Enzyme-Assisted Total Synthesis of the Optical Antipodes D-*myo*-Inositol 3,4,5-Trisphosphate and D-*myo*-Inositol 1,5,6-Trisphosphate: Aspects of Their Structure−Activity Relationship to Biologically Active Inositol Phosphates†

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Unambiguous total syntheses of both optical antipodes of the enantiomeric pair D-*myo*-inositol 3,4,5-trisphosphate (Ins(3,4,5)P_3) and D-*myo*-inositol 1,5,6-trisphosphate (Ins(1,5,6)P_3) are described. The ring system characteristic of *myo*-inositol was constructed de novo from *p*-benzoquinone. X-ray data for the enzymatically resolved (1*S*,2*R*,3*R*,4*S*)-1,4-diacetoxy-2,3-dibromocyclohex-5-ene enabled the unequivocal assignment of the absolute configuration. Subsequent transformations under stereocontrolled conditions led to enantiopure C_2-symmetrical 1,4-(di-*O*-benzyldiphospho)conduritol B derivatives. Their synthetic potential was exploited to prepare Ins(3,4,5,6)P_4 and Ins(1,4,5,6)P_4 in three steps. With a recently identified and partially purified InsP_5/InsP_4 phosphohydrolase from *Dictyostelium discoideum*, these enantiomers could be converted to the target compounds, Ins(3,4,5)P_3 and Ins(1,5,6)P_3, on a preparative scale. An HPLC system employed for both purification of the inositol phosphates and analytical runs ensured that the products were isomerically homogeneous. The sensitivity of detection achieved by a complexometric postcolumn derivatization method indicates that the complexation properties of Ins(3,4,5)P_3/Ins(1,5,6)P_3 resemble those of Ins(1,2,3)P_3, a compound with antioxidant potential. The set of inositol phosphates synthesized was used to clarify structural motifs important for molecular recognition by p42^{IP4}, a high-affinity Ins(1,3,4,5)P_4/PtdIns(3,4,5)P_3-specific binding protein from pig cerebellum.

Introduction

In a wide variety of eukaryotic cells intracellular calcium mobilization in response to agonist stimulation is mediated by the interaction of the second messenger D-*myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) with a specific receptor, a ligand-gated calcium channel.[1] The action of this short-lived signaling molecule is modulated by an extensive metabolism.[2] Kinases and/or phosphatases produce many other inositol polyphosphates, and some of them also appear to participate in cellular signaling.[3] Although their precise functions have yet to be elucidated, the discovery of potential sites for interaction supports this idea.[4] Proteins such as diacylglycerol kinase, RAC-protein kinase B, and phospholipase C possess a pleckstrin-homology domain (PH; abbreviation: ***p***latelet and ***le***ukocyte ***C k***inase sub***str***ate prote***in***),[5−7] a region of approximately 100 amino acids that can form an electrostatically polarized tertiary structure.[7] Common ligands of this domain seem to be inositol phosphates/phosphoinositides, the β/γ-subunits of heterotrimeric G-proteins and protein kinase C.[8−10] It has been proposed that the interaction plays a role in membrane recruitment of proteins, thereby enabling a coordinated link between components of the signal-transduction pathway. In the case of inositol phosphates as possible ligands, PH domains derived from various proteins showed distinct specificities.[5] The main problem is to unravel interactions that are indeed of physiological significance.

Despite the natural occurrence of a plethora of inositol phosphate isomers in eukaryotic cells, e.g., about 25 compounds in *Dictyostelium discoideum*, the microorganism used in our studies,[11] it is obvious that not all of them fulfill essential cellular functions. One feature of a signaling molecule is the response of the intracellular level to an appropriate stimulus. The levels of the inositol tetrakisphosphates Ins(1,3,4,5)P_4 and Ins(3,4,5,6)P_4/Ins(1,4,5,6)P_4 could be stimulated by agonists in several cell lines,[12−14] and high-affinity binding sites are known[15,16] especially for Ins(1,3,4,5)P_4 (p42^{IP4},[17] GAP1,[18] centaurin-α[19]). Although some of these "receptors" have been purified, cloned, and sequenced, their cellular functions remain elusive.

The isomers Ins(1,3,4,5)P_4 and Ins(3,4,5,6)P_4 share as a common structural motif three adjacent phosphate esters in positions C-3, C-4, and C-5. Thus the enantiomers Ins(3,4,5)P_3 and Ins(1,5,6)P_3 could be useful tools for intervention in their metabolism or for binding studies. This assumption encouraged us to synthesize both optical antipodes of the enantiomeric pair Ins(3,4,5)P_3/Ins(1,5,6)P_3. We describe here a new approach based on a straightforward chemical synthesis of Ins-

† According to the recommendation of IUPAC, all given names of inositol phosphates refer to D-enantiomers; see: Nomenclature Committee of the International Union of Biochemistry (NC-IUB) Numbering of Atoms in *myo*-Inositol. Recommendations 1988. *Eur. J. Biochem.* **1989**, *180*, 485−486.

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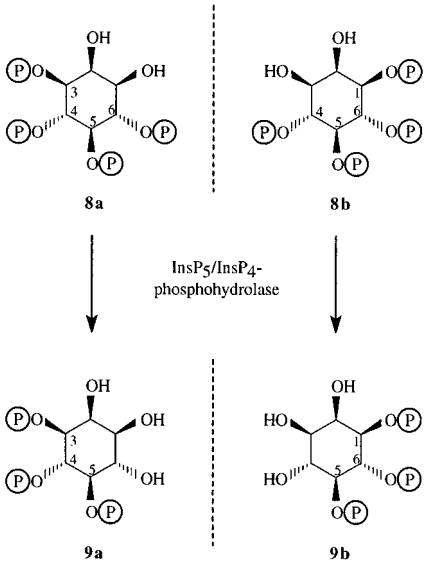
Scheme 1. Enzyme-Assisted Synthesis of the Target Compounds **9a** and **9b**



(3,4,5,6)P$_4$ (**8a**) and Ins(1,4,5,6)P$_4$ (**8b**) in combination with enzymatic conversion of the resulting compounds to Ins(3,4,5)P$_3$ (**9a**) and Ins(1,5,6)P$_3$ (**9b**).

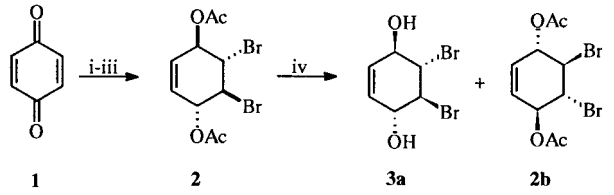
Conventional inositol phosphate synthesis usually requires extensive separation of diastereomers to yield enantiopure compounds.[20] We avoid this by using clearly defined precursors easily accessible from enzymatic resolution of the racemic diacetate **2**. Stereospecific conversion leads in only two steps to C_2-symmetric conduritol-B derivatives, which can readily be transformed into Ins(3,4,5,6)P$_4$ (**8a**) or Ins(1,4,5,6)P$_4$ (**8b**).

In the course of our investigations of the inositol phosphate metabolism in *D. discoideum*, we have previously found a cytosolic phosphatase activity that prefers InsP$_5$ and InsP$_4$ isomers as substrates and produces inositol trisphosphates. It exhibits characteristics quite different from those of published inositol phosphate-specific phosphatases.[21] One remarkable feature is the combination of high regiospecificity with low stereospecificity. Mirror positions of enantiomerically pure substrates are thus converted with nearly equal rates. This is of especially practical value for the synthesis of enantiomeric pairs, and the conversion of Ins(3,4,5,6)-P$_4$ (**8a**) and Ins(1,4,5,6)P$_4$ (**8b**) is presented in Scheme 1 as an example. The ability of the purified target compounds Ins(3,4,5)P$_3$ (**9a**) and Ins(1,5,6)P$_3$ (**9b**) to compete with Ins(1,3,4,5)P$_4$ in a displacement experiment has been investigated with a recently characterized Ins(1,3,4,5)P$_4$/PtdIns(3,4,5)P$_3$-specific receptor from pig cerebellum (p42^{IP4}),[17] a 42-kDa protein containing two PH domains.[22]

Results and Discussion

Chemical Synthesis. Knowledge of the regiospecificity of the InsP$_5$/InsP$_4$ phosphohydrolase examined suggested to us that Ins(3,4,5,6)P$_4$ (**8a**) and Ins(1,4,5,6)-P$_4$ (**8b**) should be suitable precursors of the desired inositol phosphates (Scheme 1). **8a** and **8b** have been synthesized by several groups before;[23−25] there is, however, some confusion in the assignment of the absolute configuration[26] as is apparent from the opposite signs of the optical rotation reported for the same compounds. Therefore an unambiguous synthesis of the pure enantiomers was needed, the more so as these tetrakisphosphates are interesting objects for biological investigation themselves.

Scheme 2. Enzymatic Resolution of Diacetate **2**[a]



[a] Conditions: (i) Br$_2$, CHCl$_3$, 0 °C (98%); (ii) NaBH$_4$, Et$_2$O, −20 °C to rt (88%); (iii) pyridine, acetic anhydride, overnight (68%); (iv) PPL, phosphate buffer (pH 7), 4 days (38% each).

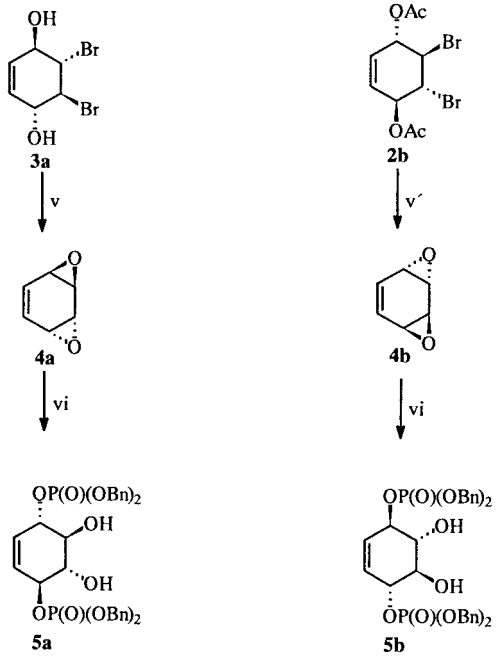
Most *myo*-inositol phosphate syntheses start from achiral *myo*-inositol and require separation of diastereomers to yield enantiomerically pure compounds in the end. In addition to the expenditure associated with this step, errors made here may lead to confounding the enantiomers. As knowledge of the correct stereochemistry is absolutely necessary for biological studies, we put special emphasis on this aspect. A de novo synthesis allows the employment of defined starting materials. Carrying out the following steps under stereocontrolled conditions ensures the identity of the product.

