited a similar stepwise increase in the emission ratio of LIBRA (Figure 3C). At the end of each experiment, 10 μM Ins(1,4,5)P_3 was applied to determine the maximal response of LIBRA. The extent of the increase in the emission ratio with 10 μM tris(PT) **3** was ~40% of the maximal elevation (Table 1). The effect of 10 μM tris(PT) **3** was mostly associated with the specific Ins(1,4,5)P_3 -binding site of LIBRA, as the effect of 10 μM tris(PT) **3** on LIBRA-N was very little (Figure 3D). From the fact that the EC_{50} of Ins(1,4,5)P_3 on the emission ratio of LIBRA is ~400 nM,[25] it is estimated that the effect of 10 μM tris(PT) **3** is comparable to that of 0.3 μM Ins(1,4,5)P_3.

The application of tris(PO) **2** increased the ratio of LIBRA (Figure 3E), but the extent of the effect was smaller than the increase induced by tris(PT) **3** (Table 1). The effect of 100 μM of tris(PO) **2** on LIBRA is comparable to that of 0.1 μM Ins(1,4,5)P_3. However, 100 μM tris(PO) **2** decreased slightly the emission ratio of LIBRA-N (Figure 3F). Therefore, the effect of tris(PO) **2** on the emission ratio of LIBRA is likely to be underestimated. No detectable increase in ratio of LIBRA was observed with 100 μM the tris(MP) **4**, tris(SA) **5**, or tris(SU) **6** (data not shown).

Cellular calcium mobilization

As the primary activity of Ins(1,4,5)P_3 is to induce cellular calcium mobilization into the cytosol, we tested the cellular responses to each of the cyclopentyl analogues in MDA-MB-435 human breast carcinoma cells. Commonly used Ins(1,4,5)P_3 analogues for eliciting calcium responses in cells have modifications at the phosphate or hydroxy positions of the inositol ring.[27,28] These analogues were able to elicit calcium responses but with reduced potencies.[29] Ins(1,4,5)P_3 analogues lacking a cyclohexyl ring structure can also mediate calcium mobilization.[30]

Thus, MDA-MB-435 cells were treated with type III histone at a final concentration of 50 μM which was followed by treatment with a given Ins(1,4,5)P_3 analogue at a final concentration of 100 μM. The histone carrier system was developed to facilitate intracellular delivery of exogenous inositol polyphosphates and phosphoinositides.[31] Cells were then analyzed for changes in cytosolic calcium levels. Figure 4 shows that tris(PO) **2**, tris(PT) **3**, and tris(MP) **4** stimulated increases in cytosolic calcium levels with the maximum response being observed for tris(PO) **2**. The increase in cytosolic calcium levels for tris(PT) **3** and tris(MP) **4** was 65% and 12% respectively of the response achieved by tris(PO) **2**. Moreover, tris(PO) **2** elicited an increase in the cytosolic calcium levels to about 66% of the response achieved by Ins(1,4,5)P_3. The other two analogues tris(SA) **5** and tris(SU) **6** caused no discernable changes in cytosolic calcium levels. Cells stimulated with histone alone at a final con-

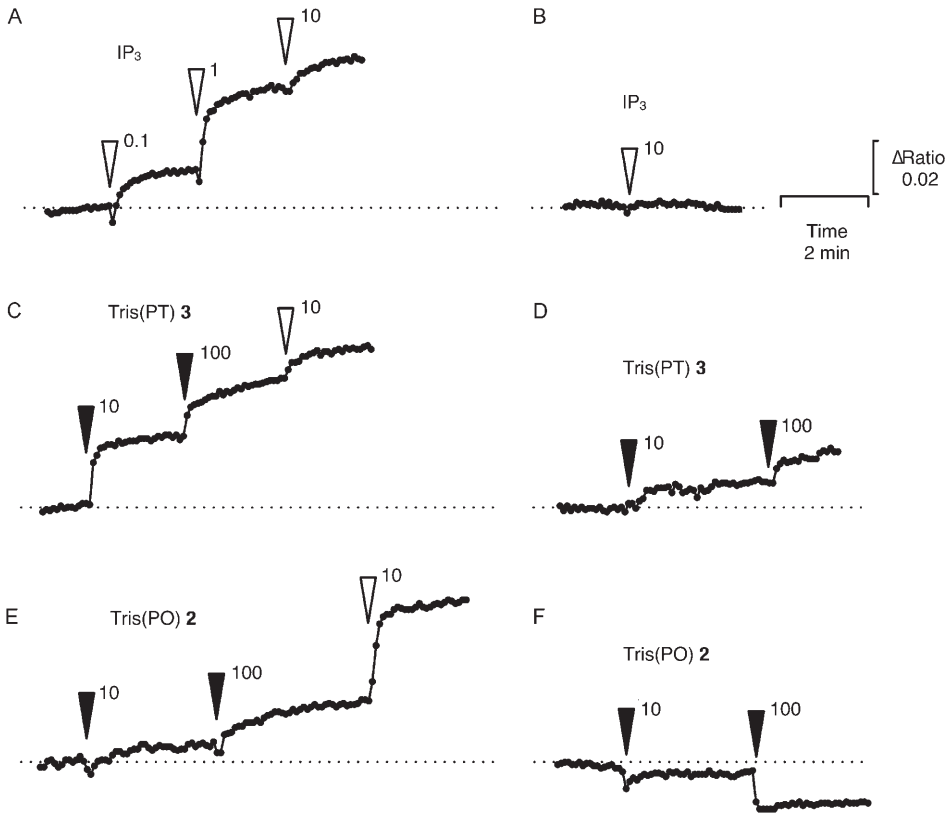


Figure 3. Effects of Ins(1,4,5)P_3 and stabilized analogues on the emission ratio (480/535 nm) of LIBRA and LIBRA-N. A, C, E: Changes in the emission ratio of LIBRA due to exposure to IP_3 (A), tris(PT) **3** (C), and tris(PO) **2** (E). B, D, F: Changes in the emission ratio of LIBRA-N due to exposure to Ins(1,4,5)P_3 (B), tris(PT) **3** (D), and tris(PO) **2** (F). Arrowheads with a number indicate the time when IP_3 (closed arrowhead) or test compounds (tris(PT) **3** or tris(PO) **2**; open arrow head) were applied and their concentrations (μM).

