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Bioorganic Chemistry 31 (2003) 44–67
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Stereo- and regiospecificity of yeast phytases–chemical synthesis and enzymatic conversion of the substrate analogues *neo*- and L-*chiro*-inositol hexakisphosphate

Stephan Adelt,[a] Michael Podeschwa,[b] Guido Dallmann,[a] Hans-Josef Altenbach,[b] and Günter Vogel[a,*]

[a] *Institut für Biochemie, Fachbereich 9-Chemie, Bergische Universität Wuppertal, Gaussstrasse 20, 42097 Wuppertal, Germany*
[b] *Institut für Organische Chemie, Fachbereich 9-Chemie, Bergische Universität Wuppertal, Gaussstrasse 20, 42097 Wuppertal, Germany*

Received 8 October 2002

Abstract

Phytases are enzymes that catalyze the hydrolysis of phosphate esters in *myo*-inositol hexakisphosphate (phytic acid). The precise routes of enzymatic dephosphorylation by phytases of the yeast strains *Saccharomyces cerevisiae* and *Pichia rhodanensis* have been investigated up to the *myo*-inositol trisphosphate level, including the absolute configuration of the intermediates. Stereoselective assignment of the *myo*-inositol pentakisphosphates (D-*myo*-inositol 1,2,4,5,6-pentakisphosphate and D-*myo*-inositol 1,2,3,4,5-pentakisphosphate) generated was accomplished by a new method based on enantiospecific enzymatic conversion and HPLC analysis. Via conduritol B or E derivatives the total syntheses of two epimers of *myo*-inositol hexakisphosphate, *neo*-inositol hexakisphosphate and L-*chiro*-inositol hexakisphosphate were performed to examine the specificity of the yeast phytases with these substrate analogues. A comparison of kinetic data and the degradation pathways determined gave the first hints about the molecular recognition of inositol hexakisphosphates by the enzymes. Exploitation of the high stereo- and regiospecificity observed in the dephosphorylation of *neo*- and L-*chiro*-inositol hexakisphosphate made it possible to establish enzyme-assisted steps for the

* Corresponding author. Fax: +49-202-439-3399.
E-mail address: vogel@uni-wuppertal.de (G. Vogel).

0045-2068/02/$ - see front matter
doi:10.1016/S0045-2068(02)00523-0

synthesis of D-*neo*-inositol 1,2,5,6-tetrakisphosphate, L-*chiro*-inositol 1,2,3,5,6-pentakisphosphate and L-*chiro*-inositol 1,2,3,6-tetrakisphosphate.

Keywords: Inositol stereoisomers; Inositol phosphates; *neo*-inositol phosphates; L-*chiro*-inositol phosphates; HPLC-analysis of inositol phosphates; Phytases; Yeast

1. Introduction

In biological systems the hydrolysis of phosphate monoesters is a crucially important process that is linked to energy metabolism, to metabolic regulation, and to many signal-transduction pathways. Despite the apparent simplicity of the task, the cleavage is accomplished by a diverse group of enzymes called phosphatases [1].

Among them a specific class of enzymes, the phytases (EC 3.1.3.8 and EC 3.1.3.26), has evolved which hydrolyze *myo*-inositol hexakisphosphate (phytic acid, *myo*-InsP_6) to less phosphorylated *myo*-inositol phosphates (in some cases to free *myo*-inositol), releasing orthophosphate [2]. Most phytases belong to the high-molecular-weight, acid-phosphatase subgroup. The reaction mechanism of these enzymes involves a covalent base-stable, acid-labile phosphohistidine adduct, which explains why the optimal pH for catalysis lies in the acidic range. Most phytases sequenced so far share a highly conserved RHGXRXP motif containing the active center histidine [3].

