Disaccharide Polyphosphates Based upon Adenophostin A Activate Hepatic D-*myo*-Inositol 1,4,5-Trisphosphate Receptors†

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ABSTRACT: The glyconucleotides adenophostin A and B are the most potent known agonists at type 1 inositol trisphosphate [Ins(1,4,5)P_3] receptors, although their stuctures differ markedly from that of Ins(1,4,5)P_3. Equilibrium competition binding with [^{3}H]Ins(1,4,5)P_3 and unidirectional $^{45}Ca^{2+}$ flux measurements were used to examine the effects of adenophostin A in hepatocytes, which express predominantly type 2 Ins(1,4,5)P_3 receptors. Both Ins(1,4,5)P_3 (K_d = 8.65 ± 0.98 nM) and adenophostin A (K_d = 0.87 ± 0.20 nM) bound to a single class of [^{3}H]Ins(1,4,5)P_3-binding site and each fully mobilized the same intracellular Ca^{2+} pool; although, adenophostin A (EC_{50} = 10.9 ± 0.7 nM) was more potent than Ins(1,4,5)P_3 (EC_{50} = 153 ± 11 nM). Working on the assumption that it is the phosphorylated glucose component of the adenophostins that mimics the critical features of Ins(1,4,5)P_3, we synthesized various phosphorylated disaccharide analogs containing this structure. The novel disaccharide-based analogs, sucrose 3,4,3′-trisphosphate [Sucr(3,4,3′)P_3], α,α′-trehalose 3,4,3′,4′-tetrakisphosphate [Trehal(3,4,3′,4′)P_4], α,α′-trehalose 2,4,3′,4′-tetrakisphosphate [Trehal(2,4,3′,4′)P_4], and methyl 3-*O*-(α-D-glucopyranosyl)-β-D-ribofuranoside 2,3′,4′-trisphosphate [Rib(2,3′,4′)P_3], were all able to mobilize the same intracellular Ca^{2+} pool as Ins(1,4,5)P_3 and adenophostin A; although, none was as potent as adenophostin A. The rank order of potency of the analogs, adenophostin A > Ins(1,4,5)P_3 ≈ Rib(2,3′,4′)P_3 > Trehal(2,4,3′,4′)P_4 > Glc(2′,3,4)P_3 ≈ Trehal(3,4,3′,4′)P_4 > Sucr(3,4,3′)P_3, was the same in radioligand binding and functional assays of hepatic Ins(1,4,5)P_3 receptors. Both Rib(2,3′,4′)P_3, which was as potent as Ins(1,4,5)P_3, and Trehal(2,4,3′,4′)P_4 bound with significantly higher affinity (∼27 and ∼3-fold, respectively) than the only active carbohydrate agonist of Ins(1,4,5)P_3 receptors previously examined [Glc(2′,3,4)P_3]. We conclude that phosphorylated disaccharides provide novel means of developing high-affinity ligands of Ins(1,4,5)P_3 receptors.

Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3,[1] (**1**), Figure 1] is the cytosolic messenger that links activation of many receptors in the plasma membrane to the release of Ca^{2+} from intracellular stores (*1*). The receptors for Ins(1,4,5)P_3 are encoded by three distinct genes (*2*) and belong to the same family of intracellular Ca^{2+} channels as ryanodine receptors (*3*). Ryanodine and Ins(1,4,5)P_3 receptors share many structural and functional properties including their regulation by phosphorylation, cytosolic Ca^{2+}, and other intracellular messengers (*2*, *3*), their association with modulatory proteins (*4*−*8*), and their ability to mediate the regenerative Ca^{2+} release that gives rise to intracellular Ca^{2+} waves (*9*). Both pharmacological (*9*) and immunological

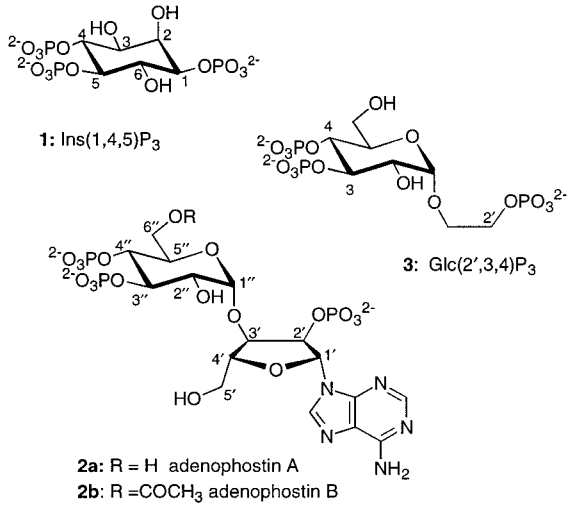

FIGURE 1: Structures of inositol 1,4,5-trisphosphate [Ins(1,4,5)-P_3], adenophostins A and B, and 2-hydroxyethyl α-D-glucopyranoside-2′,3,4-trisphosphate [Glc(2′,3,4)P_3].

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[1] Abbreviations: BSA, bovine serum albumin; CLM, cytosol-like medium; EC_{50}, concentration causing half the maximal effect; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Glc(2′,3,4)P_3, 2-hydroxyethyl α-D-glucopyranoside-2′,3,4-trisphosphate; *h*, Hill coefficient; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulfonic acid; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; Ins(4,5)P_2, D-*myo*-inositol 4,5-bisphosphate; K_d, equilbrium dissociation constant; Rib(2,3′,4′)P_3, methyl 3-*O*-(α-D-glucopyranosyl)-β-D-ribofuranoside 2,3′,4′-trisphosphate; Sucr(3,4,3′)P_3, sucrose 3,4,3′-trisphosphate; Trehal(2,4,3′,4′)P_4, α,α′-trehalose 2,4,3′,4′-tetrakisphosphate; Trehal(3,4,3′,4′)P_4, α,α′-trehalose 3,4,3′,4′-tetrakisphosphate.

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(*10*) techniques have been used to establish the respective contributions of ryanodine and Ins(1,4,5)P_3 receptors in generating the complex signals evoked in intact cells. Molecular approaches have more recently been exploited to suggest that the different Ins(1,4,5)P_3 receptor isoforms may fulfil different roles in intracellular Ca^{2+} signaling (*11, 12*). More widely applicable means of assessing the importance of Ins(1,4,5)P_3 receptors in Ca^{2+} signaling are presently hampered by an inadequate range of selective ligands. Heparin, the only available practicable antagonist of Ins(1,4,5)P_3 receptors (*13*) has many additional sites of action including ryanodine receptors (*14*) and G proteins (*15*). Until recently, the only synthetic ligands active at Ins(1,4,5)P_3 receptors were analogs of Ins(1,4,5)P_3 and, apart from some partial agonists (*16*), they have almost invariably proven to be full agonists with lesser affinity than Ins(1,4,5)P_3 (*17, 18*). However, studies of these ligands have refined our understanding of the structural determinants of inositol phosphate activity at Ins(1,4,5)P_3 receptors. In particular, it is now clear that a structure analogous to the 4,5-bis(phosphate) of Ins(1,4,5)P_3 is essential for activity, while an adjacent hydroxyl group and a third phosphate help to enhance binding affinity (*18*).

The glyconucleotides adenophostins A and B (Figure 1, **2a** and **2b**), originally isolated from *Penicillium brevicompactum* (*19*), are the most potent agonists of Ins(1,4,5)P_3 receptors so far identified (*20, 21*), and yet they show little obvious structural similarity to Ins(1,4,5)P_3 (*22*). The adenophostins bind to the same site on the receptor as Ins(1,4,5)P_3 (*21*) and mimic both the biphasic kinetics and quantal pattern of Ca^{2+} mobilization evoked by Ins(1,4,5)P_3 (*23*). Adenophostins have the additional advantages of not binding to Ins(1,3,4,5)P_4 binding sites and of being resistant to degradation by the enzymes that metabolise Ins(1,4,5)P_3 (*21*). The structure of adenophostin A has now been confirmed by total synthesis (*24, 25*).

Examination of the structure of adenophostin A suggests that its glucose 3″,4″-bis(phosphate) structure and the adjacent 2″-hydroxyl group may mimic the critical 4,5-bis(phosphate)/6-hydroxyl triad of Ins(1,4,5)P_3 (Figure 1). It is thought that this structure constitutes the essential recognition feature common to both Ins(1,4,5)P_3 and the adenophostins and that it interacts with the "anchoring domain" of the Ins(1,4,5)P_3 receptor binding site (*17*). Thus, the 1-phosphate of Ins(1,4,5)P_3 and the 2′-phosphate group of the nucleoside half in the adenophostins behave as accessories which enhance binding, perhaps by long-range electrostatic interactions with an auxiliary domain (*17*). The deletion of the 1-phosphate in Ins(1,4,5)P_3 to give Ins(4,5)P_2 (*18*) or of the 2′-phosphate in the adenophostins (*20*) does not abolish activity, but results in greatly reduced affinity for the Ins(1,4,5)P_3 receptor.

