Molecular Interactions of Endogenous D-*myo*-Inositol Phosphates with the Intracellular D-*myo*-Inositol 1,4,5-Trisphosphate Recognition Site‡

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ABSTRACT: A systematic effort was made to elucidate the mode of recognition at the inositol 1,4,5-trisphosphate-specific receptor. Eleven D-*myo*-inositol phosphates were synthesized and tested for Ca^{2+}-mobilizing and receptor-binding activities, which included Ins(1,3,4,5,6)P_5, Ins(1,2,5,6)P_4, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4, Ins(3,4,5,6)P_4, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(1,5,6)P_3, Ins(1,4)P_2, and Ins(4,5)P_2. Of these, Ins(1,4,5)P_3, Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, and Ins(4,5)P_2 were able to elicit Ca^{2+} release from rat brain microsomes. Binding experiments suggest that the ability of these polyphosphates to effect Ca^{2+} mobilization arises from interactions with the Ins(1,4,5)P_3-specific receptor. Accordingly, a model accounting for the ligand recognition is proposed. The Ins(1,4,5)P_3-binding site is presumably composed of two domains. The anchoring domain binds the 4,5-bisphosphate 6-hydroxy motif. Disruption of this structural feature abolishes the agonist activity. The auxiliary domain exerts long-range interactions with the 1-phosphate, thus enhancing the binding affinity. The stereochemical requirement for this electrostatic interaction is, however, less stringent. Evidence suggests that Ca^{2+}-mobilizing inositol phosphates are able to effect productive binding by assuming conformations displaying or mimicking these essential structural features.

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3[1]] is released from the phospholipase C-mediated breakdown of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and plays an obligatory role in stimulating intracellular Ca^{2+} mobilization (Berridge, 1993; Irvine, 1992a; Putney, 1992). It is well recognized that an elevation in cytosolic [Ca^{2+}] signals diverse cellular responses, including cell growth and development, fertilization, secretion, smooth muscle contraction, sensory perception, and neuromodulation. This signal-transducing pathway is controlled by a multitude of mechanisms in a concerted manner (Marshall & Taylor, 1993). Evidence indicates that a heterogeneous but structurally related population of Ins(1,4,5)P_3 receptors is differentially expressed in different tissues, as well as at different stages of development (Ferris & Snyder, 1992). Moreover, Ins(1,4,5)P_3, after being released into the cytosol, is rapidly metabolized by two discrete pathways mediated by a 5-phosphatase and by a 3-kinase to form Ins(1,4)P_2 and Ins(1,3,4,5)P_4, respectively (Fisher et al., 1992; Shears, 1992). While the formation of Ins(1,4)P_2 represents a "switch-off" mechanism for Ins(1,4,5)P_3, the metabolism of Ins(1,3,4,5)P_4 and its metabolites by the sequential actions of specific phosphatases and kinases produces a plethora of inositol phosphates (Menniti et al., 1993) (Scheme 1). These inositol phosphate congeners undergo dynamic turnover, and their cellular levels are sensitive to agonist stimulation.

Evidently, the rich diversity of phosphoinositols generated from such a complex metabolic network implies the physiological relevance of these molecules. Some of these inositol phosphates have been reported to exhibit interesting biochemical activities. Ins(1,3,4,5)P_4 has been shown to mobilize Ca^{2+} from the internal stores through interactions with the Ins(1,4,5)P_3-specific receptor (Wilcox et al., 1993), and many studies have implicated Ins(1,3,4,5)P_4 in the regulation of Ca^{2+} influx across the plasma membrane (Morris et al., 1987; Putney et al., 1989; Ely et al., 1990; Guse et al., 1992; Lückhoff et al., 1992; Irvine, 1992b; Smith, 1992). Ins(1,4)P_2 has been reported to exert allosteric activation of muscle-type 6-phosphofructo-1-kinase (Mayr, 1989). It has been demonstrated that Ins(4,5)P_2 and Ins(1,4,5)P_3, but not Ins(1,3,4,5)P_4, selectively inhibited the Ca^{2+}-ATPase of rat heart sarcolemma (Kuo, 1988) and of human erythrocyte membrane (Davis et al., 1991). Ins(1,3,4,6)P_4-activated Ca^{2+} mobilization has been observed in microinjected *Xenopus* oocytes (Ivorra et al., 1991) and in permeabilized human neuroblastoma cells (Gawler et al., 1991). Moreover, it has been demonstrated that Ins(1,3,4,5,6)P_5 and IP_6 were inhibitors of Ins(1,3,4,5)-P_4 3-phosphatase and Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase (Höer & Oberdisse, 1991) and that Ins(1,3,4,6)P_4 was a potent inhibitor of Ins(1,4,5,6)P_4 3-kinase (Craxton et al., 1994). The biological importance of Ins(1,4,5,6)P_4 is indicated by the recent reports that intracellular Ins(1,4,5,6)-P_4 levels were elevated upon receptor-coupled activation of phospholipase C (Barker et al., 1992) or subsequent to cell transformation with v-*src* oncogene (Mattingly et al., 1991).

Despite recent advances in the synthesis of phosphoinositols [before 1990, see reviews by Potter (1990), Reitz (Ed.) (1991), Lin et al. (1990), Bruzik and Tsai (1992), Gou et al. (1992), Kozikowski et al. (1990, 1993a), and Prestwich et al. (1991)], one major obstacle to investigating the biochemical functions

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

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[1] Abbreviations: Ins(1,4,5)P_3, 1-D-*myo*-inositol 1,4,5-trisphosphate (other inositol phosphates are abbreviated similarly); PI(4,5)P_2, phosphatidyl-D-*myo*-inositol 4,5-bisphosphate; ee, enantiomeric excess; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; DMF, dimethylformamide; DTT, dithiothreitol; PMSF, *p*-toluenesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EC_{50}, concentration causing half-maximal effect; IC_{50}, concentration causing 50% displacement of specific binding.

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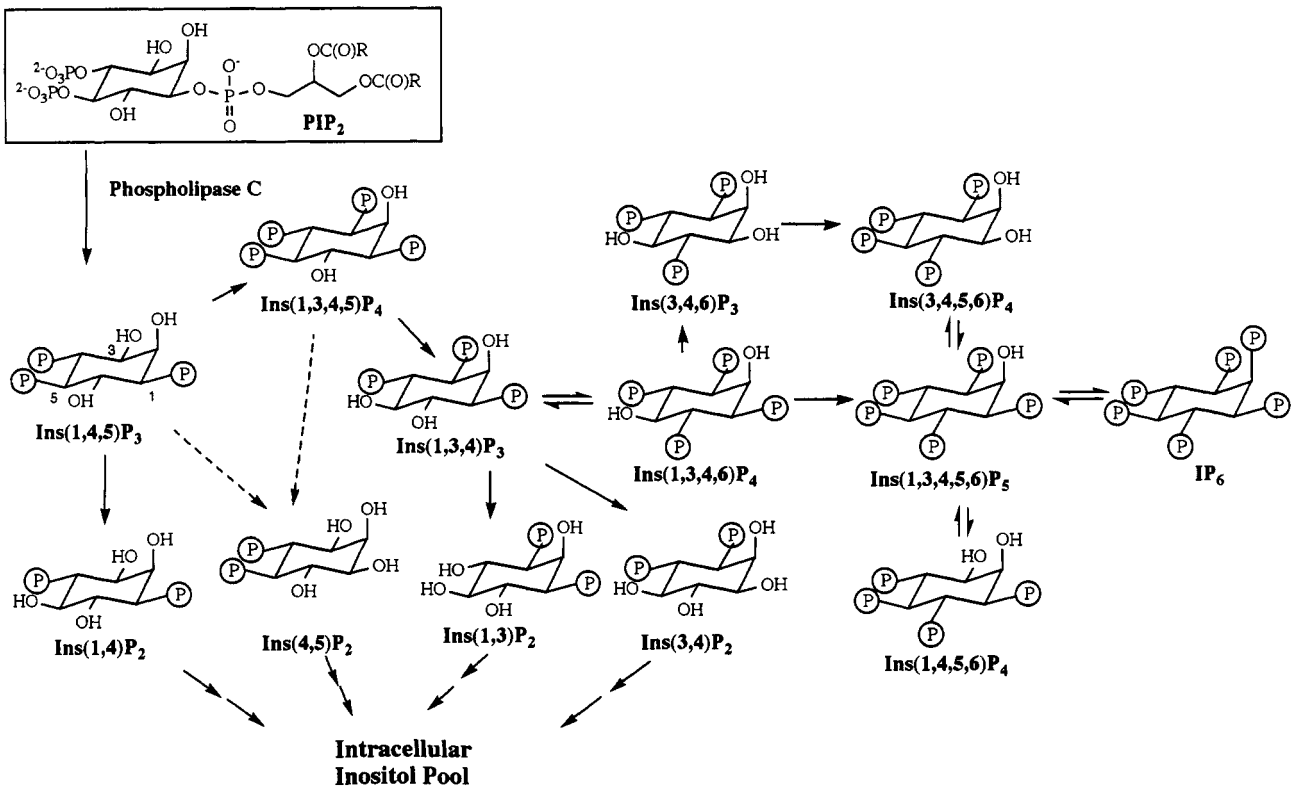
Scheme 1: Metabolism of Inositol Phosphates[a]



[a] The mechanism of Ins(4,5)P_2 formation remains unclear. It may result from phospholipase D-mediated hydrolysis of PIP_2 or from dephosphorylation of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 (Jenkinson et al., 1992).

of these polyphosphates has been the difficulty in gaining access to these compounds in high optical and chemical purity. Commercial preparations have been reported to be contaminated with other active inositol phosphates (Parker & Ivorra, 1991), and their cost often becomes a prohibitive factor for in-depth studies. Moreover, it should be noted that the use of racemic inositol phosphates may complicate the interpretation of biochemical data. For example, DL-*myo*-inositol 1,4,5,6-tetrakisphosphate is a mixture of Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4, both of which are natural inositol phosphates. Our current study shows that while Ins(1,4,5,6)P_4 is active in eliciting Ca^{2+} release from internal stores, Ins(3,4,5,6)P_4 is not.

Thus, as part of our research on the physiological significance of this intricate metabolism, we embarked on a systematic synthesis of naturally occurring D-*myo*-inositol phosphates. Since each compound was independently synthesized and fully characterized, the possibility of cross-contamination by other phosphoinositols was excluded. With these compounds in hand, we initiated a series of examinations of their roles in regulating cytosolic [Ca^{2+}]. In this account, we report the syntheses and Ca^{2+}-mobilizing activity of 11 D-*myo*-inositol phosphates. Accordingly, a working model accounting for the mode of interactions between endogenous inositol phosphates and the Ins(1,4,5)P_3 recognition site is proposed.

EXPERIMENTAL PROCEDURES

General Methods and Reagents. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded with a Brucker AM-300 spectrometer. Optical rotations were determined with a Rudolph Autopol III polarimeter. HPLC was performed using a Model 501 pump (Waters Associates) equipped with a Rheodyne injector and a Model 481 UV/vis detector (Waters Associates). Elemental analysis was conducted by M-H-W laboratories (Phoenix, AZ). Fluorescence spectrophotometric assay of Ca^{2+} release was carried out with a Hitachi F-2000 spectrometer. Racemic 1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (**2**) and racemic 1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (**32**) were prepared according to the procedures reported by Garegg et al. (1984). Ins(1,4,5)P_3, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4 were synthesized from optically active **2** according to the procedures previously reported (Gou et al., 1992). [^3H]-Ins(1,4,5)P_3 (21 Ci/mmol) was purchased from DuPont New England Nuclear.

Enzymatic Resolution of (±)-1,2:5,6-Di-O-cyclohexylidene-myo-inositol (**2**). Optically active **2** was prepared by subjecting (±)-**1** [for preparation, see Gou et al. (1992)] to enantioselective hydrolysis by porcine pancreatic lipase (PPL, Sigma type II) in a biphasic system. A solution of the substrate (14 g) in hexane–ether (10:1, 110 mL) was introduced into 0.1 M potassium phosphate buffer (pH 7.0, 60 mL) containing crude PPL powder (40 g). The mixture was stirred vigorously at 37 °C, and the reaction was monitored by TLC. After 42 h, the reaction was terminated by separating the two layers. The organic layer was dried and concentrated. Column chromatography (hexane–ethyl acetate, 15:1 → 4:1) of the residue gave (–)-**2** (5.4 g, 94% ee), $[\alpha]_D$ –17.8°), and (–)-**1** (6.9 g, 87% ee, $[\alpha]_D$ –13.2°). The remaining substrate was subjected to saponification (1M NaOH–CH_3OH, 23 °C, 3 h) to afford (+)-**2**. The optical purity of (+)- and (–)-**2** was no less than 98% ee after recrystallization. The enantiomeric purity of **2** was determined by HPLC analysis of the corresponding (*S*)-2-methoxy-2-(trifluoromethyl)phenylacetyl ester, as previously reported (Gou et al., 1992). With (+)- and (–)-**2**, 11 different D-*myo*-inositol phosphates were synthesized, including Ins(1,3,4,5,6)P_5, Ins(1,2,5,6)P_4, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4, Ins(3,4,5,6)P_4, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(1,5,6)P_3, Ins(1,4)P_2, and Ins(4,5)P_2. Syntheses of Ins(1,4,5)P_3 from (+)-**2** (Liu & Chen, 1989) and of Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 from (–)-**2** (Gou

& Chen, 1992; Gou et al., 1992) have been described elsewhere and will be not elaborated here.