Previous work led us to believe that the known dibromocyclohexenediol **3**,[27] which can easily be obtained from *p*-benzoquinone in two steps, would be an appropriate starting material. Enantiopure compounds could be received by acetylation and subsequent enzymatic resolution by pig pancreas lipase[28] in a phosphate buffer (Scheme 2). The absolute configuration of the resulting products **3a** and **2b** was determined by synthesis of optically pure conduritol-B and (+)-pinitol[28a] and was confirmed by an X-ray structure of the diacetate **2b**. The crystals examined possess a chiral space group, and the absolute structure parameter of 0.01(4) shows the (+)-isomer **2b** to be the (1*S*,2*R*,3*R*,4*S*)-enantiomer.

The resolution procedure reported offers many advantages. For example, hydrolysis stops after 50% conversion, so no reaction control is required. Furthermore, the application of relatively small quantities of enzyme is sufficient to resolve up to 100 g of racemic diacetate. The resulting products can be separated simply by their different solubility in nonpolar solvents such as dichloromethane: Diacetate **2b** dissolves easily, while dibromocyclohexenediol **3a** is almost insoluble in dichloromethane.

Enantiomeric excess after recrystallization of the crude products was determined by chiral HPLC on a Whelk S,S column to be >99% in each case. For this analysis, **3a** was converted into the corresponding diacetate **2a** with pyridine and acetic anhydride. Treatment of diol **3a** with potassium hydroxide and powdered molecular sieves in tetrahydrofuran, a slight modification of Farkas's method,[29] leads to *anti*-benzene dioxide (*trans*-1,2:3,4-diepoxycyclohex-5-ene) **4a** in good yields. Conversion of the diacetate **2b** under the same conditions was unsatisfactory. It stopped after formation of one epoxide ring and cleavage of the remaining acetate function. Longer reaction times or employment of additional base did not influence the course of the reaction.

Scheme 3. Preparation of Phosphorylated Conduritol-B Derivatives **5a** and **5b**[a]



[a] Conditions: (v) KOH, powdered molecular sieves (4 Å), 4 °C (77%); (v′) KOH, powdered molecular sieves (4 Å), methanol, 4 °C (80%); (vi) dibenzyl phosphate, abs CH_2Cl_2, 8 h (55%).

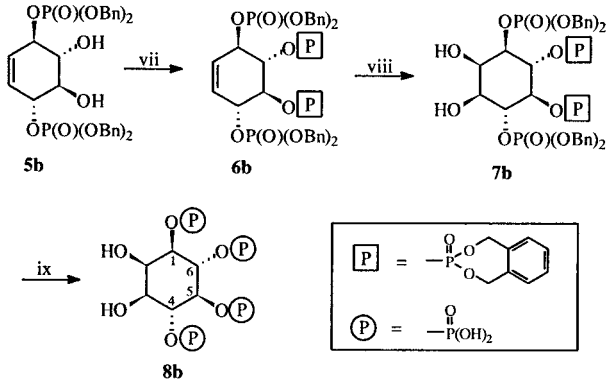
However, by addition of limited amounts of ethanol to the reaction mixture, complete reaction to **4b** could be achieved in less than 30 min (H.-J. Altenbach and A. Kaffee, unpublished results).

Double allylic epoxide opening of **4a** and **4b** by dibenzyl phosphate gives access to the phosphorylated conduritol-B derivatives **5a** and **5b** (Scheme 3). The inherent C_2-symmetry of these compounds makes them ideal for *myo*-inositol phosphate synthesis: *Cis*-dihydroxylation leads only to the *myo*-configuration, regardless of the direction of attack by the hydroxylating reagent. As modifications at the free hydroxyl groups can be carried out before the *cis*-dihydroxylation, the method described here thus allows pairwise differentiation of hydroxyl groups in *myo*-inositol systems, so that a flexible approach to a wide variety of inositol phosphates is possible. Further work on this topic is in progress and will be published elsewhere.

Ins(1,4,5,6)P_4 (and Ins(3,4,5,6)P_4 from enantiomer **5a** instead) was synthesized as shown in Scheme 4. The introduction of two additional phosphate functionalities was achieved by treatment with 3-diethylamino-2,4,3-benzodioxaphosphepane in the presence of 1*H*-tetrazole in dichloromethane. After thin layer chromatography showed absence of the starting material, the resulting phosphite was oxidized with *m*-CPBA. $^{31}P-^{1}H$-coupled NMR spectra allowed differentiation between the two different phosphate groups. The dibenzyl phosphates generate a pseudosextet ($^3J(P,H) = 7.2$ Hz) at −0.8 ppm, while the other phosphates appear as a multiplet at −2.2 ppm, as a result of the magnetic inequivalence of the two fixed methylene groups.

Dihydroxylation of the resulting tetraphosphoconduritol **6b** with osmium tetraoxide was very slow and led to unsatisfactory results. The flash dihydroxylation method,[30] in which catalytic amounts (10 mol %) of

Scheme 4. Preparation of Ins(1,4,5,6)P_4 (**8b**)[a]



[a] Conditions: (vii) 3-diethylamino-2,4,3-benzodioxaphosphepane, 1*H*-tetrazole, CH_2Cl_2 (83%); (viii) $RuCl_3$, $NaIO_4$, ethyl acetate/acetonitrile/water, 7 min (87%); (ix) Pd/C, H_2, ethanol/water (95%).

ruthenium trichloride and 1.5 equiv of sodium metaperiodate in acetonitrile/ethyl acetate/water were employed, gave good results. In the work cited, it was shown that reaction times longer than 3 min often lead to oxidative fission and/or glycol cleavage, because of the presence of metaperiodate in the aqueous layer. Examination of 1,4-disubstituted conduritol-B derivatives with large substituents showed a low tendency toward cleavage by sodium metaperiodate, though the four phosphate groups make the compounds very polar. Thus longer reaction times of 7−15 min to achieve complete conversion of the starting material could be employed. Removal of the remaining protecting groups by treatment with hydrogen and Pd/C yields the desired tetrakisphosphate **8b**, already pure by NMR spectroscopic standards.

Purification of the resulting inositol phosphates by HPLC ensures purities suitable for biological experiments. Following this independent route, Ins(3,4,5,6)-P_4 (**8a**) was identified as the (+)-enantiomer, which corresponds to the data published in refs 23 and 24.

Enzyme Preparation and Preliminary Characterization. *D. discoideum* is a cellular slime mold that shares features of animal and plant cells. Molecular phylogenetic analysis has revealed that it is possibly more closely related to the animal−fungal branch of the eukaryotic tree than green plants are.[31] The microorganism naturally occurs in soil, where the unicellular ameboid form feeds on bacteria. For laboratory use there exist axenic mutants that can grow on fluid media with doubling times of about 8 h,[32] so it is possible to obtain sufficient quantities within an acceptable time.

The partial purification of the cytosolic $InsP_5$/$InsP_4$ phosphohydrolase usually proceeded from 10^{10} cells (strain AX-2). Tests with crude *Dictyostelium* homogenates kept on ice had shown that the activity declined with a half-life of about 3−4 h. On the assumption that this was due to proteolytic degradation, the cells were incubated prior to lysis in a Mes buffer containing the nonmetabolizable sugar sucrose. Under these conditions the cells remain intact but secrete a great part of their lysosomal enzymes.[33] Cells were lysed by freezing/thawing and an additional passage through a 5-μm Nucleopore filter. A slightly alkaline lysis buffer, which was originally optimized to stabilize membranes, was used.[34] Cell debris was removed by ultracentrifugation, and the yellow-colored cytosolic extract was applied to

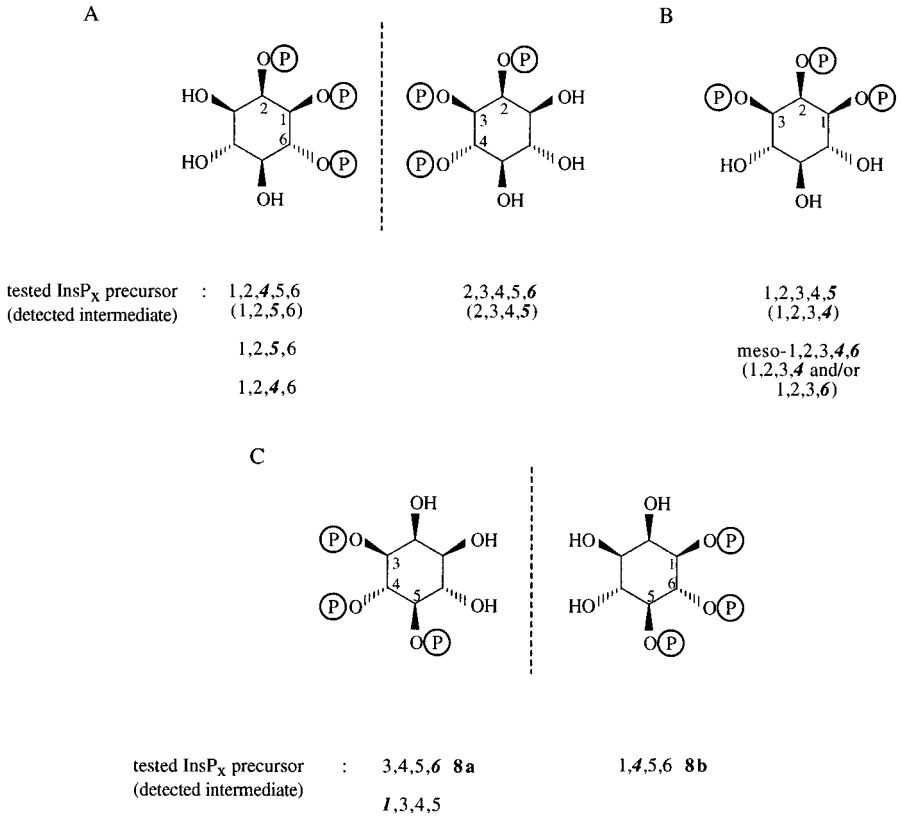


Figure 1. Synthetic potential of the partially purified InsP$_5$/InsP$_4$ phosphohydrolase. Defined inositol trisphosphates accessible from the listed InsP$_5$ and InsP$_4$ isomers (dephosphorylated positions are marked in bold and italics): A, Ins(1,2,6)P$_3$/Ins(2,3,4)P$_3$; B, *meso*-Ins(1,2,3)P$_3$; C, Ins(3,4,5)P$_3$ (**9a**)/Ins(1,5,6)P$_3$ (**9b**).

a heparin agarose column. The column was eluted stepwise with buffered NaCl solutions.

