Synthesis, Calcium Mobilizing, and Physicochemical Properties of D-*chiro*-Inositol 1,3,4,6-Tetrakisphosphate, a Novel and Potent Ligand at the D-*myo*-Inositol 1,4,5-Trisphosphate Receptor

Changsheng Liu,[†] Richard J. Davis,[‡] Stefan R. Nahorski,[‡] Stephanie Ballereau,[§] Bernard Spiess,[§] and Barry V. L. Potter*[,†]

Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K., Department of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester LE1 9HN, U.K., and Laboratoire de Pharmacochimie Moléculaire, ERS 655 du CNRS, Faculté de Pharmacie, Universite Louis Pasteur, Strasbourg, France

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The synthesis of a novel and potent ligand at the D-*myo*-*inositol* 1,4,5-trisphosphate receptor ($InsP_3R$) is described. D-*chiro*-Inositol 1,3,4,6-tetrakisphosphate (**7**) and L-*chiro*-inositol 1,3,4,6-tetrakisphosphate (*ent*-**7**) have been synthesized from D-2,5-di-*O*-benzyl-*chiro*-inositol and L-2,5-di-*O*-benzyl-*chiro*-inositol, respectively. The potency of binding and Ca^{2+} release of **7** and *ent*-**7** were examined in L15 and Lvec cells. **7** was a full agonist at the $InsP_3R$ in both cells, and *ent*-**7** was inactive. The results are compared to those from D-*myo*-inositol 1,4,5-trisphosphate (**1**), DL-*scyllo*-inositol 1,2,4-trisphosphate (**2**), DL-*myo*-inositol 1,2,4,5-tetrakisphosphate (**3**), *scyllo*-inositol 1,2,4,5-tetrakisphosphate (**4**), D-*myo*-inositol 2,4,5-trisphosphate (**5**), and D-*chiro*-inositol 1,3,4-trisphosphate (**6**). The protonation processes of **7** have also been investigated by ^{31}P NMR titration experiments.

Introduction

After more than a decade of intense study by biologists and chemists, D-*myo*-inositol 1,4,5-trisphosphate, (Figure 1a, **1**) has been established as an unambiguous intracellular second messenger.[1,2] It is well-known how it is produced, how it mobilizes Ca^{2+} via its own ligand-gated receptor ($InsP_3R$), and how the message is terminated in cells. Its receptor has been isolated,[3] cloned,[4] and sequenced,[5] and several $InsP_3R$ subtypes have been found to exist.[6,7] Now that many inositol phosphates and their metabolic enzymes have been discovered, it is clear that the area is far more complex than originally imagined. Progress in understanding this now well-developed area is greatly aided by the availability of synthetic inositol phosphates and their analogues.[8]

D-*myo*-Inositol 2,4,5-trisphosphate ($Ins(2,4,5)P_3$, **5**, Figure 1a), identified in animal tissues,[9] has been widely used in determining the role of $InsP_3$-induced Ca^{2+} mobilization, due to its poor metabolism. Contrary to previous reports, $Ins(2,4,5)P_3$ has been found recently to exhibit partial agonism.[10] To investigate whether possession of a phosphate at the 2-position or lack of it at the 1-position was responsible for the agonist properties found, we synthesized the unnatural *myo*-inositol 1,2,4,5-tetrakisphosphate ($Ins(1,2,4,5)P_4$, **3**, Figure 1a) in racemic form[11] and also as individual enantiomers.[12] This compound could be considered as a relative of $Ins(1,4,5)P_3$ with an extra phosphate group at the 2-position or related to $Ins(2,4,5)P_3$ with an extra phosphate at the 1-position. It can also be thought of as a regioisomer of $Ins(1,3,4,5)P_4$, where the phosphate at the 3-position has been transposed onto the adjacent 2-position. To further the elucidation of the structural basis for interaction of $Ins(1,4,5)P_3$ with its receptor and metabolic enzymes, we also synthesized *scyllo*-inositol 1,2,4,5-tetrakisphosphate[13] (*scyllo*-$Ins(1,2,4,5)P_4$, **4**, Figure 1a), which possesses an equatorial phosphate at C-2 rather than an axial one relative to $Ins(1,2,4,5)P_4$. The receptor binding and Ca^{2+} release properties of $Ins(1,2,4,5)P_4$ and *scyllo*-$Ins(1,2,4,5)P_4$ as well as their interactions with the metabolic enzymes of $Ins(1,4,5)P_3$ have been examined.[14,15] Both analogues were full agonists with similar potencies to that of $Ins(1,4,5)P_3$.

Related to this work, and to further our understanding of the role of phosphates at the 1- and 2-positions in binding and Ca^{2+} release, we now have designed a new related target molecule, D-*chiro*-inositol 1,3,4,6-tetrakisphosphate (D-*chiro*-$Ins(1,3,4,6)P_4$, **7**, Figure 1b). In comparison to $Ins(1,2,4,5)P_4$ and *scyllo*-$Ins(1,2,4,5)P_4$, D-*chiro*-$Ins(1,3,4,6)P_4$ also possesses the 4,5-bisphosphate motif (C-3,4 in D-*chiro*-$Ins(1,3,4,6)P_4$), which is thought to be crucial for Ca^{2+} release,[16] but differs in the arrangement of the 1,2-bisphosphate (C-1,6 in D-*chiro*-$Ins(1,3,4,6)P_4$). $Ins(1,2,4,5)P_4$ possesses one equatorial phosphate at C-1 and an axial one at C-2. In contrast to the equatorial 1,2-bisphosphates of *scyllo*-$Ins(1,2,4,5)P_4$, D-*chiro*-$Ins(1,3,4,6)P_4$ possesses nominally two axial phosphates. D-*chiro*-$Ins(1,3,4,6)P_4$ can also be thought of as a hybrid of $Ins(2,4,5)P_3$ and D-*chiro*-$Ins(1,3,4)P_3$ (Figure 1a, **6**). The latter has an EC_{50} value of 4.3 μM compared to a value of 4.2 μM for $Ins(2,4,5)P_3$[17,18] in rat basophilic leukemia (RBL) cells.

A second motive to prepare **7** was to study the conformation of this molecule in relation to its degree of protonation as a function of pH. Several NMR

* Author for correspondence. Tel: +44-1225-826639. Fax: +44-1225-826114. E-mail: B.V.L.Potter@bath.ac.uk.
[†] University of Bath.
[‡] University of Leicester.
[§] Université Louis Pasteur.