The calcium mobilization data differs somewhat from the LIBRA binding results. The three non-binding analogues **4**, **5**, and **6** were also inactive in receptor activation in cells. However, whereas tris(PT) **3** was shown to have a higher affinity than tris(PO) **2** for the Ins(1,4,5)P_3 receptor in the binding assay, the reverse order of activity was observed in the calcium mobilization assay. This could be caused by a number of factors. We excluded the possibility of influx of calcium from extracellular media by incubating the cells with the calcium chelator EGTA (calcium chelator). As there are multiple isoforms of Ins(1,4,5)P_3 receptors,[32] it is possible that tris(PO) **2** and tris(PT) **3** have different affinities for these isoforms. Alternatively, it may also be possible that tris(PT) **3** causes a decreased transient opening of the calcium channel, whereas tris(PO) **2** may induce the calcium channels to be open longer, resulting in differences in the release of calcium into the cytosol.

Table 1. Summary of data from experiments shown in Figure 3.[a]

Compound	*c* [μM]	LIBRA ΔRatio	LIBRA % max.	LIBRA-N ΔRatio
none		−0.001 ± 0.0002	−0.8 ± 0.4	−0.001 ± 0.001
Tris(PT) **3**	10	0.024 ± 0.0006	41.6 ± 0.8	0.005 ± 0.001
	100	0.041 ± 0.0017	69.9 ± 1.7	0.014 ± 0.002
Tris(PO) **2**	10	0.003 ± 0.0002	6.9 ± 0.4	−0.003 ± 0.001
	100	0.016 ± 0.0004	31.2 ± 0.4	−0.016 ± 0.001
IP3	0.1	0.012 ± 0.0004	23.4 ± 1.0	ND
	1	0.042 ± 0.0009	76.2 ± 0.2	ND
	10	0.055 ± 0.0011	100	0.003 ± 0.001

[a] Values are the mean ± SE from either three or four independent experiments.

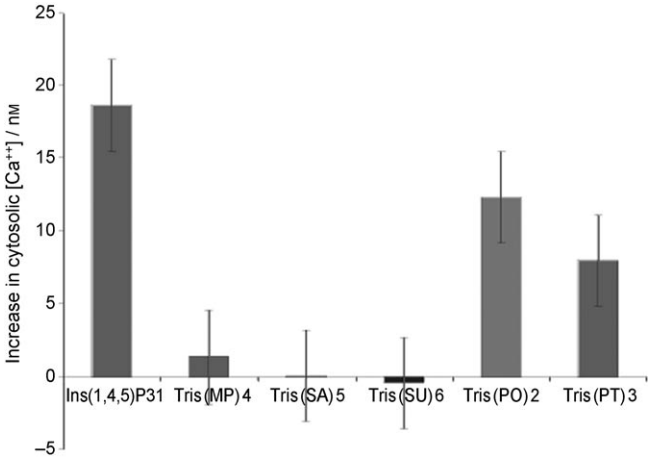


Figure 4. Cellular calcium mobilization in MDA-MB-435 cells by Ins (1,4,5)P_3 analogues. See experimental methods for details.

centration of 50 μM showed no increase in cytosolic calcium levels.

The Ins(1,4,5)P_3 analogues were also tested for their ability to stimulate increases in cytosolic calcium levels without histone as a carrier. Tris (PT) **3** stimulated an increase in cytosolic calcium levels at a comparable level to that achieved with histone (data not shown). None of the other analogues exhibited significant changes in cytosolic calcium levels following carrier-free delivery.

Together the in vitro and cellular data suggests that binding affinity of the analogues to the Ins(1,4,5)P_3 receptor may not be the only factor that influences release of calcium into the cytosol.

Taken together, we show that tris(PT) **3** analogue of the parent cyclopentane-based Ins(1,4,5)P_3 receptor ligand tris(PO)

2[6] stimulates an increase in cytosolic calcium levels in MDA-MB-435 breast carcinoma cells. It is noteworthy that only tris(PT) **3** elicited a comparable calcium response in the presence and absence of histone as a carrier. This suggests that tris(PT) **3** has an intrinsically higher rate of diffusion into cells compared to the other analogues.

Experimental Section

General synthetic procedures. Reagent chemicals were obtained from Aldrich Chemical Corporation and were used without prior purification. Solvents were reagent grade and were distilled before use: THF was distilled from sodium wire, and CH_2Cl_2 was distilled from CaH_2. Reactions were performed under Ar atmosphere, unless otherwise indicated. Chromatography refers to flash chromatography on silica gel (Whatman 230–400 mesh ASTM silica gel). NMR spectra were recorded at 400 MHz (^{1}H), 101 MHz (^{13}C), and 162 MHz (^{31}P) at 25 °C. Proton and carbon chemical shifts are given in ppm with relative to TMS as internal standard; an external standard was used for ^{31}P (85% H_3PO_4 ($\delta = 0.00$)). Low- and high-resolution spectra were obtained on HP5971AMSD and Finnigan MAT95 double focusing mass spectrometer (MS) instruments, respectively.

Methyl-2-*O*-benzyl-3-*O*-(*p*-methoxybenzyl)-4,6-*O*-(*p*-methoxybenzylidene)-α-D-glucopyranoside (8). A solution of **7** (3 g, 7.46 mmol) in anhydrous THF (100 mL) was added dropwise to a suspension of 60% NaH (0.36 g, 8.95 mmol) in anhydrous THF (250 mL) at 0 °C, and then *p*-methoxybenzyl chloride (1.1 mL, 8.10 mmol) and Bu_4NI (0.38 g, 1.03 mmol) was added. The mixture was stirred under reflux for 24 h. The solvent was concentrated and EtOAc (400 mL) was added to the residue, washed with brine (60 mL), dried over Na_2SO_4, and concentrated by rotary evaporation to afford white solid. Crystallization of the crude product from EtOH (130 mL) gave **8** as a fine white solid (3.740 g, 95.9%).