To rule out the possibility of misinterpretation, the fractions were analyzed for acid phosphatase activity,[35] Ins(1,4,5)P$_3$ 5-phosphatase activity,[36] and InsP$_5$/InsP$_4$ phosphohydrolase activity. Acid phosphatase and the 5-phosphatase are two enzymes that could probably utilize InsP$_5$ or InsP$_4$ isomers as substrates under the assay conditions employed.[37,21] These two activities were detected in the 100 mM fraction, whereas the InsP$_5$/InsP$_4$ phosphohydrolase was clearly separated and appeared in the 500 mM fraction, with an approximately 100-fold enrichment over the original cytosolic extract. The protein preparation is devoid of phosphate and InsP$_x$ metabolites, a prerequisite for the determination of the enzymatic activity by measuring liberated phosphate[38] and analyzing the products by HPLC-MDD (***m***etal-***d***ye ***d***etection).[39] We established a microplate assay with 100 μM Ins(1,2,5,6)P$_4$ as substrate to optimize reaction conditions and found the highest activity in the pH range 5.5−6.5 and in the presence of 1−2 mM Mg^{2+}. To conserve the activity for further studies and the enzyme-assisted synthesis presented below, the fraction from the heparin column was concentrated, stabilized by the addition of 25% (v/v) glycerol, and stored frozen (−83 °C).

The enzymatic activity exhibited an unusually broad substrate tolerance, releasing phosphate from various InsP$_5$ and InsP$_4$ isomers. Examination of the intermediates and products by HPLC-MDD and comparison with authentic standards led to the results illustrated in Figure 1. It is remarkable that all inositol trisphosphates identified as products bear three adjacent phosphate groups. The list of compounds tested is completed by some isomers toward which little or no activity could be measured. We can detect no conversion of InsP$_6$ and only negligible conversion of Ins(1,3,4,5,6)P$_5$. Slow dephosphorylation of Ins(1,2,4,5)P$_4$ and Ins(1,3,4,6)P$_4$ was observed.

It is obvious that all dephosphorylatable positions in the substrates investigated possess at least one vicinal *trans*-hydroxyl group, possibly a structural motif required for enzymatic activity. In summary, the synthetic potential of the enzyme preparation is such that all inositol trisphosphates phosphorylated at three adjacent positions are accessible, with the exception of Ins(4,5,6)-P$_3$.

There are only a few InsP$_5$- or InsP$_4$-specific phosphatases described in the literature.[40−42] Typically, the activities cited are measured for only one substrate, so it is impossible to reach any firm conclusions about the specificity of the enzymes. The research group of Mayr recently presented the first report of an Ins(1,2,3,4,5)-P$_5$ 5-phosphatase preparation from calf thymus, which seems to be capable of dephosphorylating the enantiomer at the same position.[43] Whether both enzymatic activities are combined in a single protein remains to be examined.

Figure 1C shows that Ins(3,4,5,6)P$_4$ (**8a**) and Ins-(1,4,5,6)P$_4$ (**8b**) are converted at their mirror positions C-6 and C-4. These reactions occur under substrate saturation of the enzyme(s) with almost equal rates (e.g., 400 μM **8a**, 10.2 mU/mL, or 400 μM **8b**, 9.7 mU/mL). To obtain further information as to whether both reactions are catalyzed by a single enzyme, the concentrated enzyme preparation was subjected to size-exclu-

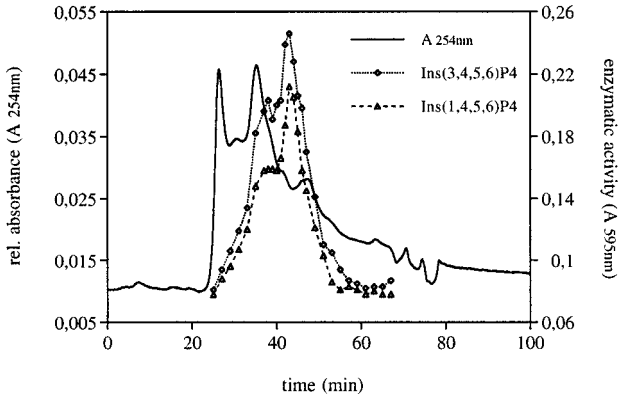


Figure 2. Gel filtration of the partially purified $InsP_5/InsP_4$ phosphohydrolase on Superdex-200 HR. The assay for phosphatase activity shows the coelution of the C-6 and C-4 position-hydrolyzing enzyme(s). Retention times for standards: aldolase (158 kDa), 42.13 min; BSA (66.2 kDa), 45.8 min; ovalbumin (45 kDa), 49.2 min; chymotrypsinogen A (25 kDa), 56.7 min; cytochrome *c* (12.5 kDa), 60.03 min.

sion chromatography. The major part of the activity eluted as a peak with an apparent molecular weight of 120 kDa (see Figure 2), determined by comparison with standard proteins run on the same column. Although a loss in total activity with little gain in specific activity was observed after chromatography, the enzyme assay clearly demonstrates that the C-6- and C-4-hydrolyzing activities coelute with exactly the same pattern (the use of Ins(1,2,4,5,6)P_5 and Ins(1,2,5,6)P_4 as substrates instead gave analogous results, not shown). This does not prove definitely that only one enzyme is responsible for the different catalytic activities observed. Nevertheless, it is an intriguing thought that the active center of a single enzyme could possibly bind and catalyze the conversion of enantiomers at their mirror positions.

Synthetic Application. In the synthetic section we noted the obvious discrepancies in reported optical rotations for Ins(3,4,5,6)P_4 (**8a**) and Ins(1,4,5,6)P_4 (**8b**). The unambiguous total synthesis presented here may help to resolve this confusion. Unfortunately, one of the very precursors that caused the misinterpretation mentioned above was used for the previously reported synthesis of Ins(1,5,6)P_3 (**9b**).[44] A different approach was exploited for the total synthesis of Ins(3,4,5)P_3 (**9a**),[45] but no value for the optical activity was quoted. We therefore set out to synthesize both optical antipodes, employing the partially purified $InsP_5/InsP_4$-phosphohydrolase as an enzymatic tool.

For this purpose the assay initially designed for the kinetic evaluation of the reaction was scaled up. The enzymatic activity remained stable at room temperature for more than 6 h. To follow the progress of the reaction, inorganic phosphate generated was measured according to the method of Lanzetta et al.[38] Usually the enzymatic conversion was stopped after 7 h by the addition of hydrochloric acid. As a consequence of denaturation, the majority of proteins coagulated and could be removed by centrifugation. The supernatant was neutralized, diluted, and applied to a Mono Q column. Separation was achieved with a linear hydrochloric acid gradient just as in the analytical runs. This has the following benefits: (a) **9a**/**9b** elutes as an individual peak not superimposing any other inositol trisphosphate currently known[39] and (b) freeze-drying the pooled fractions

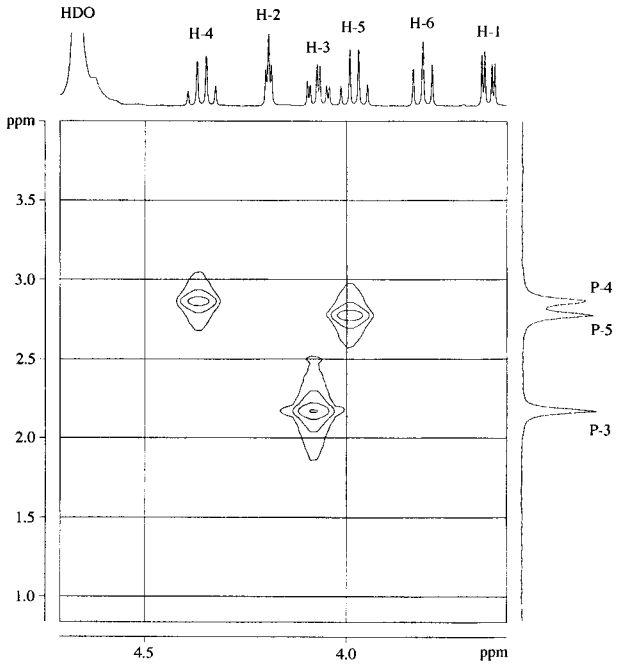


Figure 3. $^1H-^{31}P$ COSY NMR spectrum of D-*myo*-inositol 3,4,5-trisphosphate (**9a**).

led to salt-free samples. Because of remaining water, it is not appropriate to determine the yield of the protonated form by weight. Total phosphate determination after sulfuric acid hydrolysis provides a reliable quantification of phosphorylated substances. We calculated an overall yield of 70−80% for the enantiomers Ins(3,4,5)P_3 (**9a**) and Ins(1,5,6)P_3 (**9b**), equivalent to 10−12 μmol of each enantiomer in the experiments described here.