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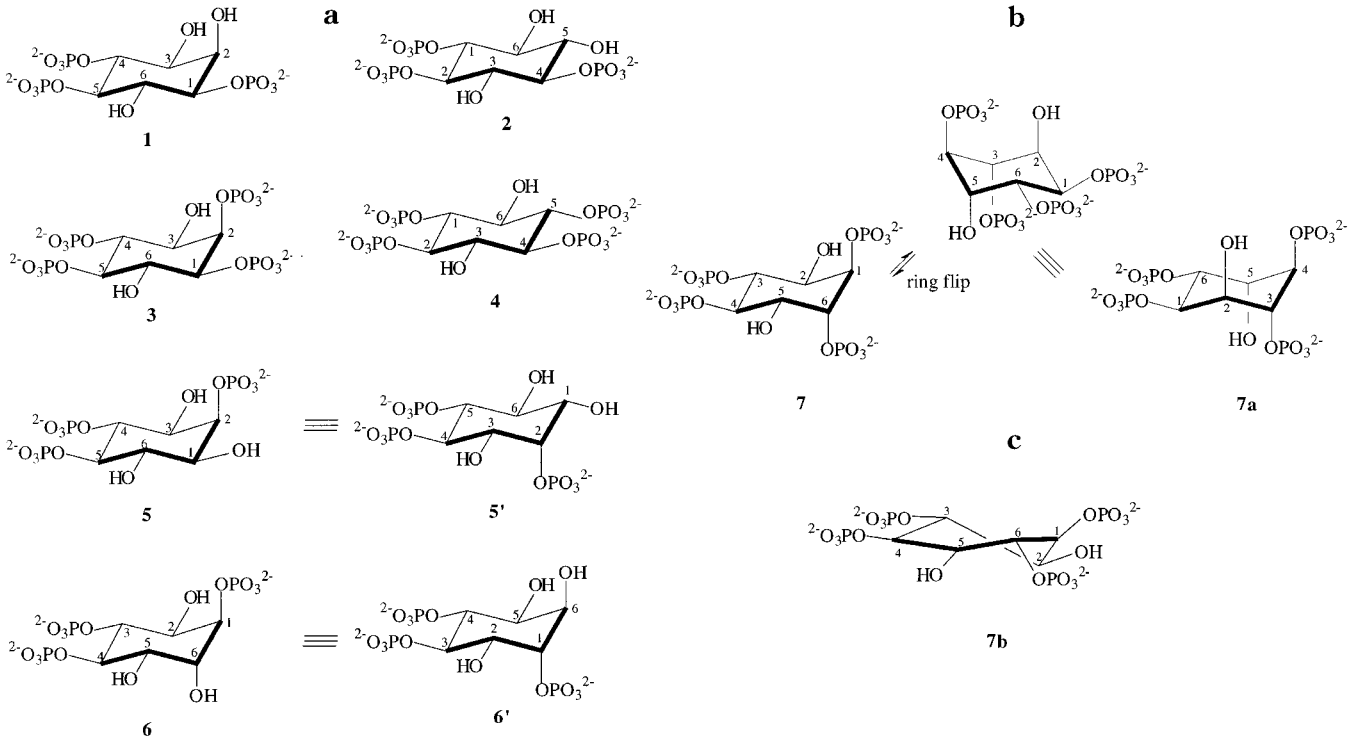


Figure 1. (a) Structures of D-*myo*-inositol 1,4,5-trisphosphate and analogues: **1**, Ins(1,4,5)P_3; **2**, L-*scyllo*-Ins(1,2,4)P_3; **3**, Ins(1,2,4,5)P_4; **4**, *scyllo*-Ins(1,2,4,5)P_4; **5**, Ins(2,4,5)P_3; **6**, D-*chiro*-Ins(1,3,4)P_3. (b) Possible chair conformations as a result of ring flipping of D-*chiro*-inositol 1,3,4,6-tetrakisphosphate. (c) Twist−boat conformation for **7**.

studies[19,20] on *myo*-inositol phosphates have been reported, including our recent report on Ins(1,3,4,5)P_4 and Ins(1,2,4,5)P_4,[21] and it is generally assumed that the conformation of naturally occurring *myo*-inositol polyphosphates is with five equatorial groups and one axial group. This has been particularly controversial for $InsP_6$,[22] where the possibility of intramolecular hydrogen bonding at low pH may stabilize the unusual five axial/one equatorial conformation. D-*chiro*-Ins(1,3,4,6)P_4, which possesses two equatorial and two axial phosphate groups in each of the two chair conformations (Figure 1b, **7**, **7a**), may offer a good chance to study a molecule with a low energy difference between the two conformations. Intriguingly also, an essential 4,5-bisphosphate in the correct orientation for Ca^{2+} release at its $InsP_3R$ is present in both conformations, **7** and **7a**.

Like *scyllo*-Ins(1,2,4,5)P_4, D-*chiro*-Ins(1,3,4,6)P_4 has a C_2-axis of symmetry, so there is only one binding orientation to consider (unlike D-*chiro*-Ins(1,3,4)P_3 and D-Ins(2,4,5)P_3). This is our third motive for making it.

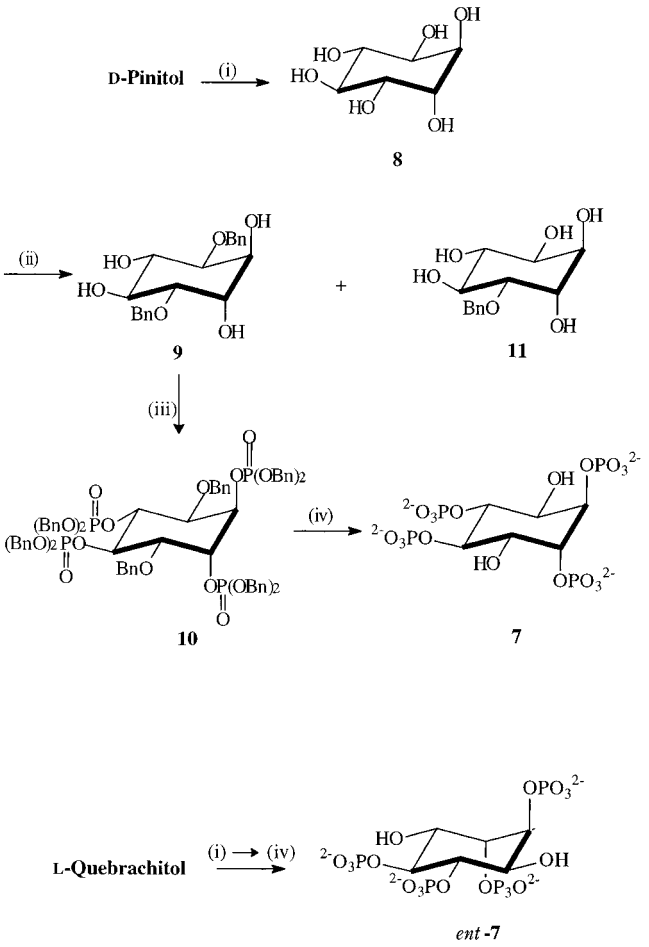
We therefore report here the synthesis of the novel inositol polyphosphate D-*chiro*-Ins(1,3,4,6)P_4 and its enantiomer L-*chiro*-Ins(1,3,4,6)P_4, together with evaluations of both its biological and physicochemical properties.