Phytases are widespread in nature, occurring in microorganisms, plants, and some animal tissues [2]. The role of this class of enzymes in these organisms seems to vary and largely to depend on the function ascribed to the substrate *myo*-inositol hexakisphosphate. In plants, phytase is induced during germination to provide the growing seedling simultaneously with orthophosphate and free *myo*-inositol, which is an important growth factor. The hydrolysis of phytate also releases bound cations, such as K^+, Mg^{2+}, Zn^{2+}, and Ca^{2+}. In microorganisms, phytase is most frequently induced and sometimes secreted in response to phosphate starvation, and it releases phosphate from surrounding and/or internal *myo*-inositol hexakisphosphate. Besides these two cases, in which *myo*-InsP_6 serves merely as a storage molecule or nutrient, there are hints that cellular *myo*-InsP_6 and its breakdown products are involved in signal-transduction pathways [4]. Two recent discoveries about *myo*-InsP_6 focus on potential functions in the nucleus: a requirement for *myo*-InsP_6 in in vitro DNA repair by non-homologous end-joining [5] and a relationship between *myo*-InsP_6 levels and nuclear mRNA export [6]. A phytase-like enzyme, multiple inositol polyphosphate phosphatase (MIPP), from mammalian tissue is capable of regulating the cellular pools of *myo*-InsP_6 and *myo*-Ins(1,3,4,5,6)P_5 [7]. Furthermore, the MIPP-generated downstream metabolites *myo*-Ins(1,3,4,5)P_4 and *myo*-Ins(1,4,5)P_3 are themselves physiologically active in cellular Ca^{2+} homeostasis [8].

Despite all that is known about the biological importance of the ubiquitous *myo*-inositol phosphates, there is little information available about the relevance of other

inositol stereoisomers and their derivatives. However, several publications have reported the occurrence of low concentrations of *scyllo*-, *chiro*-, and *neo*-inositol in animal tissue [9,10]. *chiro*-Inositol isolated from bovine-liver lipid was exclusively of the L-configuration [11]. The results of this study suggested that L-*chiro*-inositol was incorporated into the phosphatidylinositol pool. Mixtures of *scyllo*-, *chiro*-, and *neo*-inositol are present in soil as their pentakisphosphate and hexakisphosphate esters [12], but phosphate esters of these inositol stereoisomers have so far only rarely been found in living nature. One exception seems to be the parasitic amoeba *Entamoeba histolytica*, which possesses high levels of several *neo*-inositol polyphosphates [13]. The question arises whether there exists a set of enzymes responsible for the metabolism of the inositol isomers mentioned or their appearance is a consequence of a lack of specificity in transformations catalyzed by the *myo*-inositol enzyme machinery.

In a first attempt to address this question, we have carried out the total synthesis of two isomeric forms of *myo*-InsP_6, *neo*-InsP_6 (**3**), and L-*chiro*-InsP_6 ((−)-**5**) and investigated their phytase-catalyzed dephosphorylation with whole cells from the yeast strain *Saccharomyces cerevisiae* and a secreted enzyme activity from *Pichia rhodanensis*. A comparison with the conversion of *myo*-InsP_6 revealed structural motifs important for molecular recognition. Taking advantage of the unexpected, well-defined degradation routes observed for *neo*-InsP_6 (**3**) and L-*chiro*-InsP_6 ((−)-**5**), we established enzyme-assisted steps for the synthesis of D-*neo*-Ins(1,2,5,6)P_4 (**10**), L-*chiro*-Ins(1,2,3,5,6)P_5 (**11**) and L-*chiro*-Ins(1,2,3,6)P_4 (**12**).