The identity of **9a** and **9b** was confirmed by one- and two-dimensional NMR spectroscopy. It was possible to assign all the ^1H, ^{13}C, and ^{31}P signals. The NMR spectra showed no traces of other inositol trisphosphate isomers (see, for example, Figure 3). The chemical shifts of inositol ring protons are highly dependent on pH, and overlapping signals sometimes make interpretation difficult. After adjustment of the pH to 5 or 6 with ND_4-OD, complete resolution was achieved. The detection limit of the NMR method for isomeric impurities is so high that amounts up to 5% of the total may be overlooked. However, for biological investigations any uncertainty about the purity of the compounds has to be precluded.

Small differences in the net charge of inositol trisphosphates, caused by varying interactions between hydroxyl and phosphate groups, suffice for separation of up to seven regioisomers on a Mono Q column with a modified, slightly alkaline elution system (see Figure 4A).[46] In an analogous experiment, we injected amounts of Ins(1,5,6)P_3 and Ins(3,4,5)P_3 more than 60 times the detection limit (~0.15 nmol, mean value) and were unable to observe any of the possible isomeric impurities (see, for example, **9b** in Figure 4B). Purities of more than 99% are thus assured.

Complexation Properties. In the past decade research on the topic of inositol phosphates has mainly been focused on functions related to signal transduction. Besides this, there is an inherent chemical property of some *myo*-inositol polyphosphates that is reflected by

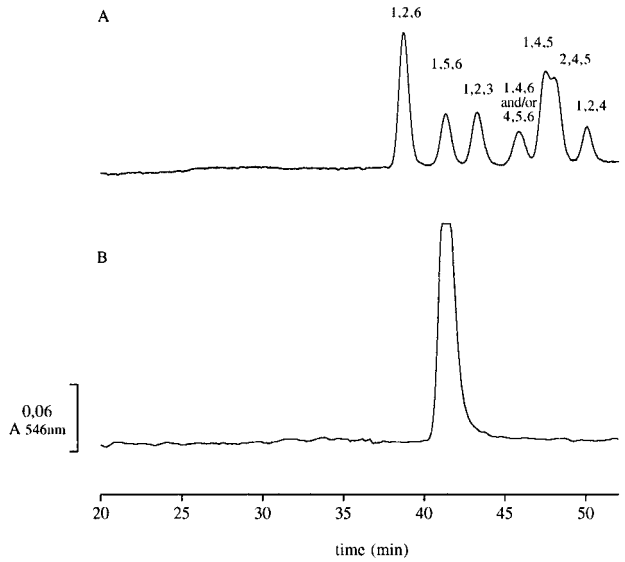


Figure 4. HPLC-MDD chromatograms showing (A) standard mixture including seven InsP$_3$ regioisomers and (B) Ins(1,5,6)-P$_3$ (**9b**), the product of the phosphatase reaction starting from Ins(1,4,5,6)P$_4$ (**8b**).

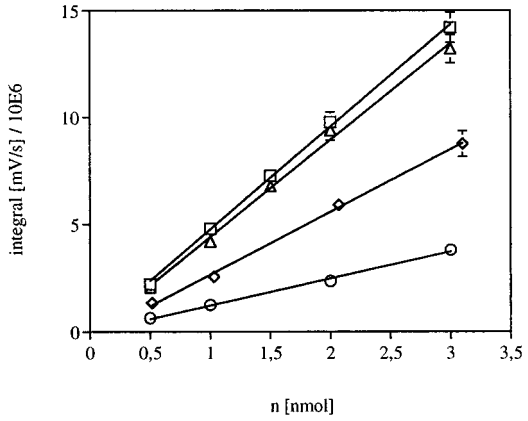


Figure 5. Calibration curves for the HCl elution system (HPLC-MDD). Sensitivity of detection reflects the complexation properties of the compounds. Regression curves calculated for the mean values of two independent experiments: Ins(1,2,3)P$_3$ (□), $y = 4.799*x - 0.038$, $r = 0.999$; Ins(3,4,5)P$_3$ (**9a**)/Ins(1,5,6)P$_3$ (**9b**) (△), $y = 4.531*x - 0.131$, $r = 0.998$; Ins-(1,2,6)P$_3$/Ins(2,3,4)P$_3$ (◇), $y = 2.918*x - 0.269$, $r = 0.999$; Ins-(1,4,5)P$_3$/Ins(3,5,6)P$_3$ (○), $y = 1.252*x - 0.028$, $r = 0.998$.

their antioxidant potential.[47] The protective effect of dietary InsP$_6$ against cancer is attributed in part to its ability to chelate iron by occupying all of the coordination sites.[48] This complete occupation seems to be the reason InsP$_6$ can effectively prevent free hydroxyl radical formation catalyzed by iron in the Haber−Weiss cycle. It has been demonstrated that not all six phosphates on *myo*-inositol are required for the inhibition and concluded that the 1,2,3-trisphosphate grouping contained the essential binding site.[49]

Complexation of transition-metal ions by *myo*-inositol (poly)phosphates is not restricted to iron. The metal-dye detection employed as a postcolumn derivatization method is a kind of "on-line complexometry", based on the ability of yttrium to reversibly bind both the cation-specific dye PAR and the polyanion InsP$_x$ with high affinity.[39] Consequently, the sensitivity of detection depends on the degree of complexation of yttrium by the inositol phosphate of interest.

The employed HPLC system is routinely calibrated for new inositol phosphate isomers, recording the peak area versus injected mass. In Figure 5 the calculated curves for Ins(1,2,3)P$_3$, Ins(3,4,5)P$_3$ (**9a**)/Ins(1,5,6)P$_3$ (**9b**), and Ins(1,2,6)P$_3$/Ins(2,3,4)P$_3$ are depicted. The inositol trisphosphates, all bearing three adjacent phosphate groups with different spatial orientations (see Figure 1), were detected with the following sensitivity: Ins(1,2,3)P$_3$ ∼ **9a**/**9b** > Ins(1,2,6)P$_3$/Ins(2,3,4)P$_3$. This indicates that the compounds **9a**/**9b** chelate yttrium about as well as Ins(1,2,3)P$_3$ does. It would thus be interesting to examine their antioxidant potential. The sensitivity decreases drastically if the block of neighboring phosphate groups is divided as is shown for Ins-(1,4,5)P$_3$ in Figure 5.

Biological Evaluation/Recognition by a Specific Receptor. The set of inositol phosphates (**8a**/**8b** and **9a**/**9b**, Scheme 1) synthesized was used to gain additional insights into structural requirements of ligand binding to p42^{IP4}, an Ins(1,3,4,5)P$_4$/PtdIns(3,4,5)P$_3$-specific receptor from pig cerebellum,[17] especially with regard to stereospecificity. The results of the displacement experiments (see Figure 6A) confirm the relevance of the phosphate groups in position C-3, C-4, and C-5, as postulated in a previous study in which racemic mixtures were employed.[50] IC$_{50}$ values for Ins(1,3,4,5)-P$_4$ and Ins(3,4,5)P$_3$ (**9a**) differ minimally (factor 2), so a phosphate group in position C-1 is not an essential motif for high-affinity binding. Like other Ins(1,3,4,5)P$_4$ binding sites described, the protein exhibited relatively poor stereoselectivity.[18,51] Ins(1,5,6)P$_3$ (**9b**) (identical to L-Ins-(3,4,5)P$_3$) was nearly one-seventh as effective as Ins-(3,4,5)P$_3$ (**9a**) at displacing specific [^{3}H]Ins(1,3,4,5)P$_4$ binding from the receptor population. Searching for a rational explanation, we note that a nearly complete superposition could be achieved by a simple rotation of Ins(1,5,6)P$_3$ (see Figure 6B). On the precondition that the orientations of the hydroxyl groups in positions C-2 (Ins(3,4,5)P$_3$)/C-4 (Ins(1,5,6)P$_3$) and in positions C-6 (Ins-(3,4,5)P$_3$)/C-2 (Ins(1,5,6)P$_3$) have only minor influences on the recognition, this could be the reason for the observed lack of stereospecificity. The binding characteristics of the inositol tetrakisphosphates indicate that additional phosphate groups in positions C-6 (**8a**) and C-4 (**8b**) clearly reduce the interaction. This tendency becomes more pronounced in going from Ins(3,4,5)P$_3$ to Ins(3,4,5,6)P$_4$ than from Ins(1,5,6)P$_3$ to Ins(1,4,5,6)P$_4$.

Conclusions

The major advantage of the approach presented here for synthesizing *myo*-inositol phosphates de novo is its versatility. As mentioned in the chemical section above, the synthetic potential of the precursors **5a** and **5b** is not restricted to the compounds described here.