Results and Discussion

Synthesis. One of the major problems in the synthesis of inositol polyphosphates is to obtain an intermediate suitable for polyphosphorylation. This requires multiple regiospecific protection of the hydroxyl groups in inositol with various permanent and temporary protective groups. Rapid and simplified routes are therefore of great advantage. *chiro*-Inositol has two axial hydroxyl groups at positions 1 and 6 while the rest are equatorial. The HO-2eq and HO-5eq positions of the vicinal *cis*-diols are the most readily alkylated. We previously synthesized L-*chiro*-inositol 2,3,5-trisphosphate via a 2,3,5-protected intermediate obtained by tin-mediated alkylation from 1L-*chiro*-inositol.[23] Using a similar approach, but controlling the amount of reagent used, benzylation could be directed only to positions 2 and 5. Thus, 1D-*chiro*-inositol was obtained by O-demethylation[24] of D-pinitol with hydriodic acid to give 1D-*chiro*-inositol (Scheme 1, **8**). Treatment of **8** under reflux first with 2.5 mol equiv of dibutyltin oxide in acetonitrile, followed by reaction of the resulting presumed bis-stannylene with dried cesium fluoride/benzyl bromide in DMF, afforded crystalline D-2,5-di-*O*-benzyl-*chiro*-inositol (**9**) (24%) and D-2-*O*-benzyl-*chiro*-inositol (**11**) (15%), together with unreacted starting material and very small amounts of other polybenzylated products.

The structure of **9** was assigned unambiguously by virtue of the symmetry of the compound. There were three signals in the spectrum, integrating for two protons each assigned to ring protons and one AB system integrating for four protons assigned to the two methylenes of the benzyl groups. Phosphitylation of **9** with bis(benzyloxy)(diisopropylamino)phosphine/1*H*-tetrazole, followed by oxidation with *m*-chloroperoxybenzoic acid (*m*CPBA), furnished the fully protected tetrakisphosphate **10**. The compound showed only two peaks in the ^{1}H-decoupled ^{31}P NMR spectrum (δ_p −0.83 and −0.92), which were to be expected for this symmetrical derivative. Catalytic hydrogenolysis (Pd/C) of **10** at atmospheric pressure gave the target 1,3,4,6-tetrakisphosphate **7**, which was purified by ion-exchange chromatography and quantified (61%) using the Briggs phosphate assay.[25]

Scheme 1. Synthesis of D-*chiro*-Inositol 1,3,4,6-Tetrakisphosphate **7** and Its Enantiomer *ent*-**7**[a]



[a] Reagents and conditions: (i) 47% HI, reflux; (ii) (a) Bu_2SnO, MeCN, reflux, (b) benzyl bromide, cesium fluoride, DMF; (iii) (a) $Pr^i_2NP(OBn)_2$, tetrazole, CH_2Cl_2, (b) *m*-chloroperoxybenzoic acid; (iv) Pd/C, H_2.

The other enantiomer, 1L-*chiro*-inositol 1,3,4,6-tetrakisphosphate (L-*chiro*-Ins(1,3,4,6)P_4, *ent*-**7**, Scheme 1), was obtained in an identical fashion to that described for the D-enantiomer, but starting from L-quebrachitol. Compounds were used as their triethylammonium salts for biological and physicochemical evaluations.

Physicochemistry. To aid the interpretation of the biological data, we carried out physicochemical studies aimed at determining both the conformation of **7** in solution and the acid−base properties of its phosphates with regard to those of Ins(1,4,5)P_3.

^{31}P NMR titration has proved to be a useful technique to give access to the protonated fraction $f_{i,p}$ of a phosphate group in position *i* on the inositol ring, provided that both potentiometric and NMR data lead to superimposed $\bar{p} = f(\mathrm{pH})$ curves, where $\bar{p}$ corresponds to the mean number of protons bound per mole of ligand.[26−29] In the case of **7**, this requirement is fulfilled from pH 3 to 9 (curves not shown), thus indicating that the ^{31}P chemical shift variations describe the changes in the protonation state of a given phosphate group. Moreover, the general shape of the NMR titration curves as well as the chemical shifts of the protonated and deprotonated forms of each phosphate give crucial information about its configuration and about the configuration of vicinal functional groups (−OH or $-OPO_3^{2-}$).

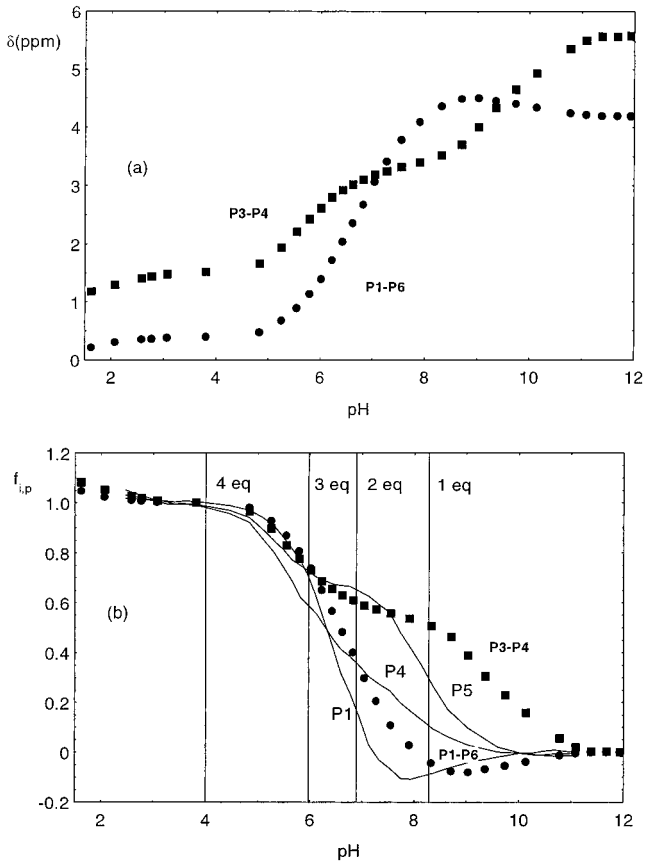


Figure 2. Chemical shifts (δ) from a ^{31}P NMR titration for **7** (a) and the corresponding protonation fraction curves $f_{i,i',p}$ (b) as a function of pH in 0.2 M KCl at 37 °C (D_2O). In panel b, the curves in solid line correspond to the protonation fraction curves of Ins(1,4,5)P_3.

The ^{31}P NMR titration curves for **7**, displayed in Figure 2a, show that only two peaks have to be considered owing to the symmetry of the molecule. The titration was carried out in D_2O with KCl (0.2 M) as supporting-electrolyte at 37 °C.

Preferential Conformation of 7 in Solution. Due to complex ^{1}H spectra, neither conclusions about the presence of conformer **7** or **7a** could be drawn nor could unambiguous assignment of the phosphorus resonances be made from 2D correlation experiments. However, it can be observed that, at pH 11.9, there are two sets of resonances for the ^{1}H spectrum, at 4.6 ppm and at about 4.2 ppm, in a 2:4 ratio, respectively. The peaks at 4.2 and 5.5 ppm in the ^{31}P spectrum correlate respectively with the resonances at 4.6 and 4.2 ppm in the ^{1}H spectrum. From the titration curves of **7** (Figure 2a), it undoubtedly appears that the resonance at 4.2 ppm belongs to an almost monophasic curve, whereas the resonance at 4.6 ppm belongs to a biphasic one. Thus, the first one can be attributed to two vicinal *trans* antiperiplanar phosphates and the second curves to two equatorial *trans* phosphates. In addition, due to the clear diphasic and monophasic character of the titration curves, it is also very likely that only one conformer does exist.

