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Synthesis and Ca^{2+}-release activity of D- and L-*myo*-inositol 2,4,5-trisphosphate and D- and L-*chiro*-inositol 1,3,4-trisphosphate

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ABSTRACT

Partial benzoylation of the 3,4-dibenzyl ethers of D- and L-*chiro*-inositol provided the 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-*chiro*-inositols. Inversion of the free axial hydroxyl group gave a mixture of chiral 1,3,4- and 1,2,4-tri-*O*-benzoyl-5,6-di-*O*-benzyl-*myo*-inositols [W. Tegge and C. E. Ballou, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 94–98]. Catalytic hydrogenolysis cleaved the benzyl ether groups of the 1,3,4-tri-*O*-benzoyl-5,6-di-*O*-benzyl-*myo*-inositols (D- and L-) to yield the 1,3,4-tri-*O*-benzoyl-*myo*-inositols, which were phosphorylated by a dibenzyl phosphoramidite method. Removal of all blocking groups gave the pure enantiomeric *myo*-inositol 2,4,5-trisphosphates. Syntheses of the *chiro*-inositol 1,3,4-trisphosphates, which are analogs of the *myo*-inositol 1,4,5-trisphosphates having an axial phosphate group at position 1, or analogs of the *myo*-inositol 2,4,5-triphosphates having an axial hydroxyl at position 1, were also devised starting with the 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-*chiro*-inositols. In a calcium-release assay with saponin-permeabilized rat basophilic leukemia cells, the D isomers of both of these analogs had EC_{50} values of 4 μM, compared with a value of 0.17 μM for D-*myo*-inositol 1,4,5-trisphosphate, whereas the L isomers had EC_{50} values of about 100 μM.

INTRODUCTION

The biological function of D-*myo*-inositol 1,4,5-trisphosphate (D-*myo*-1,4,5-IP_3) as a second messenger for the mobilization of intracellular Ca^{2+} ions is well documented[1]. D-*myo*-1,4,5-IP_3 acts by binding to a specific receptor protein in the membrane of the endoplasmic reticulum (ER) and stimulating the release of Ca^{2+} from the ER lumen into the cytosol. Several other inositol phosphates have been detected in living cells, including D-*myo*-inositol 1,3,4-trisphosphate, D-*myo*-inositol 2,4,5-trisphosphate, and D-*myo*-inositol 1,3,4,5-tetrakisphosphate, but their function is still unclear. Among the most potent of these analogs, in terms of Ca^{2+} release, is D-*myo*-inositol 2,4,5-trisphosphate (D-*myo*-2,4,5-IP_3)[2–6]. The compound has been identified in animal tissues, but whether it is formed naturally or by chemical degradation of other inositol phosphates during extraction is unknown. It could result from hydrolysis of D-*myo*-inositol 1,2-(cyclic)phosphate 4,5-bisphosphate, which is produced in small amounts by the action of phospholipase C on 1-phosphatidyl-D-*myo*-inositol 4,5-bisphosphate[7].

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Several reports have dealt with ongoing attempts to identify the relative importance of the phosphate groups at positions 1, 4, and 5 of the *myo*-inositol ring. Removal of the phosphate at position 5 completely inactivates the ligand with respect to its ability to effect Ca^{2+} release[4,8], whereas derivatization[9], cyclization[7], or removal of the 1-phosphate[4] reduces but does not eliminate activity. In previous investigations, D-*myo*-2,4,5-IP_3 was found to be 6-fold[4] or 13-fold[6] less active than D-*myo*-1,4,5-IP_3 in the Ca^{2+}-release assay, whereas synthetic D,L-*myo*-2,4,5-IP_3 was 29-fold[2] or 49-fold[5] less active. In these investigations, the *myo*-2,4,5-IP_3 was prepared by procedures that could have led to contamination with D-*myo*-1,4,5-IP_3.

Most of the methods for the chemical synthesis of inositol phosphates start with *myo*-inositol and yield racemic intermediates that require resolution[10,11]. Consequently, enantiomeric purity is open to question. We have described syntheses of D- and L-*myo*-1,4,5-IP_3, however, that start with D- and L-*chiro*-inositols and yield the pure enantiomeric *myo*-inositol derivatives without the necessity for resolution of racemic mxitures[12], while others have reported a synthesis of D-*myo*-inositol 1-phosphate[13] from quebrachitol. In an extension of this strategy, we have now synthesized pure, crystalline D- and L-*myo*-2,4,5-IP_3, as well as D- and L-*chiro*-inositol 1,3,4-trisphosphate (D- and L-*chiro*-1,3,4,-IP_3), by procedures that preclude contamination with *myo*-1,4,5-IP_3. The *chiro*-1,3,4-IP_3 can be regarded as an analog of *myo*-1,4,5-IP_3 in which the 1-phosphate group is axial instead of equatorial, or as an analog of *myo*-2,4,5-IP_3 in which the 1-hydroxyl group is axial instead of equatorial (Fig. 1). To assess the effects of these stereochemical changes on the Ca^{2+}-release potential of the analogs, we have assayed their activity with saponin-permeabilized rat basophilic leukemia (RBL) cells[14].