The finding that the presumed core pharmacophore of the adenophostins is not based upon *myo*-inositol but upon glucose suggested that a class of active analogs containing a glucose 3,4-bisphosphate structure, together with one or more accessory phosphate groups placed elsewhere in the molecule, might provide novel compounds with which to explore the structural requirements for activity at Ins(1,4,5)P_3 receptors. Furthermore, the much greater overall size of the adenophostins compared to Ins(1,4,5)P_3 suggests that phosphorylated disaccharides may be accommodated by the receptor binding site. We (*26*) and others (*22*) initially

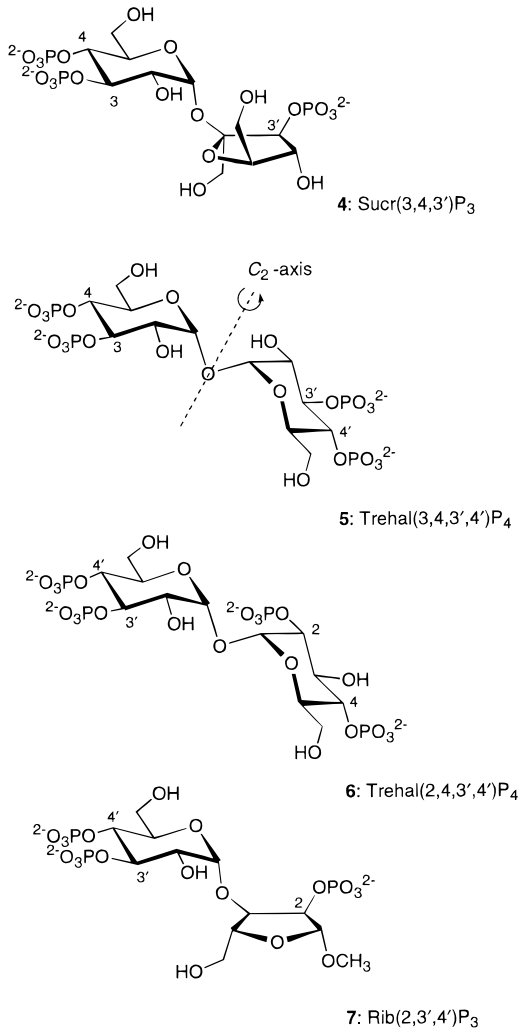


FIGURE 2: Structures of the four synthetic disaccharide polyphosphates.

synthesized the minimal structure 2-hydroxyethyl α-D-glucopyranoside 2′,3,4-trisphosphate [Glc(2′,3,4)P_3, (**3**), Figure 1] and showed it to be the first synthetic carbohydrate-based agonist at Ins(1,4,5)P_3 receptors, with ~10-fold reduced potency relative to Ins(1,4,5)P_3 in both SH-SY5Y cells (*22*) and platelets (*27*). Molecular modeling studies on **3** (*22*) confirmed that the conformationally flexible bimethylene chain did not allow the 2′-phosphate to mimic accurately the positioning of either the 1-phosphate of Ins(1,4,5)P_3 or the 2′-phosphate of the adenophostins. Presumably, this explains why **3** binds with lesser affinity than adenophostin A and Ins(1,4,5)P_3.

We have now synthesized various novel compounds incorporating the D-glucopyranosyl 3,4-bisphosphate moiety with an α-glycosidic linkage to a second sugar containing one or more phosphates. These disaccharide polyphosphates (Figure 2) are expected to be conformationally more rigid than Glc(2′,3,4)P_3, and to place their accessory phosphate group(s) in various positions within the receptor binding site. In the present paper, we describe the syntheses of three such molecules: sucrose 3,4,3′-trisphosphate, [Sucr(3,4,3′)P_3, (**4**)], α,α′-trehalose 3,4,3′,4′-tetrakisphosphate [Trehal(3,4,3′,4′)-P_4, (**5**)], and α,α′-trehalose 2,4,3′,4′-tetrakisphosphate [Trehal-(2,4,3′,4′)P_4, (**6**)]. We have also recently reported elsewhere the synthesis of another disaccharide polyphosphate, methyl 3-*O*-(α-D-glucopyranosyl)-β-D-ribofuranoside 2,3′,4′-trispho-

sphate [Rib(2,3′,4′)P_3 (**7**)], whose structure is more closely related to adenophostin A (*28*).

The effects of adenophostin have so far been shown only for the Ins(1,4,5)P_3 receptors from cerebellum (*21, 23, 27*) and platelets (*27*), each of which expresses type 1 Ins(1,4,5)-P_3 receptors (*29*). In the present study, we compare the effects of adenophostin A, Glc(2′,3,4)P_3 and the four novel disaccharide polyphosphates shown in Figure 2 (**4**-**7**) on [^{3}H]Ins(1,4,5)P_3 binding and $^{45}Ca^{2+}$ mobilization from rat hepatocytes, which express predominantly type 2 Ins(1,4,5)P_3 receptors (*30*).

EXPERIMENTAL PROCEDURES

General Methods and Reagents. Thin-layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminium sheets silica 60 F_{254}) with detection by UV light or with phosphomolybdic acid in methanol followed by heating. Flash chromatography was performed on silica gel (Sorbsil C60). Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Dichloromethane was distilled over phosphorus pentoxide and kept over 4 Å molecular sieves. *N*,*N*-Dimethylformamide was dried over 4 Å sieves. ^{1}H and ^{13}C NMR spectra (internal Me_4Si as reference) were recorded with a Jeol GX270 or EX400 FT-NMR spectrometer and ^{31}P NMR spectra (external aq 85% phosphoric acid as reference) were recorded with a Jeol EX400 or Jeol FX-90Q spectrometer. FAB mass spectra were recorded at the University of Bath using *m*-nitrobenzyl alcohol as the matrix. Microanalysis was carried out by the Microanalysis Service, University of Bath. Melting points (uncorrected) were determined using a Reichert-Jung hot stage microscope apparatus. Ion-exchange chromatography was performed on an LKB-Pharmacia medium-pressure ion-exchange chromatograph using Q-Sepharose Fast Flow resin and gradients of triethylammonium bicarbonate (TEAB) as eluent. 4,6:4′,6′-Di-*O*-benzylidene-α,α′-trehalose was prepared from α,α′-trehalose dihydrate according to the procedure of Baer and Radatus (*31*). (*N*,*N*-Diisopropylamino)dichlorophosphine was prepared by the method of Tanaka et al. (*32*) by adding 2 equiv of diisopropylamine to a solution of PCl_3 in dry ether at −78 °C. The crude product (δ_P = 166.4) was purified by distillation under reduced pressure and reaction with 2 equiv of benzyl alcohol in the presence of 2 equiv of triethylamine afforded bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (δ_P = 145.24) (*33*) which was purified by flash chromatography.

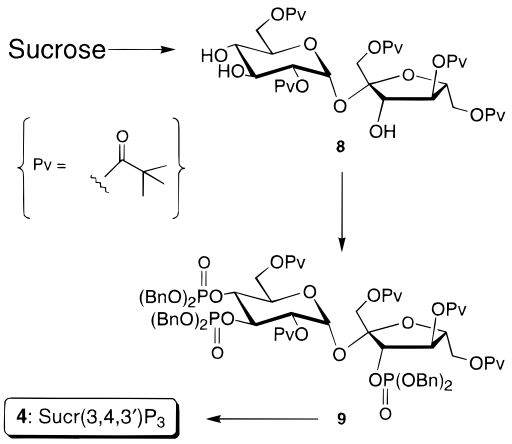
Materials for Biological Assays. [^{3}H]Ins(1,4,5)P_3 (58 Ci/mmol) was from Amersham (Little Chalfont, U.K.) and $^{45}CaCl_2$ was from ICN (Thame, U.K.). Ins(1,4,5)P_3 was from American Radiolabeled Chemicals (St. Louis, MO). Adenophostin A, purified from *Penicillium brevicompactum* (*19*), was a generous gift from Dr. M. Takahashi (Sankyo Co. Ltd., Japan). Glc(2′,3,4)P_3 (*26*) and Rib(2,3′,4′)P_3 (*28*) were synthesized as previously described. All synthetic analogs were accurately quantified using a modification of the Briggs phosphate assay (*34*). Percoll (1.13 g/mL) was from Pharmacia (Upssala, Sweden). Ionomycin was from Calbiochem (Nottingham, U.K.) and thapsigargin was from Alamone Laboratories (Jerusalem, Israel). All other reagents were from suppliers listed previously (*35*).

Preparation of Rat Liver Membranes. The liver of a male Wistar rat (200−250 g) was perfused *in situ* with 40 mL of ice-cold buffered saline (116 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 25 mM $NaHCO_3$, 1 mM EGTA, 11 mM glucose, and 5:95 CO_2:O_2, pH 7.4 at 4 °C). After excision, the liver was chopped and then homogenized in 25 mL of ice-cold buffered sucrose (250 mM sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.4 at 4 °C) using a 15 mL glass Dounce homogenizer with 10 strokes of a loose-fitting plunger and three strokes with a tighter plunger. The homogenate was made up to 50 mL in ice-cold buffered sucrose, filtered through gauze and centrifuged (2500*g*, 10 min), and the pellet then resuspended in 48 mL of ice-cold buffered sucrose containing Percoll (11.8% final v/v) (*36*). The suspension was centrifuged (35000*g*, 30 min) and membranes were harvested as a discrete fluffy band below a fatty layer at the top of each tube. The membranes were resuspended in 50 mL of ice-cold hypo-osmotic buffer (1 mM EGTA and 5 mM HEPES, pH 7.4 at 4 °C) to lyse the vesicles and then centrifuged (48000*g*, 10 min). The final membrane pellet was resuspended in binding medium (20 mM Tris and 1 mM EDTA, pH 8.3 at 4 °C) at ∼20 mg of protein/mL and stored in liquid nitrogen for up to 14 days. Protein concentrations were measured using the Bradford assay (*37*) with BSA as standard. A single liver typically provided ∼70 mg of membrane protein. Although this method produces membranes enriched in markers for plasma membrane, microsomal markers are also present (*36*) and the membranes are enriched in Ins(1,4,5)P_3 receptors whose characteristics are indistinguishable from those of permeabilized rat hepatocytes (*35*, *38*).

[^{3}H]Ins(1,4,5)P_3 Binding. Liver membranes (0.4 mg of protein/tube) were added to binding buffer (500 μL) containing [^{3}H]Ins(1,4,5)P_3 (30 nCi, final concentration = 1 nM) and the appropriate concentration of competing ligand. After 5 min at 0 °C, bound and free [^{3}H]Ins(1,4,5)P_3 were separated by centrifugation (20000*g*, 5 min, 0 °C). Previous results had established that under these conditions, binding reached equilibrium and that degradation of [^{3}H]Ins(1,4,5)-P_3 was negligible (*35*). Total binding was typically 3500 dpm/tube and nonspecific binding was approximately 30% of the total binding. [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar membranes was characterized as previously described (*39*).