2. Materials and methods

2.1. General

The following *myo*-inositol phosphates needed as reference compounds for HPLC-MDD (metal dye detection) were synthesized in our laboratories. For the synthesis of *myo*-Ins(1,2,4,5)P_4 and *myo*-Ins(1,2,4)P_3 a chemoenzymatic approach was used [14]. The isomers *myo*-Ins(1,2,5,6)P_4, *myo*-Ins(1,2,4,6)P_4, and *myo*–Ins(1,2,3,4)P_4 were prepared as decribed previously [15,16]. The *meso*-compound *myo*-Ins(2,4,5,6)P_4 was prepared by acid-catalyzed, non-specific dephosphorylation of *myo*-InsP_6 and purified by HPLC. The inositol pentakisphosphates of defined stereochemistry (*myo*-Ins(1,2,4,5,6)P_5, *myo*-Ins(2,3,4,5,6)P_5, *myo*-Ins(1,2,3,4,5)P_5, and *myo*-Ins(1,2,3,5,6)P_5) were synthesized (manuscript in preparation). Commercially available compounds were from Sigma (*myo*-InsP_6, *myo*-Ins$(SO_4)_6$), Alexis (*myo*-Ins(1,2,3,5)P_4), and Calbiochem (*myo*-Ins(2,4,5)P_3). All other chemicals used were of the highest purity available.

All NMR spectra were recorded on a Bruker ARX 400 spectrometer. Besides ^1H (400 MHz), ^{13}C (101 MHz) and ^{31}P (162 MHz) experiments, 2D COSY-(^1H–^1H, ^1H–^{13}C as well as ^1H–^{31}P), and DEPT spectra for the unequivocal correlation of the hydrogen, carbon and phosphorus atoms were recorded. The chemical shifts are given in ppm, relative to the solvents as internal standard. The multiplicity is given by the following symbols: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), Ψt

(pseudotriplet for unresolved dd), and br (broad). The coupling constant J is given in Hz. Melting points are uncorrected. IR spectra were obtained in KBr, and only noteworthy absorptions (cm^{-1}) are listed. Flash-column chromatography was performed on Merck silica gel (40–63 μm). All organic extracts were dried over $MgSO_4$, filtered and concentrated with a rotary evaporator under reduced pressure. Only distilled solvents were used. The building blocks (+)-**1** and (−)-**1** and their precursors were synthesized as described in a previously published method [16,17].

2.2. Cultivation of yeast strains

Saccharomyces cerevisae was obtained from a local grocery store (product of: Deutsche Hefewerke, Nuremberg, Germany) and *P. rhodanensis* (CBS 5518) from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). Yeast strains were grown in YPD medium consisting of 1% yeast extract (Oxoid, England), 2% peptone (Oxoid, England), and 2% glucose. The solution was autoclaved for 16 min at 121 °C before the addition of the glucose. The glucose stock solution (20%) was autoclaved separately or filtered for sterility and added afterwards. *S. cerevisae* was cultured at 30 °C, whereas *P. rhodanensis* was incubated at 25 °C, both on a rotary shaker (150 rpm). The growth of the yeast cells was monitored as optical density (OD) of the culture broth at 600 nm (*S. cerevisiae* OD $1 \approx 2.2 \times 10^8$ cells/mL).

2.3. Precipitation of an extracellular phytase from P. rhodanensis

The cells were grown overnight and harvested at an OD_{600} of 20–25. The cells were removed by centrifugation (4000*g*, 15 min, 4 °C), and the enzyme was precipitated from the supernatant with 66% (v/v) ethanol precooled to −20 °C. The precipitation was carried out in an ice/NaCl bath under stirring, and ethanol was added continuously. To preserve enzyme activity, freezing of the solution must be prevented. When the addition of ethanol is complete, the mixture should be stirred further for 15 min. The protein is sedimented by centrifugation (6000*g*, 15 min, 0 °C), resuspended in 50 mM Tris/HCl, pH 7.7, aliquoted and frozen (−20 °C). Prepared by this procedure, the enzyme maintains its activity for several months. A 50-mL culture of *P. rhodanensis* yielded 150–200 mU phytase activity with recoveries of more than 80%.

