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D-*myo*-Inositol 1,4,5-Trisphosphate Analogues as Useful Tools in Biochemical Studies of Intracellular Calcium Mobilization†

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Abstract—Two types of structural variants of D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] were prepared by a chemoenzymatic route. These 6-*O*-substituted analogues retained the biological activity of Ins(1,4,5)P_3, and were able to elicit Ca^{2+} release from porcine brain microsomes. Moreover, these derivatives allowed the preparation of Ins(1,4,5)P_3-based immunogens and affinity matrix which were successfully applied to the preparation and purification of antibodies against Ins(1,4,5)P_3. These antibodies displayed discriminative affinity towards Ins(1,4,5)P_3, and provide a useful tool to study intracellular Ca^{2+} mobilization.

Introduction

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is generated from the phospholipase C-mediated breakdown of membrane phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] in response to stimulation by hormones, neurotransmitters, and growth factors.[1] Ins(1,4,5)P_3, in turn, elicits Ca^{2+} mobilization and, subsequently, an array of intracellular events by interacting with specific receptors on intracellular organelles.[2] While many researchers assert that the biologically relevant receptors for Ins(1,4,5)P_3 distribute mainly on the membrane of the endoplasmic reticulum or nucleus, others suggest the locality on a more specialized endomembrane fraction, i.e., calciosomes.[3] In the second phase of the signaling process, i.e., Ca^{2+} entry from the extracellular medium, Ins(1,4,5)P_3 has also been implicated. The capacitative entry theory suggests that depletion of the intracellular Ca^{2+} store by Ins(1,4,5)P_3 generates a secondary signal of unknown nature that activates Ca^{2+} entry.[4–6] Thus, the mechanism of interaction between the Ins(1,4,5)P_3-sensitive Ca^{2+} pool and the Ca^{2+} channel on plasma membrane remains an important issue to be resolved.

To gain insight into this Ins(1,4,5)P_3-mediated Ca^{2+} homeostasis, one of our objectives is to develop versatile Ins(1,4,5)P_3 analogues for various biochemical applications. Although a number of Ins(1,4,5)P_3 analogues have been reported, the reactive appendages of these derivatives were attached to the parent molecule through a C-1 phosphodiester linkage.[7,8] We here report the synthesis of two novel Ins(1,4,5)P_3 analogues, **1** and **2**, with modification at the C-6 position.

†This paper is dedicated to Professor Charles J. Sih on the occasion of his 60th birthday.

The derivatized Ins(1,4,5)P_3 contains an amine or aldehyde function for further elaborations, and allows the preparation of Ins(1,4,5)P_3-based analogues for antibody induction, affinity purification, and histochemical probing.

Results and Discussion

Chemistry

In light of the delicate structure of Ins(1,4,5)P_3, the latitude in the choice of site for modification is limited. The design of these two optically active Ins(1,4,5)P_3 analogues, **1** and **2**, was based on (a) the strategic importance of the C-2,3 *cis*-dihydroxy moiety, especially the axial 2-OH, in recognizing Ins(1,4,5)P_3 versus other inositol phosphates, and (b) the intactness of the three phosphate functions in derivatized Ins(1,4,5)P_3 to achieve optimal ionic interactions with the binding proteins.

Both **1** and **2** were synthesized from (+)-2,3:4,5-di-*O*-cyclohexylidene-D-*myo*-inositol, (+)-**3**, through similar routes (Figures 1A and B). This chiral precursor was prepared by enantioselective hydrolysis of its racemic 6-*O*-butyryl ester by porcine pancreatic lipase.[9] The stannylidene-activated alkylation allowed the regioselective introduction of the substituents at the 6-*O* position to afford (+)-**4** and (+)-**9** in good yields. To prevent acid-catalyzed migration of the *cis*-cyclohexylidene group under acidic conditions, **4** and **9** were acetylated to give (–)-**5** and (+)-**10**, respectively, methanolysis of which removed the *trans*-cyclohexylidene ring and saponification of the products furnished (+)-**7** and (+)-**11**, respectively. Phosphorylation of the triol **7** by the phosphoramidite method,[10] followed by debenzylation and acid hydrolysis