$^{45}Ca^{2+}$ Release from Permeabilized Rat Hepatocytes. Hepatocytes were isolated by collagenase digestion of the livers of male Wistar rats (200−250 g), as previously described (*40*), and stored at 4 °C in Eagles medium supplemented with 26 mM $NaHCO_3$ and BSA (2% w/v) for up to 24 h. Cells were permeabilized by incubation with saponin (10 μg/mL) in a cytosol-like medium (CLM, 140 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 20 mM PIPES, pH 7.0 at 37 °C) and subsequently loaded to steady-state (1−2 nmol of Ca^{2+}/10^6 cells) by incubation (10^7-cells/mL) for 5 min at 37 °C in CLM supplemented with $CaCl_2$ (300 μM, free [Ca^{2+}] = 200 nM), ATP (7.5 mM), FCCP (10 μM) and $^{45}Ca^{2+}$ (7.5 μCi/mL). Unidirectional $^{45}Ca^{2+}$ efflux from the intracellular stores was initiated by diluting the cells (5-fold) into Ca^{2+}-containing CLM at 37 °C supplemented with thapsigargin (1 μM final). After 15 s, appropriate concentrations of Ins(1,4,5)P_3, adenophostin A or related compounds were then added, and 60 s later, the $^{45}Ca^{2+}$ content of the intracellular stores was determined after quenching in ice-cold medium (310 mM sucrose and 1 mM trisodium citrate) and than rapid filtration through

Scheme 1: Synthesis of Sucrose 3,4,3′-Trisphosphate (**4**) from Sucrose[a]



[a] Bn = benzyl, Pv = pivaloyl.

Whatman GF/C filters using a Brandel receptor-binding harvester (*38*).

Analysis of Results. Equilibrium competition binding curves were fitted to four-parameter logistic equations using a nonlinear curve-fitting program (Kaleidagraph, Synergy Software, PA):

$$R = \frac{(M - B)}{1 + \left(\frac{D}{K}\right)^h} + B$$

where R, M and B are the specific, total, and nonspecific [^{3}H]Ins(1,4,5)P_3 binding, respectively, h is the Hill coefficient, and K is the concentration of competing ligand causing 50% displacement of specific [^{3}H]Ins(1,4,5)P_3 binding (=IC_{50}) from which the K_d for each ligand was computed (*41*). Concentration-response relationships were fitted to an analogous equation from which the maximal effect, EC_{50} and h for each ligand were determined. All results are reported as means ± sem.

*Synthesis of Sucrose 3,4,3′-Trisphosphate (**4**).* Sucrose 2,6,1′,4′,6′-pentapivaloate (**8**, Scheme 1) was synthesized by regioselective pivaloylation of sucrose (*42*) and recrystallized three times from hexane to ensure maximum purity before the next step; mp 128−130 °C; $[\alpha]_D^{22} = +60$ ($c = 1$, MeOH) [Literature values (*42*): mp 120−121 °C; $[\alpha]_D = +61$ ($c =$ 1, MeOH)].

*2,6,1′,4′,6′-Penta-O-pivaloyl-sucrose 3,4,3′-Tris(dibenzylphosphate) (**9**).* To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (1.00 g, 2.90 mmol) in dry dichloromethane (10 mL) was added 1-*H*-tetrazole (400 mg, 5.71 mmol). The mixture was stirred at room temperature for 30 min and then the pure triol **8** (500 mg, 0.655 mmol) was added. The mixture was stirred for 1 h, after which a ^{31}P NMR spectrum showed signals at δ 140.7 (singlet), 140.9 (doublet, $^5J_{PP} = 5.5$ Hz), and 141.8 ppm (doublet, $^5J_{PP} =$ 5.5 Hz). The mixture was cooled to −78 °C, and *m*-CPBA (60% purity, 1.13 g, 3.93 mmol) was added. The mixture was allowed to reach room temperature and then diluted with ethyl acetate (50 mL). The solution was washed with 10% sodium sulfite solution, saturated $NaHCO_3$ solution, and brine (50 mL of each), dried ($MgSO_4$), and evaporated *in vacuo* to give an oil. Purification by column chromatography (chloroform/acetone 20:1) gave **9** as a wax (929 mg, 0.602 mmol, 92%); $[\alpha]_D^{23} = +27$ ($c = 2$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.14, 1.15, 1.22, 1.23, 1.24 (45 H, 5 s, 15 × pivaloate CH_3), 3.96 (1 H, ddd, $J = 8.2$, 5.4, 2.4 Hz, C-5′-H), 4.13 (2 H, br s, C-1′-H_2), 4.15 (1 H, dd, $J =$ 12.7, 5.4 Hz, C-6′-H_a), 4.25 (1 H, dd, $J = 12.7$, 2.0 Hz, C-6′-H_b), 4.35 (1 H, br d, $J = 9.8$ Hz, C-5-H), 4.41 (1 H, dd, $J = 12.7$, 2.0 Hz, C-6-H_a), 4.49 (1 H, dd, $J = 12.7$, 1.5 Hz, C-6-H_b), 4.77 (1 H, q*, $J \approx 9.5$ Hz, C-4-H), 4.82−5.10 (14 H, m, C-2-H, C-3-H and 6 × C$H_2C_6H_5$), 5.19 (1 H, dd, $J = 8.8$, 8.3 Hz, C-3′-H), 5.57 (1 H, dd, $J = 8.8$, 8.3 Hz, C-4′-H), 5.77 (1 H, d, $J = 3.9$ Hz, C-1-H), 7.08−7.46 (30 H, m, 6 × C_6H_5) (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 26.92. 27.05, 27.09, 27.16, 27.29 (15 × pivaloate *C*H_3), 38.62, 38.79, 38.83, 38.90 [5 × pivaloate *C*$(CH_3)_3$], 61.58, 62.75, 63.90 (C-6, C-1′, C-6′), 69.20 (*C*H), 69.28−70.06 (overlapping signals with J_{CP} couplings, 6 × *C*H_2OP), 70.65 (*C*H), 72.11 ($J_{CP} = 3.7$ Hz, *C*H), 73.84 ($J_{CP} = 5.5$, 5.5 Hz, *C*H), 76.33 ($J_{CP} = 5.5$, 5.5 Hz, *C*H), 77.52 (*C*H), 78.10 ($J_{CP} = 5.5$ Hz, *C*H), 88.53 (C-1), 102.65 ($J_{CP} = 3.7$ Hz, C-2′), 128.00−128.89 (overlapping signals, C_6H_5), 135.20−136.01 (overlapping signals with J_{CP} couplings, 6 × POC$H_2C_6H_5$, *ipso)*, 176.75, 176.83, 177.69, 177.78, 178.05 (5 × pivaloate C=O). ^{31}P NMR ($CDCl_3$, 162 MHz, ^{1}H-coupled): δ −0.43 (1 P, sextet* $J \approx 8.3$ Hz), −2.03 (1 P, sextet* $J \approx 8.0$ Hz), −2.36 (1 P, sextet* $J \approx$ 8.8 Hz) (*apparent splitting pattern). MS: *m/z* (+ ion FAB, relative intensity) 1544 [$(M + H)^+$, 60%], 363 (40), 279 (60), 91 [$(C_7H_7)^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity) 1451 [$(M - C_7H_7)^-$, 90%), 277 [$((C_6H_5CH_2O)_2PO_2)^-$, 100%]. Anal. Calcd for $C_{79}H_{101}O_{25}P_3$ (1543.58): C, 61.47; H, 6.60. Found: C, 61.2; H, 6.56.

*Sucrose 3,4,3′-Trisphosphate (**4**).* A solution of **9** (154 mg, 100 μmol) in ethanol (10 mL) was stirred vigorously with 10% palladium on carbon (100 mg) under an atmosphere of H_2 (balloon) for 2 h. The catalyst was removed by filtration and the solvents removed by evaporation under reduced pressure. The residue was redissolved in deionized water (10 mL), adjusted to pH 14 with aqueous NaOH and stirred overnight at room temperature. Purification by ion-exchange chromatography on Q Sepharose Fast Flow gave the triethylammonium salt of **4** which eluted between 470 and 550 mM of TEAB buffer, and was quantified by total phosphate assay. Yield = 57 μmol, 57%; $[\alpha]_D^{24} = +51$ (c = 0.3, H_2O, pH 5, calculated for the free acid). ^{1}H NMR (CD_3OD, 400 MHz): δ 3.57 (1 H, dd, $J = 9.3$, 3.9 Hz, C-2-H), 3.63 (2 H, br s, C-1′-H), 3.68−3.77 (2 H, m, C-6-H_a and C-6′-H_a), 3.75−3.85 (2 H, m, C-5′-H, C-6′-H_b), 3.90 (1 H, br, d, $J \approx 10$ Hz, C-5-H), 3.97 (1 H, dd, $J = 13.2$, 2.9 Hz, C-6-H_b), 4.10 (1 H, q*, $J \approx 9.5$ Hz, C-4-H), 4.15 (1 H, t*, $J = 8.3$ Hz, C-4′-H), 4.40 (1 H, q*, $J \approx 9$ Hz, C-3-H), 4.52 (1 H, t*, $J = 8.3$ Hz, C-3′-H), 5.49 (1 H, d, $J = 3.9$ Hz, C-1-H) (*apparent splitting pattern). ^{31}P NMR (162 MHz, CD_3OD, ^{1}H-coupled): δ 1.60 (1P, d, $J = 10.1$ Hz), 1.78 (1P, d, $J = 8.8$ Hz), 2.48 (1P, d, $J = 7.9$ Hz). Accurate mass FAB$^-$: *m/z* calcd for $C_{12}H_{24}O_{20}P_3^-$, 581.007. Found: 581.009.

Synthesis of α,α′*-Trehalose Tetrakisphosphates (**5** and **6**).* 4,6:4′,6′-Di-*O*-benzylidene-α,α′-trehalose (2.00 g, 3.86 mmol) was subjected to regioselective dibenzylation as described by Vicent *et al*. (*43*). The products were separated by flash chromatography (chloroform:acetone 10:1) giving first 2,3′-di-O-benzyl-4,6:4′,6′-di-*O*-benzylidene-α,α′-trehalose (**10b**, 785 mg, 1.12 mmol, 29%); mp 185−187 °C (crystals from

ethyl acetate/hexane); $[\alpha]_D^{19} = +88$ ($c = 1$, $CHCl_3$) [literature values (*43*), syrup; $[\alpha]_D = +76$ ($c = 1.1$, $CHCl_3$)]. Second to elute was the symmetrical 2,2′-*O*-benzylated product 2,2′-di-*O*-benzyl-4,6:4′,6′-di-*O*-benzylidene-α,α′-trehalose (**10a**, 1.32 g, 1.88 mmol, 49%); mp 196−199 °C (crystals from ethyl acetate/hexane); $[\alpha]_D^{19} = +99$ ($c = 1$, $CHCl_3$) [literature values (*43*), mp 197−199 °C; $[\alpha]_D = +90$ ($c = 2$, $CHCl_3$)].