(5*R*,6*S*,7*S*,8*R*,9*S*)-2-Phenyl-5,6-bis-(*p*-methoxybenzyloxy)-7-benzyloxyhexahydrocyclopenta[1,3]dioxine (10). A mixture of **9** (1.28 g, 2.59 mmol), benzaldehyde dimethyl acetal (0.43 mL, 2.86 mmol), TsOH (49.0 mg, 0.26 mmol) in DMF (10 mL) was stirred for 1 h at 50 °C. After being cooled to room temperature, the reaction mixture was diluted with EtOAc (300 mL), washed with saturated aqueous $NaHCO_3$ and brine, and dried over Na_2SO_4. After concentration, the residue was subjected to silica gel chromatography to give **10** (1.41 g, 93.5%) as a white solid. TLC (EtOAc/Hexane 1:3) $R_f = 0.27$. ^{1}H NMR ($CDCl_3$): $\delta = 2.07$–2.11 (dt, $J = 3.2$, 3.6 Hz, 1H), 3.82 (s, 3H), 3.83 (s, 3H), 3.96 (d, $J = 3.2$ Hz, 1H), 4.10–4.16 (m, 2H), 4.31–4.35 (m, 2H), 4.53–4.65 (m, 5H), 4.69–483 (m, 2H), 5.51 (s, 1H), 6.91–6.93 (m, 4H), 7.28–7.33 (m, 4H), 7.36–7.46 (m, 8H), 7.53–7.56 ppm (m, 2). ^{13}C NMR ($CDCl_3$): $\delta = 40.29$, 55.4, 65.3, 71.7, 73.0, 76.9, 77.2, 77.5, 78.3, 81.4, 87.0, 90.5, 100.6, 113.9, 113.9, 126.4, 127.9, 127.9, 128.4, 128.6, 129.2, 129.6, 129.8, 130.4, 130.9, 137.9, 138.5, 159.3, 159.3 ppm. HRMS (MALDI) (*m/z*) [*M*+Na^+]: found, 605.2483; Calcd for $C_{36}H_{38}O_7Na$, 605.2510.

(1*R*,2*S*,3*S*,4*R*,5*S*)-3-(Benzyloxyl)-5-[(benzyloxy)methyl)-4-hydroxy-1,2-bis[(*p*-methoxybenzyl)oxy]cyclopentane (11). $BH_3{\cdot}Me_2NH$ (0.46 g, 7.80 mmol) and $BF_3{\cdot}Et_2O$ (1.00 mL) were added to a solution of **10** (0.94 g, 1.61 mmol) in anhydrous CH_3CN (50 mL), at room temperature. After 4 h, the solvent was concentrated and saturated aqueous $NaHCO_3$ solution was added to the residue, extracted with EtOAc (100 mL), and then dried over Na_2SO_4, concentrated. The residue was purified by silica gel column chromatography to give **11** (0.865 g, 92%) as a white solid. TLC (EtOAc/Hexane 1:2) $R_f = 0.57$. ^{1}H NMR ($CDCl_3$): $\delta = 2.31$ (m, 1H), 3.30 (br s, 1H), 3.72–3.78 (m, 2H), 3.77–3.81 (m, 7H), 3.90 (t, $J = 5.6$ Hz, 1H), 4.06–4.09 (dd, $J = 5.6$, 9.6 Hz, 1H), 4.25 (dd, $J = 3.2$, 6.0 Hz, 1H), 4.40–4.51 (m, 4H), 4.55–4.70 (m, 4H), 6.81–6.87 (m, 4H), 7.14–7.34 ppm (m, 14H). ^{13}C NMR ($CDCl_3$): $\delta = 45.4$, 55.3, 55.3, 68.0, 71.7, 71.8, 72.0, 73.5, 75.1, 81.3, 88.0, 113.8, 113.7, 127.6, 127.8, 127.9, 127.9, 128.4, 128.5, 129.5, 129.6, 130.4, 130.6, 137.5, 138.2, 159.2, 159.2 ppm. HRMS (MALDI) (*m/z*) [*M*+Na^+]: found, 607.2667; Calcd for $C_{36}H_{40}O_7Na$, 607.2666.

(1*R*,2*S*,3*S*,4*R*,5*R*)-3-(Benzyloxyl)-5-[(benzyloxy)methyl)-1,2,4-trihydroxycyclopentane (12). A solution of **11** (0.865 g, 1.48 mmol) in EtOH (100 mL) and 1 M HCl (35 mL) was refluxed for 3 h, and EtOH were removed by evaporation in vacuo. The solution was neutralized with 0.1 M NaOH, extracted with EtOAc (300 mL), washed with brine, and dried over Na_2SO_4, concentration and then purification by chromatography to give **12** (0.42 g, 82.5%). TLC (EtOAc/MeOH 10:1) $R_f = 0.69$. ^{1}H NMR ($CDCl_3$): $\delta = 2.16$–2.23 (m, 1H), 3. 40 (s, 3H), 3.61 (dd, $J = 3.6$, 6.4 Hz, 1H), 3.67–3.74 (m, 3H), 3.82 (t, $J = 6.4$ Hz, 1H), 3.95 (dd, $J = 8.0$, 8.8 Hz, 1H), 4.17–4.20 (dd, $J = 4.0$ Hz, 1H), 4.44–4.51 (m, $J_{AB} = 12.0$ Hz, 2H), 4.59–4.70 (m, $J = 12.0$ Hz, 2H), 7.23–7.36 ppm (m, 10H). ^{13}C NMR ($CDCl_3$): $\delta = 45.9$, 68.1, 72.0, 73.5, 74.5, 76.0, 81.0, 89.2, 127.8, 127.9, 128.0, 128.4, 128.6, 137.5, 138.0 ppm; HRMS (MALDI) (*m/z*) [*M*+Na^+]: found, 367.1502; Calcd for $C_{20}H_{24}O_5Na$, 367.1516.