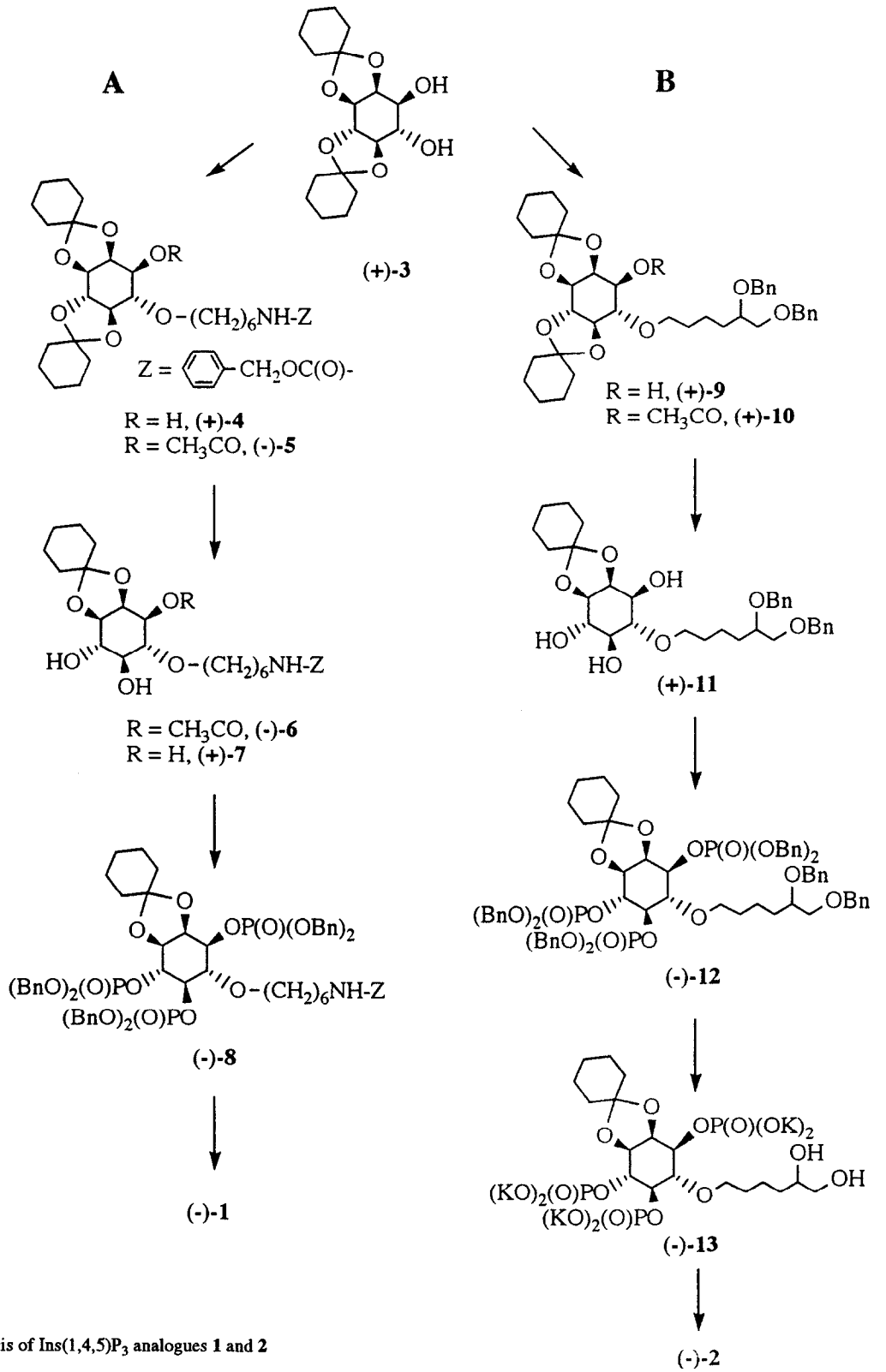


Figure 1. Synthesis of Ins(1,4,5)P_3 analogues **1** and **2**

of the *cis*-ketal, gave the trisphosphate (–)-**1**. Similar treatments of **11** but without the acid hydrolysis led to (–)-**13**. The overall yields for the synthesis of **1** and **13** from (+)-**3** were 37% and 45%, respectively. These two compounds were fully characterized by ^1H and ^{31}P NMR and mass spectrometry. The aldehyde-bearing derivative (–)-**2** was readily afforded by subjecting the vicinal diol **13** to periodate oxidation and a subsequent acid treatment. With the functionalities of amine and aldehyde in the side arms, analogues **1** and **2** allowed the coupling of Ins(1,4,5)P_3 to virtually any type of molecule. Moreover, the three phosphate functions of the resulting conjugates are fully exposed, resembling the charge state of the parent compound.