(-)-1,6-Di-O-benzyl-2,3:4,5-di-O-cyclohexylidene-myo-inositol (**3**). A solution of (+)-**2** (0.4 g, 1.1 mmol) in DMF (10 mL) was treated with NaH (100 mg, 80%, 3.3 mmol) and benzyl bromide (0.32 mL, 2.4 mmol) for 12 h at 35 °C. The excess NaH was destroyed with CH_3OH, and the mixture was diluted with ethyl acetate (50 mL). After aqueous workup, column chromatography (hexane–ether, 25:1) of the residue afforded (-)-**3** (syrup, 0.6 g, 98%): $[\alpha]_D$ −21° (*c* 1.4, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.27–1.78 (m, 20 H), 3.51 (dd, 1 H, *J* = 7.86, 9.65 Hz), 3.76 (t, 1 H, *J* = 6.60 Hz), 3.87 (dd, 1 H, *J* = 2.91, 7.80 Hz), 4.13 (dd, 1 H, *J* = 3.76, 10.6 Hz), 4.31 (t, 1 H, 7.28 Hz), 4.38 (dd, 1 H, *J* = 3.82, 6.86 Hz), 4.50–4.73 (m, 4 H), 7.26–7.38 (m, 10 H).

(+)-1,6-Di-O-benzyl-2,3-O-cyclohexylidene-myo-inositol (**4**). A solution of (-)-**3** (0.5 g, 0.96 mmol) in CH_2Cl_2–CH_3OH (3:1, 20 mL) was stirred with acetyl chloride (80 μL) at 23 °C. TLC analysis indicated that, under these conditions, the *trans*-cyclohexylidene function was totally eliminated within 10–15 min, while the cleavage of the *cis*-ketal ring was not appreciably noted until after 20 min. Thus, the solution was stirred for 10 min, triethylamine (0.3 mL) was then added, and the solution was concentrated. Column chromatography (hexane–ether, 8:1) of the residue yielded (+)-**4** (syrup, 330 mg, 78%): $[\alpha]_D$ +17° (*c* 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.26–1.78 (m, 10 H), 2.93 (br s, 2 H), 3.29 (dd, 1 H, *J* = 8.52, 10.1 Hz), 3.64–3.77 (m, 3 H), 3.88 (dd, 1 H, *J* = 5.27, 7.48 Hz), 4.27 (dd, 1 H, *J* = 3.88, 5.18 Hz), 4.74 (q, 2 H, *J* = 12.2, 13.7 Hz), 4.84 (dd, 2 H, *J* = 11.2, 77.2 Hz), 7.26–7.40 (m, 10 H).

(+)-1,6-Di-O-benzyl-2,3-O-cyclohexylidene-myo-inositol 4,5-Bis(dibenzyl phosphate) (**5**). A mixture of tetrazole (0.6 g, 8 mmol), dibenzyl *N,N*-diisopropylphosphoramidite (1.83 g, 4 mmol), and CH_2Cl_2 (20 mL) was stirred under Ar at 23 °C for 1 h, and (+)-**4** was added in one portion. The solution was kept under the same conditions for another 12 h, cooled to −40 °C, and then treated with triethylamine (2 mL) and *m*-chloroperoxybenzoic acid (50% purity, 2 g, 4.1 mmol). The mixture was stirred at −40 °C for 30 min, then allowed to attain room temperature, diluted with CH_2Cl_2 (20 mL), and subjected to aqueous workup. The product was purified by column chromatography (hexane–ethyl acetate, 20:1 → 2:1) to give (+)-**5** (syrup, 0.65 g, 85%): $[\alpha]_D$ +2.3° (*c* 1.3, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.27–1.80 (m, 10 H), 3.79 (dd, 1 H, *J* = 3.74, 7.39 Hz), 4.03 (dd, 1 H, *J* = 6.13, 7.36 Hz), 4.20 (t, 1 H, *J* = 6.83 Hz), 4.35 (dd, 1 H, *J* = 3.81, 6.43 Hz), 4.49–4.57 (m, 1 H), 7.09–7.36 (m, 30 H).

1-D-myo-Inositol 4,5-Bisphosphate [Ins(4,5)P_2]. A solution of (+)-**5** (0.65 g, 0.5 mmol) in aqueous 85% EtOH (30 mL) was shaken under H_2 (50 psi) in the presence of 10% Pd/C (0.4 g) for 24 h, filtered, and concentrated. The residue was dissolved in minimal water, and 4 equiv of 1 M KOH was added. The solution was lyophilized to afford Ins(4,5)P_2 as the tetrapotassium salt (0.28 g, 99%): $[\alpha]_D$ −10° (*c* 1, H_2O); ^1H NMR (D_2O) δ 3.42–3.46 (m, 1 H), 3.54 (dd, 1 H, *J* = 3.45, 9.60 Hz), 3.67–3.74 (m, 2 H), 3.87 (t, 1 H, *J* = 2.83 Hz), 3.99 (m, 1 H); ^{31}P NMR (D_2O, external H_3PO_4) δ 4.62, 4.77.

(-)-1,6-Di-O-acetyl-2,3:4,5-di-O-cyclohexylidene-myo-inositol (**6**). A solution of (+)-**2** (0.5 g, 1.4 mmol) in CH_2Cl_2 (10 mL) was treated with acetic anhydride (1.2 mL), triethylamine (2 mL), and 4-(dimethylamino)pyridine (100 mg) at 23 °C for 30 min. After the aqueous workup, column chromatography (hexane–ether, 20:1 → 5:1) of the residue gave (-)-**6** (syrup, 0.6 g, 96%): $[\alpha]_D$ −5.3° (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.26–1.88 (m, 20 H), 2.10 (s, 3 H), 2.12 (s, 3 H), 3.59–3.65 (m, 1 H), 4.02–4.09 (m, 1 H), 4.38 (t, 1 H, *J* = 7.58 Hz), 4.44–4.51 (m, 1 H), 5.15–5.21 (m, 2 H).

(-)-1,6-Di-O-acetyl-2,3-O-cyclohexylidene-myo-inositol (**7**). The foregoing fully protected *myo*-inositol (-)-**6** (0.6 g, 1.4 mmol) in CH_2Cl_2–CH_3OH (2:1, 15 mL) was treated with acetyl chloride (50 μL) to remove the 4,5-*trans*-cyclohexylidene ring, as described for **4**, to furnish (-)-**7** (0.43 g, 80%): $[\alpha]_D$ −25° (*c* 0.7, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.35–1.68 (m, 10 H), 2.10 (s, 6 H), 3.05 (br s, 2 H), 3.46 (t, 1 H, *J* = 8.04 Hz), 3.82 (dd, 1 H, *J* = 7.39, 9.91 Hz), 4.08 (dd, 1 H, *J* = 5.61, 7.30 Hz), 4.44 (dd, 1 H, *J* = 3.54, 5.57 Hz), 5.10–5.52 (m, 2 H).

(+)-2,3-O-Cyclohexylidene-myo-inositol (**8**). A mixture of (-)-**7** (390 mg, 1 mmol), KOH (5.6 mg, 0.1 mmol), and CH_3OH (10 mL) was stirred at 23 °C for 30 min. After aqueous workup, the residue was subjected to column chromatography (hexane–ethyl acetate, 15:1 → 2:1) to afford (+)-**8** (solid, 290 mg, 97%): mp 188–189 °C; $[\alpha]_D$ +35° (*c* 1.4, CH_3OH); ^1H NMR (CD_3OD) δ 1.36–1.66 (m, 10 H), 3.07 (dd, 1 H, *J* = 9, 10 Hz), 3.44–3.57 (m, 2 H), 3.59–3.64 (m, 1 H), 3.88 (dd, 1 H, *J* = 4.99, 7.51 Hz), 4.31 (t, 1 H, *J* = 4.94 Hz). Anal. Calcd for $C_{12}H_{20}O_6$: C, 55.37; H, 7.74. Found: C, 55.77; H, 7.59.

(-)-2,3-O-Cyclohexylidene-myo-inositol 1,4,5,6-Tetrakis(dibenzyl phosphate) (**9**). The tetrol (+)-**8** (280 mg, 1 mmol) was phosphorylated with dibenzyl *N,N*-diisopropylphosphoramidite, tetrazole, and *m*-chloroperoxybenzoic acid, as described for **5**, to yield (-)-**9** (syrup, 1 g, 77%): $[\alpha]_D$ −8.5° (*c* 2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.25–1.71 (m, 10 H), 4.26 (t, 1 H, *J* = 5.88 Hz), 4.68 (dd, 1 H, *J* = 3.62, 6.68 Hz), 4.70–4.80 (m, 2 H), 4.91–5.12 (m, 16 H), 7.18–7.29 (m, 40 H).

1-D-myo-Inositol 1,4,5,6-Tetrakisphosphate [Ins(1,4,5,6)-P_4]. A mixture of (-)-**9** (780 mg, 0.5 mmol) and 10% Pd/C (250 mg) in aqueous 85% EtOH (30 mL) was shaken under H_2 (50 psi) for 12 h, filtered, and concentrated. The residue was dissolved in the minimum amount of water, 1 M KOH (8 equiv) was added, and the mixture was lyophilized to afford Ins(1,4,5,6)P_4 as the octapotassium salt (0.47 g, 99%): $[\alpha]_D$ −6.9° (*c* 0.65, H_2O); ^1H NMR (D_2O) δ 3.95 (t, 1 H, *J* = 3.6 Hz), 4.04–4.10 (m, 2 H), 4.37 (d, 2 H, *J* = 10.8 Hz), 4.58 (d, 1 H, *J* = 10.2 Hz); ^{31}P NMR (D_2O, external H_3PO_4) δ 4.25, 4.56, 5.18, 5.33.

(-)-4-O-Allyl-1,2:5,6-di-O-cyclohexylidene-myo-inositol (**10**). A mixture of (-)-**2** (1.2 g, 3.5 mmol), Bu_2SnO (0.98 g, 3.8 mmol), and toluene (30 mL) was boiled under reflux, with azeotrophic removal of water for 1 h, and then concentrated to dryness under reduced pressure. To the residue were added DMF (10 mL), CsF (1.3 g, 8.2 mmol), and allyl bromide (0.72 mL, 8.2 mmol). The mixture was stirred under Ar at 23 °C for 16 h and then diluted with ethyl acetate (80 mL). After aqueous workup, column chromatography (hexane–ether, 20:1 → 10:1) gave (-)-**10** (syrup, 0.85 g, 63%): $[\alpha]_D$ −4.5° (*c* 0.8, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.40–1.72 (m, 20 H), 3.49 (dd, 1 H, *J* = 7.91, 10.6 Hz), 3.84 (dd, 1 H, *J* = 1.92, 7.87 Hz), 3.98–4.00 (m, 1 H), 4.10–4.28 (m, 3 H), 4.34 (t, 1 H, *J* = 6.6 Hz), 4.43 (dd, 1 H, *J* = 3.64, 7.38 Hz), 5.21 (dq, 1 H, *J* = 1.2, 2.9, 10.4 Hz), 5.33 (dq, 1 H, *J* = 1.57, 3.27, 17.2 Hz), 5.87–6.01 (m, 1 H).

(+)-4-O-Allyl-3-O-benzyl-1,2:5,6-di-O-cyclohexylidene-myo-inositol (**11**). Benzylation of (-)-**10** (0.83 g, 2.1 mmol) with benzyl bromide, as described for **3**, furnished (+)-**11** (syrup, 1 g, 99%): $[\alpha]_D$ +12.5° (*c* 0.55, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.39–1.75 (m, 20 H), 3.43 (dd, 1 H, *J* = 7.7, 10.6 Hz), 3.71 (t, 1 H, *J* = 3.28 Hz), 3.79 (dd, 1 H, *J* = 2.88, 7.76

Hz), 3.94–4.01 (m, 1 H), 4.05–4.14 (m, 2 H), 4.25–4.37 (m, 2 H), 4.72 (q, 1 H, J = 12.4, 19.3 Hz), 5.16 (dq, 1 H, J = 1.45, 3.11, 10.3 Hz), 5.26 (dq, 1 H, J = 1.55, 3.85, 17.3 Hz), 5.80–5.93 (m, 1 H), 7.27–7.40 (m, 5 H).