A defined starting material, modified by stereocontrolled reaction steps, assured the correct absolute configuration of the inositol tetrakisphosphates. The use of a regiospecific InsP$_5$/InsP$_4$ phosphohydrolase as an enzymatic tool increased significantly the number of isomers obtainable. The unusual substrate specificity of the enzymatic activity seems to allow the conversion of a series of enantiomeric pairs (Figure 1). Since dephosphorylation of the enantiomers occurs at mirror

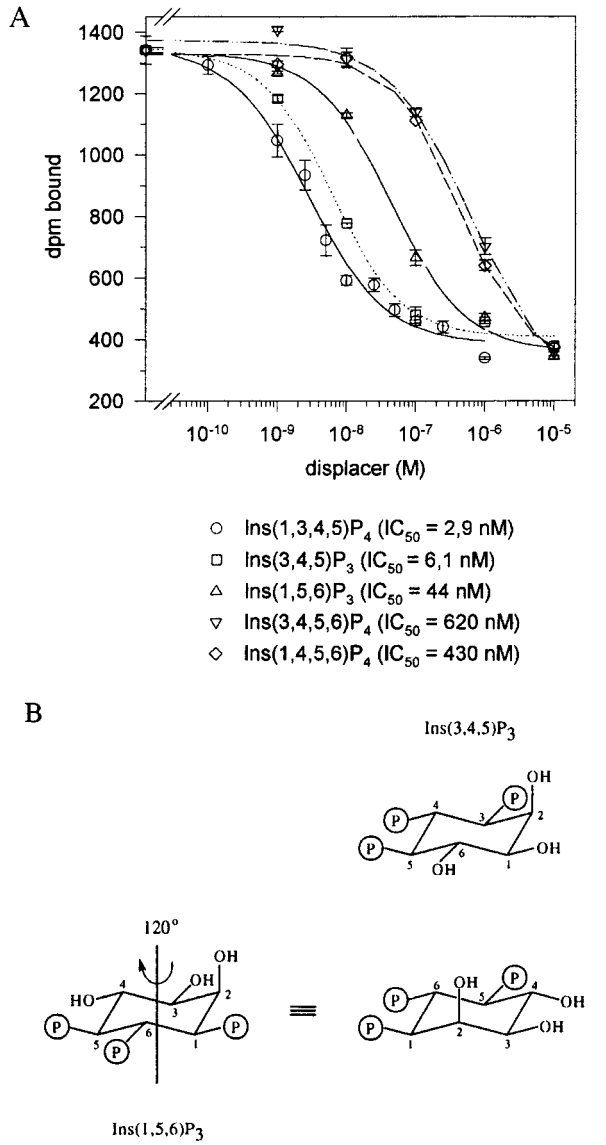


Figure 6. A: [^{3}H]Ins(1,3,4,5)P_4 displacement experiment with a membrane preparation from pig cerebellum containing p42^{IP4} (for details, see Experimental Section). B: Model for a possible interpretation of the observed lack of stereospecificity.

positions, a further enantiomeric pair or a *meso*-compound is accessible.

This strategy enabled us to synthesize two enantiomeric pairs (**8a**/**8b** and **9a**/**9b**) in a single experimental sequence. The set of enantiopure compounds was successfully exploited in a competition assay that revealed the relevance of the phosphate groups in positions C-3, C-4, and C-5 for high-affinity binding to p42^{IP4}, a Ins(1,3,4,5)P_4/PtdIns(3,4,5)P_3-specific protein. These compounds are expected to be of considerable use in unraveling aspects of inositol phosphate binding to the expanding number of eukaryotic proteins with identified PH domains.[52]

Ins(1,5,6)P_3 (**9b**), a product of the metabolism of Ins(1,3,4,5,6)P_5 from avian erythrocytes,[53] is one of a series of isomers with an unknown function in mammalian cells. Recently an inhibitory effect on the activity of SIP-110, an Ins(1,3,4,5)P_4/PtdIns(3,4,5)P_3-specific 5-phosphatase, could be demonstrated in in vitro studies.[54] It is supposed that this enzyme is involved in the control of the cellular level of PtdIns(3,4,5)P_3. The supply of Ins(1,5,6)P_3 (**9b**) is no longer limited to scarce material isolated from natural sources, so it will be possible to examine such phenomena in detail.

Experimental Section

Chemicals were purchased from Fluka, Aldrich, or Acros. Dichloromethane was dried over phosphorus pentoxide and kept over 4-Å molecular sieves. TLC was performed on precoated plates (Merck aluminum TLC sheets silica F_{254}, art. no. 5554), with detection by UV light or a solution of 1% vanillin in sulfuric acid, followed by heating. Flash column chromatography was performed on silica gel (63−200 μm; Merck). ^{1}H and ^{13}C NMR spectra (internal Me_4Si as reference) and ^{31}P NMR (external 85% phosphoric acid as reference) were recorded with an ARX 400 spectrometer (Bruker). Chemical shifts are given in ppm (δ) relative to the used reference; coupling constants are in Hz. Assignments of ^{1}H, ^{13}C, and ^{31}P NMR resonances were aided by 2D COSY and DEPT experiments. Optical rotation was determined on a Zeiss spectrometer model.

IR spectra were recorded on a Perkin-Elmer spectrometer model 1420. Melting points were determined on a Büchi 510 heating block (not corrected). Mass analysis was carried out on a Varian MAT 311 A (EI), Perkin-Elmer API 150 (ESI), or a Finnigan MAT 90 (FAB) spectrometer. High-resolution mass measurements were carried out on a Finnigan II-SQ 30. Elemental analysis was carried out on a PE model 240 B microelemental analyzer.

Inositol phosphates synthesized in our laboratories were purified by HPLC. All reagents used were obtained in the highest purity available. A membrane-associated, stereospecific InsP_6 phosphohydrolase from *D. discoideum* was exploited for the synthesis of Ins(1,2,3,4,5)P_5, Ins(1,2,4,5)P_4, Ins(1,2,4,6)-P_4, and Ins(1,2,4)P_3 (M. Knipp and M. Lammertz, manuscript in preparation). Ins(1,2,4,5,6)P_5 and Ins(1,2,5,6)P_4 were synthesized enzymatically using baker's yeast as previously described.[55] The *meso*-compounds, Ins(1,3,4,5,6)P_5, and Ins-(1,2,3,4,6)P_5 were prepared by acid-catalyzed dephosphorylation of InsP_6.[55] Ins(2,3,4,5,6)P_5 was a generous gift from C. Schultz (University of Bremen, FRG).[43] Chemically synthesized Ins(1,4,6)P_3 and Ins(4,5,6)P_3 were kindly provided by the research group of M. Schneider (University of Wuppertal, FRG). Commercially available inositol phosphates were purchased from Sigma (InsP_6, Ins(1,3,4,6)P_4, and Ins(1,4,5)P_3), Boehringer Mannheim (Ins(1,3,4,5)P_4), and Calbiochem (Ins-(2,4,5)P_3). The sources of other materials are described below.

Cell Culture and Partial Purification of InsP_5/InsP_4 Phosphohydrolase. *D. discoideum* strain AX-2 (ATCC 24397) was grown in axenic medium[32] supplemented with 0.025% dihydrostreptomycin sesquisulfate (w/v). Fernbach flasks were filled with at most 500 mL of medium, and cells were cultured at 22 °C on a rotary shaker (130 rpm). At a density of about 1×10^7 cells/mL, the cells were collected by centrifugation (1500*g*, 10 min, 4 °C), washed once with 20 mM Mes/Na^+ (pH 6.5), and resuspended to the same density in the Mes buffer containing 0.1 M sucrose.[33] After 3 h of incubation under the conditions mentioned above, the cells were harvested by centrifugation, washed once with ice-cold lysis buffer (5 mM glycine (pH 8.5), 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$), and lysed at a density of 1×10^8 cells/mL in the glycine buffer by freezing (−83 °C)/thawing. Virtually complete lysis was achieved by an additional passage through a 5-μm Nucleopore (Millipore) filter.[34] Resulting cell debris was removed by ultracentrifugation (140000*g*, 45 min, 4 °C). The following steps were performed at 4 °C. The cytosolic supernatant from a total of 10^{10} cells was carefully decanted and applied to a 15-mL heparin agarose (type II, Sigma) column, equilibrated with the lysis buffer (about 310 mg of soluble protein; flow rate 60 mL/h). The column was eluted stepwise with 50 mM Tris/HCl (pH 7.7), 1 mM EDTA containing increasing concentrations of NaCl. The following distribution of protein was typically found in the collected fractions: 210 mg (void volume/wash), 65 mg (100 mM fraction), 3 mg (200 mM fraction), 7 mg (500 mM fraction), and 2 mg (1 M fraction). The fractions were analyzed

for acid phosphatase, Ins(1,4,5)P_3 5-phosphatase, and InsP_5/InsP_4 phosphohydrolase activity. The 500 mM protein fraction was concentrated to the minimal volume attainable by the ultrafiltration method employed (Centriprep-10, Millipore), diluted 1:1 with the Tris buffer, aliquoted, and frozen (−83 °C) after the addition of 25% glycerol (v/v).

A 1.5-mg portion of this protein preparation was subjected to size-exclusion chromatography on a Superdex 200 HR 10/30 column (Pharmacia; fraction size 0.3 mL, flow rate 0.3 mL/min). The column was equilibrated and eluted with 40 mM Tris/HCl (pH 7.7), 50 mM NaCl, 1 mM EDTA. An identical run with standard proteins allowed the molecular weight to be estimated.

Enzyme Assays. 1. InsP_5/InsP_4 Phosphohydrolase. A microplate assay was established that allowed fast screening of various substrates and provided kinetic data. A standard incubation mixture contained 10 μL of assay buffer (200 mM Mes/Na^+ (pH 5.5), 4 mM $MgCl_2$, 0.4% Triton X-100 hydr. (w/v)), 10 μL of water, 10 μL of a protein fraction, and 10 μL of a 400 μM inositol phosphate solution. The samples were incubated at room temperature, with the plate placed on a reciprocal shaker (80 rpm). The enzymatic reaction was terminated after varying periods by adding 10 μL of 0.5 M HCl. No acid-catalyzed, chemical dephosphorylation of inositol phosphates could be detected after 2 h under these conditions. It was thus possible to complete an experiment before the mixtures were either analyzed for enzymatically released inorganic phosphate or alternatively subjected to HPLC-MDD analysis of inositol phosphates after neutralization.

2. Ins(1,4,5)P_3 5-Phosphatase. Activity was determined by a modification of the method of van Lookeren Campagne et al.[36] The assay was carried out on a microplate in a solution composed of the following components: 10 μL of buffer (160 mM Bis-Tris (pH 7.0), 800 mM sucrose, 1 mM EDTA, 20 mM $MgCl_2$), 10 μL of water, 10 μL of protein fraction, and 10 μL of 200 μM Ins(1,4,5)P_3 solution. The samples were treated, the reaction was stopped, and inorganic phosphate was analyzed as reported for the assay of InsP_5/InsP_4 phosphohydrolase activity.

3. Acid Phosphatase. Activity was assayed essentially as described in the literature.[35] Volume activities were calculated from the linear regions of kinetic data plots. All assays were tested for linearity with increasing enzyme concentration. Protein was measured using an assay supplied by BIO-RAD (catalog no. 500-0112) with bovine serum albumin as a standard.