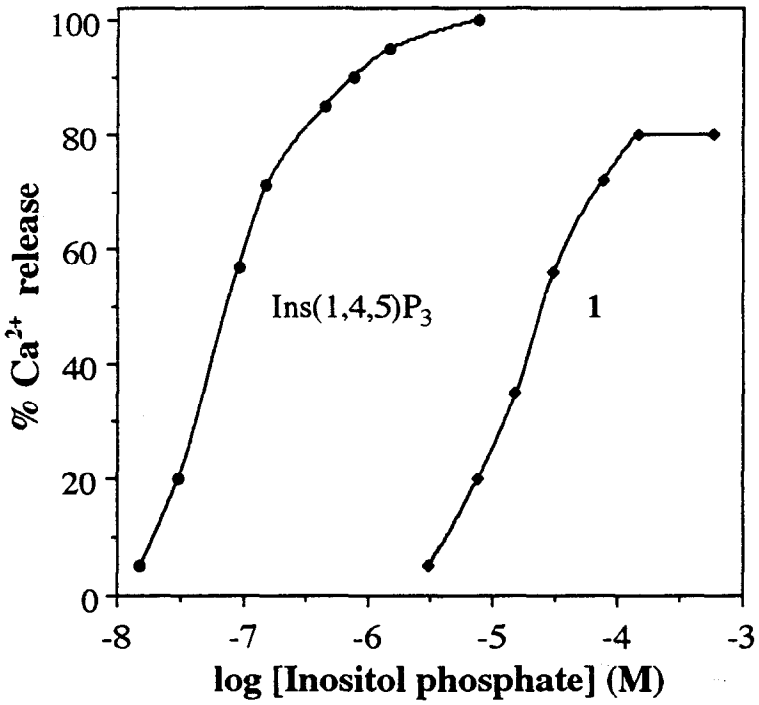


Figure 2. Inositol phosphate-induced Ca^{2+} release from porcine brain microsomes. Ca^{2+} releasing activity of Ins(1,4,5)P_3 versus analogue **1**. Ca^{2+}-loaded porcine brain microsomes were treated with Ca^{2+}-mobilizing agents, and released Ca^{2+} was monitored by a Ca^{2+}-sensitive fluorescent dye, Fura-2, according to the method described in the Experimental Section (100% = Ca^{2+} release at saturated concentrations of Ins(1,4,5)P_3). Each data point represents the means of three determinations

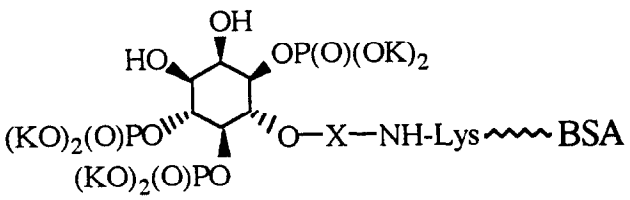
It is worthy to note that the derivatized Ins(1,4,5)P_3 retained the biological activity of Ins(1,4,5)P_3. For instance, **1** was able to mobilize 80% of Ins(1,4,5)P_3-sensitive Ca^{2+} store when porcine brain microsomes were saturated (Figure 2).

The EC_{50} values were 79 nM and 18 μM for Ins(1,4,5)P_3 and **1**, respectively. Although the Ca^{2+}-mobilizing activity of the analogue was about 200-fold less potent than that of the parent compound, it could interact functionally with the Ins(1,4,5)P_3-specific receptor(s) in the microsomes. The decrease in affinity was presumably due to the hydrophobic side arm which imposed steric and/or stereoelectronic effect on binding. It has recently been reported that the 6-OH may have an important role in the interactions with receptors and metabolic enzymes.[11,12] The finding that these 6-*O* substituted analogues were active in Ca^{2+} mobilization broadened the utility of these molecules, which is illustrated in the following two examples.

Induction of antibodies

An eminent application of these analogues was to prepare immunogens for raising Ins(1,4,5)P_3-specific antibodies. These anti-Ins(1,4,5)P_3 antibodies have important use in quantitative analysis of Ins(1,4,5)P_3 and as a biochemical probe to assess the role of Ins(1,4,5)P_3 in Ca^{2+} mobilization.

Both **1** and **2** could be coupled to carrier proteins by conventional methods. The amine-bearing derivative **1** was cross-linked to bovine serum albumin (BSA) using glutaraldehyde as a coupling agent, followed by *in situ* $NaBH_4$ reduction, to afford IP_3–C_6NC_5–BSA. The aldehyde **2** formed a Schiff base with the amino functions of BSA, which yielded the conjugate IP_3–C_5–BSA upon $NaBH_4$ reduction. The molar ratios of the bound Ins(1,4,5)P_3 to BSA were estimated to be 22 and 5 for IP_3–C_6NC_5–BSA and IP_3–C_5–BSA, respectively, according to the phosphorus contents. The distance between the coupled Ins(1,4,5)P_3 and the carrier protein was 19.7 Å and 9 Å, respectively.