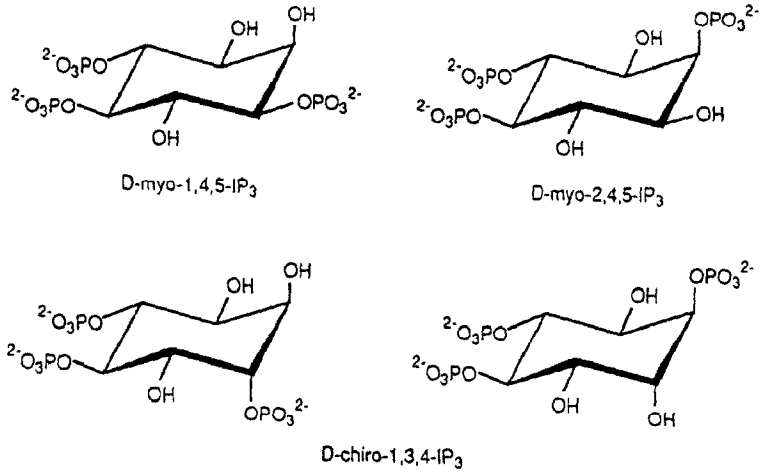


Fig. 1. Comparison of D-*myo*-1,4,5-IP_3 and D-*myo*-2,4,5-IP_3 to two representations of D-*chiro*-1,3,4-IP_3, one rotated about the axis of symmetry 180° with respect to the other.

EXPERIMENTAL

Materials. — 1,2,5-Tri-*O*-benzoyl-3,4-di-*O*-benzyl-*chiro*-inositols (D and L) and 1,3,4-tri-*O*-benzoyl-*myo*-inositols (D and L) were prepared according to our previously published procedure[12]. While optimizing the preparation of 1,3,4-tri-*O*-benzoyl-*myo*-inositols, we found it advantageous to separate the mixture of 1,2,4- and 1,3,4-tri-*O*-benzoyl-5,6-di-*O*-benzoyl-*myo*-inositol by preparative high performance liquid chromatography before hydrogenolysis of the benzyl ether groups. Like the corresponding L-isomer reported earlier[12], the crystalline 1,3,4-tri-*O*-benzoyl-D-*myo*-inositol had a broad melting point (178–185°) although it was pure according to h.p.l.c., n.m.r., and combustion analysis; $[\alpha]_{546}$ +23.5° (dioxane).

Anal. Calc. for $C_{27}H_{24}O_9$ (492.5): C, 65.85; H, 4.92. Found: C, 65.34; H, 4.92.

D,L-*myo*-Inositol 4,5-bisphosphate was a gift from Stephen Angyal[15] while glycerol 1,2- and 1,3-bisphosphates were from laboratory stocks[16].

Dibenzyl di-*N*-isopropylphosphoramidite[17] was purified by column chromatography on silica gel, using 10:1 petroleum ether–triethylamine as eluent. The compound appeared in the first fraction. *m*-Chloroperoxybenzoic acid was from CTC Organics (Atlanta) and the fluorescent calcium indicator fluo-3 (free acid) was from Molecular Probes. Most other organic reagents were from Aldrich.

Analytical and preparative methods. — Analytical h.p.l.c. was carried out on a Supelco LC18 (5 μm) stainless steel column (0.4 × 25 cm) by elution with methanol–water at a flow rate of 1.2 mL min^{-1}. An LKB 2138 Uvicord S, equipped with a microflow cell and a 254-nm filter, was used to monitor the effluent. For preparative h.p.l.c., a stainless steel column (5 × 50 cm) (HT Chemicals, St. Louis) was packed manually with Merck LiChroprep RP-18 (25–40 μm) (EM Scientific, Cherry Hill, NJ) and eluted with methanol–water at a flow rate of 25 mL min^{-1}. The UV detector was equipped with a preparative flow cell, and solvents were mixed and delivered by a Waters Prep LC3000 system. Melting points were determined in capillaries on a Thomas–Hoover apparatus and are uncorrected. Optical activity was measured on a Bendix 1100 Automatic Polarimeter having an Hg lamp (546 nm).