(1*R*,2*S*,3*S*,4*R*,5*S*)-3-(Benzyloxy)-5-[(benzyloxy)methyl]-1,2,4-tris-[[(bisbenzyloxy)phosphoryl]oxy]-cyclopentane (13). A mixture of bis(benzyloxy)(*N*,*N*-diisopropylamino)phosphine (290 mg, 0.84 mmol) and 1*H*-tetrazole (86 mg, 2.86 mmol) in anhydrous CH_2Cl_2 (1 mL) was stirred for 30 min at room temperature, and then **12** (34.4 mg, 0.10 mmol) in CH_2Cl_2 (1 mL) was added dropwise. After 3 h, *m*-CPBA (222 mg, 1.28 mmol) was added dropwise to the solution at −78 °C, followed by addition of water (0.3 mL). The solution was stirred for 2 h and extracted with CH_2Cl_2 (30 mL). The organic layer was washed with 10% *w/v* Na_2SO_3 solution (20 mL), 1 M HCl (10 mL), saturated $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated. Flash chromatography (EtOAc/Hexane, 1:1) gave the pure compound **13** (64 mg, 57%). TLC $R_f = 0.21$ (EtOAc/Hexane 2:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.09$–7.25 (m, 40H), 4.78–4.96 (m, 15H), 4.46 (s, 2H), 4.19–4.31 (m, 3H), 3.59 (d, $J = 6.8$ Hz, 2H), 2.63 (m, 1H). ^{13}C NMR ($CDCl_3$): $\delta = 138.28$, 137.70, 135.86, 128.77, 128.57, 128.49, 128.22, 128.13, 127.91, 127.79, 86.47, 85.51, 82.56, 79.04, 73.45, 72.27, 69.68, 65.92, 46.99 ppm. ^{31}P NMR ($CDCl_3$): $\delta = -0.59$ (s, 1P), −0.74 (s, 1P), −1.0 ppm (s, 1P). HRMS (MALDI) (*m/z*) [*M*+Na^+]: found, 1147.3319; Calcd for $C_{62}H_{63}O_{14}P_3Na^+$, 1147.3323.

(1*R*,2*S*,3*S*,4*R*,5*S*)-3-Hydroxy-5-(hydroxymethyl)-1,2,4-tris(phosphonooxy)cyclopentane (2). A solution of **13** (18.0 mg, 16.0 μmol), 20% $Pd(OH)_2$ (18.0 mg) in CH_3OH (2 mL), CH_2Cl_2 (2 mL), and H_2O (0.2 mL) was stirred for 48 h at 50 atm hydrogen pressure. After completion of the reaction, the mixture was filtered to remove the catalyst, and concentrated under a reduced pressure. The crude product was purified by chromatography to afford **2** (7.9 mg, 91.8%). ^{1}H NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 4.17$–4.22 (m, 1H), 4.01–4.05 (m, 1H), 3.91–3.96 (m, 1H), 3.77–3.80 (t, $J = 6$ Hz, 1H), 3.68–3.73 (m, $J = 4.4$, 6.8 Hz, 1H), 3.56–3.61 (m, $J = 4.4$, 6 Hz,1H), 2.17–2.21 ppm (m, 1H). ^{13}C NMR (CD_3OD/D_2O, 2:1, v/v): $\delta = 85.32$, 82.61, 80.48, 77.87, 62.57. ^{31}P NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 8.12$ (s, 1P), 8.03 (s, 1P), 7.18 ppm (s, 1P). MS (ESI): *m/z* 426.9 ([*M*+Na^+], 100.00). HRMS (MALDI) (*m/z*) [*M*+2Na^+]: found, 447.9342; Calcd for $C_6H_{13}O_{14}P_3Na_2^+$, 447.9308.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-(Benzyloxy)-5-[(benzyloxy)methyl]-1,2,4-tris-[[(bisbenzyloxy)thiophosphoryl]oxy]cyclopentane (14). Bis(2-cya-