(-)-4-O-Allyl-3-O-benzyl-1,2-O-cyclohexylidene-myo-inositol (**12**). Regioselective hydrolysis of the *trans*-cyclohexylidene of (+)-**11** (0.7 g, 1.5 mmol), as described for **4**, gave (-)-**12** (syrup, 0.45 g, 77%): $[\alpha]_D$ -9° (c 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.32–1.78 (m, 10 H), 2.73 (br s, 2 H), 3.22–3.32 (m, 1 H), 3.62–3.64 (m, 2 H), 3.74 (dd, 1 H, J = 7.58, 10.1 Hz), 3.91 (dd, 1 H, J = 5.21, 7.5 Hz), 4.18–4.29 (m, 2 H), 4.42–4.49 (m, 1 H), 4.75 (q, 2 H, J = 11.5, 13.7 Hz), 5.18–5.34 (m, 2 H), 5.91–6.03 (m, 1 H), 7.30–7.41 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 23.64, 23.94, 25.05, 35.17, 37.76, 65.86, 72.74, 73.18, 73.65, 73.83, 75.2, 77.23, 77.85, 78.23, 80.1, 110.73, 117.21, 127.96, 128.02, 128.48, 134.9, 138.05.

(-)-4-O-Allyl-3,5,6-tri-O-benzyl-1,2-O-cyclohexylidene-myo-inositol (**13**). Benzylation of (-)-**12** (0.4 g, 1 mmol) with benzyl bromide, as described for **3**, yielded (-)-**13** (syrup, 0.52 g, 90%): $[\alpha]_D$ -18° (c 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.37–1.78 (m, 10 H), 3.34 (dd, 1 H, J = 8.42, 9.6 Hz), 3.62 (dd, 1 H, J = 3.76, 8.11 Hz), 3.73–3.82 (m, 2 H), 4.07 (dd, 1 H, J = 5.6, 6.97 Hz), 4.23–4.37 (m, 3 H), 4.71-4.90 (m, 6 H), 5.14–5.19 (m, 1 H), 4.24–5.32 (m, 1 H), 5.91–6.05 (m, 1 H), 7.23–7.42 (m, 15 H); ^{13}C NMR ($CDCl_3$) δ 23.6, 23.88, 25.04, 35.02, 37.33, 73.07, 73.63, 73.89, 75.16, 77.09, 78.75, 80.57, 82.13, 82.80, 110.36, 116.57, 127.38 (2 C), 127.47 (2 C), 127.64 (2 C), 127.83 (2 C), 127.98 (2 C), 128.17 (2 C), 128.2 (2 C), 128.28 (2 C), 135.16, 138.39, 138.67, 138.72.

(-)-4-O-Allyl-3,5,6-tri-O-benzyl-myo-inositol (**14**). The foregoing fully protected inositol (-)-**13** (0.5 g, 0.87 mmol) was treated with acetyl chloride (50 μL) in CH_2Cl_2–CH_3OH (1:1, 25 mL) at 23 °C for 30 min, and the solution was concentrated. Column chromatography (hexane–ether, 20:1 → 10:1) of the residue gave (-)-**14** (syrup, 0.4 g, 93%): $[\alpha]_D$ -8.3° (c 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.43 (d, 1 H, J = 4.16 Hz), 2.48 (s, 1 H), 3.25–3.41 (m, 4 H), 3.37–3.47 (m, 3 H), 3.80 (dd, 2 H, J = 9.47, 19.4 Hz), 4.17 (t, 1 H, J = 2.84 Hz), 4.29–4.43 (m, 2 H), 4.66–4.82 (m, 4 H), 4.92 (t, 2 H, J = 10.6 Hz), 5.14–5.19 (m, 1 H), 5.25–5.32 (m, 1 H), 5.92–6.05 (m, 1 H), 7.26–7.37 (m, 15 H); ^{13}C NMR ($CDCl_3$) δ 69.4, 71.8, 72.8, 74.6, 75.6, 75.7, 77.3, 79.9, 81.3, 81.4, 83.2, 116.6, 127.6, 127.8 (3 C), 127.9 (4 C), 128.4 (2 C), 128.5 (3 C), 128.6 (2 C), 135.3, 137.9, 138.6.

(+)-1,4-Di-O-allyl-3,5,6-tri-O-benzyl-myo-inositol (**15**). Regioselective allylation of (-)-**14** (0.4 g, 0.8 mmol), as described for **10**, gave (+)-**15** (syrup, 0.4 g, 94%): $[\alpha]_D$ +7.8° (c 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.43 (s, 1 H), 3.25–3.41 (m, 4 H), 3.80–3.93 (m, 2 H), 4.13–4.21 (m, 2 H), 4.29–4.42 (m, 2 H), 4.73 (q, 2 H, J = 10.6, 15 Hz), 4.79–4.87 (m, 4 H), 5.14–5.20 (m, 2 H), 5.24–5.31 (m, 2 H), 5.86–6.05 (m, 2 H), 7.26–7.41 (m, 15 H).

(+)-1,4-Di-O-allyl-2,3,5,6-tetra-O-benzyl-myo-inositol (**16**). Benzylation of (+)-**15** (0.38 g, 0.67 mmol), as described for **3**, yielded (+)-**16** (syrup, 0.42 g, 95%): $[\alpha]_D$ +5.7° (c 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 3.21–3.32 (m, 2 H), 3.40 (t, 1 H, J = 9.26 Hz), 3.89–4.13 (m, 4 H), 4.26–4.43 (m, 3 H), 4.64 (q, 2 H, J = 11.8, 25.4 Hz), 4.76–4.90 (m, 6 H), 5.11–5.33 (m, 4 H), 5.83–6.04 (m, 2 H), 7.22–7.42 (m, 20 H); ^{13}C NMR ($CDCl_3$) δ 71.38, 72.90, 74.07, 74.58, 75.83, 75.94, 77.23, 80.75, 80.87, 81.5, 81.65, 83.73, 116.51 (3 C), 116.63 (3 C), 127.31 (3 C), 127.52 (3 C), 127.82 (3 C), 127.95 (3 C), 128.12 (3 C), 128.32 (3 C), 135.01, 135.53, 138.62, 139.04.

(+)-2,3,5,6-Tetra-O-benzyl-myo-inositol (**17**). A mixture of (+)-**16** (0.4 g, 0.65 mmol), 10% Pd/C (100 mg), and *p*-toluenesulfonic acid monohydrate (100 mg, 0.52 mmol) in aqueous 90% EtOH (20 mL) was stirred under reflux for 45 min and then filtered and concentrated. Column chromatography (hexane–ethyl acetate, 21:1 → 5:1) of the residue afforded (+)-**17** (syrup, 240 mg, 69%): $[\alpha]_D$ +5° (c 0.4, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.30 (d, 1 H, J = 6.0 Hz), 2.55 (d, 1 H, J = 1.5 Hz), 3.33 (dd, 1 H, J = 2.3, 9.8 Hz), 3.39 (t, 1 H, J = 9.1 Hz), 3.48–3.54 (m, 1 H), 3.78 (t, 1 H, J = 9.3 Hz), 4.06 (t, 1 H, J = 2.4 Hz), 4.15 (t, 1 H, J = 9.2 Hz), 4.57–4.78 (m, 4 H), 4.86–4.93 (m, 4 H), 7.26–7.45 (m, 20 H).

(+)-2,3,5,6-Tetra-O-benzyl-myo-inositol 1,4-Bis(dibenzyl phosphate) (**18**). The diol (+)-**17** (170 mg, 0.32 mmol) was phosphorylated, as described for **5**, to give (+)-**18** (syrup, 300 mg, 89%): $[\alpha]_D$ +5.8° (c 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 3.39-3.43 (m, 1 H), 3.50 (t, 1 H, J = 9.2 Hz), 4.07 (t, 1 H, J = 9.4 Hz), 4.24–4.27 (m, 1 H), 4.32 (m, 1 H), 4.52 (s, 2 H), 4.61–4.99 (m, 15 H), 7.03–7.31 (m, 40 H).

1-D-myo-Inositol 1,4-Bisphosphate [Ins(1,4)P_2]. The foregoing compound (+)-**18** (250 mg, 0.23 mmol) was debenzylated by hydrogenolysis, followed by KOH titration and lyophilization, as described for Ins(4,5)P_2, to generate Ins(1,4)P_2 as the tetrapotassium salt (115 mg, 99%): $[\alpha]_D$ +3.6° (c 0.5, H_2O); ^1H NMR (D_2O) δ 3.32 (t, 1 H, J = 9.3 Hz), 3.52 (dd, 1 H, J = 3, 6.45 Hz), 3.67 (t, 1 H, J = 3.9 Hz), 3.75–3.82 (m, 1 H), 4.0 (dd, 1 H, J = 7.61, 11.3 Hz), 4.08 (t, 1 H, J = 2.67 Hz); ^{31}P NMR (D_2O, external H_3PO_4) δ 3.12, 4.03.

(+)-3,4-Di-O-benzyl-1,2:5,6-di-O-cyclohexylidene-myo-inositol (**19**). Conventional benzylation of (-)-**2** (1 g, 2.9 mmol), as described for **3**, afforded (+)-**19** (1.4 g, 90%): $[\alpha]_D$ +21° (c 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.27–1.78 (m, 20 H), 3.51 (dd, 1 H, J = 7.86, 7.95 Hz), 3.76 (t, 1 H, J = 6.60 Hz), 3.87 (dd, 1 H, J = 2.9, 7.8 Hz), 4.13 (dd, 1 H, J = 7.36, 10.6 Hz), 4.31 (t, 1 H, J = 7.28 Hz), 4.38 (dd, 1 H, J = 3.81, 6.86 Hz), 4.50–4.73 (m, 4 H), 7.26–7.38 (m, 10 H).

(-)-3,4-Di-O-benzyl-1,2-O-cyclohexylidene-myo-inositol (**20**). Hydrolysis of the *trans*-cyclohexylidene ring of (+)-**19** (1.2 g, 2.31 mmol), as described for **4**, yielded (-)-**20** (syrup, 0.7 g, 70%): $[\alpha]_D$ -16.5° (c 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.27-1.78 (m, 10 H), 2.93 (br s, 2 H), 3.29 (dd, 1 H, J = 8.51, 10.1 Hz), 3.64–3.77 (m, 3 H), 3.88 (dd, 1 H, J = 5.27, 7.49 Hz), 4.27 (dd, 1 H, J = 3.88, 5.18 Hz), 4.74 (q, 2 H, J = 12.2, 13.7 Hz), 4.84 (dd, 2 H, J = 11.2, 77.2 Hz), 7.26–7.40 (m, 10 H).

(-)-5,6-Di-O-allyl-3,4-di-O-benzyl-1,2-O-cyclohexylidene-myo-inositol (**21**). Conventional allylation of (-)-**20** (0.65 g, 1.47 mmol) with allyl bromide furnished (-)-**21** (syrup, 0.73 g, 95%): $[\alpha]_D$ -26.2° (c 1.8, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.25–1.80 (m, 10 H), 3.20 (dd, 1 H, J = 8.71, 9.22 Hz), 3.58–3.64 (m, 2 H), 3.84 (t, 1 H, J = 8.35 Hz), 3.95 (dd, 1 H, J = 5.46, 6.33 Hz), 4.21–4.37 (m, 5 H), 4.69–4.84 (m, 4 H), 5.12–5.14 (m, 1 H), 5.16–5.18 (m, 1 H), 5.24–5.26 (m, 1 H), 5.29–5.32 (m, 1 H), 5.89–6.0 (m, 2 H), 7.25–7.39 (m, 10 H).

(-)-5,6-Di-O-allyl-3,4-di-O-benzyl-myo-inositol (**22**). Hydrolysis of the *cis*-cyclohexylidene of (-)-**21** (0.7 g, 1.3 mmol), as described for **14**, gave (-)-**22** (syrup, 0.56 g, 95%): $[\alpha]_D$ -15.5° (c 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.32 (br s, 2 H), 3.26 (t, 1 H, J = 9.40 Hz), 3.40 (dd, 1 H, J = 2.73, 9.4 Hz), 3.65 (t, 1 H, J = 9.5 Hz), 3.86 (t, 1 H, J = 9.48 Hz), 4.18–4.46 (m, 5 H), 4.70 (q, 2 H, J = 11.6, 12.9 Hz), 4.83 (q, 2 H, J = 10.62, 12.5 Hz), 5.14–5.33 (m, 4 H), 5.89–6.03 (m, 2 H), 7.26–7.37 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 69.4, 71.76, 72.84, 74.25, 74.3, 75.88, 77.19, 80.07, 80.95, 81.64,

82.96, 116.43, 116.94, 127.55, 127.86, 127.88, 127.99 (2 C), 128.31 (2 C), 128.5 (2 C), 135.22, 135.3, 138.01, 138.92.

(-)-1,5,6-Tri-O-allyl-3,4-di-O-benzyl-myo-inositol (**23**). Regioselective allylation of (-)-**22** (0.55 g, 1.2 mmol), as described for **10**, yielded (-)-**23** (syrup, 0.56 g, 93%): $[\alpha]_D$ -14.5° (*c* 1.1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.43 (s, 1 H), 3.15–3.26 (m, 2 H), 3.32–3.36 (m, 1 H), 3.73 (t, 1 H, *J* = 9.6 Hz), 3.87 (t, 1 H, *J* = 9.6 Hz), 4.17–4.18 (m, 3 H), 4.27–4.32 (m, 3 H), 4.71 (d, 2 H, *J* = 3.0 Hz), 4.83 (s, 2 H), 5.11–5.13 (m, 1 H), 5.15–5.17 (m, 1 H), 5.18–5.19 (m, 1 H), 5.23–5.29 (m, 2 H), 5.29–5.31 (m, 2 H), 5.84–6.04 (m, 3 H), 7.25–7.37 (m, 10 H).