X = $C_6H_{12}N(H)C_5H_{10}$, IP3-C6NC5-BSA;
C_5H_{10}, IP_3-C_5-BSA

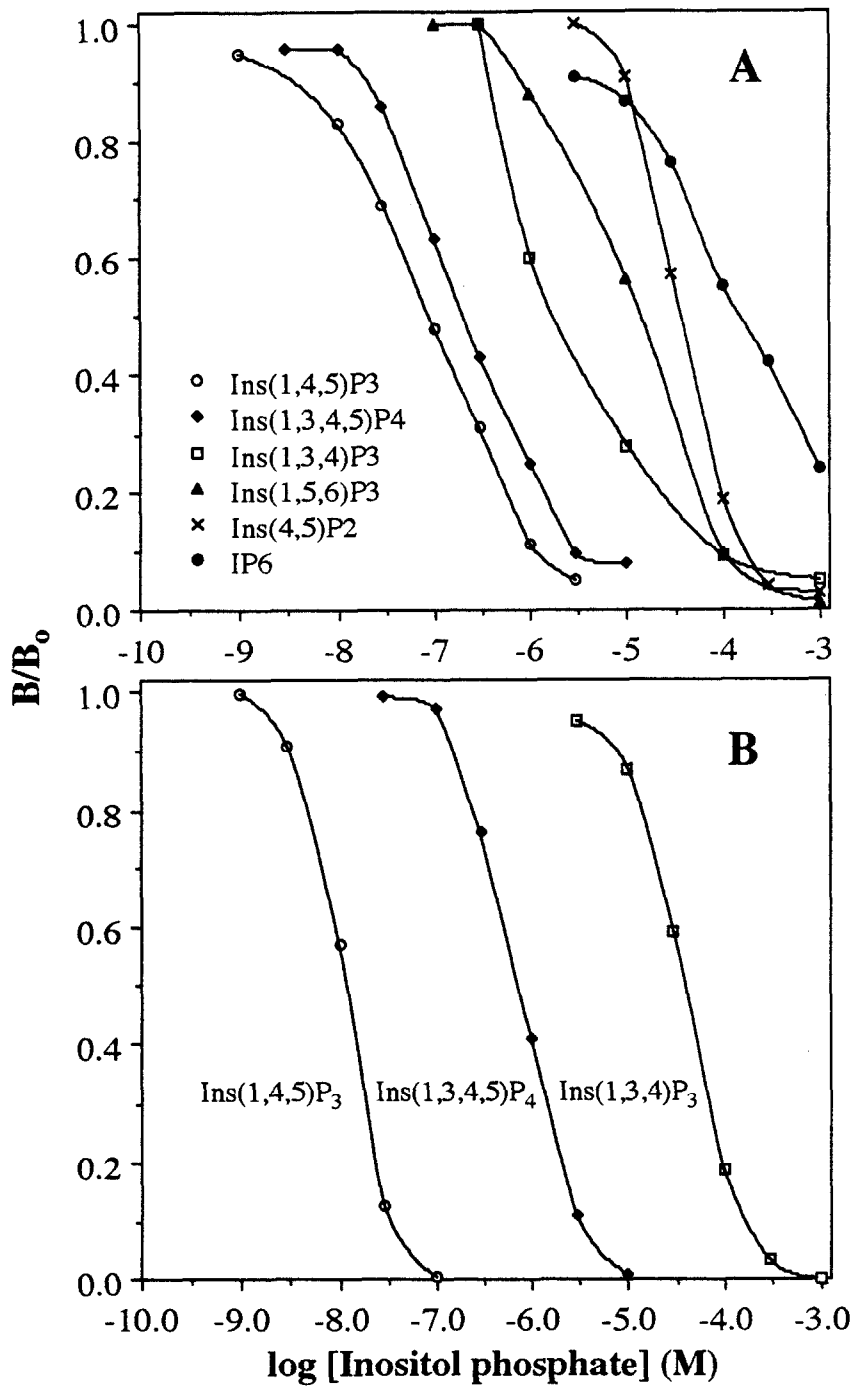


Figure 3. Inositol phosphate specificity of (A) crude rabbit serum and (B) affinity-purified antibodies. The displacement curves were generated from competitive ELISA experiments between immobilized Ins(1,4,5)P_3 and various inositol phosphates shown in the insert. Percentage total specific binding is expressed by B/B_0, where B = absorbance with competitor and B_0 = absorbance without competitor. Each data point represents the means of three determinations

Both Ins(1,4,5)P_3–BSA conjugates were used to induce antibodies in New Zealand rabbits. After 2–3 booster injections, rabbits receiving either antigen were found to produce antibodies with titers of 1:4,000. The avidity and specificity of these antisera appeared to be independent of the spacer length and IP_3 content of the antigens. The affinity toward different inositol phosphates was in the order of: Ins(1,4,5)P_3 > Ins(1,3,4,5)P_4 > Ins(1,3,4)P_3 > Ins(1,5,6)P_3 > Ins(4,5)P_2 > IP_6 (Figure 3A). The degree of discrimination between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 by these antisera appeared to be lower than that reported for Ins(1,4,5)P_3 receptors,[13] which may be accounted for by the heterogeneity in the antigen binding sites. To remove the less specific antibodies, the antiserum was subjected to affinity chromatography on Ins(1,4,5)P_3–agarose.

Affinity purification

The affinity matrix was prepared by reacting **1** with 1,1'-carbonyldiimidazole-activated agarose (Reacti-Gel®; Pierce). The Ins(1,4,5)P_3-specific antibodies displayed strong binding with the affinity matrix, and could only be eluted under alkaline conditions (100 mM $NaHCO_3$/-Na_2CO_3 buffer, pH 10.5). These affinity-purified antibodies showed much improved selectivity between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Figure 3B) with IC_{50} of 12 nM and 730 nM, respectively (Figure 3B). The differential affinity, as indicated by the ratios of IC_{50} values, increased from 2- to 60-fold. The degree of specificity for these affinity purified antibodies was in line with that reported for Ins(1,4,5)P_3-specific receptors.[13] Moreover, the span of inositol phosphate concentrations for complete displacement decreased from 3 log-units to 1 unit, indicating improved specificity of the purified antibodies. Application of this affinity matrix to the purification of the biologically relevant receptors is currently under investigation.