noethyl)diisopropylphosphorodiamidite (104 mg, 0.384 mmol) was added to a solution of **12** (40 mg, 0.116 mmol) and 1*H*-tetrazole (1.78 mL, 3 wt% in CH_3CN, 0.767 mmol) in dry CH_3CN (1 mL). After the resulting solution was stirred at room temperature for 4 h, sulfur (300 mg, 9.37 mmol) and CS_2/pyridine (3.0 mL, 1:1, *v/v*) were added. After being stirred at room temperature overnight, sulfur was filtered, EtOAc (100 mL) was added to the filtrate, and then washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by chromatography (EtOAc/CH_3OH, 50:1) to give **14** (73 mg, 95.8%) as a colorless oil. TLC $R_f = 0.28$ (EtOAc/Hexane 1:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.29$–7.40 (m, 10H), 4.78–5.02 (m, 3H), 4.70–4.75 (m, $J = 12$, 8.8 Hz, 2H), 4.19–4.31 (m, $J = 6.8$ Hz, 2H), 4.23–4.32 (m, 5H), 4.06–4.22 (m, 9H), 3.75–3.78 (m, 2H), 2.85 (m, 1H), 2.75 (t, $J = 6.0$ Hz, 4H), 2.65–2.68 (dt, $J = 2.0$, 4.0 Hz, 2H), 2.57–2.60 ppm (t, $J = 6.0$ Hz, 5H). ^{13}C NMR ($CDCl_3$): $\delta = 138.17$, 137.43, 128.80, 128.75, 128.34, 128.22, 128.14, 128.11, 127.99, 117.12, 117.07, 117.02, 116.92, 116.89, 86.93, 84.40, 83.98, 80.22, 77.68, 77.36, 77.04, 73.64, 72.22, 66.23, 63.39, 63.35, 63.27, 63.23, 63.16, 63.12, 63.07, 63.02, 47.01, 19.78, 19.66, 19.59, 19.51 ppm. ^{31}P NMR ($CDCl_3$): $\delta = 67.62$ (s, 1P), 67.51 (s, 1P), 67.48 (s, 1P). MS (ESI): *m/z* 973.11 ([*M*+Na^+], 100.00). HRMS (MALDI) (*m/z*) [*M*+Na^+]: found, 973.1389; Calcd for $C_{78}H_{45}O_{11}N_6P_3S_3Na^+$, 973.1414.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-Hydroxy-5-(hydroxymethyl)-1,2,4-tris(thiophosphonooxy)cyclopentane (3). Anhydrous ammonia (~50 mL) was condensed into a three-neck flask at −78 °C. Sodium (1 g) was added to dry the liquid NH_3 for 10 min, and then ammonia (15 mL) was distilled into a second three-neck flask. Sodium (0.2 g, 8.69 mmol) was added to the ammonia solution. After the blue solution was stirred for 10 min, a solution of **14** (15 mg, 15.7 μmol) in anhydrous 1,4-dioxane (1 mL) was added to the mixture at −78 °C. After 15 min, the reaction was quenched with MeOH (20 mL), followed by water. Ammonia and solvents were then removed by evaporation in vacuo. The residue was neutralized with 1 M HCl and purified by ion-exchange chromatography on DEAD-Sephadex eluting with a gradient of triethylammonium bicarbonate buffer (0–1 M), pH 8.0. The triethylammonium salt of **3** eluted between 0.6 and 0.8 M TEAB. Appropriate fractions containing **3**, as judged by Ellman's reagent, were combined and concentrated under reduced pressure to give the triethylammonium salt of **3**, and then the residue was treated with Dowex 50W-X8 (Na^+ form) and lyophilized to give the sodium salt of **3** (5.4 mg, 58.9%). ^{1}H NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 4.55$–4.65 (m, 3H), 4.15 (t, $J = 4$ Hz, 1H), 3.86–3.97 (m, 2H), 2.45–2.48 ppm (m, 1H). ^{13}C NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 89.12$, 84.53, 84.07, 81.05, 63.25 ppm. ^{31}P NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 50.99$ (s, 1P), 50.40 (s, 1P), 49.07 ppm (s, 1P). HRMS (MALDI) (*m/z*) [*M*−H^-]: found, 450.8917; Calcd for $C_6H_{14}O_{11}P_3S_3$, 450.8898.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-(Benzyloxy)-5-[(benzyloxy)methyl]-1,2,4-tris-[(dimethoxyphosphoryl)methoxy]cyclopentane (15). A solution of (dimethoxyphosphinyl)methyl triflate (0.297 g, 1.09 mmol) in THF (1 mL) was added dropwise to a mixture of **12** (37.58 mg, 0.11 mmol) and 60% sodium hydride (14.5 mg, 0.36 mmol) in anhydrous THF (3 mL) at 0 °C. After 20 h, the solvent was evaporated in vacuo. The resulting syrup was partitioned between water and ethyl acetate, and then washed with brine and water. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by chromatography (EtOAc/CH_3OH, 10:1) to give **15** (28.9 mg, 37.2%). TLC $R_f = 0.44$ (EtOAc/CH_3OH 3:1). ^{1}H NMR ($CDCl_3$): $\delta = 7.21$–7.28 (m, 10H), 4.54–4.63 (m, $J = 11.6$ Hz, 2H), 4.44 (s, 2H), 3.61–3.94 (m, 29H), 3.43–3.47 (t, $J = 8$ Hz, 1H), 2.40–2.44 ppm (m, 1H). ^{13}C NMR ($CDCl_3$): $\delta = 138.27$, 137.87, 128.70, 128.63, 128.11, 128.08, 127.92, 91.01, 90.89, 85.73, 85.61, 84.89, 83.26, 83.14, 73.54, 72.11, 67.07, 64.93, 64.14, 64.04, 63.27, 62.48, 62.37, 53.21, 53.14, 45.11 ppm. ^{31}P NMR ($CDCl_3$): $\delta = 24.61$ (s, 1P), 24.42 (s, 1P), 24.12 ppm (s, 1P). MS (MALDI): *m/z* 733.21 ([*M*+Na^+], 100.00). HRMS (MALDI) (*m/z*) [*M*+H^+]: found, 711.2104; Calcd for $C_{29}H_{46}O_{14}P_3$, 711.2095.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-(Hydroxy)-5-(hydroxymethyl)-1,2,4-tris[(dimethoxyphosphoryl)methoxy]cyclopentane (16). A solution of **15** (7.0 mg, 9.85 μmol) and 20% $Pd(OH)_2$ (23 mg) in CH_3OH (4 mL), CH_2Cl_2 (4 mL) and H_2O (0.08 mL) was stirred at 50 atms hydrogen pressure for 48 h. After completion of the reaction, the mixture was filtered to remove the catalyst and concentrated under reduced pressure. The crude product was purified by chromatography and afforded **16** (4.5 mg, 8.49 μmol, 86.2%). TLC $R_f = 0.48$ (EtOAc/CH_3OH 1:1). ^{1}H NMR (CD_3OD): $\delta = 4.00$–4.17 (m, 4H), 3.95–3.99 (m, 3H), 3.76–3.84 (m, 20H), 3.72–3.75 (m, 1H), 3.58–3.64 (m, 2H), 2.31–2.36 ppm (m, 1H). ^{13}C NMR (CD_3OD): $\delta = 94.49$, 87.85, 87.45, 79.77, 65.98, 65.50, 65.40, 64.32, 63.84, 63.74, 60.68, 55.15, 55.02, 54.97, 49.00 ppm. ^{31}P NMR (CD_3OD): $\delta = 25.63$ (s, 1P), 25.37 (s, 1P), 24.68 ppm (s, 1P). HRMS (MALDI) (*m/z*) [*M*+H^+]: found, 531.1170; Calcd for $C_{29}H_{46}O_{14}P_3$, 531.1156.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-(Hydroxy)-5-(hydroxymethyl)-1,2,4-tris(phosphonomethoxy)cyclopentane (4). To a solution of **16** (4 mg, 7.55 μmol) in anhydrous CH_2Cl_2 (1 mL), N,O-bis(trifluoromethylsilyl)-acetamide (40 μL, 0.15 mmol) was added and stirred for 1 h, then bromotrimethyl silane (132 μL, 1 mmol) was added. The mixture was stirred for 12 h at room temperature. After solvents were removed under reduced pressure, the residue was dried in vacuo, and then 95% methanol (1 mL) was added and stirred for 30 min, concentrated, and dried in vacuo to give **4** (3.15 mg, 93.5%). The product was treated with Dowex 50W-X8 (Na^+ form) and lyophilized to give the sodium salt of **4**. ^{1}H NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 3.83$ (m, 1H), 3.50–3.65 (m, 11H), 2.22 ppm (m, 1H). ^{13}C NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 90.35$, 84.98 (d, $J = 48.8$ Hz), 84.19, 77.49, 67.59 (d, $J = 91.6$ Hz), 66.74, 66.03, 59.03, 45.35 ppm. ^{31}P NMR (CD_3OD/D_2O, 2:1, *v/v*): $\delta = 16.44$ (s, 2P), 16.38 ppm (s, 1P). HRMS (MALDI) (*m/z*) [*M*−H^-]: found, 445.0075; Calcd for $C_9H_{20}O_{14}P_3$, 445.0071.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-(Benzyloxy)-5-[(benzyloxy)methyl]-1,2,4-tris-(sulfamoyloxy)cyclopentane (17). Formic acid (28.3 μL, 0.75 mmol) was added to $ClSO_2NCO$ (65.3 μL, 0.75 mmol) at 0 °C with rapid stirring. CH_3CN (0.5 mL) was added after 15 min and the solution was stirred for 7 h at room temperature. A solution of **12** (34.4 mg, 0.10 mmol) in *N*,*N*-dimethyl acetamide (0.9 mL) was added at 0 °C. After stirring for 2 h, the reaction was quenched by the addition of water (5 mL) at 0 °C. The aqueous layer was extracted with EtOAc (100 mL). The solution was washed with water, dried over Na_2SO_4, concentrated, and purified by chromatography on silica gel chromatography to give **17** (31 mg, 53%). TLC $R_f = 0.46$ (EtOAc/Hexanes 1.5:1). ^{1}H NMR (CD_3OD): $\delta = 7.25$–7.38 (m, 10H), 4.97–4.99 (m, $J =$ 4 Hz, 1H), 4.90–4.91 (m, 2H), 4.72 (s, 2H), 4.49–4.59 (dd, $J = 11.6$ Hz, 2H), 4.4 (m, 1H), 3.82 (d, $J = 6$ Hz, 2H), 2.80 ppm (m, 1H). ^{13}C NMR (CD_3OD): $\delta = 139.60$, 139.15, 129.54, 129.47, 129.27, 129.10, 128.95, 128.82, 88.13, 85.58, 84.67, 81.61, 74.62, 73.57, 67.14, 47.35 ppm. MS (FAB): *m/z* 582.0 ([*M*+H^+], 100.00). HRMS (MALDI) (*m/z*) [*M*+Na^+]: found, 604.0690; Calcd for $C_{20}H_{27}N_3NaO_{11}S_3$, 604.0700.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-Hydroxy-5-(hydroxymethyl)-1,2,4-tris(sulfamoyloxy)cyclopentane (5). A solution of **17** (15 mg, 25.8 μmol), 20% $Pd(OH)_2$ (15 mg) in MeOH (3 mL), CH_2Cl_2 (3 mL), and H_2O (30 μL) was stirred under 50 atms hydrogen pressure at room temperature for 24 h. The catalyst was removed by filtration and washed with MeOH. The filtrate was concentrated and the residue