(+)-1,5,6-Tri-O-allyl-2,3,4-tri-O-benzyl-myo-inositol (**24**). Conventional benzylation of (-)-**23** (0.55 g, 1.1 mmol), as described for **3**, gave (+)-**24** (syrup, 0.6 g, 93%): $[\alpha]_D$ +4.1° (*c* 2.8, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 3.15–3.36 (m, 3 H), 3.96–3.97 (m, 1 H), 4.05–4.08 (m, 1 H), 4.14–4.18 (m, 2 H), 4.28–4.33 (m, 4 H), 4.56–4.71 (m, 3 H), 4.81–4.85 (m, 3 H), 5.12–5.19 (m, 3 H), 5.23–5.26 (m, 2 H), 5.30–5.31 (m, 2 H), 5.92–5.98 (m, 3 H), 7.25–7.42 (m, 15 H).

(+)-2,3,4-Tri-O-benzyl-myo-inositol (**25**). Deallylation of the foregoing fully protected inositol (+)-**24** (0.58 g, 1 mmol) with 10% Pd/C and *p*-toluenesulfonic acid, as described for **17**, afforded (+)-**25** (syrup, 0.4 g, 86%): $[\alpha]_D$ +59.6° (*c* 0.55, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 3.33–3.39 (m, 3 H), 3.44–3.47 (m, 2 H), 3.73 (t, 1 H, *J* = 9.6 Hz), 3.85 (t, 1 H, *J* = 9.3 Hz), 4.03 (br s, 1 H), 4.65–4.76 (m, 4 H), 4.96–5.06 (m, 3 H), 7.25–7.35 (m, 15 H); ^{13}C NMR ($CDCl_3$) δ 72.38, 73.12, 73.86, 74.7, 75.06, 75.48, 77.24, 77.61, 81.01, 81.33 (2 C), 127.75 (2 C), 127.81 (2 C), 127.85 (2 C), 128.28 (2 C), 128.55 (2 C), 138.17 (2 C), 138.71 (2 C), 138.81.

(+)-2,3,4-Tri-O-benzyl-myo-inositol 1,5,6-Tris(dibenzyl phosphate) (**26**). The triol (+)-**25** (380 mg, 0.84 mmol) was phosphorylated, as described for **5**, to afford **26** (syrup, 0.9 g, 92%): $[\alpha]_D$ +8.5° (*c* 1.55, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 3.42 (dd, 1 H, *J* = 2.21, 5.96 Hz), 4.06 (t, 1 H, *J* = 9.6 Hz), 4.16–4.22 (m, 1 H), 4.40–4.49 (m, 4 H), 4.63–5.10 (m, 17 H), 6.97–7.35 (m, 45 H).

1-D-myo-Inositol 1,5,6-Trisphosphate [*Ins(1,5,6)P*$_3$]. Hydrogenolysis of (+)-**26** (0.9 g, 0.7 mmol) followed by KOH titration and lyophilization, as described for Ins(4,5)P_2, gave Ins(1,5,6)P_3 as the hexapotassium salt (0.47 g, 99%): $[\alpha]_D$ -3° (*c* 1.2, H_2O); ^1H NMR (D_2O) δ 3.47–3.52 (m, 1 H), 3.73–3.80 (m, 3 H), 3.83–3.89 (m, 1 H), 4.15–4.25 (m, 1 H), 4.29 (t, 1 H, *J* = 2.83 Hz); ^{31}P NMR (D_2O, external H_3PO_4) δ 3.56, 3.57, 4.45.

(+)-3,4-Di-O-benzyl-myo-inositol (**27**). Hydrolysis of the cyclohexylidene rings of (+)-**19** (0.7 g, 1.3 mmol), as described for **14**, generated (+)-**27** (0.46 g, 95%): $[\alpha]_D$ +6° (*c* 0.5, CH_3OH); ^1H NMR (CD_3OD) δ 3.41 (dd, 1 H, *J* = 2.71, 6.16 Hz), 3.66 (t, 1 H, *J* = 9.3 Hz), 3.74 (t, 1 H, *J* = 9.3 Hz), 4.16 (t, 1 H, *J* = 2.15), 4.66 (q, 2 H, *J* = 11.5, 30.1 Hz), 4.95 (s, 4 H), 7.26–7.40 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 68.93, 70.54, 71.46, 72.79, 73.7, 75.0, 79.74, 81.17, 126.76, 126.97, 127.20 (2 C), 127.70 (2 C), 127.83 (2 C), 138.89, 139.56.

1-D-myo-Inositol 1,2,5,6-Tetrakisphosphate [*Ins(1,2,5,6)P*$_4$]. Phosphorylation of (+)-**27** (0.35 g, 1.4 mmol), as described for **5**, furnished (+)-3,4-di-*O*-benzyl-*myo*-inositol tetrakis(dibenzyl phosphate) (+)-**28** (syrup, 1.1 g, 81%): $[\alpha]_D$ +5.8° (*c* 1.9, $CHCl_3$). Hydrogenolysis of (+)-**28** followed by KOH titration and lyophilization, as described for Ins(4,5)P_2, afforded Ins(1,2,5,6)P_4 (0.2 g, 99%): $[\alpha]_D$ -4.9° (*c* 1.3, H_2O); ^1H NMR (D_2O) δ 3.34 (dd, 1 H, *J* = 2.1, 5.2 Hz), 3.61–3.77 (m, 3 H), 4.21 (q, 1 H, *J* = 9.9, 18 Hz), 4.57–4.60 (m, 1 H); ^{31}P NMR (D_2O, external H_3PO_4) δ 4.13, 4.25, 4.37, 4.50.

(+)-3,4-Di-O-acetyl-1,2:5,6-di-O-cyclohexylidene-myo-inositol (**29**). Acetylation of (-)-**2** (0.6 g, 1.6 mmol), as described for **6**, gave (+)-**29** (syrup, 0.74 g, 99%): $[\alpha]_D$ +5.4° (*c* 1.1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.26–1.87 (m, 20 H), 2.10 (s, 3 H), 2.12 (s, 3 H), 3.59–3.65 (m, 1 H), 4.02–4.09 (m, 1 H), 4.38 (t, 1 H, *J* = 7.58 Hz), 4.44–4.51 (m, 1 H), 5.15–5.20 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 20.88, 20.98, 23.56, 23.69, 23.91, 25.04, 25.12, 25.36, 31.61, 34.32, 36.37, 36.7, 36.79, 72.38, 73.62, 75.87, 76.33, 77.27, 111.89, 113.66, 169.1, 169.39.

(-)-1,2-O-Cyclohexylidene-myo-inositol (**30**). A solution of (+)-**29** (0.7 g, 1.6 mmol) in CH_2Cl_2–CH_3OH (1:1, 14 mL) was stirred with acetyl chloride (60 μL, 0.72 mmol) at 23 °C for 5 min. Triethylamine (100 μL, 0.72 mmol) was added, the solution was concentrated, and the residue was treated with methanolic 1 M KOH for 1 h at 23 °C. After aqueous workup, column chromatography (hexane–ethyl acetate, 2:1) afforded (-)-**30** (solid, 0.35 g, 78%): $[\alpha]_D$ -35° (*c* 0.9, CH_3OH); ^1H NMR (CD_3OD) δ 1.40–1.71 (m, 10 H), 3.11 (t, 1 H, *J* = 9.31 Hz), 3.47–3.68 (m, 2 H), 3.59–3.64 (m, 1 H), 3.91 (dd, 1 H, *J* = 5.03, 7.46 Hz), 4.35 (t, 1 H, *J* = 4.46); ^{13}C NMR ($CDCl_3$) δ 24.61, 24.94, 26.11, 36.21, 39.13, 71.80, 73.86, 75.14, 77.02, 77.35, 80.25, 111.34. Anal. Calcd for $C_{12}H_{20}O_6$: C, 55.37; H, 7.74. Found: C, 55.77; H, 7.59.

(+)-1,2-O-Cyclohexylidene-myo-inositol 3,4,5,6-Tetrakis(dibenzyl phosphate) (**31**). Phosphorylation of (-)-**30** (0.3 g, 1 mmol), as described for **5**, gave (+)-**31** (1.4 g, 98%): $[\alpha]_D$ +8.5° (*c* 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.25–1.71 (m, 10 H), 4.26 (t, 1 H, *J* = 5.88 Hz), 4.68 (dd, 1 H, *J* = 3.62, 6.67 Hz), 4.70–4.80 (m, 2 H), 4.91–5.12 (m, 16 H), 7.18–7.29 (m, 40 H).

1-D-myo-Inositol 3,4,5,6-Tetrakisphosphate [*Ins(3,4,5,6)P*$_4$]. Hydrogenolysis of (+)-**31** (1.2 g, 0.9 mmol) followed by KOH titration and lyophilization, as described for Ins(4,5)P_2, furnished Ins(3,4,5,6)P_4 as the octapotassium salt (0.72 g, 99%): $[\alpha]_D$ +7.2° (*c* 2.3, H_2O); ^1H NMR (D_2O) δ 3.95 (t, 1 H, *J* = 3 Hz), 4.04–4.10 (m, 2 H), 4.36 (br d, 2 H, *J* = 10.2 Hz), 4.50 (br d, 1 H, *J* = 9 Hz); ^{31}P NMR (D_2O, external H_3PO_4) δ 3.32, 3.59, 3.78, 4.38.

(±)-3,6-Di-O-allyl-1,2:4,5-di-O-cyclohexylidene-myo-inositol (**33**). Allylation of racemic 1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (**32**) (1.5 g, 4.4 mmol), as described for **21**, provided (±)-**33** (syrup, 1.75 g, 94%): ^1H NMR ($CDCl_3$) δ 1.25–1.76 (m, 20 H), 3.31 (dd, 1 H, *J* = 9.2, 10.5 Hz), 3.63 (dd, 1 H, *J* = 6.5, 10.5 Hz), 3.76 (dd, 1 H, *J* = 4.1, 25.2 Hz), 3.97 (t, 1 H, *J* = 6.45 Hz), 4.06 (dd, 1 H, *J* = 4.95, 6.60 Hz), 4.21–4.37 (m, 4 H), 4.45 (t, 1 H, *J* = 3.9 Hz), 5.15–5.36 (m, 1 H), 5.86–6.05 (m, 2 H).

(±)-3,6-Di-O-allyl-1,2-O-cyclohexylidene-myo-inositol (**34**). Selective hydrolysis of the *trans*-cyclohexylidene of (±)-**33** (1 g, 2.3 mmol), as described for **4**, gave (±)-**34** (solid, 0.8 g, 98%): ^1H NMR ($CDCl_3$) δ 1.26–1.78 (m, 10 H), 2.79 (br s, 2 H), 3.36 (t, 1 H, *J* = 9.36 Hz), 3.44–3.51 (m, 2 H), 3.90 (t, 1 H, *J* = 9.37 Hz), 4.06 (dd, 1 H, *J* = 5.09, 5.95 Hz), 4.16–4.30 (m, 3 H), 4.39–4.47 (m, 2 H), 5.17–5.25 (m, 2 H), 5.26–5.36 (m, 2 H), 5.89–6.04 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 23.61, 23.92, 25.01, 35.24, 37.71, 71.29, 71.52, 72.34, 73.21, 73.44, 77.26, 78.93, 82.13, 110.59, 117.08, 117.59, 134.85, 135.02. Anal. Calcd for $C_{18}H_{28}O_6$: C, 63.51; H, 8.29. Found: C, 63.6; H, 8.25.

(±)-3,4,6-Tri-O-allyl-1,2-O-cyclohexylidene-myo-inositol (**35**). Regioselective allylation of (±)-**34** (0.8 g, 2.3 mmol) with allyl bromide, Bu_2SnO, and CsF, as described for **10**, gave (±)-**35** (syrup, 0.58 g, 65%): ^1H NMR ($CDCl_3$) δ 1.26–

1.80 (m, 10 H), 2.70 (s, 1 H), 3.57 (dd, 1 H, J = 7.91, 9.72 Hz), 3.54–3.66 (m, 3 H), 4.06 (dd, 1 H, J = 5.69, 6.89 Hz), 4.18–4.28 (m, 3 H), 4.32–4.43 (m, 3 H), 5.12–5.21 (m, 2 H), 5.26–5.27 (m, 2 H), 5.32–5.33 (m, 2 H), 5.87–6.03 (m, 3 H); ^{13}C NMR ($CDCl_3$) δ 23.59, 23.92, 25.07, 35.1, 37.28, 72.08, 72.29, 73.23, 73.38, 74.17, 77.20, 78.54, 80.13, 81.45, 110.51, 116.99, 117.22, 117.38, 134.93, 134.98 (2 C).