^{31}P-N.m.r. spectra, referenced to external 85% phosphoric acid, were determined on a Bruker 400 MHz spectrometer, and ^{1}H-n.m.r. spectra, measured from internal tetramethylsilane or acetone (2.225 p.p.m.), were determined on a Bruker 400 or 500 MHz spectrometer in the Chemistry Department, University of California, Berkeley. Combustion analyses were performed in the Chemistry Department Microchemical Analysis laboratory. Hydrogenolyses were carried out under one atmosphere of H_2 in a boat mounted on an Eberbach reciprocal shaker. The calcium ion-dependent fluorescence of fluo-3 was measured with an SLM-8000 fluorimeter interfaced to an IBM PC. Measurements were made in 1-cm acrylic cuvets (Sarstedt), with excitation at 488 nm and emission measurement at 520 nm.

Determination of the calcium-release potential in permeabilized rat basophilic leukemia cells. — The calcium-release potential of the different phosphate compounds was determined in the system described by Meyer *et al*[14]. RBL cells were harvested after

4–5 days, treated with trypsin, and diluted into 16 mL of a Ca^{2+}- and Mg^{2+}-free buffer containing 20mM HEPES (pH 7.4), 140mM NaCl, and 5mM KCl. Cells were spun down, washed once with the same buffer, and suspended in 24 mL of assay buffer containing 2 mg L^{-1} of metal-free fluo-3 to give a final concentration of 0.8×10^8 cells per mL. This concentration of fluo-3 gave a fairly linear response to the highest calcium levels tested with a small nonlinearity at the highest concentration giving an error of about 3.5%, for which the data were corrected. After trypsinization, the cells were kept at room temperature and used within 90 min, and no time-dependent change was observed over this period. For each data point, 2 mL of the cell suspension was used in a one-way plastic cuvet containing a magnetic stirrer that had been washed with M HCl and water. Magnesium ion (4 μL of M solution) and ATP (4 μL of 0.4M solution) were added to give concentrations of 2mM and 0.8mM, respectively. The cells were preincubated for 5 min at 37°, whereupon saponin was added to a concentration of 85 μg mL^{-1} to allow uptake of calcium into intracellular stores. After 5 min at 37°, a stable baseline was obtained and a Ca^{2+}-free solution of the inositol compound was added, typically 10–50 μL. The data were corrected for the volume change. After 3 min, saturating D-*myo*-1,4,5-IP_3 (10 μL of mM solution) was added to determine maximum Ca^{2+} release, which was 80–90% of the total releasable Ca^{2+}, as determined by the addition of μM calcium ionophore A23187.

*Phosphorylation of 1,3,4-tri-*O*-benoyl-*myo*-inositol.* — 1,3,4-Tri-*O*-benzoyl-L-*myo*-inositol (625 mg, 1.27 mmol), 750 mg of 1*H*-tetrazole, and 3 mL of dibenzyl di-*N*-isopropylphosphoramidite in 10 mL of dry dichloromethane were stirred for 21 h at 23°. The mixture was then cooled to −78°, 3 g of *m*-chloroperoxybenzoic acid was added, and the mixture was allowed to warm up to 23° overnight. Toluene (200 mL) was added and the solution was extracted with aqueous sodium hydrogencarbonate, and water. The organic layer was dried with magnesium sulfate and the solvents were evaporated. The residue, dissolved in acetonitrile, was fractionated by preparative h.p.l.c. using a step-gradient starting at 4:1 methanol–water. The 2,4,5-tris-*O*-(dibenzyloxyphosphinyl)-1,3,6-tri-*O*-benzoyl-D-*myo*-inositol was eluted at 310 min with 9:1 methanol–water and concentrated to give 837 mg (52%) of a syrup, $[\alpha]_{546}$ −17.1° (95% EtOH); ^{1}H-n.m.r. ($CDCl_3$): δ 4.38–5.11 (13 H, m, CH_2, H-1 1 or 3), 5.35–5.57 (4 H, m, H-2, 4, 5, 1 or 3), 6.18–6.24 (1 H, dd as ψt, J 10.1 Hz, H-6), and 6.80–8.16 (45 H, m, Ph-H; ^{31}P-n.m.r. ($CDCl_3$, broadband decoupled): δ −1.55 (1 P, s), −1.92 (1 P, s), and −1.95 (1 P, s).