2.4. P. rhodanensis phytase assay

The incubation mixtures contained 100 μL assay buffer (125 mM sodium acetate (pH 4.6), 1.25 mM *myo*-$InsP_6$) and 25 μL enzyme solution. The assays were incubated at 56 °C, and at varying intervals 10-μL aliquots were withdrawn. The reaction was stopped by the addition of 10 μL 2 N HCl to the aliquots, and the enzymatically released inorganic phosphate was determined. In case of a high phosphate background, the samples were analyzed with HPLC-MDD after neutralization.

Enzyme activities were calculated from the linear regions of kinetic data plots. Protein was measured by an assay supplied by BIO-RAD (catalog no. 500-0112) with bovine serum albumin as a standard.

2.5. Kinetic measurements with myo-$InsP_6$, neo-$InsP_6$ (**3**), and L-chiro-$InsP_6$ ((−)-**5**)

Measurements were performed by HPLC-MDD quantification of inositol hexakisphosphate remaining after enzymatic conversion. *S. cerevisiae* cells were harvested and washed twice with acetate buffer (3000*g*, 10 min., 4 °C). The cells (20–60 mg) were resuspended in 700 μL of 75 mM sodium acetate (pH 4.6) containing 1 mM of the corresponding substrate. In the case of *P. rhodanensis*, ethanol-precipitated phytase (20 mU/mL, released P_i from 1 mM *myo*-$InsP_6$, 56 °C) was used instead of yeast cells. For inhibition studies the reaction mixtures also contained up to 100 μM inositol hexakissulfate. The mixture was incubated in a 24-well tissue-culture plate at 45 °C in a water-bath shaker. To allow temperature equilibration, the plate was preincubated for two minutes before the analysis was started. 50-μL aliquots were taken, *S. cerevisiae* cells were removed by centrifugation (15,000*g*, 1 min, 4 °C) and the supernatants were frozen (liquid nitrogen). In the experiments with the phytase of *P. rhodanensis* the samples were frozen immediately after removal.

2.6. Inorganic phosphate determination

A colorimetric assay was employed for the determination of nanomolar amounts of inorganic phosphate [18]. Reducing the volume to a microplate scale made it possible to handle a great number of samples simultaneously. The method was applicable to following the progress of the enzymatic phosphatase reactions and to quantifying the mass of purified inositol phosphates. In the former case, the acidic solution (10–50 μL) was mixed with 100 μL of malachite green reagent, and after 1 min 10 μL of 34% (w/v) sodium citrate·$2H_2O$ 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 0.3–3 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 (approx. 1–2 mM) 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 were assayed for inorganic phosphate. Mass determinations for each substance were done in triplicate.

2.7. Purification and analysis of inositol phosphates (HPLC-MDD)

Inositol phosphates were purified and analyzed with the HPLC-MDD method described previously [16,19]. The compounds were separated by anion-exchange chromatography on a MonoQ HR10/10 column (Pharmacia). To purify inositol phosphates and identify enzymatic degradation products, a linear gradient of HCl

was applied (0 min 0.2 mM HCl; 70 min 0.5 M HCl; flow rate 1.5 mL/min). For the kinetic evaluation of the enzymatic reactions with *myo*-$InsP_6$, *neo*-$InsP_6$ (**3**), and L-*chiro*-$InsP_6$ ((−)-**5**), a shorter linear acidic gradient was used (0 min 0.375 M HCl; 15 min 0.5 M HCl; flow rate 1.5 mL/min). In analytical runs photometric detection at 546 nm was achieved with a 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). In purification steps, where on-line detection was impossible, an analogous experiment was carried out on a microplate. In brief, a 0.2–2-μL portion of the collected fraction was mixed with 100 μL of metal-dye reagent, and the absorbance at 540 nm was measured.

To separate the products of degradation of *myo*-Ins(1,2,3,4,5)P_5/*myo*-Ins(1,2,3,5,6)P_5 by the phytase of *Dictyostelium discoideum*, a modified, slightly alkaline elution system was established. 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; 68 min, 75% B; 73 min, 100% B; 80 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 PAR, 30 μM YCl_3, and 10% (v/v) MeOH (flow rate 0.75 mL/min).