(±)-3,4,6-Tri-O-allyl-5-O-benzyl-1,2-O-cyclohexylidene-myo-inositol (**36**). Benzylation of (±)-**35** (0.72 g, 1.8 mmol), as described for **3**, afforded (±)-**36** (syrup, 0.74 g, 81%): ^{1}H NMR ($CDCl_3$) δ 1.27–1.77 (m, 10 H), 3.30 (dd, 1 H, J = 8.67–9.67 Hz), 3.56 (dd, 1 H, J = 6.18, 8.70 Hz), 3.62 (dd, 1 H, J = 7.20, 8.65 Hz), 3.73 (t, 1 H, J = 8.40 Hz), 4.04 (dd, 1 H, J = 5.40, 5.47 Hz), 4.17–4.40 (m, 6 H), 4.77 (s, 2 H), 5.13–5.21 (m, 3 H), 5.24–5.34 (m, 3 H), 5.88–6.04 (m, 3 H), 7.27–7.40 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 23.58, 23.91, 25.07, 35.23, 37.37, 72.37, 72.98, 73.85, 74.20, 75.4, 77.22, 78.74, 80.47, 82.21, 82.46, 110.47, 116.69, 116.71, 117.30, 127.61, 128.14 (2 C), 128.31 (2 C), 135.11, 135.24, 135.37, 138.72.

(±)-3,4,6-Tri-O-allyl-5-O-benzyl-myo-inositol (**37**). Hydrolysis of the *cis*-ketal of (±)-**36** (0.7 g, 1.48 mmol) with acetyl chloride in CH_2Cl_2–CH_3OH, as described for **14**, furnished (±)-**37** (syrup, 0.5 g, 86%): ^{1}H NMR ($CDCl_3$) δ 2.48–2.52 (br s, 2 H), 3.28–3.38 (m, 2 H), 3.43–3.49 (m, 1 H), 3.62–3.76 (m, 2 H), 4.09–4.44 (m, 5 H), 4.81 (dd, 2 H, J = 10.7, 15.4 Hz), 5.13–5.34 (m, 6 H), 5.86–6.02 (m, 3 H), 7.26–7.37 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 69.49, 71.75, 71.84, 74.35, 74.58, 75.71, 77.23, 79.7, 80.85, 81.27, 83.16, 93.03, 116.61, 117.19, 117.67, 128.02, 128.39, 134.67, 135.08, 135.33, 138.66.

1,3,4,6-Tetra-O-allyl-5-O-benzyl-myo-inositol (**38**). Regioselective allylation of (±)-**37** (0.36 g, 0.92 mmol) with allyl bromide, Bu_2SnO, and CsF, as described for **10**, gave (±)-**35** (syrup, 0.33 g, 84%): ^{1}H NMR ($CDCl_3$) δ 2.65 (br s, 1 H), 3.21–3.35 (m, 3 H), 3.73 (t, 1 H, J = 9.06 Hz), 4.16–4.19 (m, 3 H), 4.29–4.32 (m, 3 H), 4.81 (s, 2 H), 5.12–5.34 (m, 10 H), 5.85–6.04 (m, 4 H), 7.27–7.38 (m, 5 H).

1,3,4,6-Tetra-O-allyl-2,5-di-O-benzyl-myo-inositol (**39**). Conventional benzylation of **38** (0.31 g, 0.72 mmol), as described for **3**, provided **39** (syrup, 0.36 g, 96%): ^{1}H NMR ($CDCl_3$) δ 3.18 (dd, 2 H, J = 2.3, 9.8 Hz), 3.32 (t, 1 H, J = 9.21 Hz), 3.82 (t, 2 H, J = 9.5 Hz), 3.96 (t, 1 H, J = 2.64 Hz), 4.06–4.10 (m, 4 H), 4.24–4.38 (m, 4 H), 4.81 (s, 2 H), 4.82 (m, 1 H), 5.10–5.33 (m, 8 H), 5.83–6.03 (m, 4 H), 7.24–7.41 (m, 10 H).

2,5-Di-O-benzyl-myo-inositol (**40**). Deallylation of **39** (0.5 g, 0.95 mmol) with 10% Pd/C and *p*-toluenesulfonic acid, as described for **17**, furnished **40** (solid, 0.2 g, 58%): mp 270–272 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.02 (t, 1 H, J = 9.0 Hz), 3.28–3.31 (m, 2 H), 3.54–3.62 (m, 2 H), 3.71 (t, 1 H, J = 2.4 Hz), 4.73 (d, 2 H, J = 4.8 Hz), 4.76 (s, 4 H), 4.81 (d, 2 H, J = 4.8 Hz), 7.19–7.42 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 72.07 (2 C), 72.87 (2 C), 73.4, 73.96 (2 C), 81.44, 83.98, 126.69 (2 C), 126.82 (2 C), 127.28 (2 C), 127.65 (2 C), 127.72 (2 C), 139.71. Anal. Calcd for $C_{18}H_{20}O_6$: C, 65.05; H, 6.06. Found: C, 65.48; H, 5.99.

2,5-Di-O-benzyl-myo-inositol 1,3,4,6-Tetrakis(dibenzyl phosphate) (**41**). The tetrol **40** (0.2 g, 0.55 mmol) was phosphorylated, as described for **5**, to afford **41** (syrup, 0.7 g, 91%): ^{1}H NMR ($CDCl_3$) δ 3.45–3.51 (m, 2 H), 4.21–4.28 (m, 2 H), 4.64–5.09 (m, 22 H), 7.08–7.28 (m, 50 H).

myo-Inositol 1,3,4,6-Tetrakisphosphate [*Ins(1,3,4,6)P$_4$*]. Hydrogenolysis of the foregoing intermediate **40** (0.6 g, 0.42 mmol) followed by KOH titration and lyophilization, as described for Ins(4,5)P_2, gave Ins(1,3,4,6)P_4 as the octapotassium salt (0.34 g, 99%): ^{1}H NMR (D_2O) δ 3.39 (t, 1 H, J = 9 Hz), 3.74–3.80 (m, 2 H), 4.08 (q, 2 H, J = 9.3, 13.9 Hz), 4.45 (t, 1 H, J = 1.25 Hz); ^{31}P NMR (D_2O, external H_3PO_4) δ 3.23 (2 P), 4.05 (2 P).

(±)-1,2-O-Cyclohexylidene-myo-inositol (**42**). Selective hydrolysis of the *trans*-cyclohexylidene of (±)-**32** (1.5 g, 4.4 mmol) with acetyl chloride in CH_2Cl_2–CH_3OH, as described for **4**, gave (±)-**42** (solid, 1 g, 82%): mp 173–174 °C; ^{1}H NMR (CD_3OD) δ 1.47–1.71 (m, 10 H), 3.11 (t, 1 H, J = 9.3 Hz), 3.47–3.68 (m, 3 H), 3.91 (dd, 1 H, J = 5.03, 7.46 Hz), 4.35 (t, 1 H, J = 4.47 Hz); ^{13}C NMR (CD_3OD) δ 24.61, 24.94, 26.11, 36.21, 39.13, 71.8, 73.85, 75.14, 77.01, 77.35, 80.25, 111.36. Anal. Calcd for $C_{12}H_{20}O_6$: C, 55.37; H, 7.47. Found: C, 55.17; H, 7.89.

(±)-3,4,5,6-Tetra-O-allyl-1,2-O-cyclohexylidene-myo-inositol (**43**). Exhaustive allylation of (±)-**42** (0.9 g, 3.2 mmol), as described for **21**, provided (±)-**43** (syrup, 1.3 g, 92%); ^{1}H NMR ($CDCl_3$) δ 1.28–1.80 (m, 10 H), 3.16 (dd, 1 H, J = 8.75, 8.85 Hz), 3.49–3.59 (m, 2 H), 3.67 (t, 1 H, J = 8.75), 4.00 (dd, 1 H, J = 5.45, 6.25 Hz), 4.16–4.37 (m, 9 H), 5.12–5.20 (m, 4 H), 5.24–5.33 (m, 4 H), 5.87–6.03 (m, 4 H).

(±)-3,4,5,6-Tetra-O-allyl-myo-inositol (**44**). Hydrolysis of the *cis*-ketal of (±)-**43** (1.3 g, 3.2 mmol) with acetyl chloride in CH_2Cl_2–CH_3OH, as described for **14**, furnished (±)-**44** (syrup, 0.9 g, 85%): ^{1}H NMR ($CDCl_3$) δ 2.65 (br s, 2 H), 3.16–3.26 (m, 2 H), 3.40 (dd, 1 H, J = 2.7, 6.75 Hz), 3.56–3.69 (m, 2 H), 4.16–4.44 (m, 9 H), 5.12–5.33 (m, 8 H), 5.85–6.03 (m, 4 H).

1,3,4,5,6-Penta-O-allyl-myo-inositol (**45**). Regioselective allylation of (±)-**44** (0.85 g, 2.3 mmol) with allyl bromide, Bu_2SnO, and CsF, as described for **10**, gave **45** (syrup, 0.9 g, 95%): ^{1}H NMR ($CDCl_3$) δ 2.45 (br s, 1 H), 3.16–3.21 (m, 3 H), 3.67 (t, 1 H, J = 9.3 Hz), 4.15–4.19 (m, 6 H), 4.27–4.30 (m, 6 H), 5.11–5.20 (m, 5 H), 5.23–5.33 (m, 5 H), 5.86–6.03 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 71.71 (2 C), 74.0, 74.51 (2 C), 74.61, 74.74, 80.45 (2 C), 81.33 (2 C), 83.32, 116.36 (4 C), 127.26, 127.92 (2 C), 128.07 (2 C), 135.12 (2 C), 135.61 (2 C), 135.67 (2 C), 139.13.

1,3,4,5,6-Penta-O-allyl-2-O-benzyl-myo-inositol (**46**). Conventional benzylation of **45** (0.8 g, 2 mmol), as described for **3**, provided **46** (syrup, 0.8 g, 81%): ^{1}H NMR ($CDCl_3$) δ 3.11–3.19 (m, 3 H), 3.75 (t, 2 H, J = 6.6 Hz), 3.94 (t, 1 H, J = 2.4 Hz), 4.01–4.13 (m, 4 H), 4.22–4.35 (m, 6 H), 4.83 (s, 2 H), 5.10–5.17 (m, 5 H), 5.22–5.32 (m, 5 H), 5.82–6.03 (m, 5 H), 7.22–7.42 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 71.46 (2 C), 72.51 (2 C), 74.4, 75.37, 80.32, 127.92, 128.0 (2 C), 128.4 (2 C), 137.7.

2-O-Benzyl-myo-inositol (**47**). Deallylation of **46** (0.6 g, 1.2 mmol) with 10% Pd/C and *p*-toluenesulfonic acid, as described for **17**, furnished **47** (solid, 0.28 g, 80%): mp 248–250 (dec); ^{1}H NMR (D_2O) δ 3.10 (t, 1 H, J = 8.5 Hz), 3.49–3.61 (m, 4 H), 3.88 (t, 1 H, J = 2.6 Hz), 4.69 (s, 2 H), 7.23–7.35 (m, 5 H). Anal. Calcd for $C_{12}H_{16}O_6$: C, 56.24; H, 6.29. Found: C, 56.46; H, 6.47.

2-O-Benzyl-myo-inositol 1,3,4,5,6-Pentakis(dibenzyl phosphate) (**48**). The foregoing intermediate **47** (0.2 g, 0.69 mmol) was phosphorylated, as described for **5**, to afford **48** (syrup, 1 g, 91%): ^{1}H NMR ($CDCl_3$) δ 4.25–4.42 (m, 3 H), 4.73–4.75 (m, 3 H), 4.89–5.07 (m, 22 H), 7.13–7.25 (m, 55 H).

myo-Inositol 1,3,4,5,6-Pentakisphosphate [*Ins(1,3,4,5,6)-P$_5$*]. Hydrogenolysis of **48** (0.8 g, 0.5 mmol) followed by KOH titration and lyophilization, as described for Ins(4,5)-P_2, gave Ins(1,3,4,5,6)P_5 as the decapotassium salt (0.46 g, 99%): ^{1}H NMR (D_2O) δ 3.96 (t, 1 H, J = 3.3 Hz), 4.05 (br d, 1 H, J = 9.6 Hz), 4.37 (br s, 1 H), 4.41 (br s, 2 H), 4.45

(t, 1 H, J = 2.7 Hz); ^{31}P NMR (D_2O, external H_3PO_4) δ 4.49 (2 P), 4.89 (1 P), 5.42 (2 P).

Preparation of Crude Rat Brain Microsomes. Adult rat brain (250 g) was homogenized (Polytron, setting 7 and 5 strokes) in ice-cold buffer A, consisting of 0.32 M sucrose, 10 mM Tris-HCl (pH 7.3), 1 mM dithiothreitol (DTT), and 0.5 mM *p*-toluenesulfonyl fluoride (PMSF). The homogenate was centrifuged at 500*g* at 4 °C for 20 min, and the supernatant was first centrifuged at 12000*g* at 4 °C for 20 min to remove the mitochondria and then pelleted by centrifugation at 35000*g* at 4 °C for 40 min. The pellet was resuspended in buffer B, consisting of 20 mM Hepes–KOH (pH 7.3), 150 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT. The washing procedure was repeated two times. The resulting final pellet was suspended in buffer B to a protein concentration of 2–3 mg/mL.