The L-isomer, prepared from 367 mg of 1,3,4-tri-*O*-benzoyl-D-*myo*-inositol (yield 551 mg, 58%), had $[\alpha]_{546}$ +16.4° (95% EtOH).

D- *And* L-myo-*inositol 2,4,5-trisphosphate.* — 2,4,5-Tris-*O*-(dibenzyloxyphosphinyl)-1,3,6-tri-*O*-benzoyl-D-*myo*-inositol (837 mg, 657 μmol) was hydrogenolyzed in 19:1 ethanol–water over 190 mg of palladium hydroxide. After 17 h the catalyst was removed and the solvent was evaporated. After the addition of water, the solution was adjusted to pH 14 with NaOH and left overnight to saponify the benzoyl ester groups. Both deprotection steps were quantitative. The cations were removed on Dowex 50 (H^+), cyclohexylamine was added to bring the pH to 11, and the solution was evaporated to dryness. The solid was dissolved in 2 mL of water, and 2-propanol and acetone

were added to cause crystallization of the D-*myo*-inositol 2,4,5-trisphosphate hexakis (cyclohexylamine) salt after several days at 5°; yield 467 mg (68%); $[\alpha]_{546}$ −8.05° (H_2O); ^{1}H-n.m.r. (D_2O, pH 9): δ 1.11–2.05 (60 H, m, CH_2 of C_6H_{11}), 3.07–3.19 (6 H, m, H-1 of C_6H_{11}), 3.44–3.49 (1 H, dd, $J_{1,6}$ 9.8, $J_{1,2}$ 2.1 Hz, H-1), 3.67–3.72 (1 H, br. dd, H-3), 3.79–3.86 (1 H, ddd as ψq, $J_{H,P}$ 8.5, $J_{H,H}$ 8.5 Hz, H-5), 3.88–3.94 (1 H, dd as ψt, J 9.5 Hz, H-6), 4.15–4.24 (1 H, ddd as ψq, $J_{H,P}$ 8.8, $J_{H,H}$ 8.8 Hz, H-4), and 4.43–4.47 (1 H, ddd as ψdt, $J_{H,P}$ 7.3 Hz, H-2). The assignments were made on the basis of COSY experiments. ^{31}P-N.m.r. (D_2O, pH 9, broadband decoupled): δ 5.08 (2 P, s) and 5.21 (1 P, s).

Anal. Calc. for $C_6H_{15}O_{15}P_3 \cdot 6\,C_6H_{11}NH_2 \cdot 2\,H_2O$ (1051): C, 47.99; H, 9.00; N, 7.99; P, 8.85. Found: C, 48.04; H, 9.69; N, 7.26; P, 8.12.

The L-*myo*-isomer, made in the same manner, had $[\alpha]_{546}$ +9.5° [hexakis(cyclohexylamine) salt]; found: C, 45.67; H, 8.92; N, 7.21; P, 8.62.

*1,2,5-Tri-O-benzoyl-*chiro*-inositols.* — The 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-D-*chiro*-inositol (1 g, 2.03 mmol) was hydrogenolyzed in acetic acid over 1 g of Pd–C (5% Pd, Aldrich). After 18 h the catalyst was filtered off and the acetic acid was removed by lyophilization. The yield of 1,2,5-tri-*O*-benzoyl-D-*chiro*-inositol was quantitative. The compound was crystallized from 100 mL of 1:1 ethanol–water; m.p.228–230°; $[\alpha]_{546}$ +61.5° (dioxane).

Anal. Calc. for $C_{27}H_{24}O_9$ (492.5): C, 65.85; H, 4.92. Found: C, 65.23; H, 4.98.

The L-isomer, prepared the same way, had m.p. 228–231°; $[\alpha]_{546}$ −67.5° (dioxane); found: C, 65.75; H, 5.08.

^{1}H-N.m.r. ($CDCl_3$, Me_2SO-d_6): δ 4.04–4.10 (1 H, ddd as ψdt, $J_{3,4}$, $J_{2,3}$ 9.8, $J_{3,OH}$ 4.4 Hz, H-3), 4.14–4.20 (1 H, ddd as ψdt, $J_{3,4}$, $J_{4,5}$ 9.6, $J_{4,OH}$ 4.7 Hz, H-4), 4.26–4.29 (1 H, ddd as ψq, J 3.3 Hz, H-6), 4.67–4.69 (1 H, d, J 4.4 Hz, O*H*-3), 4.72–4.74 (1 H, d, J 4.8 Hz, O*H*-4), 5.25–5.29 (1 H, dd, $J_{5,6}$ 3.2, $J_{4,5}$ 10.0 Hz, H-5), 5.53–5.57 (1 H, dd, $J_{1,2}$ 3.3, $J_{2,3}$ 10.1 Hz, H-2), 5.59–5.62 (1 H, d, J 4.7 Hz, O*H*-6), 5.62–5.65 (1 H, dd as ψt, J 3.75 Hz, H-1), and 7.24–8.10 (15 H, m, Ph-H). Unambigous assignment of the inositol ring protons was done by 2D-n.m.r. (COSY).