At this stage of the investigation, the question of the type of conformer remains. However, it can be considered that in conformer **7** there are two equatorial protons, whereas in conformer **7a** there are four equatorial protons. Since equatorial protons are usually down-

Table 1. Percentages of Protonation of the Phosphates for **7** and Ins(1,4,5)P_3 at pH 7.40

chiro-Ins(1,3,4,6)P_4	$f_{i,p}$(%)	Ins(1,4,5)P_3	$f_{i,p}$(%)
P4	55%	P5	60%
P3	55%	P4	25%
P6	15%	P1	5%

field shifted, it is therefore likely from the ^{1}H spectrum, even badly resolved, that conformer **7** is present. In addition, if we keep this conclusion, the chemical shift for both P3 and P4 protonated forms is $\delta_{3,4,p} = 1.6$ ppm. This is the mean value of $\delta_{4,p} = 1.8$ ppm and $\delta_{5,p} = 1.2$ ppm of P4 and P5 for Ins(4,5)P_2[26] where the phosphates have the same configuration and hydroxyl environment. If, on the contrary, conformer **7a** would be the right one, we would expect a value of $\delta_{1,6,p}$ closer to 0.4 ppm than to 1.6 ppm, due to the relative position of the vicinal hydroxyls. Indeed, for L-*chiro*-Ins(1,2,3)P_3, where P1 has a vicinal OH in a *trans* antiperiplanar position as for P1 of **7a**, $\delta_{1,p} = 0.4$ ppm.[30]

Microscopic Acid−Base Properties of *chiro*-Ins(1,3,4,6)P_4 with Regard to Ins(1,4,5)P_3. Assuming the above assignment, it appears that P3 and P4 interact, whereas P1 and P6 behave independently. Also, from pH 9 a slight reshielding is observed for P1−P6 as has been previously observed for P1 in Ins(1,4,5)-P_3.[26] This can be interpreted as follows. As long as a proton remains between P3 and P4, both phosphates contribute to stabilize it. This allows P1 and P6 to partially interact with HO-2 and HO-5, respectively. With the neutralization of the last proton on the molecule, P3 and P4 bearing two negative charges will repel each other, thus moving P1 and P6 away from their neighboring OH group. The consequence of increasing the distance between a phosphate group and a vicinal hydroxyl always results in a highfield shift of the phosphorus resonance. This observation is also in line with the presence of conformer **7**, since for conformer **7a** such an effect is hardly expected.

The reshielding of P1−P6 prevents the interpretation of the titration curves in terms of microprotonation constants. Nevertheless, since the potentiometric and NMR calculated $\bar{p} = f$(pH) curves satisfactory superimpose until pH 9, some conclusions can be drawn from the calculated protonation fraction curves shown in Figure 2b. From these curves it can be seen that the first added equivalent of protons will be taken almost only by P3 and P4 which thus appear to be the most basic phosphates. In contrast, the fourth equivalent of protons is equally shared by all the phosphates to reach the monoprotonated state at pH 4.

In Figure 2b are superimposed the $f_{i,p} = f$(pH) curves of Ins(1,4,5)P_3. As expected, the tetrakisphosphate exhibits a higher basicity than the trisphosphate. The difference is especially high for P3 (P4 in Ins(1,4,5)P_3). However, at physiological pH (7.4), there is not a large difference in the protonation state of P4 for **7** (55%, Table 1) and P5 for Ins(1,4,5)P_3 (60%). This should not disfavor **7** to too large an extent with regard to Ins(1,4,5)P_3 in terms of binding properties, at least as far as the ionization state of this phosphate is concerned which has been shown to play an important role.[26,31]

Macroscopic Acid−Base Properties of *chiro*-Ins(1,3,4,6)P_4 with Regard to Ins(1,2,4,5)P_4. The

Table 2. Logarithms of the Protonation Constants of **7** and Ins(1,2,4,5)P_4 in 0.2 M KCl at 37 °C (H_2O)[a]

log K_y	*chiro*-Ins(1,3,4,6)P_4	*myo*-Ins(1,2,4,5)P_4
$y = 1$	9.15 ± 0.02	9.39
$y = 2$	7.33 ± 0.04	7.65
$y = 3$	6.32 ±0.04	6.06
$y = 4$	5.38 ± 0.05	5.23

[a] The uncertainties are estimates of the standard deviation as calculated by Superquad.[43]

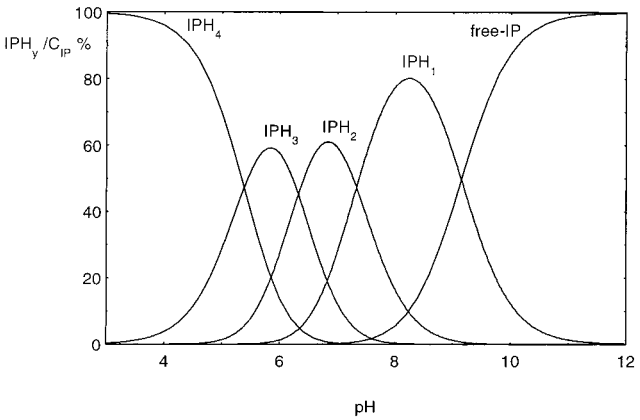


Figure 3. Distribution curves of the protonated macrospecies of **7** in 0.2 M KCl at 37 °C plotted against pH.

macroscopic stepwise protonation constants K_y ($y = 1-4$) related to the equilibria $H_{y-1}L^{(8-y+1)-} + H^+ \Leftrightarrow H_yL^{(8-y)-}$ were determined in KCl (0.2 M) at 37 °C. The logarithms of these constants along with those of Ins(1,2,4,5)P_4, previously published, are listed in Table 2.[21] It can be seen that log K_1 and log K_2 values for the *chiro* derivative are slightly lower than those of the *myo* derivative, whereas the inverse occurs for log K_3 and log K_4. Such a result could be expected since *chiro*-Ins(1,3,4,6)P_4 carries two axial phosphates with regard to Ins(1,2,4,5)P_4 which has only one. Now, the axial phosphate groups always show a lower basicity than equatorial phosphates.[27,28] The distribution curves of the protonated species calculated from the constants of Table 2 are shown in Figure 3.

Biology. D-*chiro*-Ins(1,3,4,6)P_4 was able to fully displace [^{3}H]Ins(1,4,5)P_3 from the type 1 Ins(1,4,5)P_3 receptor of L15 cell membranes (Table 3, Figure 4). Although Lvec cells endogenously express predominantly the type 1 InsP_3R isoform, the level of receptor protein present was ∼7.5-fold lower than that found in L15 cells (0.578 ± 0.013 pmol/mg Lvec protein to 4.227 ± 0.108 pmol/mg L15 protein, data not shown), such that it was too low to obtain accurate displacement data. D-*chiro*-Ins(1,3,4,6)P_4 was shown to fully displace [^{3}H]-Ins(1,4,5)P_3, albeit with approximately 5-fold lower affinity than Ins(1,4,5)P_3. Cooperativity in receptor binding describing the steepness of the binding curve (bound verses free radioligand) was determined from the obtained nonlinear Hill slopes. Ins(1,4,5)P_3 and D-*chiro*-Ins(1,3,4,6)P_4 exhibited Hill slopes of approximately 1 (see Table 3 for mean ± SEM).