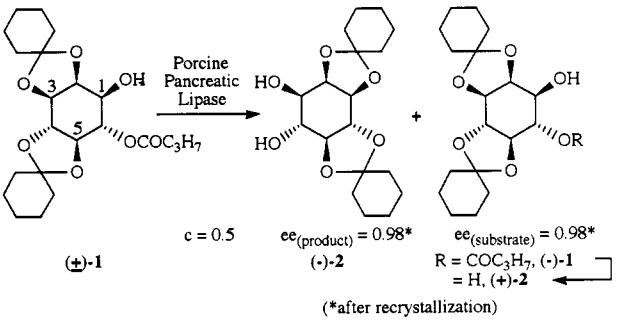
Ca^{2+} Release Assay. Measurements of free Ca^{2+} concentrations in incubation media were performed by using a Ca^{2+}-sensitive fluorescent dye, Fura-2, in a Hitachi F-2000 spectrofluorimeter. The assay medium consisted of 40 units of creatine kinase, 20 mM creatine phosphate, 5 μg of oligomycin, and 0.5 μM-Fura 2 free acid in 2 mL of buffer B containing 0.2–0.25 mg of microsomal proteins. 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. $[Ca^{2+}]_i$ was calculated according to the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where F_{max} and F_{min} are readings in the presence of 2.5 mM $CaCl_2$ and 1 mM EGTA, respectively; K_d denotes the apparent dissociation constant of the Ca^{2+}–dye complex. The Fura-2 fluorescence ratio signal was calibrated as described by Grynkiewicz et al. (1985).

Ins(1,4,5)P_3 Receptor-Binding Assay. Rat cerebellar microsomes were prepared according to the preceding procedure and suspended at 2 mg/mL in 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 1 mM EDTA, and 0.25 mM DTT. The [^{3}H]Ins(1,4,5)P_3-binding assay was performed by the membrane filtration method (Spät et al., 1986; Worley et al., 1987), in which 50 μg of microsomes in 250 μL of the same buffer was incubated with 2 nM [^{3}H]Ins(1,4,5)P_3 and individual inositol phosphates at the indicated concentrations for 30 min at 4 °C. Nonspecific binding was measured in the presence of 10 μM cold Ins(1,4,5)P_3. Incubations were terminated by filtration through phosphate-presoaked GF/C filters (Whatman), followed by 3 × 3 mL washes with the ice-cold incubation buffer. The receptor-bound radioactivity was analyzed by liquid scintillation spectrometry.

RESULTS

Systematic Synthesis of D-*myo-Inositol Polyphosphates.* Our key synthetic strategy focused on the preparation of a pair of enantiomerically active 1,2:5,6-dicyclohexylidene-*myo*-inositols, (+)- and (–)-**2**, as common precursors. Both enantiomers have a wide scope of application and allow the synthesis of virtually all of the inositol phosphates and inositol phospholipids. Preparation of the pure enantiomers of **2** was achieved by a facile enzymatic resolution, shown in Scheme 2. The 4-butyryl monoester of **2**, (±)-**1**, was subjected to enantiospecific hydrolysis by porcine pancreatic lipase in a biphasic mixture consisting of hexane–0.1 M potassium phosphate buffer (pH 7.0). Enantiospecificity of the enzymatic hydrolysis was greater in the biphasic system than in aqueous solutions in part due to the inhibition of contaminating proteases and esterases by the organic phase. Both product

Scheme 2: Enzymatic Preparation of (+)- and (–)-**2**



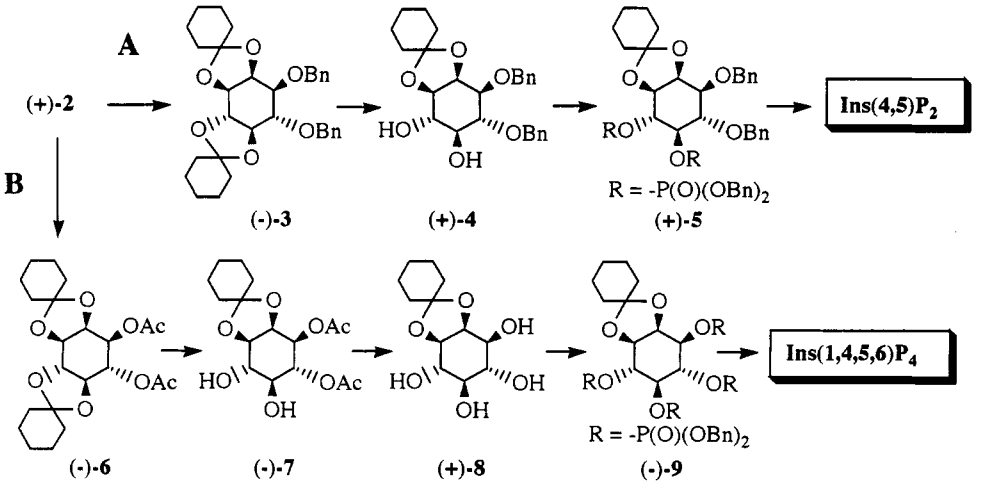
(–)-**2** and substrate (–)-**1** fractions were obtained with satisfactory optical purity (ee = 0.98) after recrystallization. This expedient and inexpensive method permitted the preparation of both antipodes of **2** in good quantities.

The synthetic versatility of **2** is underscored by the preferential removal of the *trans*-5,6-cyclohexylidene ring vs the *cis*-ketal and the regiospecific distinction of the vicinal diols (1- vs 6-OH) via the corresponding stannylene acetal. Accordingly, the combination of selective protections and deprotections led to a variety of useful intermediates for the synthesis of target inositol phosphates, the details of which are described in the following sections. The syntheses of Ins(1,4,5)P_3 from (+)-**2** and of Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 from (–)-**2** have been described elsewhere (Gou et al., 1992) and will not be elaborated here.

Synthetic Utility of (+)-2. As shown in Scheme 3, (+)-**2** provided a convenient route to both Ins(4,5)P_2 and Ins(1,4,5,6)P_4. Exhaustive benzylation of (+)-**2** in tandem with the selective hydrolysis of the *trans*-cyclohexylidene group afforded a key intermediate, (+)-**4**. Phosphorylation of the diol **4** by the phosphoramidite method, followed by debenzylation and removal of the *cis*-cyclohexylidene group, gave Ins(4,5)P_2 with a 65% overall yield from (+)-**2**. Efficient use of (+)-**2** to prepare Ins(1,4,5,6)P_4 was made by selectively removing the *trans*-cyclohexylidene via the 1,6-diacetyl ester (–)-**6**, followed by alkaline hydrolysis to yield (+)-**8**. Accordingly, the tetrol **8** was transformed into Ins(1,4,5,6)P_4 [77% from (+)-**2**].

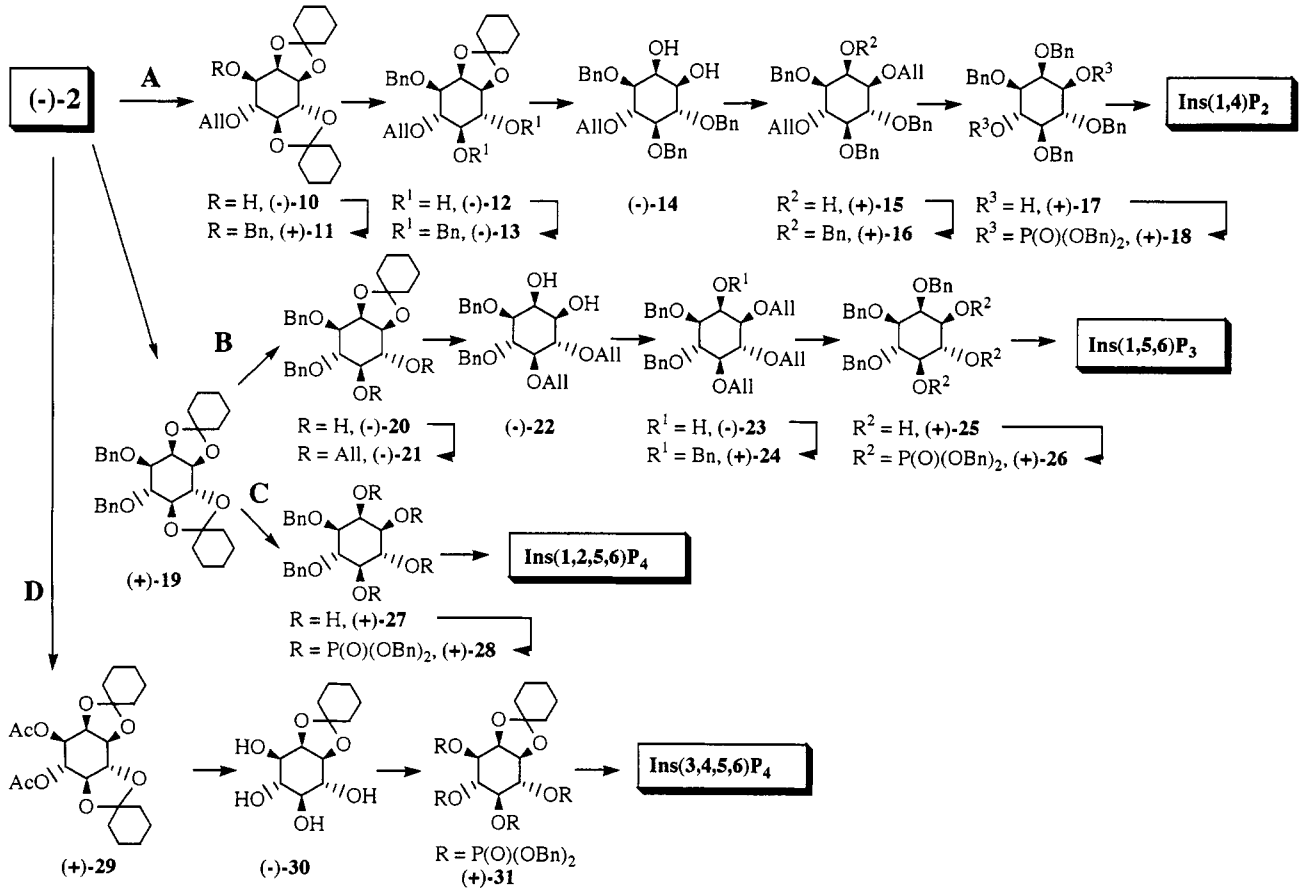
Synthetic Utility of (–)-2. Retrosynthetically, Ins(1,4)P_2, Ins(1,5,6)P_3, Ins(1,2,5,6)P_4, and Ins(3,4,5,6)P_4 could be obtained from (–)-**2**, as illustrated in Scheme 4. Regiospecific introduction of allyl and benzyl groups to the C-4 and C-3 hydroxyl functions, respectively, of (–)-**2** gave the fully protected derivative (+)-**11**. Selective removal of the *trans*-ketal, followed by exhaustive benzylation, afforded (–)-**13**, the methanolysis of which provided the diol (–)-**14**. 1-*O*-Allylation of **14** yielded (+)-**15**, and subsequent benzylation at the 2-OH followed by deallylation with Pd/C and *p*-toluenesulfonic acid furnished a crucial diol, (+)-**17**. Phosphorylation and debenzylation of **17** gave Ins(1,4)P_2 [21% from (–)-**2**]. With regard to the synthesis of Ins(1,5,6)P_3, (–)-**2** was subjected to exhaustive benzylation to give (+)-**19**, and the selective hydrolysis of the *trans*-cyclohexylidene group yielded (–)-**20**. Di-*O*-allylation of **20** provided (–)-**21**, and removal of the *cis*-ketal by methanolysis gave the diol (–)-**22**. 1-*O*-Allylation of **22** provided (–)-**23**, and benzylation at the 2-OH followed by deallylation furnished a pertinently protected triol (+)-**25**, which afforded Ins(1,5,6)P_3 accordingly, with an overall yield of 39% from (–)-**2**. Alternatively, (+)-**19** could undergo exhaustive methanolysis to afford 3,4-di-*O*-benzyl-D-*myo*-inositol (+)-**27**, which was then converted to Ins(1,2,5,6)P_4 [77% from (–)-**2**]. The use of (–)-**2** to prepare Ins(3,4,5,6)P_4 was made in a straightforward manner. To

Scheme 3: Synthetic Utility of (+)-2[a]



[a] Route A, Ins(4,5)P_2 synthesis; route B, Ins(1,4,5,6)P_4 synthesis.

