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Regiospecific Phosphohydrolases from *Dictyostelium* as Tools for the Chemoenzymatic Synthesis of the Enantiomers D-*myo*-Inositol 1,2,4-Trisphosphate and D-*myo*-Inositol 2,3,6-Trisphosphate: Non-physiological, Potential Analogues of Biologically Active D-*myo*-Inositol 1,3,4-Trisphosphate

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Abstract—A new de novo synthesis of the enantiomeric pair D-*myo*-inositol 1,2,4-trisphosphate and D-*myo*-inositol 2,3,6-trisphosphate is described. Starting from enantiopure dibromocyclohexenediol, several C_2 symmetrical building blocks were synthesized which gave access to D-*myo*-inositol 1,2,4,5-tetrakisphosphate and D-*myo*-inositol 1,2,3,6-tetrakisphosphate. Exploiting the high regiospecificity of two partially purified phosphohydrolases from *Dictyostelium*, a 5-phosphatase and a phytase, the inositol tetrakisphosphates were converted enzymatically to the target compounds. Their potential to modulate the activity of Ins(3,4,5,6)P_4 1-kinase was investigated and compared with the effects of D-*myo*-inositol 1,3,4-trisphosphate. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

myo-Inositol phosphates are involved in essential processes in eucaryotic cells.[1] Interdependent kinases and phosphohydrolases construct a complex metabolic network that allows a fine-tuning of cellular inositol phosphate levels. This is especially important for isomers participating in cellular signaling.

Ins(3,4,5,6)P_4 is an inhibitor of calcium activated chloride channels in the plasma membrane.[2] These ion channels contribute to the homoeostatic control of salt and fluid secretion with impact on osmoregulation and pH balance. The cellular accumulation of Ins(3,4,5,6)P_4 is known to correlate well with receptor-dependent changes in phospholipase C activity, but the molecular mechanisms that link these two events have only recently been elucidated.[3] It was shown that Ins(1,3,4)P_3, a downstream-product of the phosphoinositide signaling cascade, is involved in the regulation of Ins(3,4,5,6)P_4 levels.[4] Ins(1,3,4)P_3 is a competing substrate for the Ins(3,4,5,6)P_4 1-kinase and is phosphorylated by the enzyme at 5- and 6-positions.[5] Certain diseases, such as cystic fibrosis, may be treated by either up- or down-regulation of calcium activated chloride secretion,[6] and for that reason the design of effective Ins(3,4,5,6)P_4 agonists and antagonists for pharmacological intervention in the signaling actions of Ins(3,4,5,6)P_4 is of great interest. In this context it was found that a racemic mixture of Ins(1,2,4)P_3/Ins(2,3,6)P_3 (both non-physiological isomers) was only 4-fold less potent than Ins(1,3,4)P_3 as an inhibitor of Ins(3,4,5,6)P_4 phosphorylation by the kinase. We therefore set out to clarify the relative potencies of Ins(1,2,4)P_3 (**8a**) and Ins(2,3,6)P_3 (**8b**) as 1-kinase inhibitors.

Their first total synthesis described here combines a straightforward chemical synthesis of Ins(1,2,4,5)P_4 (**5**) and Ins(1,2,3,6)P_4 (**7**) with subsequent regiospecific enzymatic dephosphorylations to obtain Ins(1,2,4)P_3

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(**8a**) and Ins(2,3,6)P_3 (**8b**). The target compounds were tested for their ability to influence the activity of recombinant human Ins(3,4,5,6)P_4 1-kinase and the reason for this effect was investigated.

Chemistry

Enantiopure dibromocyclohexenediol (+)-**1** serves as starting material. It can be prepared on a large scale in only four steps from *p*-benzoquinone.[7] Treatment of dibromodiol **1** with sodium benzylate in benzyl alcohol leads in a stereocontrolled transformation to conduritol-B derivative **2** (Scheme 1). A fast *cis*-dihydroxylation step with $RuCl_3/NaIO_4$ gives compound **3** with *myo*-inositol stereochemistry, which can be phosphorylated by reaction with 3-diethylamino-2,3,4-benzodioxaphosphepane in the presence of 1*H*-tetrazole and subsequent oxidation with *m*CPBA. Complete deprotection of the Ins(1,2,4,5)P_4 derivative **4** can be achieved in one step by a Pd-catalyzed hydrogenation. The product Ins(1,2,4,5)P_4 (**5**) was subjected to enzymatic conversion as described below.[8]

As published previously starting from the same precursor **1** it is possible to synthesize the isomer Ins(1,2,3,6)P_4 (**7**) via a different conduritol-B derivative **6** (Scheme 1).[9] It has to be mentioned that the enantiomers of these inositol tetrakisphosphates are also accessible, illustrating the versatility of this synthetic strategy.