Figure 5 shows the $^{45}Ca^{2+}$ releasing ability of Ins(1,4,5)P_3 and D-*chiro*-Ins(1,3,4,6)P_4 from saponin-permeabilized L15 and Lvec cells. Both permeabilized cell lines displayed ATP-dependent $^{45}Ca^{2+}$ loading of their Ca^{2+} stores, with uptake reaching a steady-state equilibrium within 15 min. D-*chiro*-Ins(1,3,4,6)P_4 was a full

Table 3. $InsP_3R$ Analogue [^{3}H]Ins(1,4,5)P_3 Displacement Binding pIC_{50} Values in L15 Membranes and pEC_{50} Value Determinations of Steady-State $^{45}Ca^{2+}$ Release from Saponin-Permeabilized L15 and Lvec Cell Lines at 22 °C by Ins(1,4,5)P_3 and D-*chiro*-Ins(1,3,4,6)P_4[a]

Ins(1,4,5)P_3 analogue	IC_{50} (nM)	Hill slope	EC_{50} (nM)	Hill slope	cell line
Ins(1,4,5)P_3	33.5 ± 2.5	1.000 ± 0.073	123 ± 8.2	0.960 ± 0.062	L15
			247 ± 21.5	1.168 ± 0.110	Lvec
D-*chiro*-Ins(1,3,4,6)P_4	172 ± 25	1.290 ± 0.212	420 ± 112	1.217 ± 0.093	L15
			906 ± 140	1.253 ± 0.151	Lvec
L-*chiro*-Ins(1,3,4,6)P_4			no release at 10 μM		L15
Ins(2,4,5)P_3			740 ± 100		L15
Ins(1,4,5)P_3[a]	4.43		51.6		SH-SY5Y
DL-*scyllo*-Ins(1,2,4)P_3[a]	52.0		171.4		SH-SY5Y
DL-Ins(1,2,4,5)P_4[a]	26.5		163.3		SH-SY5Y
scyllo-Ins(1,2,4,5)P_4[a]	14.0		76.9		SH-SY5Y
Ins(1,4,5)P_3[b]			170		RBL
Ins(2,4,5)P_3[b]			4300		RBL
D-*chiro*-Ins(1,3,4)P_3[b]			4200		RBL

[a] Data from Wilcox et al.[14] [b] Data from Tegge et al.[17]

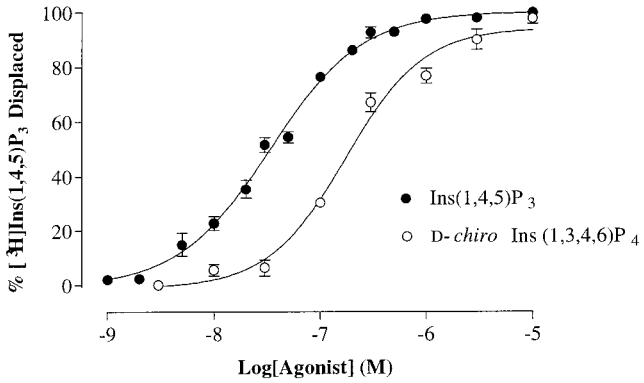


Figure 4. Displacement of [^{3}H]Ins(1,4,5)P_3 receptor binding to the type 1 $InsP_3R$ in L15 membranes, by increasing concentrations of Ins(1,4,5)P_3 (●) and D-*chiro*-Ins(1,3,4,6)P_4 (○). Nonspecific binding was defined by the addition of 10 μM cold Ins(1,4,5)P_3. D-*chiro*-Ins(1,3,4,6)P_4 fully displaced [^{3}H]Ins-(1,4,5)P_3. Displacement binding was not determined in Lvec cells due to the low levels of endogenous $InsP_3R$ protein expressed. Experiments were performed at 4 °C, in the presence of EGTA. Results are denoted as mean ± SEM, characteristic of at least three independent experiments, performed in duplicate.

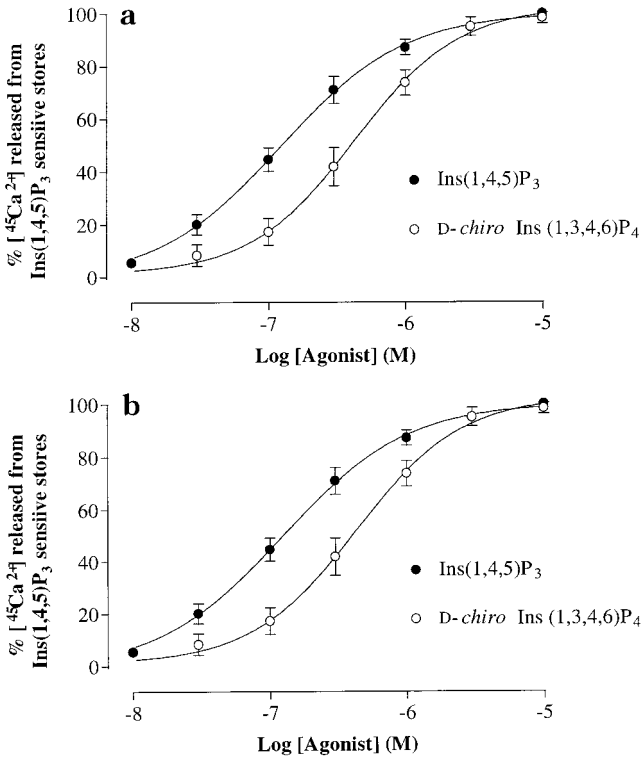


Figure 5. Percentage of steady-state $^{45}Ca^{2+}$ released from Ins(1,4,5)P_3-sensitive pool of saponin-permeabilized L15 (a) and Lvec (b) cell lines at 22 °C by Ins(1,4,5)P_3 (●) and D-*chiro*-Ins(1,3,4,6)P_4 (○). D-*chiro*-Ins(1,3,4,6)P_4 was a full agonist at the $InsP_3R$ in both cell lines. The maximal Ins(1,4,5)P_3-sensitive pool (100%) was defined by the amount of $^{45}Ca^{2+}$ released of that loaded, by a maximally effective concentration (10 μM) of Ins(1,4,5)P_3 in each cell line. Results are denoted as mean ± SEM, characteristic of at least four independent experiments, performed in duplicate.

agonist at the $InsP_3R$ and appeared approximately 4-fold less potent in releasing Ca^{2+} in a dose-dependent fashion than Ins(1,4,5)P_3 in both L15 and Lvec cells (see Table 3 for mean ± SEM, EC_{50}, and Hill slopes). Neither Ins(1,4,5)P_3 nor D-*chiro*-Ins(1,3,4,6)P_4 exhibited any cooperativity in their ability to release $^{45}Ca^{2+}$ under steady-state conditions. L-*chiro*-Ins(1,3,4,6)P_4, as expected, was inactive. No $^{45}Ca^{2+}$ release was detected even at concentrations of 10 μM.