was purified by chromatography to afford **5** (7.5 mg, 72.5%) as a colorless oil. TLC R_f=0.30 (EtOAc/CH_3OH 30:1). ^1H NMR (CD_3OD): δ=4.68–4.72 (dt, 3H), 4.36 (t, J=5.6 Hz, 1H), 3.78 (d, J=4.8 Hz, 2H), 2.71 ppm (m, J=6 Hz, 1H). ^{13}C NMR (CD_3OD): δ=89.42, 84.44, 83.46, 78.21, 58.75 ppm. MS (negative-ion ESI, M-H$^-$): *m/z* 400.0 ([*M*−H$^-$], 100.00). HRMS (MALDI) (*m/z*) [*M*+Na$^+$]: found, 423.9755; Calcd for $C_6H_{15}N_3NaO_{11}S_3$, 423.9761.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-(Benzyloxy)-5-[(benzyloxy)methyl]-1,2,4-tris-(hydrogensulfate)cyclopentane (18). Chlorosulfonic acid (49.7 μL, 0.748 mmol) was added to a solution of **12** (32.1 mg, 0.093 mmol) in anhydrous pyridine (2 mL) at 0 °C. After 6 h, 5% Na_2CO_3 solution (3 mL) was added. The solution was condensed in vacuo to remove the solvents, and then purified by flash chromatography ($CHCl_3$/CH_3OH, 2:1) to give **18** as a white solid (49.3 mg, 90.7%). TLC R_f=0.37 ($CHCl_3$/CH_3OH 1.3:1). ^1H NMR (CD_3OD): δ=7.31–7.41 (m, 10H), 4.81 (t, J=3.6 Hz, 1H), 4.65–4.68 (m, 3H), 4.50 (m, 2H, J=12 Hz), 4.37 (t, J=3.2 Hz, 1H), 4.79 (d, J=5.6 Hz, 1H), 2.69 ppm (m, J=6.4 Hz, 1H). ^{13}C NMR (CD_3OD): δ=135.85, 131.17, 127.49, 127.37, 127.09, 126.95, 110.68, 91.48, 84.84, 82.92, 81.49, 77.99, 71.89, 71.02, 64.67, 44.17 ppm. MS (MALDI): *m/z* 673.00 [*M*+H$^+$]. HRMS (MALDI) (*m/z*) [*M*+Na$^+$]: found, 672.9702; Calcd for $C_{20}H_{21}Na_4O_{14}S_3$, 672.9679.

(1*R*,2*R*,3*S*,4*R*,5*S*)-3-Hydroxy-5-(hydroxymethyl)-1,2,4-tris(hydrogensulfate)cyclopentantriol (6). A solution of **18** (10 mg, 15.4 μmol), 20% $Pd(OH)_2$ (10 mg) in MeOH (2 mL), CH_2Cl_2 (2 mL) and H_2O (0.2 mL) was stirred under 50 atms hydrogen pressure at room temperature for 48 h. The catalyst was removed by filtration and washed with water. The filtrate was concentrated and the residue was treated with $CHCl_3$ to afford **6** (6.5 mg, 89.8%). TLC R_f=0.2 ($CHCl_3$/CH_3OH 1:1). ^1H NMR (CD_3OD): δ=4.71 (m, 2H), 4.65 (t, J=4 Hz, 1H), 4.39 (t, J=4 Hz, 1H), 3.88 (t, J=6.8, 3.6 Hz, 1H), 2.66 ppm (m, J=6.4 Hz).^{13}C NMR (CD_3OD): δ=88.93, 83.56, 82.24, 79.76, 59.76, 45.66 ppm. MS (negative-ion ESI, *M*−H$^-$): *m/z* 446.9 [*M*−Na$^-$], 100.00). HRMS (MALDI) (*m/z*) [*M*+2Na$^+$]: found, 447.9029; Calcd for $C_6H_{10}O_{14}S_3Na_2$, 447.9022.