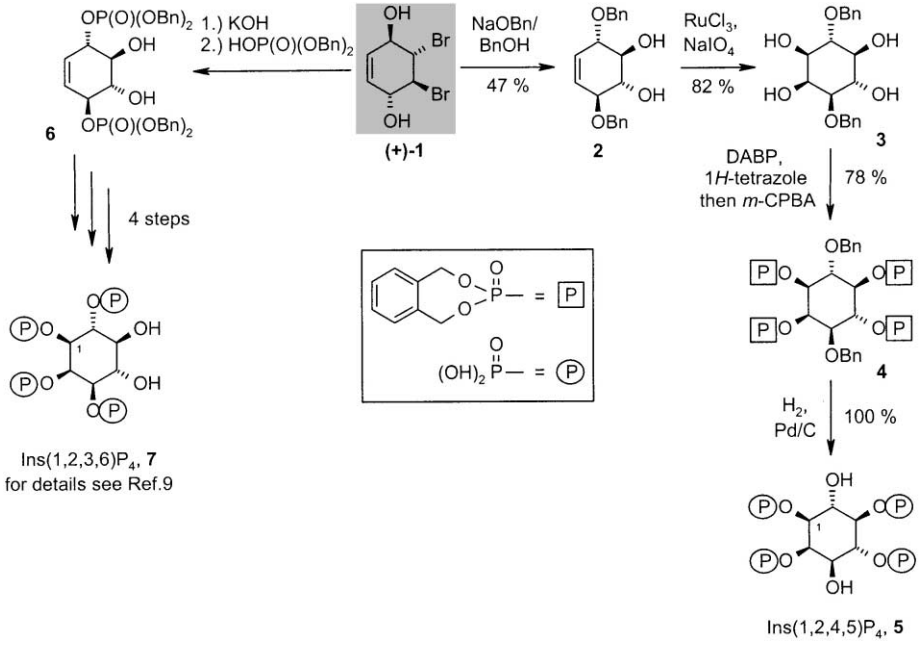
The tetrakisphosphates **5** and **7** were already pure by NMR spectroscopic standards. Purification of the products by HPLC ensures purities suitable for biological experiments. Total phosphate determination after sulfuric acid hydrolysis allows a reliable quantification of the hygroscopic substances.[7]

Synthetic Application of Phosphohydrolases

One focus of our research is the establishment of new, preparatively applicable enzymatic reactions in inositol phosphate synthesis.[7,9] We direct our closest attention to conversions producing enantiomers of current biological interest which are only difficult to obtain by conventional organic synthetic methods.

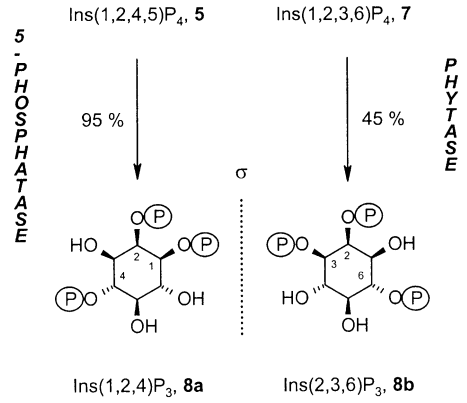
Previous work on the substrate specificity of two partially purified enzymes from the cellular slime mold *Dictyostelium discoideum* encouraged us to utilize them for preparation of the target compounds. An easily to cultivate axenic mutant of this microorganism (strain AX-2) was used as enzyme source.[10] Cells were lysed by freezing/thawing and the homogenate was separated into a cytosolic fraction and a membrane-enriched fraction by ultracentrifugation. The supernatant contains an enzyme of the Ins(1,4,5)P_3 5-phosphatase family and the sediment an enzyme with characteristics of a phytase ($InsP_6$-phosphohydrolase) that could be solubilized with nonionic detergent. Both protein solutions were subjected to column chromatography to gain enriched enzyme preparations, which are devoid of interfering phosphate, $InsP_x$ metabolites and unspecific phosphohydrolase activities.[11,12]