Scheme 4: Synthetic Utility of (−)-2[a]

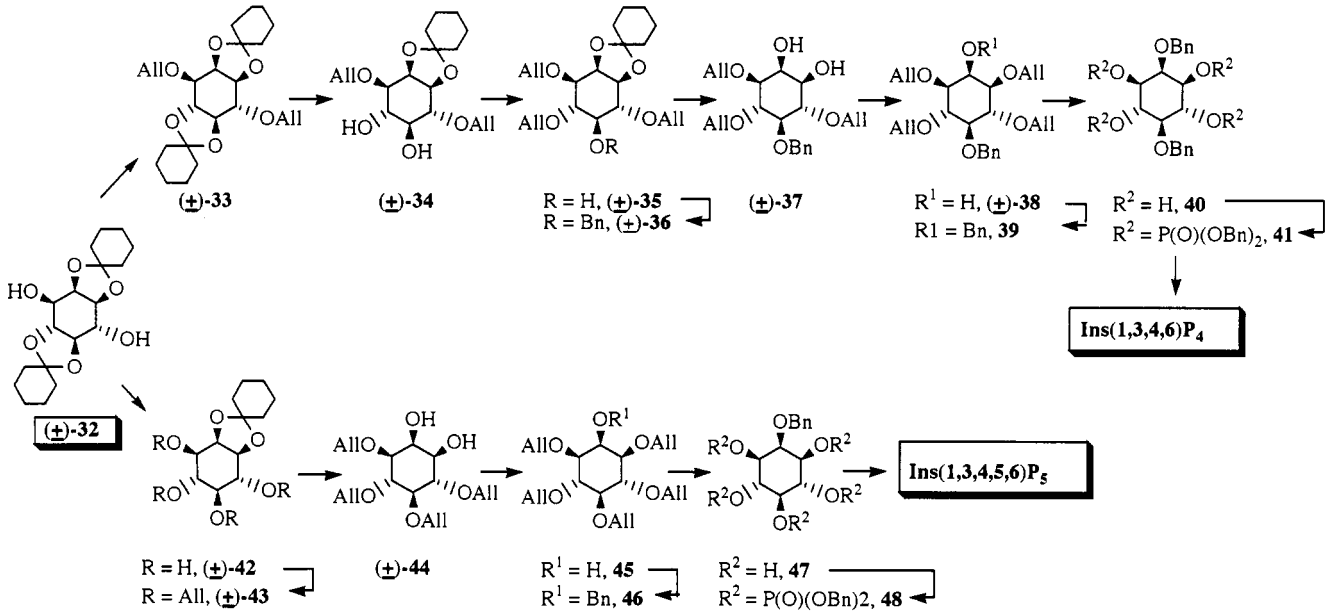


[a] Route A, Ins(1,4)P_2 synthesis; route B, Ins(1,5,6)P_3 synthesis; route C, Ins(1,2,5,6)P_4 synthesis; route D, Ins(3,4,5,6)P_4 synthesis.

prevent ketal migration, (−)-**2** was subjected to acetylation to afford (+)-**29**. Selective removal of the *trans*-cyclohexylidene group furnished the tetrol (−)-**30**, the phosphorylation and then debenzylation of which provided the target molecule with an overall yield of 79% from (−)-**2**.

Syntheses of Ins(1,3,4,6)P_4 and Ins(1,3,4,5,6)P_5. In view of the symmetric nature of Ins(1,3,4,6)P_4 and Ins(1,3,4,5,6)-P_5, the syntheses did not call for the use of (+)- or (−)-**2** as a chiral starting material. Thus, racemic 1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (**32**), a major byproduct from the preparation of **2**, was employed as a precursor to Ins-(1,3,4,6)P_4 and Ins(1,3,4,5,6)P_5 (Scheme 5). Di-*O*-allylation of **32** in conjunction with the selective removal of the *trans*-cyclohexylidene group gave the diol **34**. Regiospecific allylation of **34** at the C-6 hydroxyl and subsequent benzylation led to the fully protected derivative **36**, the methanolysis of which afforded **37**. The diol **37** underwent regioselective allylation at the 1-OH position. This was followed by 2-*O*-benzylation and subsequent deallylation to furnish the tetrol **40**. Phosphorylation and then debenzylation of **40** provided Ins(1,3,4,6)P_4 (17% from **32**). The pentakisphosphate Ins-(1,3,4,5,6)P_5 was synthesized in a similar manner. Selective removal of the *trans*-ketal of **32** followed by exhaustive allylation and subsequent methanolysis afforded the diol **44**. Again, regiospecific introduction of allyl and benzyl groups to the hydroxyl functions at C-1 and C-2, respectively, provided

Scheme 5: Synthesis of Ins(1,3,4,6)P_4 and Ins(1,3,4,5,6)P_5



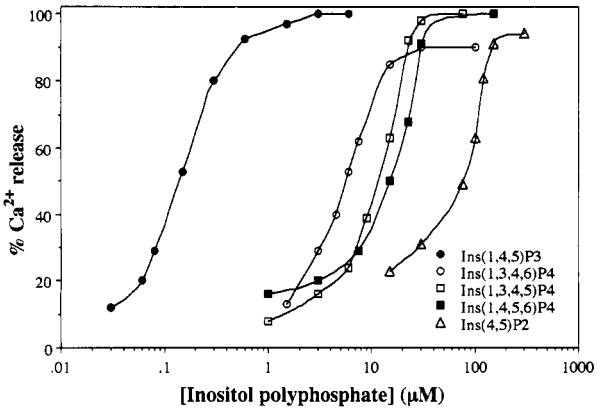


FIGURE 1: Inositol phosphate-induced Ca^{2+} release from rat brain microsomes. Ca^{2+}-loaded rat brain microsomes were treated with Ca^{2+}-mobilizing agents, and the released Ca^{2+} was monitored by a Ca^{2+}-sensitive fluorescent dye, Fura-2, according to the method described in the Experimental Procedures [100% = Ca^{2+} release at saturated concentrations of Ins(1,4,5)P_3]. Each data point represents the mean of three determinations.

a fully protected derivative **46** which, after deallylation, led to 2-*O*-benzyl-*myo*-inositol (**47**). Phosphorylation and then debenzylation of **47** gave Ins(1,3,4,5,6)P_5 with an overall yield of 35% from **32**.

The chemical purity of these synthetic inositol phosphates was greater than 97% according to ^1H and ^{31}P NMR spectroscopy. The amounts of isomeric impurities were negligible, as indicated by these NMR spectra.

Inositol Phosphate-Induced Ca^{2+} Release from Rat Brain Microsomes. Ca^{2+}-loaded rat brain microsomes were treated with individual synthetic D-*myo*-inositol phosphates at 37 °C, and the released Ca^{2+} was monitored by bulk fluorimetry using Fura-2 as an indicator. Of the 11 phosphoinositols examined, Ins(1,4,5)P_3, Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, and Ins(4,5)P_2 exhibited Ca^{2+}-mobilizing activity in a dose-dependent manner (Figure 1), with apparent EC_{50} values of 0.13, 5, 13, 15, and 60 μM, respectively. It is worth noting that Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, and Ins(4,5)P_2 were capable of mobilizing the entire Ins(1,4,5)P_3-sensitive Ca^{2+} store of rat brain microsomes, albeit at much lower potency. The Ca^{2+}-mobilizing activities of Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, and Ins(4,5)P_2 were 38-fold, 100-fold, 115-fold, and 460-fold, respectively, lower than that of Ins(1,4,5)P_3. Ins(1,3,4,6)P_4 (Ivorra et al., 1991; Gawler et al., 1991), Ins(1,3,4,5)P_4 (Wilcox et al., 1993), and Ins(4,5)P_2 (Burgess et al., 1984; Irvine et al., 1984) have been reported to be active in Ca^{2+} mobilization. Their reported potencies were in line with those of this study.

Other inositol phosphates including Ins(1,4)P_2, Ins(1,5,6)P_3, Ins(1,3,4)P_3, Ins(3,4,5,6)P_4, Ins(1,2,5,6)P_4, and Ins(1,3,4,5,6)P_5 failed to exert appreciable Ca^{2+} release from the microsomal preparation, even at concentrations up to 100 μM.

Inositol Phosphate Displacement of Specific [^3H]Ins(1,4,5)P_3 Binding in Rat Cerebellar Membranes. To assess the binding of inositol phosphates to the Ins(1,4,5)P_3 receptor, displacement of specific [^3H]Ins(1,4,5)P_3 binding was carried out using rat cerebellar membrane preparations. Figure 2A,B illustrates the displacement curves for the inositol phosphates examined. Accordingly, the mean IC_{50} values for individual compounds were determined as follows: Ins(1,4,5)P_3, 0.031 μM; Ins(1,4,5,6)P_4, 1.7 μM; Ins(1,3,4,5)P_4, 1.8 μM; Ins(1,3,4,6)P_4, 2.3 μM; Ins(4,5)P_2, 24.3 μM; Ins(1,3,4,5,6)P_5, 41 μM; Ins(3,4,5,6)P_4, 58 μM; Ins(1,2,5,6)P_4, 63 μM; Ins(1,3,4)P_3, 125 μM; Ins(1,4)P_2, 315 μM; Ins(1,5,6)P_3, 375 μM. For the inositol phosphates capable of effecting Ca^{2+} mobilization, the relative potency of inhibiting [^3H]Ins(1,4,5)P_3 binding to cerebellar membranes was in line with the order of the EC_{50} values. Those molecules that failed to elicit Ca^{2+} mobilization were weak ligands for the receptor, presumably through nonspecific electrostatic interactions. The IC_{50} values were 1500–12000-fold higher than that of Ins(1,4,5)P_3.

DISCUSSION

The metabolic turnover of inositol phosphates in various cell types generates more than 20 different metabolites (Majerus et al., 1988; Fisher et al., 1992; Shears, 1992; Menniti et al., 1993). To date, studies on the biochemical relevance of these inositol phosphates have primarily focused on the roles of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in modulating cytosolic [Ca^{2+}] (Berridge, 1993). However, evidence is mounting that other inositol phosphates may also participate in important cellular functions. As part of our systematic effort to unveil the biological utility of these phosphoinositols, in this paper we examined the molecular recognition of endogenous inositol

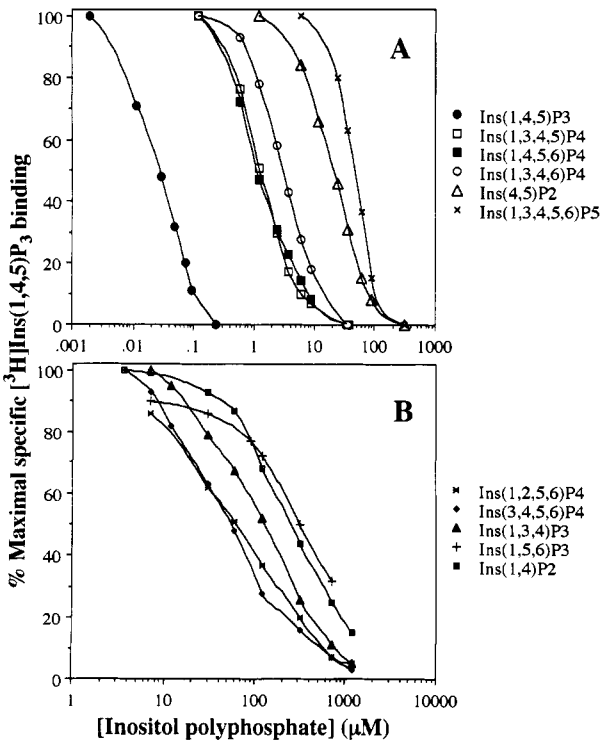


FIGURE 2: Inhibition of specific [^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar microsomes by increasing concentrations of inositol phosphates: (A) Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4,6)-P_4, Ins(4,5)P_2, and Ins(1,3,4,5,6)P_5; (B) Ins(1,2,5,6)P_4, Ins(3,4,5,6)P_4, Ins(1,3,4)P_3, Ins(1,5,6)P_3, and Ins(1,4)P_2. Nonspecific binding was measured in the presence of 10 μM cold Ins(1,4,5)P_3. Each data point represents the mean of at least three determinations.

phosphates at the Ins(1,4,5)P_3-specific receptor. Eleven inositol phosphate congeners, including Ins(1,3,4,5,6)P_5, Ins-(1,2,5,6)P_4, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4, Ins-(3,4,5,6)P_4, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(1,5,6)P_3, Ins(1,4)-P_2, and Ins(4,5)P_2, were synthesized, many of which are unavailable from commercial sources. All but Ins(1,5,6)P_3 and Ins(1,2,5,6)P_4 are naturally occurring compounds. These optically active inositol phosphates were independently synthesized from (+)- or (−)-**2** in good yields and were fully characterized by ^{1}H and ^{31}P NMR, with both chemical and optical purities greater than 97%.