The Ins(1,4,5)P_3 receptor shows considerable stereo- and regiospecificity in its interaction with Ins(1,4,5)P_3 analogues. However, of the three phosphate groups and three hydroxyl groups, the presence of the vicinal 4,5-bisphosphate motif has been thought to be crucial for Ca^{2+} mobilizing activity[8,16] and the 6-OH group plays a significant role in receptor binding or fixing the conformation of Ins(1,4,5)P_3.[32,33] Surprisingly, all results to date show that the 3-hydroxyl group plays a relatively insignificant role[34−36] compared with the 6-OH. If the potential roles of the phosphate group at the 1-position and hydroxyl group at the 2-position are considered without concerning the 4,5-bisphosphate motif and 3-OH and 6-OH of Ins(1,4,5)P_3, there are five known related analogues of Ins(1,4,5)P_3 worthy of consideration: L-*scyllo*-Ins(1,2,4)P_3 (**2**), D-Ins(1,2,4,5)P_4 (**3**), *scyllo*-Ins(1,2,4,5)P_4 (**4**), D-Ins(2,4,5)P_3 (**5**), and D-*chiro*-Ins-(1,3,4)P_3 (**6**). They can be divided into two groups depending upon whether the molecule possesses an equatorial 1-phosphate group. Table 3 shows that members of the first group, *scyllo*-Ins(1,2,4)P_3 (**2**), Ins-(1,2,4,5)P_4 (**3**), and *scyllo*-Ins(1,2,4,5)P_4 (**4**), which all possess the requirement of an equatorial 1-phosphate, are apparently potent full agonists at the Ins(1,4,5)P_3 receptor giving respective EC_{50} values (in the same cell system) of: *scyllo*-Ins(1,2,4,5)P_4 (IC_{50} 14 nM, EC_{50} 77 nM), DL-Ins(1,2,4,5)P_4 (IC_{50} 26 nM, EC_{50} 163 nM), DL-

scyllo-Ins(1,2,4)P_3 (IC_{50} 52 nM, EC_{50} 171 nM), compared to Ins(1,4,5)P_3 (IC_{50} 4 nM, EC_{50} 52 nM). When we consider that D-*scyllo*-Ins(1,2,4)P_3 and L-Ins(1,2,4,5)P_4 are essentially inactive with respect to binding to the $InsP_3R$ and Ca^{2+} mobilization, L-*scyllo*-Ins(1,2,4)P_3 and D-Ins(1,2,4,5)P_4 are most likely to be as equally effective as *scyllo*-Ins(1,2,4,5)P_4. There are therefore no significant effects at the Ins(1,4,5)P_3 receptor whether an axial phosphate group (Ins(1,2,4,5)P_4), an equatorial phosphate group (*scyllo*-Ins(1,2,4,5)P_4), or an equatorial hydroxyl group at the 2-position (L-*scyllo*-Ins(1,2,4)P_3) is present.

It is not surprising that Ins(2,4,5)P_3 (**5**) and D-*chiro*-Ins(1,3,4)P_3 (**6**), which form the second group, are weaker agonists (ca. 25-fold) relative to Ins(1,4,5)P_3 because both possess an axial rather than an equatorial phosphate at the pseudo 2-position (Figure 1a, **5** or **6**) or pseudo 1-position (Ins(1,4,5)P_3 numbering, depending upon the relative true binding orientation) (Figure 1a, **5′** or **6′**). We hypothesize that **5′** or **6′** may be the predominant orientation, relative to **1,** recognized by the Ins(1,4,5)P_3 receptor, since the axial 1-phosphate could interact more efficiently with the receptor site normally occupied by the equatorial 1-phosphate of Ins(1,4,5)P_3 rather than **5** or **6** which possesses the axial 2-phosphate. The biological data from D-*chiro*-inositol 1,3,4,6-tetrakisphosphate (Figure 1b, **7**) have improved understanding of the site of Ins(1,4,5)P_3 receptor which binds the 1-phosphate and 2-OH of Ins(1,4,5)P_3. Without an equatorial 1-phosphate but with an axial one at the site of the receptor, the analogue was relatively well-recognized (IC_{50} 172 nM), with an affinity about 6-fold less than that of Ins(1,4,5)P_3 and a potency to mobilize Ca^{2+} with an EC_{50} only 3.5-fold greater than that of Ins(1,4,5)P_3. It is useful to compare Ins(1,2,4,5)P_4 (**3**) with Ins(1,4,5)P_3 (**1**) and D-*chiro*-Ins(1,3,4,6)P_4 (**7**) with D-*chiro*-Ins(1,3,4)P_3 (**6**). For the former pair, that Ins(1,2,4,5)P_4 was found to display a slightly lower activity with respect to receptor binding and Ca^{2+} release was ascribed to the existence of the extra axial 2-phosphate. For the latter ones, surprisingly, the introduction of the axial 2-phosphate of D-*chiro*-Ins-(1,3,4,6)P_4 (**7**) increased the potency significantly compared to that of D-*chiro*-Ins(1,3,4)P_3 (**6**) (across two cell lines). The fact that the axial 2-phosphate in Ins-(1,2,4,5)P_4 and D-*chiro*-Ins(1,3,4,6)P_4 have apparently different effects consolidates previously published evidence that the area of Ins(1,4,5)P_3 receptor around the 2-hydroxyl and 1-phosphate group is quite open.[8] When an analogue possesses an equatorial 1-phosphate group which enhances binding and Ca^{2+} release, any change rather than an axial hydroxyl group at the 2-position only acts to decrease the activity of the analogue. Without an equatorial 1-phosphate, an axial 1-phosphate group may cause an analogue to bind much more weakly, as is shown in D-*chiro*-Ins(1,3,4)P_3 where the axial 1-phosphate binds to the site of the Ins(1,4,5)P_3 receptor where presumably the equatorial 1-phosphate would normally be. However, in the case where an additional 2-phosphate is introduced, the data for D-*chiro*-Ins(1,3,4,6)P_4 (**7**) suggest that the axial 2-phosphate strengthens the binding ability considerably, and some adjustment of the molecular conformation may take place to facilitate the binding needed with the complementary centers of the receptor. The data for D-*chiro*-Ins(1,3,4,6)P_4 (**7**) (EC_{50} 420 nM) and Ins(2,4,5)P_3 (EC_{50} 740 nM) observed using the same cell system also lead to the same hypothesis.

Note that in our arguments we have adopted a simplistic approach and have not considered the possibility that **7** might exist in solution or be bound to the receptor in, for example, a twist−boat type conformation (Figure 1c, **7b**). This might enable a phosphate group conformation more akin to that of *scyllo*-Ins(1,2,4,5)P_4 to be reached. Nevertheless, the observation from the physicochemical studies of two cooperative phosphate groups and an independent pair implies that distortion of **7** from a chair conformation, if any, must be minimal.