*3,3′-Di-O-benzoyl-2,2′-di-O-benzyl-4,6:4′,6′-di-O-benzylidene-α,α′-trehalose (**11a**).* The symmetrical diol **10a** (500 mg, 0.716 mmol) was benzoylated under standard conditions. Aqueous workup followed by flash chromatography (ethyl acetate/hexane 1:1) gave **11a** (586 mg, 0.646 mmol, 90%) as a highly crystalline white solid; mp 230−231 °C (from ethyl acetate/hexane); $[\alpha]_D^{20} = +78$ ($c = 1$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.68 (2 H, dd, $J = 10.3$, 9.8 Hz, C-6/6′-H_{ax}), 3.73 (2 H, t*, $J = 9.8$ Hz, C-4/4′-H), 3.86 (2 H, dd, $J = 9.8$, 3.9 Hz, C-2/2′-H), 4.11 (2 H, dd, $J = 10.3$, 4.9 Hz, C-6/6′-H_{eq}), 4.37 (2 H, td*, $J = 9.8$, 4.9 Hz, C-5/5′-H), 4.62, 4.69 (4 H, ABq, $J_{AB} = 12.2$ Hz, CH_2Ph), 5.28 (2 H, d, $J = 3.9$ Hz, C-1/1′-H), 5.43 (2 H, s, C*H*Ph), 5.92 (2 H, t*, $J = 9.8$ Hz, C-3/3′-H), 7.17−7.46 (24 H, m, C_6H_5), 7.56 [2 H, t, $J = 7.3$ Hz, OC(O)C_6H_5 *para*], 8.05 (4 H, d, $J = 7.8$ Hz, OC(O)C_6H_5 *ortho*) (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 62.95 (2 × *C*H), 68.84 (C-6/6′), 71.65 (2 × *C*H), 72.64 (2 × O*C*H$_2$C$_6$H$_5$), 76.17, 79.55 (2 × *C*H), 94.79 (C-1/1′), 101.61 (2 × Ph*C*H), 126.35, 127.91, 128.02, 128.25, 128.49, 128.84, 129.86 (C_6H_5), 130.17 [2 × OC(O)C_6H_5 *ipso*], 132.84 [2 × OC(O)C_6H_5 *para*], 136.96, 137.20 (4 × C_6H_5 *ipso*), 165.09 (2 × *C*═O). MS: *m/z* (+ ion FAB, relative intensity) 907 [(M + H)$^+$, 60%], 445 [+ fragment ion from cleavage of glycosidic bond, 60%], 323(80), 91 [(C_7H_7)$^+$, 100%]. Anal. Calcd for $C_{54}H_{50}O_{13}$ (906.98): C, 71.51; H, 5.56. Found: C, 71.6; H, 5.56.

*2,3′-Di-*O*-benzoyl-2′,3-di-*O*-benzyl-4,6:4′,6′-di-O-benzylidene-α,α′-trehalose (**11b**).* The asymmetrical diol **10b** (1.00 g, 1.43 mmol) was benzoylated as for **10a**. Purification by flash chromatography (ethyl acetate/hexane 1:2) gave **11b** (1.13 g, 1.25 mmol, 87%) as a white foam; $[\alpha]_D^{26} = +148$ ($c = 1$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 270 MHz): δ 3.40 (1 H, t*, $J = 10.3$ Hz, C-6-H_{ax}) 3.51 (1 H, dd, $J = 10.3$, 4.9 Hz, C-6-H_{eq}), 3.60 (1 H, t*, $J = 9.5$ Hz, C-4-H), 3.71−3.95 (4 H, C-2′-H, C-4′-H, C-6′-H_{ax}, C-5-H), 4.12 (1 H, dd, $J = 10.3$, 4.9 Hz, C-6′-H_{eq}), 4.33 (1 H, ddd, $J = 10.1$, 9.3, 4.9 Hz, C-5′-H), 4.43 (1 H, t*, $J = 9.5$ Hz, C-3-H), 4.59, 4.68 (2 H, ABq, $J_{AB} = 12.3$ Hz, CH_2Ph), 4.83, 4.95 (2 H, ABq, $J_{AB} = 12.3$ Hz, CH_2Ph), 5.21−5.28 (3 H, m, C-1′-H, C-2-H, C*H*Ph), 5.50 (1 H, d, $J = 3.84$ Hz, C-1-H), 5.58 (1 H, s, C*H*Ph), 5.85 (1 H, t*, $J = 9.5$ Hz, C-3′-H), 7.17−7.60 (26 H, m, C_6H_5), 8.02−8.09 (4 H, m, C_6H_5) (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 63.15 (2 × *C*H), 68.18, 68.80 (C-6, C-6′), 71.45, 73.02, 75.86, 75.99, 79.11, 82.08 (6 × *C*H), 72.77, 75.13 (2 × O*C*H$_2$C$_6$H$_5$), 94.20, 94.53 (C-1, C-1′), 101.11, 101.57 (2 × Ph*C*H), 126.26, 127.56, 128.02, 127.83, 127.94, 128.02, 128.16, 128.27, 128.35, 128.55, 128.78, 128.95, 129.74, 129.85 (C_6H_5) 128.84, 129.99 [2 × OC(O)C_6H_5 *ipso*], 133.05, 133.43 [2 × OC(O)C_6H_5 *para*], 136.99, 137.03, 137.29, 138.28 (C_6H_5 *ipso*), 165.47, 165.93 (2 × *C*═O). MS: *m/z* (+ ion FAB, relative intensity) 907 [(M + H)$^+$, 80%], 445 [+ fragment ion from cleavage of glycosidic bond, 20%], 323(10), 91 [(C_7H_7)$^+$, 100%]. Anal. Calcd for $C_{54}H_{50}O_{13}$ (906.98): C, 71.51; H, 5.56. Found: C, 71.3; H, 5.50.

*3,3′-Di-O-benzoyl-2,6,2′,6′-tetra-O-benzyl-α,α′-trehalose (**12a**).* To a solution of sodium cyanoborohydride in THF (14 mL of a 1 M solution, 14 mmol) containing 3 Å molecular sieves was added compound **11a** (500 mg, 0.551 mmol). The mixture was stirred under N_2 at room temperature, and a 1 M solution of hydrogen chloride in dry ether was added dropwise until evolution of gas ceased. TLC (chloroform/acetone 10:1) showed the reaction to be complete with total conversion of starting material ($R_f = 0.55$) to a product at $R_f = 0.24$. The mixture was diluted with dichloromethane (50 mL), washed with water, saturated $NaHCO_3$ solution and brine (50 mL of each), and dried over $MgSO_4$. Evaporation under reduced pressure gave an oil which was purified by flash chromatography to provide the symmetrical **12a** as a white foam (404 mg, 0.443 mmol, 80%); $[\alpha]_D^{25} = +158$ ($c = 1$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.99 (2 H, d, $J = 6.4$ Hz, D_2O ex., C-4/4′-OH), 3.59 (4 H, narrow ABX system, C-6/6′-H), 3.69 (4 H, dd, $J = 9.8$, 3.5 Hz, C-2/2′-H), 3.74 (2 H, m, D_2O ex. gives t*, $J = 9.5$ Hz, C-4/4′-H), 4.21 (2 H, dt*, $J = 10$, 3.4 Hz, C-5/5′-H), 4.50 (4 H, narrow ABq, OCH_2C$_6$H$_5$), 4.57, 4.63 (4 H, ABq, $J_{AB} = 12.3$ Hz, OCH_2C$_6$H$_5$), 5.35 (2 H, d, $J = 3.5$ Hz, C-1/1′-H), 5.63 (2 H, t*, $J = 9.5$ Hz, C-3/3′-H), 7.14−7.19 (10 H, m, CH$_2$C$_6$H$_5$), 7.24−7.29 (10 H, m, CH$_2$C$_6$H$_5$) 7.45 [4 H, dd, $J = 7.9$, 7.1 Hz, OC(O)C_6H_5 *meta*], 7.59 [2 H, tt, $J = 7.1$, 1.5 Hz, OC(O)C_6H_5 *para*], 8.04 [4 H, dd, $J = 7.9$, 1.5 Hz, OC(O)C_6H_5 *ortho*] (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 68.89 (C-6/6′), 69.38, 71.73, 72.52 (6 × *C*H), 73.64, 75.22 (4 × O*C*H$_2$C$_6$H$_5$), 76.17 (2 × *C*H), 93.45 (C-1/1′), 127.60, 127.70, 127.83, 127.89, 128.33 (C_6H_5), 129.76 [2 × C(O)C_6H_5 *ipso*], 129.93 [4 × OC(O)C_6H_5 *ortho*], 133.23 [2 × OC(O)C_6H_5 *para*], 137.88, 136.99 (4 × CH$_2$$C_6H_5$ *ipso*), 167.14 (2 × *C*═O). MS: *m/z* (+ ion FAB, relative intensity) 911 [M$^+$, 40%], 537(80), 447 [+ fragment ion from cleavage of glycosidic bond, 10%), 91 [(C_7H_7)$^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity) 1063 [(M + NBA-H)$^-$, 35%], 121 [($C_7H_5O_2$)$^-$, 100%]. Anal. Calcd for $C_{54}H_{54}O_{13}$ (911.01): C, 71.19; H, 5.97. Found: C, 71.0; H, 5.97.