Experimental Section

General methods

^1H, ^{13}C, and ^{31}P NMR spectra were recorded with a Bruker AM-300 spectrometer. Optical rotations were determined at 23 °C with a Rudolph Autopol III polarimeter. Fast atom bombardment mass spectra (FAB-MS) were obtained from the Chemical Instrument Center, Yale University. Elemental analysis was performed by M-H-W laboratories (Phoenix, AZ). Fluorescence spectrophotometric assay of Ca^{2+} release was carried out with a Hitachi F-2000 spectrometer.

Materials

(1,4,5)IP_3, (1,3,4)IP_3, and (1,3,4,5)IP_4 were synthesized from optically active 1,2:5,6-di-*O*-cyclohexylidene-inositol **1** (optical purity > 98% enantiomeric excess) according to previously described procedures.[9,14,15] (4,5)IP_2 and (1,5,6)IP_3 were prepared by following a similar approach with pertinent modifications. The chemical purity of these chiral inositol phosphates was greater than 95% according to ^1H and ^{31}P NMR spectroscopy. The amount of isomeric impurities was negligible as indicated by these NMR spectra. Phytic acid was purchased from Sigma.

*(+)-6-*O*-(ω-Benzyloxycarbonylaminohexyl)-2,3:4,5-di-*O*-cyclohexylidene*-myo-*inositol* **4**

A mixture of (+)-2,3:4,5-di-*O*-cyclohexylidene-D-*myo*-inositol **3** (1 g, 2.9 mmol), Bu_2SnO (840 mg, 3 mmol), and toluene (25 ml) was boiled under reflux with azeotrophic removal of water for 1 h, then concentrated to dryness under reduced pressure. To the residue were added *N,N*-dimethylformamide (10 ml) and ω-benzyloxycarbonylaminohexyl bromide (2 g, 8.5 mmol). The mixture was stirred at 23 °C overnight, then diluted with CH_2Cl_2 (50 ml), washed with saturated aq. NaCl, dried with Na_2SO_4, and concentrated. Column chromatography of the residue on a silica gel column (hexane–ether, 15:1) gave **4** (1.1 g, 65%). $[\alpha]_D$ +4 9° (*c* 1.7, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.25–1.71 (m, 28H), 2.66 (d, 1H, *J* = 1 Hz), 3.14–3.21 (m, 2H), 3.40–3.48 (m, 1H), 3.50–3.59 (m, 1H), 3.63–3.73 (m, 2H), 3.93–3.94 (m, 1H), 4.12–4.18 (m, 1H), 4.32 (t, 1H, *J* = 6 Hz), 4.37–4.41 (m, 1H), 4.86 (m, 1H), 5.08 (s, 2H), 7.28–7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 15.16, 23.37, 23.65, 23.73, 25.03, 25.09, 25.69, 26.42, 29.59, 29.87, 33.52, 36.35, 36.57, 36.67, 41.07, 53.28, 65.69, 66.53, 69.78, 72.35, 75.34, 76.74, 77.19, 78.19, 78.84, 80.40, 111.17, 112.71, 127.96, 128.42, 136.79, 156.39.

*(–)-1-*O*-Acetyl-6-*O*-(ω-benzyloxycarbonylaminohexyl)-2,3-*O*-cyclohexylidene*-myo-*inositol* **6**

A solution of **4** (1 g, 1.7 mmol) in CH_2Cl_2 (10 ml) was treated with acetic anhydride (0.4 ml, 3.5 mmol), and 4-dimethylaminopyridine (208 mg, 1.7 mmol) at 23 °C for 30 min. The mixture was washed with aq. $NaHCO_3$ and water, dried, and concentrated to afford (–)-1-*O*-acetyl-6-*O*-(ω-benzyloxycarbonylaminohexyl)-2,3:4,5-di-*O*-cyclohexylidene-*myo*-inositol **5** (syrup) in quantitative yield. ^{13}C NMR ($CDCl_3$) δ 13.97, 15.18, 21.10, 22.57, 23.49, 23.70, 23.87, 25.03, 25.09, 25.65, 26.42, 29.54, 29.89, 31.52, 34.27, 36.47, 36.63, 41.12, 64.10, 65.72, 66.54, 70.01, 72.81, 73.41, 77.19, 78.38, 79.75, 111.62, 113.02, 127.99, 128.45, 136.86, 156.41, 169.43. A solution of the oily compound in CH_2Cl_2–CH_3OH (1:1, 10 ml) was stirred with acetyl chloride (0.1 ml, 1.2 mmol) at 23 °C for 10 min. Triethylamine (0.3 ml) was added, and the solution was concentrated. Column chromatography (hexane–ether, 20:1) of the residue yielded (–)-**6** (syrup, 0.67 g, 75%). $[\alpha]_D$ –14.8° (*c* 1.1, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.30–1.72 (m, 18H), 2.14 (s, 3H), 3.15–3.21 (m, 2H), 3.33–3.39 (m, 1H), 3.44–3.51 (m, 2H), 3.56 (t, 1H, *J* = 9 Hz), 3.64–3.78 (m, 3H), 4.01–4.05 (m, 1H), 4.39–4.42 (m, 1H), 4.85 (m, 1H), 5.03–5.09 (m, 3H), 7.30–7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.97, 15.16, 21.02, 23.56, 23.97, 25.03, 25.65, 26.35, 29.85, 29.97, 34.99, 37.58, 66.67, 71.64, 72.48, 73.17, 73.70, 74.92, 77.19, 78.30, 79.49, 110.97, 113.02, 127.99, 128.04, 128.50, 156.52, 170.02.