2.8. Stereochemical assignment of myo-inositol pentakisphosphates by means of a phytase from D. discoideum

A Triton X-100 solubilized membrane-associated phytase from *D. discoideum*, partially purified by sequential biochromatography (Q-Sepharose ff and Source 15Q, both from Amersham-Pharmacia) was used as the enzymatic tool [14].

A microplate assay was established that provided kinetic data. The incubation mixture in one cavity contained 10 μL assay buffer (200 mM Mes/Na^+ (pH 5.15), 0.2% (w/v) Triton X-100 hydr.), 10 μL water, 10 μL phytase (45 mU/mL, P_i-release from 1 mM *myo*-$InsP_6$) from *D. discoideum* and 10 μL of a 400-μM *myo*-inositol phosphate solution. The samples were incubated at room temperature on a reciprocal shaker (80 rpm). The enzymatic reaction was terminated at varying intervals by the addition of 10 μL of 2 N HCl, and the inorganic phosphate released was analyzed. For the stereochemical assignment of the *myo*-inositol pentakisphosphates the assay was scaled up to a test volume of 120 μL. The reaction was stopped with 30 μL 2 N HCl when 24% P_i of the total organic phosphate had been released. After neutralization, the samples were analyzed by HPLC-MDD with an acidic (*myo*-Ins(1,2,4,5,6)P_5 and *myo*-Ins(2,3,4,5,6)P_5) or an alkaline (*myo*-Ins(1,2,3,4,5)P_5 and *myo*-Ins(1,2,3,5,6)P_5) elution system.

*2,3,4,5-Tetra-O-acetyl-neo-inositol,(+)-**2**.* (1*S,2R,3R,4S*)-1,4-diacetoxy-2,3-dibromocyclo-hex-5-ene (10 g, 28 mmol) (+)-**1** was added to a hot solution of 10 g potassium acetate or sodium acetate in 100 mL acetic acid and 5 mL water.The mixture was stirred for 10 days at 130 °C. The solvent was removed under reduced pressure, and the residue was dried under high vacuum. The residue was suspended in anhydrous dichloromethane, and acetic anhydride (30 mL) and DMAP (200 mg) were added. The mixture was stirred overnight. For work-up the mixture was

added slowly to a vigorously stirred aqueous solution of $NaHCO_3$ (16 g, 0.19 mol, 100 mL water). The aqueous phase was extracted several times with dichloromethane. The combined organic phase was washed with saturated aqueous $NaHCO_3$ and then with brine. After evaporation of the solvent the raw product (10 g, oil) was purified by flash chromatogaphy (cyclohexane/EtOAc 3:2) to yield (+)-**2a** (6.5 g, 70%) as a colorless oil which crystallized after one day. R_f 0.35 (cyclohexane/EtOAc 3:2); $[\alpha]_D^{20} = +243°$ ($c = 1.27$, $CHCl_3$); [20] $[\alpha]_D^{20} = +244°$ ($c = 1.6$, $CHCl_3$). Racemic **2a** was purified without flash chromatogaphy simply by recrystallization from EtOH to yield pure **2a** (6 g, 67%) as a colorless solid. The analytical data is presented elsewhere [21].