*Phosphorylation of 1,2,5-tri-*O*-benzoyl-*chiro*-inositols.* — 1,2,5-Tri-*O*-benzoyl-D-*chiro*-inositol (461 mg, 936 μmol) was phosphorylated in the same manner as the *myo*-inositol derivative, and the 1,3,4-tris-*O*-(dibenzyloxyphosphinyl)-2,5,6-tri-*O*-benzoyl-D-*chiro*-inositol was eluted on h.p.l.c. at 190 min with 88:12 methanol–water to give 1.0 g (84%) of a syrup, $[\alpha]_{546}$ +19° (dioxane); ^{1}H-n.m.r. ($CDCl_3$): δ 4.40–5.40 (15 H, m, 6 × CH_2, H-1, 3, 4), 5.72–5.79 (1 H, dd as br. d, J 9.15 Hz, H-2 or 5), 5.79–5.85 (1 H, dd, J 3, 9.15 Hz, H-2 or 5), 5.95–5.99 (1 H, dd as ψt, J 3.75 Hz, H-6), and 6.76–8.15 (45 H, m, Ph-H); ^{31}P-n.m.r. ($CDCl_3$, broadband decoupled); δ −1.0 (1 P, s), −1.08 (1 P, s), and −1.45 (1 P, s).

Anal. Calc. for $C_{69}H_{63}O_{18}P_3$ (1273.1): C, 65.10; H, 4.99; P, 7.31. Found: C, 62.63; H, 5.41; P, 6.69.

The L-*chiro*-isomer (429 mg) gave 77% of syrup having $[\alpha]_{546}$ −17.5° (dioxane).

D- *And* L-chiro-*inositol 1,3,4-trisphosphate.* — 1,3,4-Tris-*O*-(dibenzyloxyphosphinyl)-2,5,6-tri-*O*-benzoyl-D-*chiro*-inositol (970 mg, 762 μmol) was hydrogenolyzed in 19:1 ethanol–water over Pd–C, the benzoyl groups were saponified with NaOH, and the

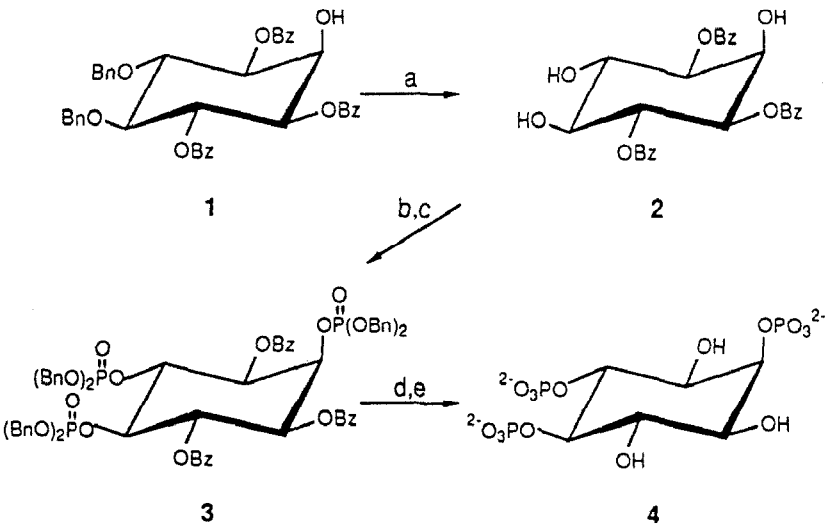
cyclohexylamine salt of the D-*chiro*-inositol 1,3,4-trisphoshpate was crystallized from water–2-propanol (566 mg, 71%). The free acid, after removal of cations by Dowex 50 (H^+), had $[\alpha]_{546}$ +37.1°; ^{1}H-n.m.r. [D_2O, hexakis(acyclohexylamine) salt, pH 9]: δ 1.10–2.06 (60 H, m, C*H*$_2$ of C_6H_{11}), 3.08–3.19 (6 H, m, H-1 of C_6H_{11}), 3.92–3.99 (1 H, br. m, C*H* of inositol), 3.99–4.05 (1 H, m, C*H* of inositol), 4.09–4.23 (2 H, m, C*H* of inositol), 4.24–4.28 (1 H, m, C*H* of inositol), and 4.30–4.36 (1 H, m, C*H* of inositol); ^{31}P-n.m.r. (D_2O, cyclohexylamine salt, pH 9, broadband decoupled): δ 3.9–4.1 (1 P, s), 4.5–4.9 (1 P, s), and 5.0–5.3 (1 P, s).