*(+)-6-*O*-(ω-Benzyloxycarbonylaminohexyl)-2,3-*O*-cyclohexylidene*-myo-*inositol* **7**

The acetate **6** (0.9 g, 1.7 mmol) was treated with methanolic 1 M NaOH for 1 h at 23 °C, and the solvent was evaporated under reduced pressure. Column chromatography (hexane–ether, 5:1) of the residue afforded (+)-**7** (0.77 g, 93%). $[\alpha]_D$ +11.6° (*c* 2.4, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.34–1.72 (m, 18H), 2.84 (br s, 1H), 3.13–3.20 (m, 2H), 3.30–3.35 (m, 1H), 3.44–3.51 (m, 2H), 3.69–3.85 (m, 5H), 3.99–4.03 (m, 1H), 4.34–4.37 (m, 1H), 5.01 (br s, 1H), 5.08 (br s, 2H), 7.27–7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 23.61, 24.03, 25.08, 25.66, 26.38, 29.88, 30.00, 34.75, 37.52, 41.07, 66.71, 70.47, 72.16, 74.04, 75.08, 75.26, 77.10, 78.29, 81.90, 110.73, 128.09 (2C), 128.54 (2C), 136.83, 156.64.

(–)-6-O-(ω-Benzyloxycarbonylaminohexyl)-2,3-O-cyclohexylidene-myo-*inositol 1,4,5-tris-(dibenzyl phosphate) 8*

A mixture of 1*H*-tetrazole (2.5 g, 35 mmol), dibenzyl *N,N*-di-isopropyl-phosphoramidite (6 g, 7 mmol), and CH_2Cl_2 (40 ml) was stirred under Ar at 23 °C for 1 h, and (+)-**7** (0.75 g, 1.5 mmol) was added in one portion. The solution was kept under the same conditions for another 12 h, cooled to –15 °C, and then treated with *m*-chloroperoxybenzoic acid (50% purity, 7 g, 17 mmol). The mixture was stirred at –15 °C for 30 min, then allowed to attain room temperature, diluted with CH_2Cl_2 (80 ml), washed with aq. Na_2SO_3, aq. $NaHCO_3$, and water, dried, and concentrated. Column chromatography (hexane–ether, 15:1) of the residue furnished (–)-**8** (syrup, 1.6 g, 83%). $[\alpha]_D$ –3.4° (*c* 3, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.11–1.74 (m, 18H), 3.08 (q, 2H, *J* = 6.5 and 12.9 Hz), 3.43–3.48 (m, 1H), 3.54–3.60 (m, 1H), 3.97 (q, 1H, *J* = 5.4 and 7.8 Hz), 4.24 (t, 1H, *J* = 6.6 Hz), 4.48–4.58 (m, 2H), 4.67–4.74 (m, 1H), 4.87–5.10 (m, 16H), 7.20–7.32 (m, 35H).

(–)-6-O-(ω-Aminohexyl)-D-myo-*inositol 1,4,5-trisphosphate 1*

A solution of (–)-**8** (1.5 g, 1.2 mmol) in aq. 80% ethanol was shaken under H_2 (50 psi) in the presence of 10% Pd/C (0.5 g) for 15 h, then filtered, and concentrated to dryness. To the residue was added 2 ml of glacial acetic acid, and the resulting solution was stirred at 23 °C for 2 h, and concentrated. The residue was triturated with CH_2Cl_2–ether (1:1), the resulting white precipitate was dissolved in the minimum amount of water, and 1 M KOH (6 equiv.) was added. The solution was lyophilized to afford **1** as the hexapotassium, acetate salt (0.92 g, 99%) $[\alpha]_D$ –14° (*c* 1.1, H_2O). ^1H NMR (D_2O) δ 1.10–1.23 (m, 4H), 1.23–1.38 (m, 2H), 1.40–1.53 (m, 2H), 2.48 (br s, 2H), 3.43–3.72 (m, 4H), 3.91–4.14 (m, 4H); ^{31}P NMR (D_2O, external H_3PO_4, broadband decoupled): 2.28, 2.64, and 3.18. FAB-MS m/q 790 [$C_{12}H_{22}NO_{15}P_3K_6$ (M) - H_2O], 752 [M - K + 1H], 714 [M - 2K + 2H], 672 [M - 2K - HOAc + 2H], 591 [M - P(O)(OK)$_2$].