A solution of trifluoroperacetic acid was prepared by adding 1.4 mL H_2O_2 (85%, 42 mmol) slowly to an ice-cooled solution of 7 mL trifluoroacetic anhydride (7 mL, 50 mmol) in 20 mL dichloromethane. The solution was stirred for 1 h at RT. This solution, freshly prepared, was added dropwise at 0 °C over 15 min to a suspension of tetra-*O*-acetyl-conduritol-E (+)-**2a** (1.2 g, 3.8 mmol) and $NaHCO_3$ (1.4 g, 16 mmol) in 35 mL dichloromethane. The mixture was refluxed for 2 h and stirred for 12 h at room temperature. The reaction mixture was added slowly to a cooled and stirred mixture of 30 mL saturated aqueous $NaHCO_3$, 8.4 g $NaHCO_3$, 100 mL brine, and 200 mL dichloromethane. After addition of aqueous thiosulfate solution (50 mL, 20%) the mixture was extracted four times with dichloromethane or EtOAc. The combined organic layer was washed with brine and then dried. Evaporation of the solvent gave a yellow foam (1.2 g). The raw product was purified by fractionated silica-gel filtration (first cyclohexane/EtOAc 8:2 until educt and epoxide could not be detected by TLC, then EtOAc). The EtOAc fraction was evaporated to yield (+)-**2** (900 mg, 2.7 mmol, 71%) as a colorless foam. R_f 0.03 (cyclohexane/EtOAc 1:1); $[\alpha]_D^{20} = +12.7°$ ($c = 1.1$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$): δ 1.99 (s, 6 H, 2 CH_3), 2.13 (s, 6 H, 2 CH_3), 3.27 (br, 2 H, OH), 4.01 (s, 2 H, H-1, H-6), 5.20 (s, 2 H, H-3, H-4), 5.62 (s, 2 H, H-2, H-5); ^{13}C-NMR ($CDCl_3$): δ 20.58 (2 CH_3), 20.70 (2 CH_3), 68.33 (2 CH), 68.57 (2 CH), 70.19 (2 CH), 170.17 (2 C=O), 170.66 (2 C=O); MS (EI, 70 eV, *m*/*z* (%)): 271 (2), 228 (1), 210 (5), 168 (16), 157 (9), 126 (27), 115(16), 60 (18), 43 (100); calcd. for $C_{14}H_{20}O_{10}$: C, 48.28; H, 5.79. Found: C, 48.18; H, 6.00.

neo-Inositol hexakisphosphate ***3***. 1.9 g (5.5 mmol) Tetra-*O*-acetyl-*neo*-inositol (+)-**2** was suspended in anhydrous methanol (50 mL) under argon and cooled to 4 °C. 500 μL (2.25 mmol) of a 5.5 M sodium methanolate solution was added dropwise over 10 min. The solution was allowed to warm to room temperature and stirred for 12 h. The solvent was removed, and for complete turnover the residue was dissolved in 20 mL of 0.25 M aqueous NaOH and refluxed for 2 h. The hot solution was neutralized by addition of ion exchanger (H^+, Dowex 50-X) and filtered, and the Dowex material was washed with boiling water. The filtrate was first concentrated in high vacuum and then lyophilized. Recrystallization from water (50 mL) yielded free *neo*-inositol (1 g, 99%) as a colorless solid. The analytical data is presented elsewhere [22].

514 mg (2.26 mmol) 3-Diethylamino-2,4,3-benzodioxaphosphepane was added to a suspension of 50 mg (0.27 mmol) *neo*-inositol thus obtained and 315 mg (4.5 mmol) 1*H*-tetrazole in 20 mL anhydrous dichloromethane/acetonitrile (1:1);

the solution was stirred at 40 °C for three days. For work-up the solution was cooled to −40 °C, and an anhydrous solution of 1.5 g (6 mmol) *m*-CPBA (70%) in dichloromethane (15 mL) was added. The solution was allowed to warm to room temperature, and the stirring was continued for another hour. The reaction mixture was diluted with dichloromethane (150 mL) and washed consecutively with aqueous sodium thiosulfate (20%, 2 × 100 mL), saturated $NaHCO_3$ (3 × 100 mL) and brine. After evaporation of the solvent the resulting colorless foam was purified by flash chromatography on silica gel (R_f 0.22 (CH_2Cl_2/MeOH 95:5)) to yield hexa-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)-*neo*-inositol (75 mg, 21%) as a colorless foam. ^{1}H-NMR ($CDCl_3$): δ 5.15 (m, 4 H, H-1, H-3, H-4, H-6), 5.58 (m, 2 H, H-2, H-5), 5.00–5.82 (m, 24 H, 6 (CH_2)$_2$C$_6$H$_4$), 7.26–7.43 (m, 24 H, Ph–H); ^{13}C-NMR ($CDCl_3$): δ 69.19–69.63 (m, 6 (CH_2)$_2$C$_6$H$_4$), 72.86 (m, 4 C, C-1, C-3, C-4, C-6), 77.06 (2 C, C-2, C-5), 128.94–129.48 ($C_{arom.}$), 134.54, 135.63, 135.77 (C_{ipso}); ^{31}P-NMR{^{1}H} ($CDCl_3$): δ −1.48 (2 P, P-2, P-5), −2.39 (4 P, P-1, P-3, P-4, P-6).