*2,3′-Di-O-benzoyl-3,6,2′,6′-tetra-O-benzyl-α,α′-trehalose (**12b**).* Regioselective reduction of the benzylidene acetals in **11b** (400 mg, 0.441 mmol), as described for **11a**, and purification by flash chromatography (ethyl acetate/hexane 1:2) gave asymmetrical **12b** (314 mg, 0.345 mmol, 78%) as a hygroscopic white foam; $[\alpha]_D^{23} = +149$ ($c = 2.5$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.58 (1 H, d, $J = 3.4$ Hz, C-4-OH), 2.73 (1 H, d, $J = 3.4$ Hz, C-4′-OH), 2.99 (1 H, dd, $J = 10.7$, 2.4 Hz, C-6-H_a), 3.07 (1 H, dd, $J = 10.7$, 2.4 Hz, C-6-H_b), 3.57 (2 H, narrow ABX system, C-6′-H), 3.68 (2 H, dd, $J = 9.8$, 3.4 Hz, C-2′-H), 3.76−3.83 (3 H, m, C-4-H, C-4′-H, C-5-H), 4.14−4.28 (4 H, m, C-3-H, C-5′-H, OCH_2C$_6$H$_5$), 4.46−4.66 (4 H, two overlapping AB systems, OCH_2C$_6$H$_5$), 4.84, 4.90 (2 H, ABq, $J_{AB} = 11.7$ Hz, OCH_2C$_6$H$_5$), 5.19 (1 H, dd, $J = 10.3$ Hz, 3.9 Hz, C-2-H), 5.29 (1 H, d, $J = 3.4$ Hz, C-1′-H), 5.47 (1 H, d, $J = 3.4$ Hz, C-1-H), 5.60 (1 H, t*, $J = 9.3$ Hz, C-3′-H), 7.09−7.62 (26 H, m, C_6H_5), 8.03−8.06 [4 H, m, OC(O)C_6H_5 *ortho*] (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 100.4 MHz): δ 68.31, 69.29, (C-6, C-6′), 70.08 70.48, 70.96, 71.09 (4 × *C*H), 72.35, 73.43, 73.63, 75.37 (4 × O*C*H$_2$C$_6$H$_5$), 75.42, 75.73 79.39 (3 × *C*H), 93.49 (C-1, C-1′), 127.58, 127.67, 127.71, 127.82, 127.94, 128.09, 128.18, 128.29,

128.36, 128.44, 128.51, 128.64 (C_6H_5), 129.13, 129.66 [C(O)C_6H_5 *ipso*], 129.75, 129.96 [OC(O)C_6H_5 *ortho*], 133.37, 133.48 [OC(O)C_6H_5 *para*], 137.27, 137.53, 137.87, 138.24 ($CH_2C_6H_5$ *ipso*), 165.47, 167.39 (2 × *C*═O). MS: *m/z* (+ ion FAB, relative intensity): 933 (30), 537 (40), 447 [+ fragment ion from cleavage of glycosidic bond, 60%], 181 (60), 91 [$(C_7H_7)^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity): 1063 [(M + NBA−H)$^-$, 100%]. Anal. Calcd for $C_{54}H_{54}O_{13}$ (911.01): C, 71.19; H, 5.97. Found: C, 70.8; H, 6.12.

*2,6,2′,6′-Tetra-O-benzyl-α,α′-trehalose (**13a**).* Sodium hydroxide pellets (100 mg, 2.5 mmol) were added to a solution of **12a** (400 mg, 0.439 mmol) in methanol (25 mL), and the mixture was heated at reflux for 30 min. TLC (chloroform methanol 5:1) showed complete conversion of starting material ($R_f = 0.82$) to a product ($R_f = 0.50$). The solution was allowed to cool and then neutralized with solid CO_2. The solvents were removed by evaporation under reduced pressure and the solid residue was extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with water (100 mL), dried over $MgSO_4$, and evaporated to give an oil. Purification by flash chromatography (ethyl acetate) gave **13a** (263 mg, 0.374 mmol, 85%) as a white crystalline solid; mp 135−137 °C (from ethyl acetate/hexane); $[\alpha]_D^{18} = +127$ ($c = 1$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 270 MHz): δ 3.37 (2 H, dd, $J = 9.7$, 3.1 Hz, C-2/2′-H), 3.53 (2 H, td*, $J = 9.5$, ∼5 Hz, D_2O ex. gives t*, $J = 9.5$ Hz, C-4/4′-H), 3.52−3.65 (4 H, m, C-6/6′-H_2), 3.64, (2 H, br d, $J \approx 5$ Hz, D_2O ex., C-4/4′-OH), 4.02−4.12 (4 H, m, C-3/3′-H, C-5/5′-H), 4.30 (2 H, br s, D_2O ex. C-3/3′-OH), 4.44−4.66 (8 H, 2 × AB systems, 4 × OC$H_2$$C_6H_5$), 5.12 (2 H, d, $J = 3.1$ Hz, C-1/1′-H), 7.22−7.27 (20 H, m, $CH_2C_6H_5$) (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 67.8 MHz): δ 69.31 (C-6/6′), 70.72, 71.28, 72.41 (6 × CH), 72.54, 73.55 (4 × O*C*$H_2C_6H_5$), 78.09 (2 × CH), 93.92 (C-1/1′), 127.57, 127.66, 128.04, 128.09, 128.30 (C_6H_5), 137.05, 137.96 (4 × $CH_2C_6H_5$ *ipso*). MS: *m/z* (+ ion FAB, relative intensity) 703 [(M + H)$^+$, 10%], 685 (56), 433 (24), 343 [+ fragment ion from cleavage of glycosidic bond, 92%], 91 [$(C_7H_7)^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity) 1404 [2M$^-$, 20%], 869 (100), 855 [(M + NBA)$^-$, 65%], 701 [(M − H)$^-$, 30%]. Anal. Calcd for $C_{40}H_{46}O_{11}$ (702.80): C, 68.36; H, 6.60. Found: C, 68.2; H, 6.6.

*2,6,3′,6′-Tetra-O-benzyl-α,α′-trehalose (**13b**).* Removal of the benzoyl groups from **12b** (200 mg, 0.220 mmol) as described for **12a**, followed by flash chromatography (ethyl acetate/hexane 2:1) gave **13b** (140 mg, 0.199 mmol, 90%) as a waxy solid; $[\alpha]_D^{25} = +104$ ($c = 0.7$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.65 (1 H, d, $J = 4.0$ Hz, D_2O ex. OH), 3.04 (1 H, d, $J = 6.4$ Hz, D_2O ex. OH), 3.36 (1 H, dd, $J = 9.5$, 3.4 Hz, C-2-H or C-2′-H), 3.50−3.70 (10 H, m, 8 × CH and 2 × OH), 3.94−4.07 (3 H, m, C-3-H, C-5-H, C-5′-H), 4.44−4.84 (8 H, 4 × overlapping AB systems, 4 × OC$H_2$$C_6H_5$), 5.12 (2 H, d, $J = 3.7$ Hz, C-1-H or C-1′-H), 5.21 (2 H, d, $J = 3.4$ Hz, C-1-H or C-1′-H), 7.20−7.36 (20 H, m, $CH_2C_6H_5$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 69.31, 69.77 (C-6 and C-6′), 70.70, 70.87, 70.96, 71.10, 72.75 (6 × CH), 72.66, 73.46, 73.68, 74.36 (4 × O*C*$H_2C_6H_5$), 78.25, 82.21 (2 × CH), 92.19, 94.46 (C-1 and C-1′), 127.69, 127.82, 127.91, 127.98, 128.09, 128.40, 128.55 (C_6H_5), 137.43, 137.84, 137.89, 138.66 (4 × $CH_2C_6H_5$ *ipso*). MS: *m/z* (+ ion FAB, relative intensity) 725 (90), 703 [(M + H)$^+$, 26%], 685 (22), 437 (40), 181 (20), 91 [$(C_7H_7)^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity) 1404 [2M$^-$, 30%], 869 (20), 855 [(M + NBA)$^-$, 100%], 701 [(M − H)$^-$, 25%].

*2,6,2′,6′-Tetra-O-benzyl-α,α′-trehalose 3,4,3′,4′-Tetrakis-(dibenzylphosphate) (**14a**).* To a solution of bis(benzyloxy)-*N*,*N*-diisopropylaminophosphine (550 mg, 1.59 mmol) in dry dichloromethane (2 mL) was added 1-*H*-tetrazole (223 mg, 3.18 mmol). The mixture was stirred at room temperature for 10 min, and then the tetraol **13a** (140 mg, 0.199 mmol) was added. The mixture was stirred for another 1 h, after which a ^{31}P NMR spectrum showed signals at δ 140.7 and 141.1 ppm (AB system $^5J_{PP} = 4.9$ Hz). The mixture was cooled to −78 °C and *m*-CPBA (60% purity, 480 mg, 1.67 mmol) was added. The mixture was allowed to reach room temperature and then diluted with ethyl acetate (50 mL). The solution was washed with 10% sodium sulfite solution, 1 M HCl, saturated $NaHCO_3$ solution, and brine (50 mL of each), dried ($MgSO_4$) and evaporated *in vacuo* to give an oil. Purification by column chromatography (chloroform/acetone 10:1) gave **14a** as a colorless oil (315 mg, 0.181 mmol, 91%); $[\alpha]_D^{26} = +46$ ($c = 1$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.28 (2 H, br d, $J \approx 11$ Hz, C-6/6′-H_a), 3.47 (2 H, dd, $J = 11$, 3.5 Hz, C-6/6′-H_b), 3.63 (2 H, dd, $J = 9.8$, 3.7 Hz, C-2/2′-H), 4.21 (2 H, obscured by AB system, C-5/5′-H), 4.20, 4.37 (4 H, ABq, $J_{AB} = 11.9$ Hz, 2 × OC$H_2$$C_6H_5$), 4.52, 4.60 (4 H, ABq, $J_{AB} = 11.9$ Hz, 2 × OC$H_2$$C_6H_5$), 4.70 (2 H, q*, $J = 9.5$ Hz, C-4/4′-H), 4.84−5.13 (18 H, m, CH_2-OP AB systems and C-3/3′-H), 5.16 (2 H, d, $J = 3.7$ Hz, C-1/1′-H), 7.07−7.27 (60 H, m, 12 × C_6H_5) (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 67.34 (*C*-6/6′-H), 69.18 ($J_{CP} = 5.5$ Hz, 4 × *C*H_2OP), 69.42 ($J_{CP} = 5.5$ Hz, 2 × *C*H_2OP), 69.66 ($J_{CP} = 5.5$ Hz, 2 × *C*H_2OP), 69.69 (2 × *C*H), 72.59 (2 × OC$H_2$$C_6H_5$), 73.05 (2 × O*C*$H_2$$C_6H_5$), 74.07 ($J_{CP} = 5.5$, 3.7 Hz, C-3/3′ or C-4/4′), 76.89 (2 × CH), 78.39 ($J_{CP} = 5.5$, 3.7 Hz, C-3/3′ or C-4/4′), 93.44 (C-1/1′), 127.22, 127.33, 127.46, 127.59, 127.79, 127.88, 127.93, 127.99, 128.12, 128.23, 128.28, 128.37 (C_6H_5), 135.69 ($J_{CP} = 7.4$ Hz, 2 × POCH_2*C*$_6H_5$ *ipso*), 135.89, 136.00, 136.12 ($J_{CP} = 7.3$ Hz, 6 × POCH_2*C*$_6H_5$ *ipso*), 137.33, 137.90 (4 × OC$H_2$$C_6H_5$ *ipso*). ^{31}P NMR ($CDCl_3$, 162 MHz, ^{1}H-decoupled): δ −1.94 (2 P), −2.20 (2 P). MS: *m/z* (+ ion FAB, relative intensity) 1744 [(M + H)$^+$, 85%], 1654 (20), 459 (40), 307 (85), 221 (50), 91 [$(C_7H_7)^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity) 1742 [(M − H)$^-$, 30%], 1652 [(M − C_7H_7)$^-$, 80%], 277 [$((C_6H_5CH_2O)_2PO_2)^-$, 100%]. Anal. Calcd for $C_{96}H_{98}O_{23}P_4$ (1743.71): C, 66.13; H, 5.66. Found: C, 66.0; H, 5.75.