Molecular docking experiments. Docking experiments were carried out on a SGI Octane workstation and Windows PC by AutoDock3.0.5 with AutoDockTools (ADT), a graphical user interface of AutoDock program. The crystal structure of type I $InsP_3R$ complex with Ins(1,4,5)P_3, coded 1N4K was obtained from PDB bank (http://www.rcsb.org/). The interaction of ligands with $InsP_3R1$ was summarized by LIGPLOT 4.4.2. Firstly, a 3D structural model of $InsP_3R1$ binding core was constructed and used for docking small molecules by AutoDock3.0. Small ligands, water and nonpolar hydrogen were removed from the crystal structure (1N4K), and then the structure was converted to a pdbqs format file with information of charge and solution by ADT. Ins(1,4,5)P_3 was used as a model molecule to study the reliability and reproducibility of the docking procedure. AutoDock program with ADT was employed to dock Ins(1,4,5)P_3 to $InsP_3R1$. All atom values were generated automatically by ADT. Using AutoGrid, a grid of 30×30×35 Å^3 with 0.375 Å spacing was calculated around the docking area for relative ligand atom types of Ins(1,4,5)P_3. Separate docking computations were performed using the Lamarckian Genetic Algorithm Local Search (GALS) method. The docking conformations were clustered on the basis of root-mean-square deviation (rmsd) and ranked on the basis of free energy of binding. The top-ranked conformations were visually studied for good chemical geometry. The interactions of the original complex and the docking complex were determined by LIGPLOT 4.4.2. The result showed a hydrogen bond network of both complexes remained almost in the same region with Arg 265, Gly 268, Arg 269, Lys 508, Arg 511, Arg 568, and Lys 569. Furthermore, the overlapping of docked Ins(1,4,5)P_3 and original Ins(1,4,5)P_3 suggested a similar binding conformation with good rmsd (1.02 Å). Under the same docking parameters, the docking program was performed for ten times to test the reproducibility of the docking procedure. The overlapped 50 top-score conformations showed good identity. These results indicated the reliability of the docking program used for studies on the associations between the ligands and $InsP_3R1$. The same docking parameters were employed for docking of the reference compound **2** and analogue **3**. Residues Arg 265, Gly 268, Arg 269, Lys 508, Arg 511, Arg 568, and Lys 569 were revealed to participate the binding with ligands.

Cell culture and transfection. COS-7 cells obtained from RIKEN Cell Bank (Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium with 1000 mg mL^{-1} glucose (Sigma), and supplemented with 10% fetal calf serum (Gibco BRL, Rockville, MD, USA), 100 units mL^{-1} penicillin (Gibco BRL) and 100 μg mL^{-1} streptomycin (Gibco BRL). Cells were transfected with 5 μg mL^{-1} of plasmid using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Measurement of fluorescence. COS-7 cells were grown in experimental chambers consisting of 7 mm plastic cylinders and fibronectin-coated cover slips. Permeabilization was performed by exposing cells to intracellular-like medium (ICM; 125 mM KCl, 19 mM NaCl, 10 mM Hepes-KOH, pH 7.3, 1 mM EGTA, and 330 μM $CaCl_2$) containing 200 μg mL^{-1} (*w/v*) saponin (ICN, Cleveland, OH, U.S.A.) for ~90 s. Permeabilized cells were washed with ICM and then exposed to ICM containing various concentrations of IP_3 or other reagents.

Fluorescence images were captured using a dual-wavelength ratio imaging system consisting of a EM-CCD camera (C9135-special; Hamamatsu photonics, Shizuoka, Japan) and W-View (Hamamatsu photonics) optics coupled to a Nikon TE2000 inverted fluorescence microscope equipped with a Nikon S Fluor 60 oil immersion objective (NA 1.25). Fluorescence of LIBRA and LIBRA-N were monitored with excitation at 425 nm and dual-emission at 480 nm and 535 nm. Data were analyzed with AQUACOSMOS 2.6 software (Hamamatsu photonics). All experiments were performed at room temperature.

Cell culture. MDA-MB-435 cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 1% nonessential amino acids, and 1 mM sodium pyruvate. Cells were cultured on Corning tissue culture flasks at 37 °C with 5% CO_2. Cells were passaged using 0.25% trypsin and 2 mM EDTA solution in Ca^{2+}/Mg^{2+}-free PBS. No antibiotics and antimycotics were used.

Calcium imaging. MDA-MB-435 cells grown on coverslips were incubated in serum-free media for 3 h prior to loading with 5 μM Fura-2 AM (Fura-2-acetoxymethyl ester; Molecular Probes; Eugene, Oregon) from a 5 mM DMSO stock solution for 90 min. At the end of the incubation period the coverslips were washed twice with serum free media and transferred to a plate with an 8 mm hole and sealed with vacuum grease, forming a well to which 100 μL of media was added. The cells were imaged for the cellular calcium levels using the Incyt Im dual fluorescence imaging system (Intracellular Imaging Inc; Cincinnati OH). Cells were illuminated with a xenon lamp and images were collected with a camera controlled by the imaging software. Multiple cells within the field of view were individually tracked as a separate data set. Images were collected at one second intervals and ratio values saved. Excitation wavelengths were 340 nm (calcium bound to dye) and 380 nm (calcium free dye) and the emission wavelength was 510 nm. Nu-

merical data from emission ratios at 510 nm resulting from excitation at 340 nm or 380 nm were used to calculate cytosolic calcium levels.

Prior to stimulation with each Ins(1,4,5)P_3 analogue, ratiometric data was collected for approximately 1–2 min to establish a stable baseline. Then, a given analogue (**2**, **3**, **4**, **5**, or **6**) was added to cell media without carrier and data collected (data not shown). For delivery of charge neutralized complex of Ins(1,4,5)P_3 or the analogue, type III histone (Sigma; St Louis, MO) was added to give a final concentration of 50 μM followed by the addition of the metabolically stabilized analogues at a final concentration of 100 μM. Histone facilitates intracellular delivery of charged inositol polyphosphates and phosphoinositides.[31,33] Calcium ionophore (Ionomycin; Calbiochem, San Diego, CA) was added at the end of the experiment to demonstrate that all cells were still able to mobilize calcium.