Anal. Calc. for $C_6H_{15}O_{15}P_3 \cdot 6\ C_6H_{11}NH_2 \cdot 2\ H_2O$ (1051): C, 47.99; H, 9.00; N, 7.99; P, 8.85. Found: C, 47.74; H, 9.09; N, 7.67; P, 9.08.

The L-*chiro*-isomer, made in the same manner, had $[\alpha]_{546}$ −43.4°; found: C, 47.43; H, 9.00; N, 7.55; P, 8.82.

RESULTS AND DISCUSSION

Synthesis of inositol trisphosphates. — For the synthesis of the enantiomers of *myo*-1,4,5-IP_3 (ref. 12), we converted D- and L-*chiro*-inositol into the 1,2:5,6-di-*O*-cyclohexylidene-*chiro*-inositols, which were then benzylated at positions 3 and 4. Acid hydrolysis removed the cyclohexylidene groups to give the 3,4-dibenzyl ethers of D- and L-*chiro*-inositol. Partial benzoylation of these ethers yielded the 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-*chiro*-inositols, which were used for the present syntheses of D- and L-*chiro*-1,3,4-IP_3 (see below). Inversion of the free hydroxyl group in these compounds, by way of the trifluoromethanesulfonate esters, gave two tri-*O*-benzoyl-5,6-di-*O*-benzyl-*myo*-inositols. The synthesis of D- and L-*myo*-2,4,5-IP_3 started with the chiral 1,3,4-tri-



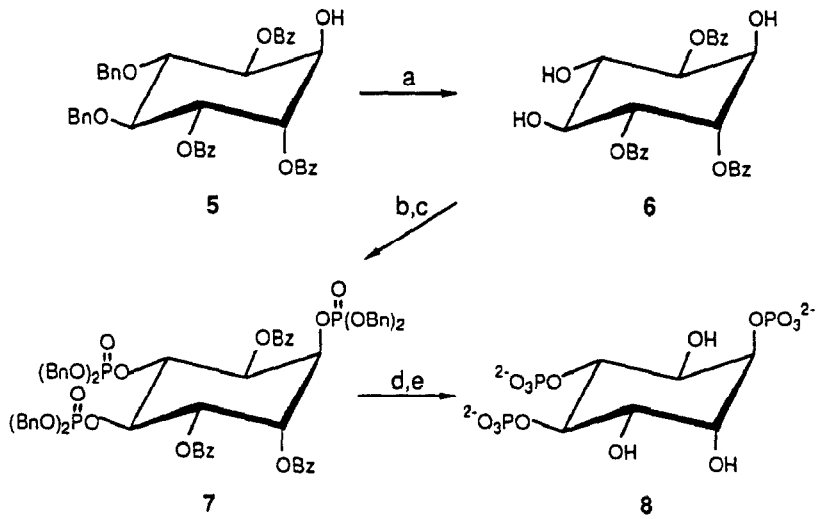
Scheme 1. Pathway for the synthesis of 4. The reagents are: (a) H_2, Pd–C, acetic acid; (b) *i*-$Pr_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2; (c) *m*-chloroperoxybenzoic acid; (d) H_2, Pd–C, EtOH; (e) M NaOH, 18 h, 25°.

O-benzoyl-5,6-di-*O*-benzyl-*myo*-inositols (**1**, Scheme 1; only one enantioner is shown), each minor product obtained in the inversion reaction. The two inversion products were separated prior to debenzylation, because benzoyl migration occurred during attempted preparative h.p.l.c. purification of the 1,3,4-tri-*O*-benzoyl-*myo*-inositols. In contrast, benzoyl migration was not noticeable on h.p.l.c. of 1,2,4-tri-*O*-benzoyl-*myo*-inositol.