*3,6,2′,6′-Tetra-O-benzyl-α,α′-trehalose 2,4,3′,4′-Tetrakis-(dibenzylphosphate) (**14b**).* The tetraol **13b** (100 mg, 0.142 mmol) was phosphitylated as described for **13a**. A ^{31}P NMR spectrum of the tetrakisphosphite triester intermediate showed signals at δ 140.35 and 141.70 ppm (AB system $^5J_{PP} = 4.9$ Hz), 140.18 and 140.97 (two singlets). Oxidation and workup as before, followed by flash chromatography (chloroform/acetone 20:1) gave **14b** as a colorless oil (238 mg, 0.136 mmol, 96%); $[\alpha]_D^{26} = +54$ ($c = 1$, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.30 (1 H, dd, $J \approx 11$, 1.5 Hz, C-6-H_a), 3.39 (1 H, dd, $J = 11.0$, 4.3 Hz, C-6-H_b), 3.60 (1 H, br d, $J \approx 11$ Hz, C-6′-H_a), 3.67 (1 H, dd, $J = 9.5$, 3.7 Hz, C-2′-H), 3.86 (1 H, dd, $J = 11.3$, 2.1 Hz, C-6′-H_b), 4.11 (1 H, t*, $J = 9.3$ Hz, C-3-H), 4.13 (1 H, m, C-5-H), 4.23−4.27 (3 H, m, C-5′-H and C$H_2$$C_6H_5$), 4.34−4.41 (3 H, m, C-2-H and C$H_2$$C_6H_5$), 4.53−4.61 (3 H, m C-4-H and C$H_2$$C_6H_5$), 4.70−5.17 (20 H, m, 9 × C$H_2$$C_6H_5$, C-3′-H and

C-4′-H), 5.25 (1 H, d, J = 3.7 Hz, C-1′-H), 5.35 (1 H, d, J = 3.7 Hz, C-1-H), 7.04−7.28 (58 H, m, C_6H_5), 7.38−7.40 (2 H, m, C_6H_5) (*apparent splitting pattern). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 67.32, 67.59 (*C*-6, C-6′), 69.13 −69.62 (overlapping signals with J_{CP} couplings, 8 × *C*H_2-OP), 70.07 (2 × *C*H), 72.62, 73.15, 73.30, 74.85 (4 × O*C*$H_2C_6H_5$), 73.94 (J_{CP} = 5.5 Hz, 5.5 Hz, *C*H), 75.02 (J_{CP} = 5.5 Hz, 5.5 Hz, *C*H), 75.94 (J_{CP} = 5.5 Hz, *C*H), 77.05, 78.13, 78.74 (3 × *C*H), 92.79, 93.23 (*C*-1, *C*-1′), 127.18−128.53, (overlapping signals, C_6H_5), 135.35−136.25 (overlapping signals with J_{CP} couplings, 8 × POC$H_2C_6H_5$ *ipso*), 137.40, 137.97, 138.15, 138.28 (4 × O$CH_2C_6H_5$ *ipso*). ^{31}P NMR ($CDCl_3$, 162 MHz, ^{1}H-decoupled): δ −1.34 (P), −1.85 (P), −2.11 (2P). MS: *m/z* (+ ion FAB, relative intensity) 1744 [(M + H)$^+$, 80%], 1654 (10), 459 (40), 271 (40), 91 [$(C_7H_7)^+$, 100%]. MS: *m/z* (− ion FAB, relative intensity) 1896 (80), 1802 (25), 1652 [(M − $C_7H_7)^-$, 30%], 277 [$((C_6H_5CH_2O)_2PO_2)^-$, 100%]. Anal. Calcd for $C_{96}H_{98}O_{23}P_4$ (1743.71): C, 66.13; H, 5.66. Found: C, 65.6; H, 5.67.

α,α′-*Trehalose 3,4,3′,4′-Tetrakisphosphate (**5**).* Deprotection of the tetrakisphosphate triester **14a** (135 mg, 77.4μmol) using sodium in liquid ammonia (*34*) and then purification by ion-exchange chromatography on Q Sepharose Fast Flow resin gave the glassy triethylammonium salt of **5**, which eluted between 500 and 600 mM TEAB. Yield = 50.5μmol, 65%; $[\alpha]_D^{20}$ = +124 (*c* = 0.3, H_2O, pH 5−6, calculated for the free acid). ^{1}H NMR (D_2O, 400 MHz, pH 7) δ 3.54−3.61 (4 H, m, C-2/2′-H and C-6/6′-H), 3.67−3.73 (4 H, m, C-5/5′-H and C-6/6′-H), 3.86 (2 H, q*, $J \approx$ 9.3 Hz, C-4/4′-H), 4.36 (2 H, q*, J = 9.3 Hz, C-3/3′-H), 5.03 (2 H, br s, C-1/1′-H) (*apparent splitting pattern). ^{31}P NMR (D_2O, 162 MHz, pH 7, ^{1}H-coupled): δ 0.58 (2 P, d, $^3J_{HP}$ = 8.9 Hz, 3/3′-P), 0.99 (2 P, d, $^3J_{HP}$ = 10.1 Hz, 4/4′-P). MS*: m/z* (+ ion FAB) 255 [$((C_2H_5)_3N^+H$ + NBA), 85%], 102 [$(C_2H_5)_3N^+H$, 100%]. MS: *m/z* (− ion FAB) 1323 [$2M^-$, 80%], 661 [M^-, 100%]. Accurate mass FAB$^-$ *m/z* calcd for $C_{12}H_{25}O_{23}P_4^-$: 660.974. Found: 660.982.

α,α′-*Trehalose 2,4,3′,4′-Tetrakisphosphate (**6**).* A solution of **14b** (115 mg, 66μmol) in methanol/water 4:1 (50 mL) was shaken with 10% palladium on carbon (200 mg) under H_2 (50 psi) for 20 h. The catalyst was removed by filtration and the solvents removed by evaporation under reduced pressure. The residue was redissolved in deionized water (100 mL) and purified by ion-exchange chromatography on Q-Sepharose Fast Flow resin, eluting with a gradient of TEAB buffer (0−1 M). The glassy triethylammonium salt of **6** eluted between 650 and 750 mM of buffer, and was quantified by total phosphate assay. Yield = 60 μmol (91%); $[\alpha]_D^{20}$ = +108 (*c* = 0.4, H_2O, pH 5−6, calculated for the free acid). ^{1}H NMR (CD_3OD, 400 MHz): δ 3.63 (1 H, dd, 9.3, 3.9 Hz, C-2′-H), 3.73 (1 H, br d, $J \approx$ 12 Hz, CH_2), 3.80−3.87 (2 H, m, C-3-H and CH_2), 4.02−4.18 (7 H, m, 5 × C*H* and CH_2), 4.43 (1 H, q*, $J \approx$ 9.3 Hz, C-3′-H), 5.11 (1 H, d, J = 3.9 Hz, C-1′-H), 5.32 (1 H, d, J = 2.9 Hz, C-1-H) (*apparent splitting pattern). ^{31}P NMR (109 MHz, CD_3OD, ^{1}H-decoupled): δ 1.47 (1P), 1.75 (1P), 2.51 (1P), 2.67 (1P) (overlapping doublets in ^{1}H-coupled spectrum). MS*: m/z* (+ ion FAB) 255 [$((C_2H_5)_3N^+H$ + NBA), 10%], 102 [$(C_2H_5)_3N^+H$, 100%]. MS: *m/z* (− ion FAB) 661 [M^-, 100%]. Accurate mass FAB$^-$ *m/z* calcd for $C_{12}H_{25}O_{23}P_4^-$: 660.974. Found: 660.973.