The Mg^{2+}-dependent Ins(1,4,5)P_3 5-phosphatase possesses a pH optimum at about 7. We tested the substrate specificity of the 5-phosphatase with an extensive number of inositol phosphates and phosphoinositides. A detailed list of the compounds and the results of this study will be published elsewhere. Relevant for the intended application are examined isomers modified at their functional groups in position C-1 and C-2. Dephosphorylation of Ins(1,2,4,5)P_4 (**5**) and dideoxy(1,2)Ins(4,5)P_2 (the double bond of compound **2** was hydrogenated for its synthesis, see Scheme 1) takes place with approximately 20 and 10% of the rate the



Scheme 1.

physiological substrate Ins(1,4,5)P_3 is converted. Enzymatic activity was measured in each case under substrate saturation of the enzyme. Considering that the reaction occurs highly specifically at the 5-position and that it stops after removal of one phosphate group this is still fast enough to be of practical value in enzyme-assisted synthesis. Working with the total enzyme activity obtained from 10^{10} cells (10 g wet weight) it is possible to dephosphorylate 25 μmol Ins(1,2,4,5)P_4 (**5**) in about 4 h (Scheme 2).[13] After HPLC purification and quantification (see above) we calculated an excellent overall yield of 95% for the enantiomer Ins(1,2,4)P_3 (**8a**).[14]



Scheme 2.

In contrast to the 5-phosphatase, the phytase reaction possesses a pH optimum in the range 4–5. From the substrate Ins(1,2,3,6)P_4 (**7**) phosphate is released predominantly in position C-1 (Scheme 2), but there is a tendency toward further hydrolyzation of Ins(2,3,6)P_3 (**8b**) to unidentified InsP_2 isomers. Nevertheless under optimized experimental conditions acceptable yields of about 45% for **8b** have been achieved.[14,15]

As described previously, the isolated products were checked for isomeric impurities by HPLC-MDD.[7] We were unable to detect any contaminating inositol trisphosphate isomer in **8a** (purity ≥ 99%). Less than 2% of byproducts were found in **8b**. They were identified as Ins(1,2,3)P_3 and Ins(1,2,6)P_3.

Effects on Kinase Activity

The relative potencies of non-physiological **8a** and **8b** to influence the activity of recombinant human Ins(3,4,5,6)P_4 1-kinase were determined and compared with the data for Ins(1,3,4)P_3 (Fig. 1). [^{3}H]Ins(3,4,5,6)P_4 was used as substrate and the amount of [^{3}H]Ins(1,3,4,5,6)P_5 formed in the presence and absence of each particular inositol trisphosphate was analyzed.[4] This study clearly demonstrates that it is the Ins(1,2,4)P_3 (**8a**, $IC_{50} \approx 9$ μM) which imitates the inhibitory actions of Ins(1,3,4)P_3 ($IC_{50} \approx 4$ μM) whereas the optical antipode Ins(2,3,6)P_3 is almost ineffective (**8b**, $IC_{50} \geq 300$ μM). Previous work with racemic Ins(1,2,4)P_3/Ins(2,3,6)P_3 could not answer this important issue.[4]

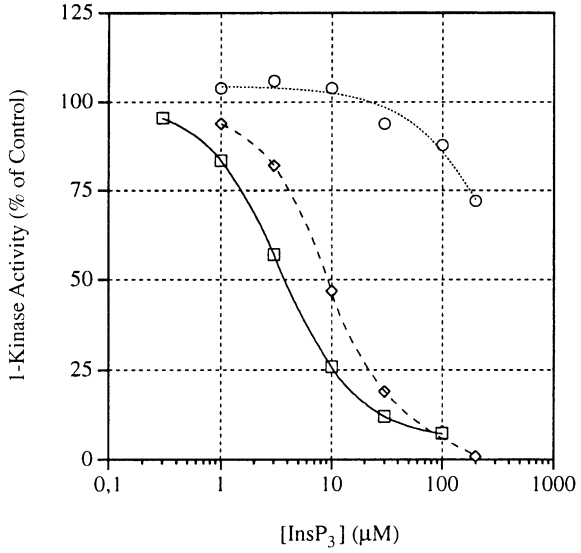


Figure 1. Effects of InsP_3 isomers upon Ins(3,4,5,6)P_4 1-kinase activity. Enzyme activity was assayed as previously described,[4] except that recombinant human kinase[5] and a higher substrate concentration of 5 μM [^{3}H]Ins(3,4,5,6)P_4 (approx. 2000 dpm/assay) were used. Activity remaining in the presence of non-radiolabeled Ins(1,3,4)P_3 (squares, solid line), Ins(1,2,4)P_3 (**8a**; diamonds, broken line) and Ins(2,3,6)P_3 (**8b**; circles, dotted line) is expressed as a percentage in relation to control.