(+)-6-O-(5',6'-Dibenzyloxyhexyl)-2:3,4:5-di-O-cyclohexylidene-myo-*inositol 9*

A mixture of **3** (1 g, 2.9 mmol), Bu_2SnO (840 mg, 3 mmol), and toluene (30 ml) was boiled under reflux for 1 h, then concentrated to dryness under reduced pressure. To the residue were added *N,N*-dimethylformamide (10 ml), CsF (1.3 g, 8.6 mmol), and 5,6-dibenzyloxyhexyl bromide (2.5 g, 6.7 mmol). The mixture was stirred at 23 °C overnight, then diluted with ethyl acetate (50 ml), washed with saturated aq. NaCl, dried (Na_2SO_4), and concentrated. Column chromatography (hexane–ether, 15:1→5:1) of the residue gave **9** (syrup, 1.5 g, 80%). $[\alpha]_D$ +3.8° (*c* 3, $CHCl_3$). ^1H NMR ($CDCl_3$) 1.38–1.72 (m, 26H), 2.59–2.63 (m, 1H), 3.40–3.73 (m, 7H), 3.90–3.93 (m, 1H), 4.12–4.19 (m, 1H), 4.29–4.38 (m, 2H), 4.53–4.71 (m, 4H), 7.26–7.35 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 22.08, 23.49, 23.77, 23.85, 23.94, 25.15, 25.21, 29.90, 31.88, 33.63, 36.47, 36.70, 36.79, 69.89, 72.05, 72.06, 72.46, 72.47, 73.09, 73.11, 73.48, 75.45, 76.87, 77.26, 78.27, 78.98, 79.00, 80.47, 111.28, 112.82, 127.46, 127.59, 127.64, 127.79, 128.31, 128.40, 138.61, 139.17.

(+)-6-O-(5',6'-Dibenzyloxyhexyl)-2:3-O-cycloxhexylidene-myo-*inositol 11*

A solution of **9** (1.5 g, 2.3 mmol) in CH_2Cl_2 (15 ml) was treated with acetic anhydride (1.2 ml, 12.7 mmol) and 4-dimethylaminopyridine (1.6 g, 13 mmol) at 23 °C for 1 h. The mixture was washed with aq. $NaHCO_3$ and water, dried, and concentrated to afford 1-*O*-acetyl-6-*O*-(5',6'-dibenzoxyhexyl)-2:3-cyclohexylidene-*myo*-inositol (**10**) in quantitative yield. Without purification, the oily compound was dissolved in a solution of CH_2Cl_2–CH_3OH (1:1, 10 ml), and treated with acetyl chloride (0.1 ml, 1.4 mmol) for 10 min. Triethylamine (0.5 ml) was added, and the solution was concentrated. To the crude residue was added 1.2 equiv. of methanolic 1 M NaOH. The solution was stirred at 23 °C for 1 h, and solvent was removed under reduced pressure. Column chromatography (hexane–ether, 10:1) of the residue afforded **11** (syrup, 0.91 g, 70%). $[\alpha]_D$ +9.9° (*c* 1.1, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.30–1.74 (m, 16H), 2.80 (dd, 1H, *J* = 4.8 and 60 Hz), 3.12–3.32 (m, 3H), 3.38–3.44 (m, 1H), 3.49–3.59 (m, 4H), 3.68–3.74 (m, 4H), 3.90–3.98 (m, 1H), 4.19–4.34 (m, 1H), 4.50–4.55 (m, 2H), 4.70 (dd, 1H, *J* = 4.8 and 10 Hz), 7.26–7.34 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 21.66, 21.71, 23.58, 23.99, 25.05, 29.92, 31.49, 34.81, 34.84, 37.56, 70.49, 71.94, 71.95, 72.17, 72.24, 72.91, 72.96, 73.48, 73.79, 75.19, 75.29, 78.20, 78.29, 81.49, 81.67, 127.60, 127.64, 127.69, 127.97, 128.04, 128.36, 128.41.

(–)-6-O-(5',6'-Dibenzyloxyhexyl)-2:3-O-cyclohexylidene-myo-*inositol 1,4,5-tris-(dibenzyl phosphate) 12*

A mixture of 1*H*-tetrazole (2.75 g, 39 mmol), dibenzyl *N,N*-di-isopropylphosphoramidite (7.49 g, 8.7 mmol), and CH_2Cl_2 (30 ml) was stirred under Ar at 23 °C for 1 h, and (+)-**11** (0.89 g, 1.6 mmol) was added in one portion. The solution was kept under the same condition for another 12 h, cooled to –40 °C, and then treated with *m*-chloroperoxybenzoic acid (50% purity, 9 g, 25 mmol). The mixture was stirred at –40 °C for 10 min, then allowed to attain room temperature, diluted with CH_2Cl_2 (80 ml), washed with aq. Na_2SO_3, aq. $NaHCO_3$, and water, dried, and concentrated. Column chromatography (hexane–ether, 15:1) of the residue furnished (–)-**12** (syrup, 2 g, 93%). $[\alpha]_D$ –3° (*c* 1.1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 1.25–1.91 (m, 16H), 3.38–3.60 (m, 5H), 3.98 (dd, 1H, *J* = 4.8 and 6.0 Hz), 4.25 (t, 1H, *J* = 6.6 Hz), 4.47–4.70 (m, 6H), 4.92–5.10 (m, 14H), 7.21–7.34 (m, 40H).