Inorganic Phosphate Determination. A colorimetric assay was employed for the determination of nanomolar amounts of inorganic phosphate.[38] Reducing the volume to a microplate scale made it possible to handle a great number of probes simultaneously. The method was applicable to follow the progress of the enzymatic phosphatase reactions and to quantify the mass of purified inositol phosphates. In the former case, the acidic solution (generally 50 μL; preparation described under Enzyme Assays) was mixed with 100 μL of malachite green reagent, and after 1 min 10 μL of 34% sodium citrate·$2H_2O$ (w/v) was added. For full color development the mixture was incubated for 20 min at room temperature. Absorbance was measured at 595 nm. The assay was linear in the range 0−3 nmol of phosphate.

Covalently bound organic phosphate had to be liberated by treatment with hot sulfuric acid. In brief, a 10-μL aliquot of an inositol phosphate solution (approximately 1−2 mM InsP_3 or InsP_4) was pipetted into 50 μL of 10 N H_2SO_4, mixed, and heated for 5 h at 170 °C (borosilicate glass tubes of hydrolysis class 1 are required; Macherey-Nagel). The residue was diluted 100-fold with 1 N H_2SO_4, and 50-μL portions of the solution were assayed for inorganic phosphate. Mass determinations for each substance were done in triplicate, and the results were validated by HPLC-MDD analysis (HCl gradient). On injection of the same amounts of enantiomers in two independent experiments, the recorded peak areas differed less than 5%.

Purification and Analysis of Inositol Phosphates. Inositol phosphates were purified and analyzed using the HPLC-MDD method described previously.[39] The compounds were separated by anion-exchange chromatography on a Mono Q HR 10/10 column (Pharmacia). To distinguish between InsP_4 and InsP_5 isomers and to purify **8a**/**8b** and **9a**/**9b**, a linear gradient of HCl was applied to elute the inositol phosphates (0 min, 0.2 mM HCl; 70 min, 0.5 M HCl; flow rate 1.5 mL/min). In analytical runs photometric detection at 546 nm was achieved using a modified metal-dye reagent (2 M Tris/HCl (pH 9.1), 200 μM 4-(2-pyridylazo)resorcinol (PAR), 30 μM YCl_3, 10% (v/v) MeOH; flow rate 0.75 mL/min). Where on-line detection was impossible (e.g., purification steps), an analogous experiment could be carried out on a microplate. This allowed fast screening for the presence of inositol phosphates. In brief, a 0.2−10-μL portion of the sample (depending on the InsP_x concentration) was mixed with 100 μL of metal-dye reagent, and the absorbance was measured at 540 nm.

The isomeric purity of the inositol trisphosphates **9a**/**9b** was controlled by use of a modified, slightly alkaline elution system.[46] The gradient composed of 50 mM Tris/HCl (pH 8.5; solvent A) and 50 mM Tris/HCl, 0.4 M KCl (pH 8.5; solvent B) had the following characteristics: 0 min, 30% B; 2 min, 40% B; 16 min, 42% B; 20 min, 50% B; 38 min, 60% B; 48 min, 75% B; 50 min, 100% B; 60 min, 100% B (flow rate 1.5 mL/min). The metal-dye reagent consisted of 2 mM NH_4OAc/AcOH (pH 5.0), 200 μM 4-(2-pyridylazo)resorcinol, 30 μM YCl_3, and 10% (v/v) MeOH (flow rate 0.75 mL/min).

Peaks were assigned to specific compounds by cochromatography with commercially available standards or with preparatively synthesized and purified inositol phosphates, which were identified by NMR analysis.

Chemical Syntheses. (5*RS*,6*RS*)-5,6-Dibromocyclohex-2-ene-1,4-dione. Bromine (159.8 g, 1.0 mol) in 400 mL of $CHCl_3$ was added dropwise over 2 h to a cooled solution of *p*-benzoquinone (**1**) (108.1 g, 1.0 mol) in 900 mL of $CHCl_3$. The reaction mixture was allowed to warm to room temperature and stirred for another hour. The chloroform was evaporated to give 262.5 g (98 %) of crude 2,3-dibromo-1,4-benzoquinone, which was immediately used for reduction: mp: 82−83 °C (lit.[29] mp 82−84 °C, lit.[56] mp 85.5−86 °C); ^{1}H NMR ($CDCl_3$, 400 MHz) 4.80 (s, 2H, H−C5 and H−C6), 6.75 (s, 2H, H−C2 and H−C3); ^{13}C NMR ($CDCl_3$, 101 MHz) 45.15 (2 CH, C5 and C6), 136.89 (2 CH, C2 and C3), 188.02 (2 C, C1 and C4); IR (KBr) 3045 (=C−H), 3000, 2975 (−C−H), 1750, 1690 (C=O); MS (EI, 70 eV, rel intensity) m/z = 266/268/270 [(M^+), 6], 187/189 [(M^+ − Br), 68], 159/161 [(M^+ − Br − CO), 3], 108 [(M^+ − 2 Br), 5].

(1*RS*,2*SR*,3*SR*,4*RS*)-2,3-Dibromocyclohex-5-ene-1,4-diol (3). A solution of 5,6-dibromocyclohex-2-ene-1,4-dione (200 g, 0.75 mmol) in 2.5 L of diethyl ether was cooled to −15 °C. During 1 h a solution of sodium borohydride (60 g, 1.6 mol) in 1 L of water was added to the vigorously stirred solution. The reaction mixture was allowed to warm to room temperature and stirred for another 2 h. The ether phase was separated, and the aqueous layer was extracted five times with ethyl acetate. The combined organic layers were evaporated to yield 178.6 g (0.66 mol, 88%) of **3**: mp 149 °C (lit.[27] mp 149 °C); ^{1}H NMR (400 MHz, MeOD) 5.72 (2 H, s, H−C5 and H−C6), 4.42 (2 H, m, H−C1 and H−C4), 4.11 (2 H, m, H−C2 and H−C3); ^{13}C NMR (101 MHz, MeOD) 61.22 (CH, C2 and C3), 74.23 (CH, C1 and C4), 131.42 (CH, C5 and C6); IR (KBr) 3300 (O−H), 2960, 2890 (C−H), 1055 (C−O); MS (EI, 70 eV, rel intensity) m/z = 191/193 [(M^+ − Br), 8], 173/175 [(M^+ − Br − H_2O), 8], 111 [(M^+ − 2 Br − 1), 100]. Anal. ($C_6H_8O_2Br_2$) C, H.

(1*RS*,2*SR*,3*SR*,4*RS*)-1,4-Diacetoxy-2,3-dibromocyclohex-5-ene (2). Compound **3** (178.6 g, 0.66 mol) was dissolved in a cooled mixture of 200 mL of pyridine and 200 mL of acetic anhydride. The reaction mixture was stirred for 12 h. Ice (300 g) was added, and after stirring for 15 min dichloromethane (300 mL) was added. The layers were separated, and the aqueous layer was extracted four times with dichloromethane. The combined organic layer was washed with saturated aqueous $NaHCO_3$ (3 × 200 mL), 0.75 N HCl (3 × 200 mL), and brine (200 mL). After evaporation, the resulting residue was recrystallized from ethanol to yield **2** (159.0 g, 0.45 mol,

68%): mp 94 °C (lit.[57] mp 91−92 °C); ^{1}H NMR ($CDCl_3$, 400 MHz) 5.61 (s, 2 H, H−C5 and H−C6), 5.68 (2 H, mc, H−C1 and H−C4), 4.26 (2 H, mc, H−C2 and H−C3), 2.20 (2 CH_3, $COCH_3$); ^{13}C NMR ($CDCl_3$, 101 MHz) 20.70 (CH_3, $COCH_3$), 52.65 (CH, C2 and C3), 73.29 (CH, C1 and C4), 128.12 (CH, C5 and C6), 169.68 (C, C=O); IR 2980, 2940 (−C−H), 1750 (C=O), 1220, 1040 (C−O−C), 715 (C−Br); MS (EI, 70 eV, rel intensity) *m/z* = 295/297/299 [(M^+ − OC(O)CH_3), 1], 257/277 [(M^+ − Br), 2], 173/175 [(M^+ − Br − 2 OC(O)CH_3), 33], 153 [(M^+ − 2 Br − OC(O)CH_3), 33]. Anal. ($C_{10}H_{12}Br_2O_4$) C, H.

(1*S*,2*R*,3*R*,4*S*)-Diacetate 2b and (1*R*,2*S*,3*S*,4*R*)-Diol 3a. Powdered racemic diacetate **2** (100.0 g, 0.28 mol) and pig pancreas lipase (54.7 g) were suspended in 1.2 L of 0.1 M phosphate buffer (pH 7) and 120 mL of diethyl ether. The mixture was stirred vigorously for 4 days. Ethyl acetate (500 mL) was added, and the enzyme was filtered over a pad of Celite. The residue was washed with ethyl acetate (4 × 150 mL) and water (4 × 150 mL). The combined aqueous phase was extracted with ethyl acetate (3 × 150 mL). The organic phase was evaporated, and the resulting solid was suspended in dichloromethane (600 mL). Diol **3a** remained insoluble as a white, crystalline solid, while diacetate **2b** dissolved totally. The solid was filtered off and recrystallized from toluene to yield **3a** in 38% (28.9 g, 0.1 mol). The dichloromethane was evaporated, and the resulting solid was recrystallized from ethanol to give **2b** as colorless needles (37.9 g, 0.1 mol, 38%). **2b**: mp 110 °C (lit.[28b] mp 107−109 °C); $[\alpha]^{20}_D$ +11.3° (*c* = 5.1, CH_2Cl_2), lit.[28b] $[\alpha]^{20}_D$ +11.7° (*c* = 1.1, CH_2Cl_2); HPLC (Whelk, S,S; heptane/2-propanol, 90:10, flow 0.8 mL/min) *t* = 9.08 min (other enantiomer **2a**, *t* = 10.24 min); for spectral data see racemic compound **2**. **3a**: mp 164 °C (lit.[28b] mp 164−166 °C), $[\alpha]^{20}_D$ +41.8 ° (*c* = 5.0, MeOH), lit.[28b] $[\alpha]^{20}_D$ +45.8° (*c* = 1.2, acetone); for spectral data see racemic compound **3**.