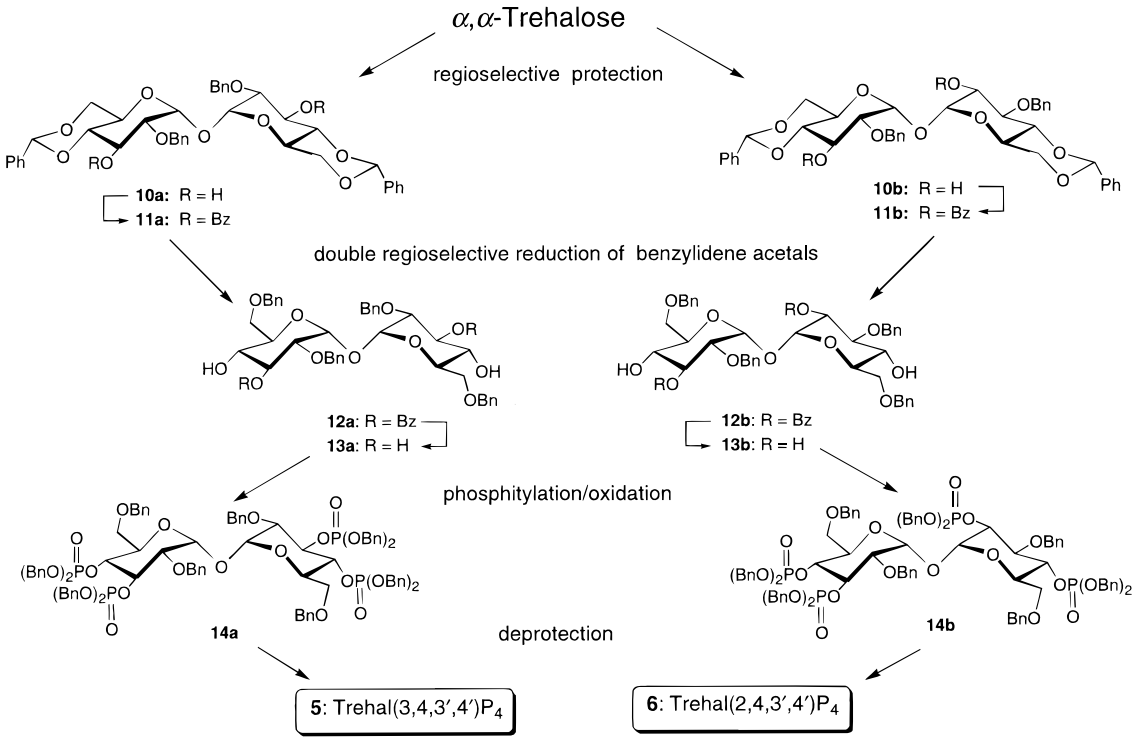
RESULTS

Synthesis of Disaccharide Polyphosphates. Disaccharides offer plenty of scope as frameworks for the synthesis of novel polyphosphates, but the difficulties of protecting the hydroxyl groups selectively are often much greater than those encountered with simple inositols. One solution is to protect selected hydroxyl groups in two monosaccharides and then couple the two together. This was the strategy used to prepare Rib(2,3′,4′)P_3 (**7**) (*28*) and has the advantage that, with sufficient ingenuity, any desired disaccharide polyphosphate can be assembled. Thus, **7** was designed to mimic, as closely as possible, the structure of adenophostin A, but with the adenine removed. This approach also allows the glucose and ribose components to be coupled to various other moieties or a range of monosaccharides may be coupled combinatorially in pairs, thereby generating a number of analogs. The chief disadvantages of this strategy are the time taken to prepare the selectively protected intermediates and the lack of stereospecificity in the coupling reactions. Another solution is simply to choose an existing disaccharide with the required anomeric configuration, selectively protect some of its hydroxyl groups, phosphorylate the unprotected positions, and then deprotect. This strategy has the disadvantage that a precise target structure may not be accessible because the required disaccharide is unavailable, but it has the advantage that the polyphosphates may be synthesized much more rapidly from cheap and abundant starting materials, potentially on a large scale. Given the range of available disaccharides, the strategy may have potential for the discovery of lead compounds with novel properties, and for the generation of a range of structurally diverse analogs required to provide data for molecular modeling studies. This method was chosen for compounds **4**, **5,** and **6**.

*Sucrose 3,4,3′-Trisphosphate (**4**).* Sucrose is the most abundant carbohydrate, and indeed, the cheapest chiral material available (*44*). Unfortunately, with its eight free hydroxyl groups and acid-labile glycosidic linkage, sucrose presents considerable difficulties in selective protection and manipulation. However, a comparison of the structure of sucrose with the disaccharide part of adenophostin A suggested that an investigation of the previously unknown sucrose 3,4,3′-trisphosphate (**4**) might be worthwhile if a sufficiently concise route could be devised. A literature search revealed that an appropriately protected triol (**8**, Figure 3) was obtainable in multigram quantities directly from sucrose by a strategy of regioselective pivaloylation, which exploits slight differences in reactivity between the hydroxyl groups (*42*). It was then straightforward to prepare the fully protected trisphosphate (**9**) by phosphitylation followed by oxidation. A two-step deprotection method, removing first the benzyl protecting groups on phosphates and then the pivaloyl esters from the five hydroxyl groups allowed the isolation of pure Sucr(3,4,3′)P_3 (**4**) in an overall yield of 52% from **8**.

Trehalose Tetrakisphosphates. Assuming that the basic Ins(1,4,5)P_3-like activity of the adenophostins originated in their D-glucose 3,4-bis(phosphate) component, we reasoned that the novel analog (**5**) consisting simply of two copies of this structure joined by an α-glycosidic linkage might also be active. Either bis(phosphate) of this C_2-symmetrical molecule could (potentially) be recognized by the anchoring domain of the Ins(1,4,5)P_3 receptor binding site, leaving the

Scheme 2: Synthesis of Symmetrical (**5**) and Asymmetrical (**6**) α,α′-Trehalose Tetrakisphosphates from α,α′-Trehalose[a]



[a] Bn = benzyl, Bz = benzoyl, Ph = phenyl.

other phosphate groups to interact with the postulated accessory domain. Moreover, **5** could be synthesized from the readily available disaccharide α,α′-trehalose, a common metabolite and energy store in a variety of bacteria, fungi, plants and insects. Molecular dynamics studies have shown α,α′-trehalose to be a particularly rigid disaccharide, and this may be related to its suggested role as a protectant of enzymes and membranes under conditions of heat and desiccation (*45*).

The chosen synthetic strategy (Scheme 2) employed the symmetrically protected trehalose derivative **10a**, available from α,α′-trehalose in only two steps *via* the regioselective tin-mediated dibenzylation of 4,6:4′,6′-di-*O*-benzylidene-α,α′-trehalose (*43*). The byproduct of this reaction, the asymmetrically protected **10b**, would lead to another, potentially interesting, asymmetrical trehalose tetrakisphosphate **6**, in which the position of a single phosphate group on one glucose residue is altered, leaving the other glucose 3,4-bis(phosphate) component unchanged. A comparison of the biological properties of **5** and **6** would enable us to examine the biological consequences of this slight alteration in structure.

The key step in the synthesis of **5** was the simultaneous regioselective reduction of both benzylidene acetals in **11a**, leaving the two equivalent hydroxyl groups at positions 4 and 4′ exposed for later phosphorylation, and both primary hydroxyls protected as benzyl ethers. This reaction was successfully carried out using sodium cyanoborohydride-hydrogen chloride (*46*). It was found that, in order to obtain the required regioselectivity and thereby avoid a complex mixture of products, it was necessary first to protect positions 3 and 3′, adjacent to the acetals. This was easily achieved using benzoyl esters as temporary protecting groups, which were removed after the reduction step to give the symmetrical tetraol **13a**. Phosphitylation/oxidation followed by deprotection using sodium in liquid ammonia and ion-exchange chromatography on Q-Sepharose Fast Flow resin gave the

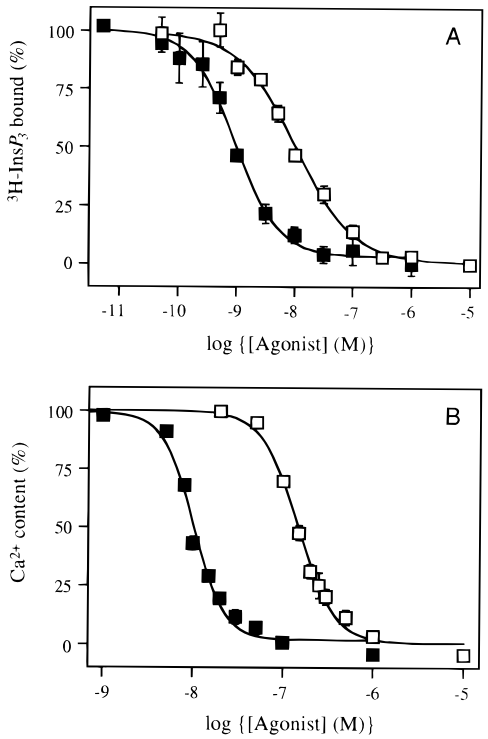


FIGURE 3: Effects of Ins(1,4,5)P_3 and adenophostin A on [^{3}H]Ins-(1,4,5)P_3 binding and Ca^{2+} mobilization. (A) Specific [^{3}H]Ins-(1,4,5)P_3 binding to rat hepatic membranes is shown in the presence of the indicated concentrations of Ins(1,4,5)P_3 (□) or adenophostin A (■). Results (% specific binding in the absence of competing ligand) are expressed as mean ± sem of three or more independent experiments. (B) The results show the effects of the indicated concentrations Ins(1,4,5)P_3 (□) or adenophostin A (■) on the Ca^{2+} content of Ins(1,4,5)P_3-sensitive Ca^{2+} stores of permeabilized hepatocytes. Results [% Ins(1,4,5)P_3-sensitive Ca^{2+} stores] are expressed as mean ± sem of 3 or more independent experiments.

Table 1: Effects of Ins(1,4,5)P_3, Adenophostin A, and Phosphorylated Carbohydrates on [^{3}H]Ins(1,4,5)P_3 Binding and Ca^{2+} Mobilization[a]

	binding		Ca^{2+} mobilization	
	K_d (nM)	h	EC_{50} (nM)	h
adenophostin A	0.87 ± 0.20	1.28 ± 0.37	10.9 ± 0.7	2.18 ± 0.39
Ins(1,4,5)P_3	8.65 ± 0.89	0.96 ± 0.04	153 ± 11	2.25 ± 0.20
Rib(2,3′,4′)P_3	8.42 ± 2.5	0.89 ± 0.10	213 ± 7.4	2.49 ± 0.18
Trehal(2,4,3′,4′)P_4	81.0 ± 23	0.88 ± 0.31	1271 ± 46	2.53 ± 0.43
Glc(2′,3,4)P_3	225 ± 28	0.74 ± 0.10	1867 ± 64	2.76 ± 0.10
Trehal(3,4,3′,4′)P_4	228 ± 69	0.90 ± 0.26	2466 ± 29	2.37 ± 0.27
Sucr(3,4,3′)P_3	343 ± 71	0.69 ± 0.08	5409 ± 110	2.60 ± 0.08

[a] Experiments similar to those shown in Figure 3 were used to determine the K_d from equilibrium competition binding experiments with [^{3}H]Ins(1,4,5)P_3 and EC_{50} from $^{45}Ca^{2+}$ flux assays for each of the indicated compounds. Results are expressed as means ± sem of three to seven independent experiments with duplicate determinations for each concentration of compound in each.

target tetrakisphosphate **5**, whose structure was confirmed by, *inter alia*, the presence of a symmetry element in the ^{1}H, ^{31}P and ^{13}C NMR spectra. Similarly, the symmetry element in the spectra of **10a**, **11a**, **12a**, and **13a** made identification particularly easy. The same sequence of reactions was then applied with equal success to **10b** to yield fully protected **14b**. Deprotection of **14b** using catalytic hydrogenation and purification as before gave asymmetrical **6** in an overall yield of 55% from **10b**.