To a suspension of 50 mg (39 μmol) hexa-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)-*neo*-inositol in 30 mL ethanol/water (1:2) was added Pd/C (50 mg, Degussa RW 10). The mixture was stirred at room temperature under H_2 overnight. The catalyst was filtered off, and the filtrate was concentrated in high vacuum and then lyophylized to give 24 mg (99%) of **3** as a colorless, very hygroscopic foam. Purification by HPLC assured purity >99% for the following reactions. ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 4.37 (d, $J = 4.6$ Hz, 4 H, H-1, H-3, H-4, H-6), 4.89 (d, $J = 9.2$, 2 H, H-2, H-5); ^{13}C-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 74.24 (4 C, C-1, C-3, C-4, C-6), 76.57 (2 C, C-2, C-5). ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 1.89 (2 P, P-2, P-5), 2.07 (4 P, P-1, P-3, P-4, P-6); HR-MS(MALDI-FTMS): *m*/*z*: 658.8541 $[M - H]^+$ calcd. for $C_6H_{17}O_{24}P_6$: 658.8527.

Alternative preparation: 70 mg (0.38 mmol) of *neo*-inositol was dissolved in 0.22 mL phosphoric acid at 60 °C. Polyphosphoric acid (4 mL) was added, and the mixture was heated to 150 °C under vacuum for 12 h. The mixture was diluted with water and purified by an ion exchanger (Cl^--form, DEAE sepharose) and eluted with a linear gradient of hydrochloric acid (0–1 N HCl). Purification by HPLC yielded 80 mg (31%) of *neo*-InsP_6 (**3**).

*2,3,4,5-Tetra-O-acetyl-1-O-benzyl-*D*-neo-inositol* (−)-***6***. A solution of 350 mg (1 mmol) tetra-*O*-acetyl-D-*neo*-inositol (+)-**2** in 8.5 mL of anhydrous dichloromethane/cyclohexane (2:1) was cooled to 0 °C. 2,2,2-trichloro-acetamidic acid benzyl ester (550 mg, 2.2 mmol) and trifluoromethanesulfonic acid (33 mg, 0.22 mmol) were added slowly; the reaction mixture was allowed to warm to room temperature and was stirred for another 5 h. The solvent was evaporated, and the residue was suspended in 1.5 mL hexane/dichloromethane (2:1). The solid was filtered off, and the filtrate was concentrated under reduced pressure. The crude product was purified by flash chromatography to yield (−)-**6** (100 mg, 0.22 mmol, 22%, R_f 0.21 (cyclohexane/EtOAc 1:1)) as a colorless solid. The dibenzyl product was also isolated, but was still strongly contaminated with trichloroacetamide. $[\alpha]_D^{20} = -8.4°$ ($c = 0.54$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$): δ 2.01 (s, CH_3), 2.03 (s, CH_3), 2.13 (s, CH_3), 2.14 (s, CH_3), 3.81