It only recently emerged that the actions of Ins(1,3,4)P_3 upon Ins(3,4,5,6)P_4 1-kinase activity result from a competition for phosphorylation and the enzyme is now reclassified as Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase.[5] Therefore the question arises whether Ins(1,2,4)P_3 (**8a**) is indeed a competitive inhibitor or a competing substrate. To clarify this aspect we have identified potential phosphorylation products by HPLC-MDD and evaluated the kinetics of the reaction. Ins(1,3,4)P_3 is phosphorylated at the expected positions (Fig. 2B) leading to Ins(1,3,4,6)P_4 (65%) and

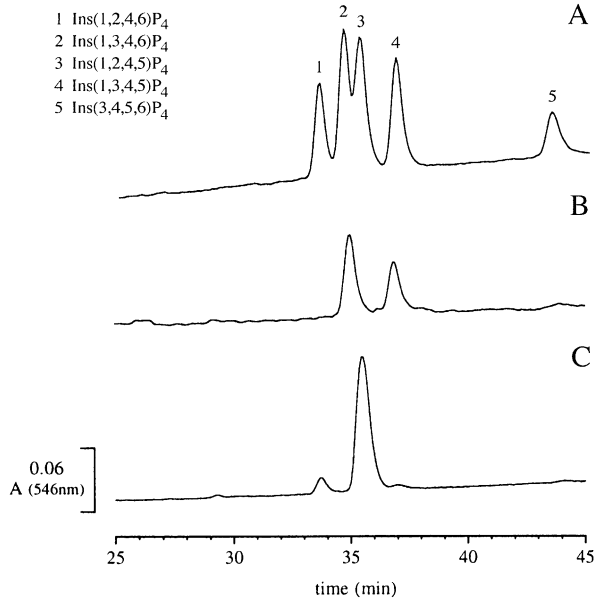


Figure 2. Identification of the kinase products by HPLC-MDD analysis after complete conversion of Ins(1,3,4)P_3 (B) and Ins(1,2,4)P_3 (**8a**, C). Chromatogram A shows the separation of authentic standards.

Ins(1,3,4,5)P_4 (35%). We also observed rapid phosphorylation of Ins(1,2,4)P_3 (**8a**), but with a remarkable difference in regiospecificity [7% Ins(1,2,4,6)P_4 and 93% Ins(1,2,4,5)P_4; see Figure 2C]. Consistent with the experiment presented above, Ins(2,3,6)P_3 (**8b**) is not a substrate. Inspection of the molecular models and first calculations confirm a structural relationship between Ins(1,3,4)P_3 and Ins(1,2,4)P_3 (**8a**). According to molecular modeling analysis the axial phosphate group in 2-position of Ins(1,2,4)P_3 (**8a**) can be nearly superimposed on the equatorially orientated 3-phosphate group of Ins(1,3,4)P_3 by rotating the corresponding phosphate groups around the C–O bonds. The structural resemblance may explain why both compounds are converted with nearly equal rates [specific activities at substrate saturation: 36.9 mU/mg for Ins(1,3,4)P_3 and 39.8 mU/mg for Ins(1,2,4)P_3 (**8a**)]. Further progress in understanding substrate recognition and varying specificities of the kinase for different substrates will be best addressed by determination of the structure of the active site.

Concluding Remarks

In vitro Ins(1,2,4)P_3 (**8a**) delays the off-switch reaction of the signaling molecule Ins(3,4,5,6)P_4 catalyzed by Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase. Unexpectedly, the non-physiological trisphosphate **8a** functions as a substrate and not as an inhibitor of the enzyme. Phosphorylation of **8a** leads predominantly to Ins(1,2,4,5)P_4, a compound with potential to act as an Ins(1,4,5)P_3-receptor agonist.[16] In vivo Ins(1,2,4,5)P_4 is most likely dephosphorylated by a member of the 5-phosphatase family, thereby regenerating Ins(1,2,4)P_3. The synthesis of a cell-permeant, bioactivatable analogue of Ins(1,2,4)P_3 (**8a**) for studies with intact cells could be a promising new starting point for the development of drugs that might intervene in the signal transduction pathways with therapeutic benefit.[4]