(1*R*,2*R*,3*R*,4*R*)-*anti*-Benzene Dioxide (4a). Compound **3a** (8.2 g, 30.4 mmol) was dissolved in 300 mL of absolute THF under argon and cooled to 4 °C. During 30 min a mixture of powdered KOH (20 g) and powdered 4-Å molecular sieves (13 g) was added in portions. The reaction mixture was stirred for 1 h and allowed to warm to room temperature. To complete conversion, another 5 g of the mixture (2 g KOH and 3 g molecular sieves) was added. Stirring was continued for another hour. Then 400 mL of diethyl ether was added, and the solution was filtered. The residue was washed with diethyl ether (3 × 100 mL), and the combined organic phase was evaporated to yield **4a** as a colorless solid (2.57 g, 23.3 mmol, 77%): mp 59 °C; $[\alpha]^{20}_D$ −320.2° (*c* = 0.75, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) 3.03 (m, 2H, H−C2 and H−C3), 3.69 (m, 2H, H−C1 and H−C4), 6.02 (s, 2 H, H−C5 and H−C6); ^{13}C NMR ($CDCl_3$, 101 MHz) 46.38 (CH, C2 and C3), 53.64 (CH, C1 and C4), 129.48 (CH, C5 and C6); MS (EI, 70 eV, rel intensity) *m/z* = 111 [$(M + H)^+$, 4], 110 [(M^+), 79], 109 [($M - H)^+$, 11], 82 [23], 81 [100], 68 [4], 55 [27], 54 [45], 42 [5]. Anal. ($C_6H_6O_2$) C, H.

(1*S*,2*S*,3*S*,4*S*)-*anti*-Benzene Dioxide (4b). Compound **2b** (10.1 g, 28.4 mmol) was dissolved in 300 mL of absolute THF under argon and cooled to 4 °C. Over 15 min a mixture of powdered KOH (10 g) and powdered 4-Å molecular sieves (6.5 g) was added in portions, followed by addition of 2 mL of absolute methanol and subsequently a mixture of powdered KOH (10 g) and powdered 4-Å molecular sieves (6.5 g). The reaction mixture was stirred for 15 min and allowed to warm to room temperature. To complete conversion, stirring was continued for 1 h. Then 400 mL of diethyl ether was added, and the solution was filtered. The residue was washed with diethyl ether (3 × 100 mL), and the combined organic layers were evaporated to yield **4b** as a colorless solid (2.50 g, 22.7 mmol, 80%): mp 53 °C (lit.[58] mp 53−54 °C); $[\alpha]^{20}_D$ +320.6 ° (*c* = 1.44, $CHCl_3$), lit.[58] +170° (*c* = 0.3, $CHCl_3$); NMR and MS data were identical to those for the other enantiomer **4a**.

(1*S*,2*S*,3*S*,4*S*)-1,4-Bis(di-*O*-benzylphospho)conduritol-B (5a). To a solution of **4a** (1.98 g, 18 mmol) in absolute dichloromethane (150 mL) under argon was added dibenzyl phosphate (10.5 g, 37.8 mmol). The solution was stirred for 12 h and evaporated. Crystallization from ethyl acetate gave 6.6 g (10 mmol, 55%) of a colorless, voluminous solid: mp 157 °C; $[\alpha]^{20}_D$ +56.6° (*c* = 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) 3.75 (dd, 2H, *J* = 2.4 Hz, 5.2 Hz, H−C2 and H−C3), 3.97 (s−b, 2H, OH), 4.85 (m, 2H, H−C1 and H−C4), 5.00−5.14 (m, 8H,-O−CH_2-), 5.62 (s, 2H, H−C5 and H−C6), 7.37−7.28 (m, 20H, −C_6H_5); ^{13}C NMR ($CDCl_3$, 101 MHz) 69.91 (m, CH_2, CH_2−C_6H_5), 74.25 (d, CH, *J* = 3.8 Hz, C3 and C4), 78.80 (d, CH, *J* = 5.1 Hz, C1 and C4), 127.95, 127.99, 128.51, 128.56, 128.59 (5 CH, C_6H_5), 135.53, 135.60 (d, 2C, *J* = 3.1 Hz, C_6H_5); ^{31}P NMR ($CDCl_3$, 162 MHz) 0.58 (2P); IR (KBr) 3380 (O−H), 3065, 3020 (=C−H), 2940, 2880 (−C−H), 1250 (P=O), 1135 (C−O−C), 1020 (P(O)-OR), 730, 690 (=C−H); MS (ESI, rel intensity) *m/z* = 667.7 [$(M + H)^+$, 100]. Anal. ($C_{34}H_{36}O_{10}P_2$) C, H.

(1*R*,2*R*,3*R*,4*R*)-1,4-Bis(di-*O*-benzylphospho)conduritol-B (5b). The (+)-enantiomer **4b** (1.5 g, 13.6 mmol) was treated as described for **4a** and yielded **5b** as a colorless solid (5.0 g, 7.5 mmol, 55%): mp 157 °C; $[\alpha]^{20}_D$ −55.2° (*c* = 1.0, $CHCl_3$); NMR, IR, and MS data were identical to those for the other enantiomer **5a**.

(1*S*,2*S*,3*S*,4*S*)-1,4-Bis-*O*-(di-*O*-benzylphospho)-2,3-bis-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)conduritol-B (6a). To a solution of **5a** (300 mg, 0.45 mmol) and 1*H*-tetrazole (508 mg, 3.6 mmol) in anhydrous dichloromethane (30 mL) was added 3-diethylamino-2,4,3-benzodioxaphosphepane (432 mg, 1.8 mmol), and the mixture was stirred at room temperature for 2 h. The mixture was cooled to −60 °C, and a solution of *m*-CPBA (350 mg) in dichloromethane (5 mL) was added. Stirring was continued for 30 min at −60 °C and for another hour at room temperature. The reaction mixture was diluted with 100 mL of dichloromethane and washed consecutively with aqueous sodium bisulfite (50 mL), saturated aqueous $NaHCO_3$ (2 × 40 mL), and brine (40 mL). After evaporation of dichloromethane the resulting oil was purified by flash chromatography (CH_2Cl_2/MeOH, 97:3) to yield **6a** in 83% (385 mg, 0.37 mmol): mp 135 °C; $[\alpha]^{20}_D$ +19.8° (*c* = 1.32, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) 4.92−5.30 and 5.52−5.59 (m, 20H, C*H*$_2C_6H_5$ and C*H*$_2C_6H_4$, H−C1, H−C2, H−C3 and H−C4), 5.93 (s, 2H, H−C5 and H−C6), 7.43−7.30 and 7.11−7.16 (m, 28H, C_6H_5 and C_6H_4); ^{13}C NMR ($CDCl_3$, 101 MHz) 68.92, 69.21 (2 CH_2, d, *J* = 6.8 Hz, $OCH_2C_6H_5$), 69.73, 69.94 (2 CH_2, m, $OCH_2C_6H_4$), 76.19 (CH, C2 and C3), 77.92 (CH, C1 and C4), 127.15 (CH, C5 and C6), 128.00, 128.16, 128.57, 128.61, 128.63 (5 CH, 4 C_6H_5), 128.94, 129.03 (2 CH, d, *J* = 4.1 Hz, 2 C_6H_4), 135.31, 135.44 (2C, 4 C_6H_5), 135.48, 135.50, 135.55, 135.57 (4 C, C_6H_4); ^{31}P NMR ($CDCl_3$, 162 MHz) −0.84 (2P, P−C1 and P−C4), −2.18 (2P, P−C2 and P−C3); IR (KBr) 3470 (O−H), 3065, 3035 (=C−H), 2960, 2930, 2895 (−C−H), 1290 (P=O), 1045, 1020 (C−O−C), 730, 695 (=C−H); MS (ESI, rel intensity) *m/z* = 1049.5 [(M^+ + H_2O), 37], 1031.5 [(M^+ + H), 100], 425.6 [10], 367.5 [8], 151.1 [12], 120.1[23], 79.0 [18]; MS *m/z* = 1031.2125 $[M + H]^+$ calcd for $C_{50}H_{51}O_{16}P_4$ 1031.2128. Anal. ($C_{50}H_{50}O_{16}P_4$): C, H.

(1*R*,2*R*,3*R*,4*R*)-1,4-Di-*O*-(di-*O*-benzylphospho)-2,3-bis-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)conduritol-B (6b). The (−)-enantiomer **5b** (500 mg, 0.75 mmol) was phosphitylated as described for **5a**. Oxidation and purification as before gave **6b** (649 mg, 0.63 mmol, 84%): mp 135 °C; $[\alpha]^{20}_D$ −19.7° (*c* = 0.98, $CHCl_3$); NMR, IR, and MS data were identical to those for the other enantiomer **6a**.