Purified **1** was debenzylated by hydrogenolysis to yield the tribenzoyl derivative **2** in quantitative yield, and this was phosphorylated by the dibenzyl phosphoramidite procedure, a method that avoids the formation of cyclic phosphates from 1,2-diols. Deprotection of **3** in two steps (hydrogenolysis, saponification) furnished the free inositol trisphosphate **4**, which was crystallized as the hexakis(cyclohexylammonium) salt by adding 2-propanol to a concentrated aqueous solution. N.m.r. spectroscopy confirmed the structure and revealed that the crystals contained 2-propanol, which was lost after several weeks at room temperature.

The synthesis of *chiro*-1,3,4-IP_3 started with 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-*chiro*-inositol **5**, (Scheme 2), which was debenzylated quantitatively to **6**. Phosphorylation provided **7**, and this was deprotected in two steps to furnish **8** as the crystalline cyclohexylammonium salt.

The *enantiomeric* purity of all of the trisphosphates should be high because the starting compounds, D-pinitol and L-quebrachitol, were pure. Moreover, the syntheses involved numerous crystallizations of intermediates whereby minor contaminants were removed. The *isomeric* purity of the *myo*-2,4,5-IP_3, in terms of contamination with *myo*-1,4,5-IP_3, depends on the separation of the two tribenzoyl-dibenzyl-*myo*-inositol isomers and the further purification steps in the synthesis. The isomers were separated by preparative h.p.l.c. and each was recrystallized to a sharp melting point. After debenzylation, the product was again recrystallized. The phosphorylated compound



Scheme 2. Pathway for the synthesis of **8**. The reagents are the same as in the legend of Scheme 1.

was purified by preparative h.p.l.c. and the unblocked trisphosphate was finally crystallized as a cyclohexylammonium salt. Because our preparation of this compound is less active than others have reported and because its activity is similar to that of D-*chiro*-1,3,4-IP_3, we conclude that the D-*myo*-2,4,5-IP_3 must be of high isomeric purity. The ^1H- and ^{31}P-n.m.r. spectra were consistent with this conclusion.

Determination of Ca^{2+}-release activity. — The Ca^{2+}-release potential of the synthesized compounds was determined in permeabilized RBL cells, using fluo-3 as the Ca^{2+} indicator (Fig.2). Addition of the compound being tested (arrow 1) caused a fast

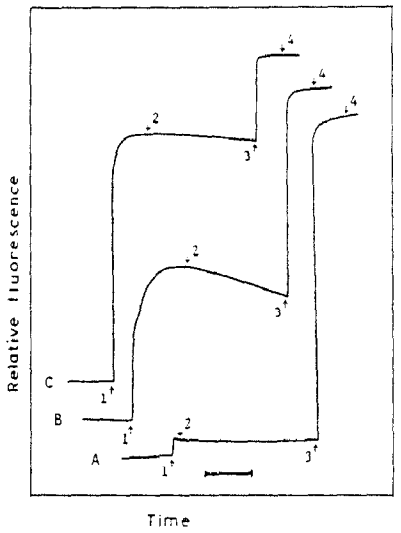


Fig. 2. Representative Ca^{2+}-release curves for D-*myo*-1,4,5-IP_3. Different amounts of IP_3 were added at arrow 1 and the fluorescence was measured at arrow 2. Saturating amounts of IP_3 were added at arrow 3 and the maximum response was measured by the fluorescence at arrow 4. The *bar* indicates an elapsed time of 1 min.

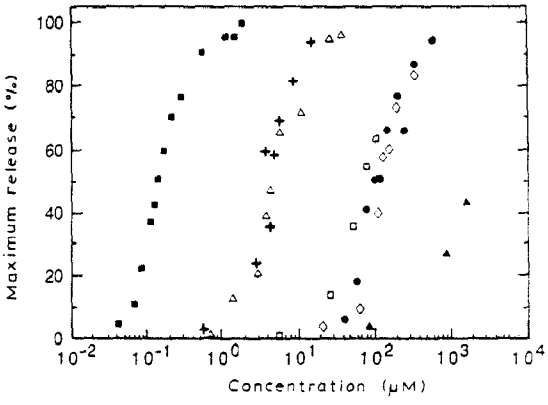


Fig. 3. Semilogarithmic plot of Ca^{2+}-release curves of D-*myo*-1,4,5-IP_3 and its polyol phosphate analogs. The symbols are: ■, D-*myo*-1,4,5-IP_3; +, D-*myo*-2,4,5-IP_3; △, D-*chiro*-1,3,4-IP_3; ◇, L-*chiro*-1,3,4-IP_3; ●, L-*myo*-2,4,5-IP_3; □, D,L-*myo*-4,5-IP_2; ▲, L-glycerol-1,2-P_2.

release of Ca^{2+}, followed by a slower additional response, a behavior that has been well documented[14] for D-*myo*-1,4,5-IP_3 and that is most strongly pronounced at concentrations giving an intermediate response (Fig. 2, curve B). After reaching a maximum (arrow 2), the calcium concentration declines due probably to metabolism of the inositol compound, mainly by the 5-phosphatase[14]. After 3 min, saturating IP_3 was added (arrow 3) to determine the maximum response (arrow 4). That we observe the same biphasic kinetics and similarly shaped dose–response curves for the analogs (Fig. 3) suggests that they all act at the same site or sites.