Acknowledgements

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8. ^{1}H NMR data for compound **5** [D_2O, 400 MHz, pH adjusted to 6.0 (ND_4OD)]: δ ppm 3.56 (dd, $J=2$, 10.2 Hz, H-C3); 3.78 (Ψt, $J\approx9.6$ Hz, H-C6); 3.86 (Ψq, $J\approx8.7$ Hz, H-C5); 3.92 (dΨt, $J=2$, 7.4 Hz, H-C1); 4.15 (Ψq, $J\approx9.2$ Hz, H-C4); 4.68 (H-C2, partially under HDO-signal); ^{13}C NMR (101 MHz): δ ppm 70.61 (s, C3); 71.77 (dd, C6); 74.39 (dd, C1); 74.86 (d, C4); 77.01 (dd, C2); 78.04 (m, C5); $[\alpha]_D^{20}$ −26.3° ($c=0.7$, H_2O, free acid). This agrees with published data: Mills, St. J.; Potter, B. V. L. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1279.
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11. The cytosolic 5-phosphatase was enriched by chromatography on heparin agarose (type II, Sigma) and DEAE-Sepharose ff (Amersham-Pharmacia). The fraction with highest activity was concentrated by ultrafiltration (Centriprep-10, Millipore).
12. Membrane-associated phytase was solubilized with 1% hydrogenated Triton X-100 (Calbiochem). The enzyme was partially purified by sequential biochromatography (Q-Sepharose ff and Source 15Q, both from Amersham-Pharmacia).
13. The conversion of Ins(1,2,4,5)P_4 (**5**) catalyzed by the 5-phosphatase was carried out at room temperature in a volume of 12 mL [40 mM Bis-Tris (pH 7.0), 200 mM sucrose, 5 mM $MgCl_2$, 0.25 mM EDTA] on a reciprocal shaker (60 rpm). At an initial substrate concentration of 400 μM **5** and a volume activity of about 10 mU/mL (total activity obtained from 10^{10} cells), it took approximately 0.5 h for a complete conversion. New substrate was added four times from a 30 mM stock solution. After additional 1.5 h of incubation the reaction was stopped with 2 mL 2.5 M HCl. The pH of the mixture was adjusted to 7, and the denaturated protein was removed by centrifugation (6000*g*, 15 min, rt). Overall yield after purification by HPLC was 95%, equivalent to 22.8 μmol **8a**. For details about reaction control, purification by HPLC and quantification of inositol phosphates see ref 7.
14. ^{1}H NMR data for compound **8a** (D_2O, 400 MHz, pH adjusted to 6.0 (ND_4OD)): δ ppm: 3.5 (Ψt, $J\approx9.2$ Hz, H-C5); 3.65 (dd, $J=1.5$, 9.5 Hz, H-C3); 3.84 (Ψt, $J\approx9.6$ Hz, H-C6); 4.0 (dΨt, $J=1.8$, 9.7 Hz, H-C1); 4.18 (Ψq, $J\approx9.3$ Hz, H-C4); 4.73 (H-C2, partially covered by HDO-signal); ^{13}C NMR (101 MHz): δ ppm: 70.68 (m, C3); 71.92 (d, C6); 74.04 (s, C5); 74.6 (m, C1); 75.23 (d, C2); 77.54 (d, C4); $[\alpha]_D^{20}$ (**8a**) −15.7° ($c=3.78$, H_2O, free acid); $[\alpha]_D^{20}$ (**8b**) +19.6° (c = 1.23, H_2O, free acid).
15. The dephosphorylation of Ins(1,2,3,6)P_4 (**7**) catalyzed by the phytase was carried out at room temperature in a volume of 13 mL (50 mM Mes/Na^+ (pH 5.1), 0.05% Triton X-100 hydr) with an initial substrate concentration of 310 μM and a volume activity of 1.4 mU/mL (total activity obtained from 5×10^9 cells) on a reciprocal shaker (40 rpm). Every 1.5 h new substrate was added from a 20 mM stock solution (three times). After 9 h of incubation the reaction was stopped with 3 mL 2.5 N HCl. The pH of the reaction-mixture was adjusted to 7, and the denaturated protein was removed by centrifugation (6000*g*, 15 min, rt). Overall yield after purification by HPLC was 45%, equivalent to 7.2 μmol Ins(2,3,6)P_3 (**8b**).
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