6-O-(5',6'-Dihydroxyhexyl)-2:3-O-cyclohexylidene-myo-*inositol 1,4,5-trisphosphate 13*

A solution of **12** (0.2 g, 0.15 mmol), and 1 M KOH (0.9 ml, 0.9 mmol) in aq. 80% ethanol was shaken under H_2 (50 psi) in the presence of 10% Pd/C (1 g) for 24 h, then filtered, and concentrated. The residue was dissolved in water (10 ml), and lyophilized to afford **13** as the

hexapotassium salt (0.13 g, 99%). ^{1}H NMR data (D_2O): δ 1.15–1.76 (m, 16H), 3.25–3.38 (m, 1H), 3.40–3.51 (m, 1H), 3.52–3.80 (m, 3H), 4.06–4.18 (m, 1H), 4.23–4.35 (m, 2H), 4.42–4.55 (m, 2H), 4.65–4.80 (m, 1H). ^{31}P (D_2O, external H_3PO_4, broadband decoupled): 3.44, 4.11, and 4.75. FAB-MS m/q 845 [$C_{18}H_{29}O_{17}P_3K_6$ (M)], 807 [M - K + 1H], 769 [M - 2K + 2H].

Preparation of crude porcine brain microsomes

The brain from an adult pig was homogenized (Polytron, setting 7 and 5 strokes) in ice-cold buffer A, consisting of 20 mM-Hepes/KOH, 110 mM-KCl, 10 mM-NaCl, 2 mM-$MgCl_2$, 5 mM-KH_2PO_4, 2 mM-EGTA, 1 mM-dithiothreitol (DTT), and 0.5 mM-*p*-toluenesulfonyl fluoride (PMSF). The homogenate was centrifuged at 500 *g* for 15 min at 4 °C, and the supernatant was pelleted by centrifugation at 35,000 *g* for 25 min. The pellet was resuspended in buffer B (as buffer A, but without EGTA and PMSF). The washing procedure was repeated twice. The resulting final pellet was suspended in buffer B to a protein concentration of 2.1 mg/ml .

Ca^{2+} release assay

Measurements of free Ca^{2+} concentrations in incubation media were performed by using a Ca^{2+}-sensitive fluorescent dye, Fura-2, in a Hitachi F-2000 spectrofluorimeter. The assay medium consisted of 40 units of creatine kinase, 20 mM-creatine phosphate, 5 μg of oligomycin, and 0.5 μM-Fura-2 free acid in 2 ml of buffer B containing 0.4 mg of microsomal protein. The mixture was incubated for 5 min, and treated with 1 mM-ATP to allow the loading of Ca^{2+} stores. Until the external Ca^{2+} concentration returned to a near-base level, the microsomes were stimulated with the Ca^{2+}-mobilizing agents. Experiments were carried out at 37 °C. Excitation and emission wavelengths were 340 and 510 nm, respectively. The Fura-2 fluorescence ratio signal was calibrated as described by Grynkiewicz *et al.*[16]

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References

1. Berridge, M. J. *Nature* **1993**, *361*, 315; and references cited therein.

2. Meldolesi, J.; Villa, A.; Volpe, P.; Pozzan, T. In *Advances in Second Messenger and Phosphoprotein Research*, Vol. 26, pp. 187–208, Putney, Jr, J. W., Ed.; Raven Press; New York, 1992.

3. Volpe, P.; Kraus, K. H.; Sadamitsu, H.; Zorzato, F.; Pozzan, T.; Meldolesi, J.; Low, P. B. *Proc. Natl Acad. Sci. U.S.A.* **1988**, *85*, 1091.

4. Putney, Jr, J. W. *Cell Calcium* **1986**, *7*, 1.

5. Takemura, H.; Putney, Jr, J. W. *Biochem. J.* **1989**, *258*, 409.

6. Putney, Jr, J. W. In *Advances in Second Messenger and Phosphoprotein Research*, Vol. 26, pp. 143–160, Putney, Jr, J. W., Ed.; Raven Press; New York, 1992.

7. Prestwich, G. D.; Marecek, J. F.; Mourey, R. J.; Theibert, A. B.; Ferris, C. D.; Danoff, S. K.; Snyder, S. H. *J. Am. Chem. Soc.* **1991**, *113*, 1822.

8. Tegge, W.; Ballou, C. E. *Carbohydr. Res.* **1992**, *230*, 63.

9. Gou, D.-M.; Liu, Y.-C.; Chen, C.-S. *Carbohydr. Res.* **1992**, *234*, 51.

10. Yu, K.-L.; Fraser-Reid, B. *Tetrahedron Lett.* **1988**, *29*, 979.

11. Hirata, M.; Watanabe, Y.; Yoshida, M.; Koga, T.; Ozaki, S. *J. Biol. Chem.* **1993**, *268*, 19260.

12. Kozikowski, A. P.; Ognyanov, V. I.; Fauq, A. H.; Nahorski, S. R.; Wilcox, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 4429.

13. Wilcox, R. A.; Challiss, R. A. J.; Baudin, G.; Vasella, A.; Potter, B. V. L.; Nahorski, S. R. *Biochem. J.* **1993**, *294*, 191.

14. Liu, C.-Y.; Chen, C.-S. *Tetrahedron Lett.* **1989**, *30*, 1617.

15. Gou, D.-M.; Chen, C.-S. *Tetrahedron Lett.* **1992**, *33*, 721.

16. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440.