D-*myo*-3,6-Bis-*O*-(di-*O*-benzylphospho)-4,5-bis-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)inositol (7a). To a vigorously stirred solution of **6a** (400 mg, 0.38 mmol) in ethyl acetate/acetonitrile (1:1, 20 mL) was added a solution of 125 mg (0.58 mmol) of sodium metaperiodate and ruthenium trichloride (10 mg, 10 mol %) in 2 mL of water. The stirring was continued until TLC showed absence of **6a** (CH_2Cl_2/MeOH, 95:5) (about 10 min). The reaction was quenched by addition of saturated $Na_2S_2O_3$ (20 mL). The aqueous layer was separated and extracted with dichloromethane (3 × 20 mL). The combined organic layers were evaporated, and the product was purified by flash chromatography (CH_2Cl_2/MeOH, 97:3) to give **7a** as a white solid (348 mg, 0.33 mmol, 86%): mp 168 °C; $[\alpha]^{20}_D$ −5.7° (*c* = 4.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) 3.71 (dd, 1H, *J* = 2.5 Hz, 9.3 Hz,H−C1) = 4.39 (ddd, 1H, *J* = 2.6 Hz, 10.0 Hz, 6.1 Hz, H−C3), 4.51 (t, 1H, *J* = 2.5 Hz, H−C2),

4.77 (m, 1H, H−C6), 4.82 (m, 1H, H−C5), 4.59−5.53 (m, 8H, $-CH_2$), 5.35 (q, 1H, J = 9.2 Hz, H−C4), 7.40−7.22 and 7.05−6.92 (m, 28H, C_6H_5 and C_6H_4); ^{13}C NMR ($CDCl_3$, 101 MHz) 70.24 (CH, C1 and C2), 76.07 (m, CH, C3), 76.88 (m, CH, C4), 77.83 (m, CH, C5), 79.83 (m, CH, C6), 69.92, 70.07(d, 2 CH_2, J = 5.3 Hz, $-OCH_2C_6H_5$), 69.10, 68.71 (m, 2 CH_2, $-OCH_2C_6H_4$), 128.05, 128.15, 128.22, 128.37, 128.56, 128.64, 128.73, 128.81, 128.88, 128.94 (10 CH, C_6H_5 and C_6H_4), 135.25, 135.30, 135.42, 135.51, 135.55, 135.58, 135.60, 135.68 (8 C, C_6H_5 and C_6H_4); ^{31}P NMR ($CDCl_3$, 162 MHz) 0.61 (P−C4), −1.42 (P−C3), −1.78 (P−C5), −1.93 (P−C6); IR (KBr) 3400 (O−H), 3080, 3057, 3035 (=C−H), 2950, 2910, 2885 (C−H), 1285 (P=O), 1050, 1025, 1015 (C−O−C), 730, 693 (C=C); MS (+ ion FAB, rel intensity) m/z =1065.5 [(M^+ + H), 100], 1084.8 [(M + H_3O^+), 45], 425.6 [80], 120 [61]; MS m/z = 1065.2189 [M + H]$^+$, calcd for $C_{50}H_{53}O_{18}P_4$ 1065.2182. Anal. ($C_{50}H_{52}O_{18}P_4$) C, H.

D-*myo*-1,4-Bis-*O*-(di-*O*-benzylphospho)-5,6-bis-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)inositol (7b). The (−)-enantiomer **6b** (500 mg, 0.49 mmol) was *cis*-dihydroxylated as described for **6a**. Workup and purification as before gave **7b** (437 mg, 0.42 mmol, 87%): mp 173 °C; $[\alpha]^{20}_D$ +5.5° (c = 4.5, $CHCl_3$); NMR, IR, and MS data were identical to those for the other enantiomer **7a**.

D-*myo*-Inositol 3,4,5,6-Tetrakisphosphate (8a). To a suspension of **7a** (300 mg, 0.28 mmol) in ethanol/water (1:2) was added 10% Pd/C (50 mg). The mixture was stirred at room temperature under H_2 overnight. The catalyst was filtered off, and the filtrate was lyophilized to give 132 mg (0.27 mmol, 95%) of **8a**. Further purification by HPLC assured purity >99% for the following reaction: $[\alpha]^{20}_D$ +4.1° (c = 2.7, H_2O, free acid), lit.[24] $[\alpha]^{24}_D$ +9.8 ° (c = 1.43, H_2O, sodium salt), lit.[25] $[\alpha]^{20}_D$ −5.6 ° (c = 0.2, H_2O), lit.[23] $[\alpha]_D$ +6.2 ° (c = 2.15, H_2O, pH 9.5); ^{1}H NMR (D_2O, 400 MHz, pH adjusted to 5 (ND_4OD)) 3.75 (dd, 1H, J = 3.1 Hz, 9.7 Hz, H−C1), 4.12 (dt, 1H, J = 3.1 Hz, 9.7 Hz, H−C3), 4.14 (q, 1H, J = 9.7 Hz, H−C4), 4.25 (t, 1H, J = 3.1 Hz, H−C2), 4.33 (q, 1H, J = 9.6 Hz, H−C6), 4.45 (q, 1H, J = 9.5 Hz, H−C6); ^{13}C NMR (D_2O, 101 MHz, pH adjusted to 5 (ND_4OD)) 70.35 (d, CH, J = 2.7 Hz, C1), 71,23 (s, CH, C2), 74.76 (m, CH, C3), 76.47 (m, CH, C4), 77.07 (m, CH, C6), 77.71 (m, CH, C5); ^{31}P NMR (D_2O, 162 MHz, pH adjusted to 5 (ND_4OD)) 1.37 (P−C3), 2.28 (P−C4), 2.32 (P−C6), 2.36 (P−C5); MS (ESI-neg, rel intensity) m/z = 499.1 [(M − H)$^-$, 75], 419.0 [18], 321.0 [31] 158.9 [158.9]; MS m/z = 500.934 [M + H]$^+$, calcd for $C_6H_{17}O_{18}P_4$ 500.9365.

D-*myo*-Inositol 1,4,5,6-Tetrakisphosphate (8b). Deprotection of **7b** (200 mg, 0.19 mmol) as described for **7a** led to **8b** as a colorless foam (89 mg, 0.18 mmol, 96%): $[\alpha]^{20}_D$ −4.8° (c = 2.7, H_2O, free acid), lit.[24] $[\alpha]^{20}_D$ −10.2° (c = 2.46, H_2O, sodium salt); NMR and MS data were identical to those for the other enantiomer **8a**.

D-*myo*-Inositol 3,4,5-Trisphosphate (9a). The enzymatic reaction was carried out at room temperature in a volume of 12 mL on a reciprocal shaker (40 rpm). The final buffer composition was identical to that used for the analytical assay of $InsP_5$/$InsP_4$ phosphohydrolase activity. At a substrate concentration of 300 μM **8a** and a volume activity of about 3−3.5 mU/mL (equivalent to 50−75% of the total activity obtained from 10^{10} cells), it took approximately 1.5 h to convert all the inositol tetrakisphosphate. Depending on the phosphate release measured, new substrate was added three times from a 30 mM stock solution. To verify complete conversion of the substrate, an aliquot was subjected to HPLC-MDD (HCl gradient: t(**9a**) = 30.0 min, t(**8a**) = 44.4 min) before the reaction was stopped by addition of 3 mL of 0.5 M HCl. The pH of the mixture was adjusted to 6, and the precipitate formed was removed by centrifugation (6000*g*, 15 min, room temperature). The supernatant was diluted 10-fold with water, and the inositol phosphates were separated by HPLC. After freeze-drying, overall yields were in the range of 70−80% (10−12 μmol) for **9a** (quantification based on inorganic phosphate determination): $[\alpha]^{20}_D$ −4.6° (c = 1.4, H_2O, free acid); ^{1}H NMR (D_2O, 400 MHz, pH adjusted to 6 (ND_4OD)) 3.64 (dd, 1H, J = 3.0 Hz, 10.1 Hz, H−C1), 3.80 (t, 1H, J = 9.6 Hz, H−C6), 3.98 (q, 1H, J = 9.0 Hz, H−C5), 4.07 (dt, 1H, J = 3.0 Hz, 9.8 Hz, H−C3), 4.20 (t, 1H, J = 3.0 Hz, H−C2), 4.37 (q, 1H, J = 9.3 Hz, H−C4); ^{13}C NMR (D_2O, 101 MHz, pH adjusted to 6 (ND_4OD)) 72.60 (d, CH, J = 2.7 Hz, C1), 73.75 (s, CH, C2), 74.04 (d, CH, J = 3.0 Hz, C6), 77.05 (dd, CH, J = 2.0 Hz, 6.0 Hz, C3), 78.42 (m, CH, C4), 80.76 (dd, CH, J = 3.0 Hz, 6.1 Hz, C5); ^{31}P NMR (D_2O, 162 MHz, pH adjusted to 6 (ND_4OD)) 2.17 (P−C3), 2.77 (P−C5), 2.87 (P−C4); MS (+ionFAB, rel intensity) m/z = 420.9 [(M^+) 16], 277.2 [5], 202.1 [22], 185.1 [80], 110.2 [100]; MS m/z = 420.966 [M + H]$^+$, calcd for $C_6H_{16}O_{15}P_3$ 420.9702.

D-*myo*-Inositol 1,5,6-Trisphosphate (9b). Dephosphorylation of **8b** was carried out as described for **8a**, yielding **9b** in 70−80%: $[\alpha]^{20}_D$ +2.2° (c = 2.3, H_2O, free acid), lit.[44] $[\alpha]^{20}_D$ −2.8 ° (c = 1.43, H_2O, sodium salt); NMR and MS data were identical to those for the other enantiomer **9a**.

Binding Assays. Membranes containing p42^{IP4} were prepared from pig cerebellum in the presence of protease inhibitors (0.2 mM Pefabloc SC, 1 mM EGTA, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 1 mM benzamidin) as described previously.[59] The binding assays were performed in a final volume of 400 μL in microcentrifuge tubes. Membranes (300 μg of protein/assay) were incubated in 10 mM HEPES/KOH (pH 7.0) containing 1.2 nM (22 500 dpm) [^{3}H]Ins(1,3,4,5)P_4 (777 gBq/mmol; DuPont-NEN), 0.25% BSA, 1 mM EDTA, 20 mM NaCl, and 100 mM KCl. The samples were incubated in the presence of different concentrations of unlabeled Ins(1,3,4,5)P_4 (Boehringer Mannheim), Ins(3,4,5)P_3 (**9a**), Ins(1,5,6)P_3 (**9b**), Ins(3,4,5,6)P_4 (**8a**), or Ins(1,4,5,6)P_4 (**8b**) for 20 min on ice. Bound ligand was separated from free ligand by addition of 100 μL of γ-globulin (10 mg/mL) and 500 μL of 5% PEG-8000, further incubation for 15 min, and then centrifugation (14000*g*, 15 min, 4 °C). The pellets were washed with 100 μL of binding buffer and solubilized with 2% SDS; the bound radioactivity was measured. Nonspecific binding was determined in the presence of 1 μM Ins(1,3,4,5)P_4. All determinations were made in duplicate and were repeated at least twice with two different membrane preparations.

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Supporting Information Available: X-ray data for compound **2b**. This information is available free of charge via the Internet at http://pubs.acs.org.

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