TABLE I

Ca^{2+}-Release activities of polyol phosphates

Compound	EC_{50} (μM)[a]
D-*myo*-1,4,5-IP_3	0.17
D-*myo*-2,4,5-IP_3	4.3
D-*chiro*-1,3,4-IP_3	4.2
D,L-*myo*-4,5-IP_2	70
L-*myo*-2,4,5-IP_3	110
L-*chiro*-1,3,4-IP_3	120
L-Glycerol-1,2-P_2	1900
L-*myo*-1,4,5-IP_3	>2000
Glycerol-1,3-P_2	>2000

[a] Concentration for half-maximal response.

Fig. 1. Comparison of D-*myo*-1,4,5-IP_3 and D-*myo*-2,4,5-IP_3 to two representations of D-*chiro*-1,3,4-IP_3, one rotated about the axis of symmetry 180° with respect to the other.

The concentration dependence of Ca^{2+} release is sigmoidal. A low fluorescence increase at low ligand concentrations is followed by a steep rise to about 80% of maximum release, followed again by a lower response. The EC_{50} values (Table I) show that D-*myo*-1,4,5-IP_3 is by far the most potent agonist (EC_{50} 0.17μM). Others have reported values between 0.1μM and 1.0μM in various systems[18]. D-*myo*-2,4,5-IP_3 (**4**) and D-*chiro*-1,3,4-IP_3 (**8**) have EC_{50} values of 4.3 and 4.2μM, corresponding to a 25-fold decrease with respect to D-*myo*-1,4,5-IP_3. The lower activity of our preparation of D-*myo*-2,4,5-IP_3, compared to values previously reported, may reflect a higher purity of our compound. The similar activities of **4** and **8** lead to the conclusion that the receptor recognizes predominantly the arrangement of the phosphate groups and is not sensitive to the orientation of the 1-hydroxyl group in **4**. L-*myo*-2,4,5-IP_3 and L-*chiro*-1,3,4-IP_3 were similar to each other in activity but, as expected, they had a lower activity than the D-isomers. Although the observed Ca^{2+}-release activities of these two compounds could reflect contamination, we conclude that the activities are real because the parallel synthesis of L-*myo*-1,4,5-IP_3 led to a product with no detectable activity. It is not reasonable to expect that the synthesis of the latter could be effected with no contamination by D-*myo*-1,4,5-IP_3, while the syntheses of the other two isomers led to such contamination and to precisely the same extent. This view is also supported by the

similar high Ca^{2+}-release activities of D-*myo*-2,4,5-IP_3 and D-*chiro*-2,3,4-IP_3, an unlikely consequence of equal and fortuitous contamination. Conversely, the simiar activities of these two isomers are expected owing to their structural similarities noted in Fig. 1.

The Ca^{2+}-release activity of the pure L-*myo*-2,4,5-IP_3 has not been tested previously, but it is similar to that of synthetic D,L-*myo*-inositol 4,5-biphosphate (D, L-*myo*-4,5-IP_2). L-Glycerol 1,2-biphosphate (L-glycerol-1,2-P_2) gave an EC_{50} value of 2μM, while L-*myo*-1,4,5-IP_3 and glycerol 1,3-biphosphate showed no activity, even at concentrations that caused a reduction in the fluorescence due to competition with the fluo-3 for Ca^{2+}. The relatively high activity of L-*myo*-1,4,5-IP_3 found by others (EC_{50} values of 220μM in bovine aortic smooth muscle cells[2] and 30μM in 3T3 cells[8]) suggests possible contamination with the D isomer. In this regard, the relatively high Ca^{2+}-release activities of the L-*myo*-2,4,5-IP_3 and L-*chiro*-1,3,4-IP_3 may reflect the fact that these isomers have an axial phosphate group, which introduces conformational flexibility into the molecules and allows an induced fit to the active site of the receptor. Alternatively, the axial phosphate group in these compounds may be close enough to one of the equatorial groups to mimic the 4,5-biphosphate motif of the natural ligand.

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