Effects of Adenophostin A on Hepatic Ins(1,4,5)P_3 Receptors. The results shown in Figure 3 demonstrate that Ins(1,4,5)P_3 binds to a single class of site on hepatic membranes and that the same sites bind adenophostin A with ∼10-fold greater affinity (Table 1). These results are similar to those obtained with cerebellar membranes in which adenophostin A (K_d = 0.91 ± 0.08 nM, n = 5) bound with ∼7-fold greater affinity than Ins(1,4,5)P_3 (K_d = 6.57 ± 1.36 nM, n = 8). We conclude that the predominantly type 2 Ins(1,4,5)P_3 receptors of rat hepatocytes (*30*) and the type 1 Ins(1,4,5)P_3 receptors of cerebellum bind adenophostin A with similar affinity.

A maximally effective concentration of Ins(1,4,5)P_3 (10 μM) released 50 ± 3% (n = 7) of the intracellular Ca^{2+} stores of saponin-permeabilized hepatocytes. The same amount of Ca^{2+} was released by a maximally effective concentration of adenophostin A (1 μM) which evoked 102 ± 2% (n = 3) of the response evoked by Ins(1,4,5)P_3. Addition of Ins(1,4,5)P_3 (10 μM) 30 s after adenophostin A (1 μM) evoked no further Ca^{2+} release (not shown), confirming that each compound released the same intracellular Ca^{2+} store. Adenophostin A, however, was ∼14-fold more potent than Ins(1,4,5)P_3 in evoking Ca^{2+} release and, as with Ins(1,4,5)P_3, the responses were positively cooperative (h > 2) (Figure 3 and Table 1).

These results demonstrate that adenophostin A and Ins(1,4,5)P_3 interact with the same receptors to evoke Ca^{2+} release, but that adenophostin A binds with ∼10-fold greater affinity. These results with hepatocytes are comparable to those obtained previously from cells expressing largely type 1 Ins(1,4,5)P_3 receptors (*21*, *23*, *27*).

Effects of Phosphorylated Carbohydrate Analogs on Ins(1,4,5)P_3 Receptors. Figure 4 illustrates the concentration-dependent effects of five phosphorylated carbohydrate analogs on the $^{45}Ca^{2+}$ content of the intracellular Ca^{2+} stores in permeabilized rat hepatocytes. The results are summarized

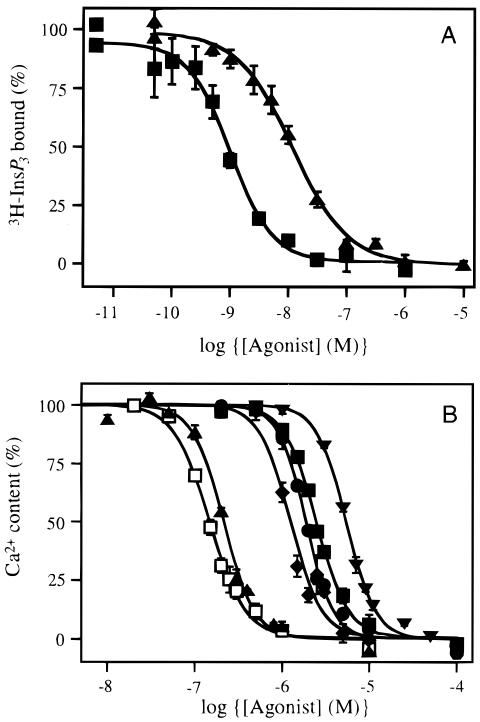


FIGURE 4: Effects of synthetic phosphorylated carbohydrates on [^{3}H]Ins(1,4,5)P_3 binding and Ca^{2+} mobilization. (A) Specific [^{3}H]-Ins(1,4,5)P_3 binding to rat hepatic membranes is shown in the presence of the indicated concentrations of adenophostin A (■) or Rib(2,3′,4′)P_4 (▲) (means ± sem of three independent experiments). The equivalent radioligand binding results for the other analogs, which are not shown graphically because too many of the curves overlapped, are summarized in Table 1. (B) Experiments similar to those shown in Figure 3B were used to examine the effects of Ins(1,4,5)P_3 (□) and the following synthetic phosphorylated carbohydrates on the Ca^{2+} content of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores of permeabilized hepatocytes: Rib(2,3′,4′)P_4 (▲); Trehal-(2,4,3′,4′)P_4 (◆); Glc(2′,3,4)P_3 (●); Trehal(3,4,3′,4′)P_4 (■); Sucr-(3,4,3′)P_3 (▼). Results [% Ins(1,4,5)P_3-sensitive Ca^{2+} stores] are expressed as mean ± sem of three or more independent experiments.

in Table 1. Each analog released the same fraction of the intracellular Ca^{2+} stores as was released by 10 μM Ins(1,4,5)-P_3 (50 ± 3%) (Table 1), and addition of Ins(1,4,5)P_3 (10μM) to cells that had previously been stimulated (30s) with a maximally effective concentration of each analog failed to evoke further Ca^{2+} release (not shown). These results demonstrate that Ins(1,4,5)P_3 and each of the analogs release the same intracellular Ca^{2+} stores.

In equilibrium competition binding studies with hepatic membranes, each of the phosphorylated carbohydrate analogs completely displaced specific [^{3}H]Ins(1,4,5)P_3 binding (Table 1). The rank order of potency of the analogs [adenophostin A > Ins(1,4,5)P_3 ≈ Rib(2,3′,4′)P_3 > Trehal(2,4,3′,4′)P_4 > Glc(2′,3,4)P_3 ≈ Trehal(3,4,3′,4′)P_4 > Sucr(3,4,3′)P_3] was the same in radioligand and functional assays (Table 1). Both Rib(2,3′,4′)P_3 and Trehal(2,4,3′,4′)P_4 bound with significantly greater affinity (∼27- and ∼3-fold, respectively) than the only active carbohydrate agonist of Ins(1,4,5)P_3 receptors previously examined [Glc(2′,3,4)P_3]. Indeed, the most potent of the carbohydrate analogs, Rib(2,3′,4′)P_3, was as active as the endogenous agonist, Ins(1,4,5)P_3.

DISCUSSION

All the synthetic analogs described in this paper contain the same phosphorylated glucose component, identical to that

found in the adenophostins. The observed differences in activity must, therefore, be wholly attributable to the influence of the second component, which bears the accesssory phosphate group(s). Glc(2′,3,4)P_3, the first carbohydrate mimic of Ins(1,4,5)P_3, was designed to place the accessory phosphate group two carbons away from an α-glycosidic oxygen, as in the adenophostins. However, it soon became clear that this was not sufficient even for Ins(1,4,5)P_3-like activity and that the precise orientation and position of the phosphate needed to be controlled in some way. It is likely that the bimethylene chain of Glc(2′,3,4)P_3 tends to adopt extended conformations, placing the phosphate group too distant from the ring, and this has been demonstrated by a molecular dynamics simulation (*22*). The observation that increasing the chain length and/or inversion of anomeric configuration in related xylopyranoside trisphosphates further reduces activity (*47*) supports this hypothesis.

Trehal(3,4,3′,4′)P_4 is unique in that either half of the molecule can mimic the 4,5-bis(phosphate)/6-hydroxyl of Ins(1,4,5)P_3. It undoubtedly gives greater rigidity than Glcl-(2′,3,4)P_3, although its potency is similar. It is likely that one glucose bis(phosphate) residue of Trehal(3,4,3′,4′)P_4 interacts with the receptor binding site in the same way as does this component in the other analogs and that neither phosphate group on the second glucose residue is placed close enough to the active bis(phosphate) to give increased activity over Glc(2′,3,4)P_3. Trehal(2,4,3′,4′)P_4, is an asymmetrical regioisomer of Trehal(3,4,3′,4′)P_4 in which a single phosphate group is relocated to a position two carbons removed from the glycosidic oxygen. This slight modification leads to a significant gain in activity, suggesting that the 2-phosphate group is better-placed than either the 3 or 4 phosphates. It is also possible that the alteration in the substitution pattern on one glucose residue may influence the conformation about the glycosidic linkage. The fact that Trehal(2,4,3′,4′)P_4 is more potent than Glcl(2′,3,4)P_3, despite its increased size, validates the general approach of increasing rigidity.

The finding that Rib(2,3′,4′)P_3 still does not approach the potency of the adenophostins suggests a role for the adenine, either by engaging in its own stabilizing interactions with a nearby area of the Ins(1,4,5)P_3 receptor or by further optimizing the positioning of the 2′-phosphate at the binding site. However, Rib(2,3′,4′)P_3 is equipotent to Ins(1,4,5)P_3, and much more potent than the other disaccharides. This supports the idea that accurate positioning of the accessory phosphate is important in achieving Ins(1,4,5)P_3-like activity. The weak activity of Sucr(3,4,3′)P_3 compared to all the other analogues is surprising, considering its apparent structural resemblance to Rib(2,3′,4′)P_3 (Figure 2). It could be that steric hindrance from one or both hydroxymethyl groups on the fructofuranoside in Sucr(3,4,3′)P_3 interferes with binding or that overlapping anomeric effects (*48*), not present in Rib-(2,3′,4′)P_3, influence the conformation about the glycosidic linkage. Finally, the presence of the quaternary furanosyl anomeric center in Sucr(3,4,3′)P_3 may lead to increased flexibility about the fructofuranosyl linkage (*49*).

It remains to be established whether adenophostin-like activity can be achieved without the adenine, simply by constraining the phosphate group in the (presently unknown) position that it adopts at the receptor binding site. The possibility must be considered that, although Rib(2,3′,4′)P_3 can attain the necessary conformation, it is still too flexible. Alongside a study of adenine-containing analogs, therefore, it will also be important to investigate conformationally restricted analogs lacking the adenine. Molecular modeling studies may aid the development of these, although simulating the combined influence of highly charged phosphate groups, anomeric effects in disaccharides and the environment at the receptor binding site presents many difficulties. In this respect, detailed NMR studies should be useful.

In summary, we have demonstrated that adenophostin A is a potent agonist of the type 2 Ins(1,4,5)P_3 receptor expressed in hepatocytes and have synthesized four disaccharide polyphosphates, all of which bind to and activate hepatic Ins(1,4,5)P_3 receptors. Rib(2,3′,4′)P_3, whose structure is closely related to adenophostin A, is as potent as Ins-(1,4,5)P_3 and more potent than most conventional inositol-based agonists. We conclude that phosphorylated disaccharides provide novel opportunities to develop high affinity ligands of Ins(1,4,5)P_3 receptors.

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