The present data clearly show that many of the inositol phosphates derived from Ins(1,4,5)P_3 metabolism are capable of eliciting intracellular Ca^{2+} release, however, at lower potency than the parent compound. Of the 11 compounds examined, the Ca^{2+}-releasing inositol phosphates include, in the order of EC_{50} values, Ins(1,4,5)P_3, Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, and Ins(4,5)P_2. Binding experiments using rat cerebella membrane as a source of Ins(1,4,5)P_3-specific receptor further suggest that the ability of these molecules to elicit Ca^{2+} mobilization arises from interactions with the Ins-(1,4,5)P_3-specific receptor. As noted in Figure 2A, for these inositol phosphates, the range of concentrations for complete displacement of [^{3}H]Ins(1,4,5)P_3 binding was approximately 1 log unit, which is a good indication of specific binding. Moreover, it is worth mentioning that Ins(1,3,4,6)P_4 is a potent agonist at the Ins(1,4,5)P_3 receptors of rat cerebella microsomes. The Ca^{2+}-releasing activity of Ins(1,3,4,6)P_4 has also been observed in microinjected *Xenopus* oocytes (Ivorra et al., 1991) and in permeabilized human neuroblastoma cells (Gawler et al., 1991), with potencies comparable to that found in this study (EC_{50} = 5 μM). With the unique symmetric nature of the molecule, Ins(1,3,4,6)P_4 may assume alternative binding conformations to the receptor, in which the important recognition features of Ins(1,4,5)P_3 can be mimicked (Potter & Nahorski, 1992; Mills et al., 1993). Previously, Gawler et al. (1991) indicated that DL-Ins(1,4,5,6)P_4 did not exhibit Ca^{2+}-releasing activity at concentrations up to 10 μM. As mentioned earlier, racemic Ins(1,4,5,6)P_4 is a mixture of Ins-(1,4,5,6)P_4 and Ins(3,4,5,6)P_4. In view of the facts that the latter compound is inactive in Ca^{2+} mobilization and that Ins(1,4,5,6)P_4 has an EC_{50} value of 15 μM, the Ca^{2+}-mobilizing activity of the racemic mixture at 10 μM might not be appreciably noticed.

The structure–activity relationships between inositol phosphates and the Ins(1,4,5)P_3 recognition sites have been the focus of discussion in many papers (Kozikowski et al., 1990, 1993a,b; Safrany et al., 1992a,b; Potter & Nahorski, 1992; Mills et al., 1993). It has generally been perceived that the vicinal 4,5-bisphosphate motif assumes an obligatory role in effecting productive binding with the Ins(1,4,5)P_3 receptor. The current study indicates that the absence of either the 4- or 5-phosphate results in the complete loss of agonist property, as evidenced by the lack of activity in Ins(1,4)P_2, Ins(1,3,4)-P_3, Ins(1,5,6)P_3, and Ins(1,2,5,6)P_4 even at concentrations up to 100 μM. The importance of the 1-phosphate is implied by the potency of Ins(4,5)P_2 relative to that of Ins(1,4,5)P_3. Comparison of the Ca^{2+} release and receptor-binding data indicates that Ins(4,5)P_2 is a 460-fold weaker agonist and a 800-fold weaker ligand than Ins(1,4,5)P_3. Moreover, it is noteworthy that structural variants of Ins(1,4,5)P_3 with modification of the phosphate position, such as Ins(2,4,5)P_3 (Burgess, 1984; Loomis-Husselbee & Dawson, 1993), Ins-(1:2cyc,4,5)P_3 (Wilson et al., 1985; Irvine et al., 1986), and Ins(1,2,4,5)P_4 (Mills et al., 1993b; Hirata et al., 1994), were reported to retain full agonist and ligand properties. These results suggest that the 1-phosphate group contributes substantially to the binding through charge–charge interactions and/or hydrogen bonding with the binding domain. The ionic interaction is thought to be of a long-range nature to account for the relaxed stereochemical and positional specificities in recognizing this phosphate moiety.

Previously, Kozikowski et al. (1993a) proposed that the vicinal 2,3-dihydroxyl functions played a nonessential role in receptor recognition on the basis of the finding that D-3-deoxy-, D-2,3-dideoxy-, and D-3-deoxy-3-fluoro-Ins(1,4,5)P_3 exhibited very potent ligand and agonist properties. Nevertheless, the present evidence indicates that Ins(1,3,4,5)P_4 is a much weaker agonist than Ins(1,4,5)P_3. Presumably, electrostatic repulsion of the three adjacent phosphate functions alters the conformation of the 4,5-bisphosphate motif, which weakens the binding affinity. In contrast, the contribution of the 6-hydroxyl function appears to be prominent. One result from the work of Kozikowski et al. (1993a,b) on D-2,3,6-trideoxy-Ins(1,4,5)-P_3 supports this premise, as it was shown to be a much weaker agonist than 2,3-dideoxy-Ins(1,4,5)P_3. Also, we previously prepared 6-*O*-(ω-aminohexyl)-Ins(1,4,5)P_3 for the preparation of anti-Ins(1,4,5)P_3 antibodies (Gou et al., 1994). The Ca^{2+}-mobilizing activity of this 6-*O*-substituted molecule was about 200-fold less than that of the parent molecule. Structural analysis indicates that all Ca^{2+}-mobilizing inositol phosphates, including Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4, mimic or display a structure similar to that of the 4,5-bisphosphate 6-hydroxy motif of Ins(1,4,5)P_3 (Figure 3).

On the basis of these findings, we propose a binding model to account for ligand recognition at the Ins(1,4,5)P_3 receptor (Figure 4). The binding site of the Ins(1,4,5)P_3 receptor is assumed to be composed of two domains. The anchoring domain interacts with the 4,5-bisphosphate 6-hydroxy motif, attributing to the Ca^{2+}-mobilizing activity. Disruption of this

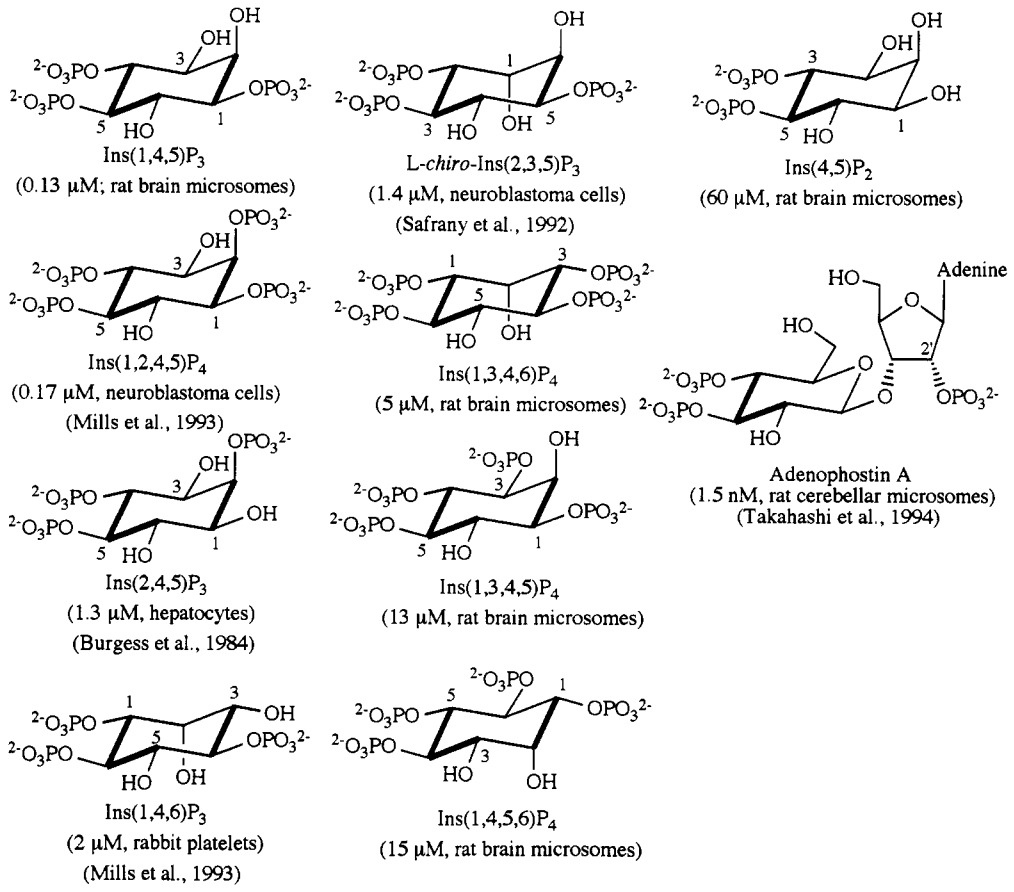


FIGURE 3: Structures of Ca^{2+}-mobilizing inositol phosphates and adenophostin A. All of these molecules assume conformations sharing or mimicking the structural feature (marked in bold) of the 4,5-bisphosphate 6-hydroxy and 1-phosphate motifs of Ins(1,4,5)P_3. Indicated in the parentheses are the EC_{50} value and the source of Ins(1,4,5)P_3 receptor for the binding assay.

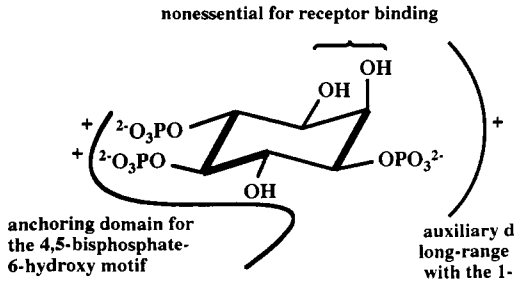


FIGURE 4: Ligand recognition at the Ins(1,4,5)P_3-binding site. The 4,5-bisphosphate 6-hydroxy and 1-phosphate motifs (marked in bold) of Ins(1,4,5)P_3 represent structural features essential to its Ca^{2+}-mobilizing and binding properties. Evidence suggests that the 2- and 3-OH functions do not have a crucial role in receptor binding.

structural feature, especially the bisphosphate moiety, significantly diminishes or even abolishes the agonist activity. The auxiliary domain exerts long-range electrostatic interactions with the 1-phosphate group, which enhances the binding affinity. The stereochemical requirement for this phosphate recognition appears to be less stringent. As mentioned earlier, structural variants with modification of the position and stereochemistry of the 1-phosphate, such as Ins(2,4,5)P_3, still retain full agonist activity. Moreover, due to the symmetric nature of the *myo*-inositol ring and the nonessential role of the 2- and 3-hydroxyl functions in receptor binding, inositol phosphates are able to assume alternative conformations, some of which mimic those structural motifs essential for productive binding. This supposition is supported by the Ca^{2+}-releasing activity of Ins(1,3,4,6)P_4 and Ins(1,4,6)P_3 (Mills et al., 1993a). Comparison of the structures of both molecules to that of Ins(1,4,5)P_3 (Figure 3) indicates that the 1,6-bisphosphate 5-hydroxy structure resembles the 4,5-bisphosphate 6-hydroxy motif, while the 4-phosphate corresponds to the 1-phosphate. On the basis of a similar analogy, one would also expect Ins(1,3,6)P_3 to be a partial agonist.

The proposed model accounts for the high potency of adenophostins (Figure 3) in Ca^{2+} mobilization, which was reported recently by Takahashi et al. (1994). Adenophostin A and B, metabolites isolated from *Penicillium brevicompactum*, showed Ca^{2+}-releasing and receptor-binding activities 100-fold stronger than those of Ins(1,4,5)P_3. Although adenophostins and inositol phosphates are structurally unrelated molecules, one may envisage that these phosphoglucose derivatives display structural features for Ins(1,4,5)P_3 receptor recognition. Moreover, the fact that the auxiliary domain accommodates the bulky 2′-phosphoadenosine moiety suggests the size of the Ins(1,4,5)P_3-binding cavity. This information lends support to our supposition that the 1-phosphate of the bound Ins(1,4,5)P_3 exerts a long-range ionic interaction with the receptor. For adenophostins, tight binding due to close proximity between the 2′-phosphate and the charged group on the receptor may account for their strong agonist activity. It is also worth noting that the removal of the 2′-phosphate function on the ribose ring of adenophostin A attenuated the activity by 1000-fold, which is in line with the relative potency of Ins(4,5)P_2 to Ins(1,4,5)P_3 (Takahsshi et al., 1994).

Heparin (Ghosh et al., 1988) and related sulfated polysaccharides and decavanadate (Foehr et al., 1989) are known to competitively inhibit Ins(1,4,5)P_3 binding to its receptor. However, none of these antagonists is specific. It is likely that these negatively charged molecules gain access to the receptor through nonspecific electrostatic interactions with the binding domains, thus blocking the access of Ins(1,4,5)P_3 to the pocket.

In summary, we have demonstrated that many intracellular inositol phosphates are capable of effecting Ca^{2+} mobilization by interacting with Ins(1,4,5)P_3-specific receptors. At present, the biochemical implication of this observation remains to be explored since information on their intracellular distribution and concentrations as well as susceptibility to agonist stimulation is still lacking. One school of thought is that these inositol phosphates exert a concerted, although redundant, effort to maintain cytosolic [Ca^{2+}]. However, one can argue that the Ca^{2+}-mobilizing activity of these molecules is coincidental due to the sharing of common structural motifs. Several lines of evidence have indicated that some of these polyphosphates may be involved in cellular functions distinct from Ca^{2+} mobilization. Thus, further examinations of the biochemical functions of these intracellular inositol phosphates constitute the focus of this investigation.

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