Thus, we have described a synthetic route to the novel D-*chiro*-Ins(1,3,4,6)P_4 and its enantiomer and have presented physicochemical data on the ionization state of the phosphate groups in this molecule, which may also suggest a preferred conformation in solution. Finally, we have demonstrated that **7** is potent in Ca^{2+} release and in Ins(1,4,5)P_3 receptor binding and is more potent than Ins(2,4,5)P_3 and probably D-*chiro*-Ins(1,3,4)P_3.

Experimental Section

General Methods. Chemicals were purchased from Aldrich London, and pinitol was purchased from New Zealand Pharmaceuticals Ltd. Dichloromethane was dried over calcium hydride. TLC was performed on precoated plates (Merck TLC aluminum sheets silica $60F_{254}$, Art. No. 5554). Spots were visualized by spraying of plates with phosphomolybdic acid in methanol followed by heating. Flash chromatography was carried out using Sorbsil C60 silica gel. NMR spectra were recorded on either JEOL FX-90Q, JEOL JNM GX-270 NMR, or JEOL EX-400 NMR spectrometers. Chemical shifts were measured in ppm relative to tetramethylsilane (TMS). ^{31}P chemical shifts were measured in ppm relative to external 85% H_3PO_4 and are positive when downfield from this reference. *J* values are given in Hz. Mp (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler block. Microanalysis was carried out by the University of Bath microanalysis service. Optical rotations were measured using an Optical Activity Ltd. AA-10 polarimeter, and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Mass spectra were recorded at the Mass Spectrometry Service of the University of Bath. Ion-exchange chromatography was performed on a LKB-Pharmacia medium-pressure ion-exchange chromatograph using Q Sepharose Fast Flow with gradients of triethylammonium hydrogen carbonate (TEAB) as eluent. Column fractions containing inositol polyphosphate analogues were assayed for total phosphate by a modification of the Briggs test as described.[25,37] (Diisopropylamino)dichlorophosphine was prepared by the method of Tanaka et al.,[38] 2 mol equiv of benzyl alcohol in the presence of 2 mol equiv of triethylamine afforded bis(benzyloxy)-(diisopropylamino)phosphine (δ_P 145.24) which could be purified by flash chromatography. Ins(2,4,5)P_3 was synthesized from D-1,3,6-tri-*O*-benzyl-*myo*-inositol and was a gift of Dr. S. J. Mills.

Ca^{2+} Release. Tris(hydroxymethyl)methylamine (2-amino-2-hydroxymethylpropane-1,3-diol) (tris), disodium ATP, EGTA·2Na, saponin, and dithiothreitol (DTT) were purchased from Sigma (Poole). $^{45}CaCl_2$ was from Amersham International (Amersham, Bucks). [^{3}H]Ins(1,4,5)P_3 was from NEN-DuPont. GF/B glass fiber filters were from Whatman International Ltd. (Maidstone, Kent). Chemically synthesized Ins(1,4,5)P_3 (K^+ salt)[39] was obtained from the University of Rhode Island, Foundation Chemistry Group. Dow Corning silicone oils 550 and 556 were from BDH (Leicester). Chelex 100 resin was purchased from Bio-Rad (U.K.).

Cell Culture. L15 cells, a stably transfected L-mouse fibroblast cell line overexpressing approximately 8-fold the levels of essentially homogeneous populations of type 1 $InsP_3$

receptor protein, and Lvec cells (the vector control cell line)[40] were cultured in 175-cm^2 flasks, at 37 °C, in a 5% CO_2 incubator. Media used was Dulbecco's MEM (containing 25 mM Hepes, sodium pyruvate, 1000 mg of L-glucose), and it was supplemented with 10% fetal calf serum (v/v), 2 mM glutamine, 50 IU mL^{-1} penicillin and 50 μg mL^{-1} streptomycin.

Preparation of L15 Cell Membranes for [^{3}H]InsP_3 Displacement Binding. Preparation of L15 membranes and the [^{3}H]Ins(1,4,5)P_3 binding displacement assay were essentially as previously described.[41] After incubation for 30 min at 4 °C, in EGTA, bound and free [^{3}H]Ins(1,4,5)P_3 were separated by vacuum filtration through Whatman glass fiber filters using a Millipore Corp. manifold vacuum filtration unit. Associated radiolabel was determined by liquid scintillation counting. Specifically bound [^{3}H]Ins(1,4,5)P_3 (approximately 2000 dpm/assay) and nonspecific binding (approximately 100 dpm/assay) were defined by the addition of 10 μM Ins(1,4,5)P_3.

Ins(1,4,5)P_3/Analogue-Induced $^{44}Ca^{2+}$ Release End Point Assay. $^{45}Ca^{2+}$ mobilization from saponin-permeabilized cells was performed at 22 °C as previously described.[42] Experiments were conducted in an intracellular-like buffer (ICB) consisting of 120 mM KCl, 2mM KH_2PO_4, 5 mM sodium succinate, 20 mM Hepes (free acid), 2 mM ATP·2Na, and 2.4 mM $MgCl_2$·$6H_2O$ (ratio 5:6), pH 7.2 at room temperature, with 20% (w/v) KOH and 1 mM HCl. The free ICB [Ca^{2+}] was adjusted to between 80 and 150 nM by the addition of EGTA. Retained $^{45}Ca^{2+}$ was determined by liquid scintillation analysis. The total releasable $InsP_3$-sensitive pool (100%) was determined by the addition of 10 μM Ins(1,4,5)P_3.

Data Analysis. IC_{50} and EC_{50} values are concentrations producing half-maximal inhibition and stimulation, respectively, and Hill slope estimates were by computer-assisted curve fitting using Version 2.1 of GraphPad Prism (GraphPad Software). All experiments were performed in duplicate and are representative of at least three separate experiments. The combined results were then expressed as mean ± SEM.

Physicochemistry. Potentiometric and NMR determinations were carried out as previously described.[26,27] The triethylammonium salt of **7** was converted into its acidic form by passage on an Amberlyst IRN(H^+) column; 0.45 mL of a 3 × 10^{-3} mol·dm^{-3} solution of **7** in D_2O added with 0.2 M KCl was first potentiometrically titrated with KOD. This experiment provided both the analytical concentration of **7** and the pH values at which the ^{31}P NMR spectra have to be taken. Then, for the NMR titration, the same initial volume of sample solution and the same additions of KOD were used. ^{31}P NMR spectra were recorded at 121.497 MHz on a Bruker 300 DPX FT spectrometer. Chemical shifts were measured relative to an external 85% orthophosphoric acid reference. Resonance peaks of **7** were assigned by performing proton phosphorus 2D correlation experiments at pH 12 and with the help of the ^{31}P NMR titration curve (see above discussion). To determine the protonation constants, potentiometric titrations were performed as previously indicated[26,27] but on a volume of 2 mL of solution in aqueous 0.2M KCl instead of 0.45 mL. Processing of the pH measurements by the program SUPERQUAD[43] yielded the macroscopic protonation constants.