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[1] R. L. Patterson, D. Boehning, S. H. Snyder, *Annu. Rev. Biochem.* **2004**, *73*, 437 – 465.
[2] A. M. Riley, A. J. Laude, C. W. Taylor, B. V. L. Potter, *Bioconjugate Chem.* **2004**, *15*, 278 – 289.
[3] A. M. Riley, S. A. Morris, E. P. Nerou, V. Correa, B. V. Potter, C. W. Taylor, *J. Biol. Chem.* **2002**, *277*, 40290 – 40295.
[4] S. Takahashi, T. Kinoshita, M. Takahashi, *J. Antibiot.* **1994**, *47*, 95 – 100.
[5] C. N. Borissow, S. J. Black, M. Paul, S. C. Tovey, S. G. Dedos, C. W. Taylor, B. V. L. Potter, *Org. Biomol. Chem.* **2005**, *3*, 245 – 252.
[6] D. J. Jenkins, A. M. Riley, B. V. Potter, *J. Org. Chem.* **1996**, *61*, 7719 – 7726.
[7] C. S. Rye, J. B. Baell, *Curr. Med. Chem.* **2005**, *12*, 3127 – 3141.
[8] J. Gajewiak, Y. Xu, S. A. Lee, T. G. Kutateladze, G. D. Prestwich, *Org. Lett.* **2006**, *8*, 2811 – 3.
[9] Y. Xu, S. A. Lee, T. G. Kutateladze, D. Sbrissa, A. Shisheva, G. D. Prestwich, *J. Am. Chem. Soc.* **2006**, *128*, 885 – 897.
[10] H. Zhang, N. Markadieu, R. Beauwens, C. Erneux, G. D. Prestwich, *J. Am. Chem. Soc.* **2006**, *128*, 16464 – 16465.
[11] G. D. Prestwich, Y. Xu, L. Qian, J. Gajewiak, G. Jiang, *Biochem. Soc. Trans.* **2005**, *33*, 1357 – 1361.
[12] P. Westerduin, H. A. M. Willems, C. A. A. van Boeckel, *Tetrahedron Lett.* **1990**, *31*, 6915 – 6918.
[13] P. Westerduin, H. A. M. Willems, C. A. A. van Boeckel, *Carbohydr. Res.* **1992**, *234*, 131 – 140.
[14] J. Gajewiak, G. D. Prestwich, *Tetrahedron Lett.* **2006**, *47*, 7607 – 7609.
[15] I. Bosanac, J. R. Alattia, T. K. Mal, J. Chan, S. Talarico, F. K. Tong, K. I. Tong, F. Yoshikawa, T. Furuichi, M. Iwai, T. Michikawa, K. Mikoshiba, M. Ikura, *Nature* **2002**, *420*, 696 – 700.
[16] D. S. Goodsell, A. J. Olson, *Proteins Struct. Funct. Genet.* **1990**, *8*, 195 – 202.
[17] G. M. Morris, D. S. Goodsell, R. Huey, A. J. Olson, *J. Comput.-Aided Mol. Des.* **1996**, *10*, 293 – 304.
[18] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639 – 1662.
[19] Q. X. Jiang, E. C. Thrower, D. W. Chester, B. E. Ehrlich, F. J. Sigworth, *EMBO J.* **2002**, *21*, 3575 – 3581.
[20] K. Fukase, Y. Fukase, M. Oikawa, W. Liu, Y. Suda, S. Kusumoto, *Tetrahedron* **1998**, *54*, 4033 – 4050.
[21] R. A. Vishwakarma, S. Vehring, A. Mehta, A. Sinha, T. Pomorski, A. Herrmann, A. K. Menon, *Org. Biomol. Chem.* **2005**, *3*, 1275 – 1283.
[22] "myo-Inositol 1, 4,5,-trisphosohorothioate: a novel analogue of a biological second messenger": A. M. Cooke, R. Gigg, P. B. V. L. Potter, *J. Chem. Soc. Chem. Commun.* **1987**, 1525 – 1526.
[23] Y. Xu, M. T. Flavin, Z. Q. Xu, *J. Org. Chem.* **1996**, *61*, 7697 – 7701.
[24] M. Okada, S. Iwashita, N. Koizumi, *Tetrahedron Lett.* **2000**, *41*, 7047 – 7051.
[25] A. Tanimura, A. Nezu, T. Morita, R. J. Turner, Y. Tojyo, *J. Biol. Chem.* **2004**, *279*, 38095 – 38098.
[26] A. Nezu, A. Tanimura, T. Morita, A. Shitara, Y. Tojyo, *Biochim. Biophys. Acta Gen. Subj.* **2006**, *1760*, 1274 – 1280.
[27] C. W. Taylor, M. J. Berridge, A. M. Cooke, B. V. Potter, *Biochem. J.* **1989**, *259*, 645 – 50.
[28] R. A. Wilcox, C. Erneux, W. U. Primrose, R. Gigg, S. R. Nahorski, *Mol. Pharmacol.* **1995**, *47*, 1204 – 1211.
[29] M. Mezna, F. Michelangeli, *J. Biol. Chem.* **1996**, *271*, 31818 – 31823.
[30] C. T. Murphy, A. M. Riley, C. J. Lindley, D. J. Jenkins, J. Westwick, B. V. L. Potter, *Mol. Pharmacol.* **1997**, *52*, 741 – 748.
[31] S. Ozaki, D. B. DeWald, J. C. Shope, J. Chen, G. D. Prestwich, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 11286 – 11291.
[32] S. K. Joseph, *Cell Signal* **1996**, *8*, 1 – 7.
[33] D. B. DeWald, S. Ozaki, S. Malaviya, J. C. Shope, K. Manabe, L. Crosby, P. Neilsen, D. Johnston, S. Harihar, G. D. Prestwich, *Cell Calcium* **2005**, 38, 59 – 72.

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