D-2,5-Di-*O*-benzyl-*chiro*-inositol (9). A mixture of **8** (1.43 g, 7.9 mmol), Bu_2SnO (4.96 g, 19.9 mmol), and dry MeCN (150 mL) was heated under reflux in a Soxhlet apparatus (molecular sieve 4A) for 24 h. The solvent was evaporated, and to the resulting residue were added cesium fluoride (5.2 g, 34.2 mmol) and benzyl bromide (6.75 g, 39.5 mmol). The mixture was stirred at room temperature for 20 h and was evaporated to dryness in vacuo. The residue was taken up in ethyl acetate and washed with 1 M HCl, brine, and saturated $NaHCO_3$, and the precipitated tin derivative was removed by filtration through Celite. The filtrate was concentrated and chromatographed (ethyl acetate/EtOH, 95:5), followed by crystallization from ethyl acetate to give D-2,5-di-*O*-benzyl-*chiro*-inositol (**9**) (1.7 g, 24%) and D-2-*O*-benzyl-*chiro*-inositol (**11**) (0.8 g, 15%).

D-2,5-Di-*O*-benzyl-*chiro*-inositol (9): $[\alpha]_D$ = +37.2 (EtOH, c = 0.6); δ_H (DMSO, 400 MHz) 3.38−3.41 (2H, m, H-2 and H-5), 3.51 (2H, dd, J = 2.5 and 6.8 Hz, H-3 and H-4), 3.91 (2H, s, H-1 and H-6), 4.62, 4.59 (4H, AB, J_{AB} = 12.7 Hz, CH_2), 4.66 (2H, d, J = 3.4 Hz, OH), 4.79 (2H, d, J = 3.0 Hz, OH), 7.11−7.60 (10H, m, Ph). Anal. ($C_{20}H_{24}O_6$) C, H, N.

D-2-*O*-Benzyl-*chiro*-inositol (11): $[\alpha]_D$ = +43.7 (MeOH, c = 0.3); δ_H (DMSO, 270 MHz); 3.28 (1H, t, J = 9.2 Hz, H-3 or H-4), 3.35 (1H, dd, J = 2.6 and 9.8 Hz, H-2 or H-5), 3.44 (1H, dd, J = 3.3 and 9.5 Hz, H-5 or H-2), 3.46 (1H, t, J = 9.3 Hz, H-4 or H-3), 3.67 (1H, t, J = 2.7 Hz, H-1 or H-6), 3.89 (1H, t, J = 2.8 Hz, H-6 or H-1), 4.56, 4.60 (2H, AB, J_{AB} = 12.3 Hz, CH_2), 7.23−7.41 (5H, m, Ph). Anal. ($C_{13}H_{18}O_6$) C, H, N.

L-2,5-Di-*O*-benzyl-*chiro*-inositol (*ent*-9) was obtained in an identical fashion to that described for **9** in crystalline form: NMR spectra as for **9**; $[\alpha]_D$ = −35.9 (EtOH, c = 0.4). Anal. ($C_{20}H_{24}O_6$) C, H, N.

L-2-*O*-Benzyl-*chiro*-inositol (*ent*-11) was obtained in an identical fashion to that described for **11** in crystalline form: NMR spectra as for **11**; $[\alpha]_D$ = −42.6 (MeOH, c = 0.3). Anal. ($C_{13}H_{18}O_6$) C, H, N.

D-2,5-Di-*O*-benzyl-*chiro*-inositol 1,3,4,6-Tetrakis(*O*,*O*-dibenzyl phosphate) (10). Compound **9** (0.18 g, 0.5 mmol) and tetrazole (0.35 g, 5 mmol) in dry dichloromethane (10 mL) were stirred at room temperature for 10 min, and then bis-(benzyloxy)(diisopropylamino)phosphine (1.84 g, 2.4 mmol) was added to the mixture. The mixture was stirred for 1 h and cooled to 0 °C followed by addition of *m*-chloroperoxybenzoic acid (1.01 g, 3 mmol). After further stirring for 30 min, the mixture was diluted with ethyl acetate (50 mL) and washed with 10% sodium metabisulfite (50 mL), aqueous $NaHCO_3$, brine, and water (50 mL each). The organic phase was dried over magnesium sulfate and evaporated to dryness. The residue was chromatographed on silica gel to give compound **10** (0.49 g, 0.35 mmol, 70%): $[\alpha]_D$ = +12.9 ($CHCl_3$, c = 2.0); δ_H ($CDCl_3$, 400 MHz) 3.86 (2H, br, 2 CH), 4.45 and 4.52 (4H, AB, J_{AB} = 11.3 Hz, CH_2), 4.74−5.15 (20H, m, 8 CH_2Ph and 4 CH), 7.05−7.39 (50H, m, CH_2Ph); δ_P ($CDCl_3$, 36 MHz, ^{1}H-decoupled) −0.83 (2P), −0.92 (2P); m/z (+ve ion FAB) 1402 [(M + H)$^+$ 85%], 1311 (30), 712 (10), 271 (35), 91 (100). Acc. Mass for $C_{76}H_{77}O_{18}P_4$, 1401.4072; found, 1401.4072.

L-2,5-Di-*O*-benzyl-*chiro*-inositol 1,3,4,6-tetrakis(*O*,*O*-dibenzyl phosphate) (*ent*-10) was obtained in an identical fashion to that described for **10**: NMR spectra as for **10**; $[\alpha]_D$ = −11.1 ($CHCl_3$, c = 4.0). Acc. Mass for $C_{76}H_{77}O_{18}P_4$, 1401.4072; found, 1401.4072.

D-*chiro*-Inositol 1,3,4,6-Tetrakisphosphate (7). To 5% Pd/C (1.2 g), freshly prepared by hydrogenation in EtOH (20 mL) at atmospheric pressure for 1 h at room temperature, was added **10** (0.3 g, 0.21 mmol) in EtOH (10 mL). After being shaken under H_2 for a further 12 h at room temperature, the mixture was filtered and concentrated in vacuo. The residue was purified by ion-exchange chromatography on Pharmacia Q Sepharose Fast Flow with a gradient of TEAB buffers (0.1−1 M) as eluent. The triethylammonium salt of compound **7** eluted between 610 and 720 mM (0.13 mmol, 61%): $[\alpha]_D$ = −3.4 (MeOH, c = 2.0); δ_H (CD_3OD, 400 MHz) 4.02 (2H, d (br), J = 7.3 Hz, H-2 and H-5), 4.30 (2H, dddd, J = 7.5, 8.3, 9.2 and 9.7 Hz, H-3 and H-4), 4.68 (2H, ddd, J = 3.9, 3.9 and 9.8 Hz, H-1 and H-6); δ_P (CD_3OD, 160 MHz, ^{1}H-decoupled) 2.50 (2P), 0.92 (2P); δ_P (CD_3OD, 160 MHz, ^{1}H-coupled) 2.52 (2P, d, J = 7.5 Hz), 0.95 (2P, d, J = 8.4 Hz); m/z (−ve ion FAB) 498 [(M − H)$^-$ 100%], 998 (40). Acc. Mass for $C_6H_{15}O_{18}P_4$, 498.9211; found, 498.9211.

L-*chiro*-Inositol 1,3,4,6-tetrakisphosphate (*ent*-7) was obtained in an identical fashion to that described for **7**: NMR spectra as for **7**; $[\alpha]_D$ = +3.0 (MeOH, c = 3.0). Acc. Mass for $C_6H_{15}O_{18}P_4$, 498.9211; found, 498.9211.

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