(dd, $J = 3.1$ Hz, $J = 10.2$ Hz, 1 H, H-1), 4.10 (dd, $J = 2.8$ Hz, $J = 9.9$ Hz, 1 H, H-6), 4.46, 4.74 (AB, $J = 10.7$ Hz, 2 H, Ph–CH_2), 5.26 (Ψs, 2 H, H-3, H-4), 5.69 (Ψt, $J = 2.3$ Hz, 1 H, H-5), 5.85 (Ψt, $J = 2.3$ Hz, 1 H, H-2), 7.24–7.46 (m, 5 H, Ph–H); ^{13}C-NMR ($CDCl_3$): δ 20.55 (2 CH_3), 20.66 (CH_3), 20.71 (CH_3), 66.55 (C-2), 67.58 (C-6), 68.17 (C-3 or C-4), 68.27 (C-3 or C-4), 69.57 (C-5), 72.06 (Ph–CH_2), 76.15 (C-1), 128.10, 128.52 ($C_{arom.}$), 136.98 (C_{ipso}), 169.93, 170.02, 170.06, 170.14 (C=O).

*1-O-Benzyl-*D*-neo-inositol* (−)-*7*. 55 mg (0.13 mmol) (−)-**6** was suspended in 10 mL anhydrous methanol and cooled to 4 °C. A sodium methanolate solution (50 μL, 5.5 M, 0.28 mmol) was added; the solution was allowed to warm to room temperature and was stirred for 3 h. The solution was neutralized by addition of ion exchanger (H^+, Dowex 50-X) and filtered, and the residue washed with methanol. The filtrate was concentrated in high vacuum to yield (−)-**7** (35 mg, 0.13 mmol, 100%) as a colorless solid. $[\alpha]_D^{20} = -14.0°$ ($c = 0.35$, DMSO); ^{1}H-NMR (d_6-DMSO): δ 3.37 (dd, $J = 3.1$ Hz, $J = 9.7$ Hz, 1 H, H-1), 3.40 (dd, $J = 2.8$ Hz, $J = 9.9$ Hz, 1 H, CH), 3.46 (dd, $J = 2.8$ Hz, $J = 9.9$ Hz, 1 H, CH), 3.66 (under HDO, 2 CH), 3.91 (Ψt, $J = 2.8$ Hz, 1 H, H-2), 4.51, 4.59 (AB, $J = 12.2$ Hz, 2 H, Ph–CH_2), 7.24–7.46 (m, 5 H, Ph–H); ^{13}C-NMR (d_6-DMSO): δ 68.98 (C-2), 69.40 (CH), 69.95 (CH), 70.08 (CH), 72.36 (CH), 70.98 (Ph–CH_2), 78.49 (C-1), 127.42, 127.82, 128.31 ($C_{arom.}$), 139.52 (C_{ipso}).

D-neo-Inositol 2,3,4,5,6-pentakisphosphate (+)-***8***. 3-Diethylamino-2,4,3,-benzodioxaphosphepane (0.2 g, 0.9 mmol) was added to a solution of 1-*O*-benzyl-D-*neo*-inositol (−)-**7** (30 mg, 0.11 mmol) and 1*H*-tetrazole (140 mg, 2 mmol) in anhydrous dichloromethane (20 mL) under argon, and the mixture was stirred at room temperature for 4 h. For work-up the solution was cooled to −40 °C, and an anhydrous solution of *m*-CPBA (1 g, 4 mmol, 70%) in dichloromethane (15 mL) was added. The solution was allowed to warm to room temperature, and the stirring was continued for another hour. The reaction mixture was diluted with dichloromethane (100 mL) and washed consecutively with aqueous sodium thiosulfate (20%, 2 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL) and brine. After evaporation of the solvent the resulting colorless foam was converted without further purification.

To this raw product in 40 mL ethanol/water (1:1) 50 mg Pd/C (Degussa RW 10) was added. The mixture was stirred at room temperature under H_2 for 12 h. The catalyst was filtered off, and the filtrate was concentrated in high vacuum and then lyophilized. Purification by HPLC yielded 30 mg (50%, purity >99%) of **8** as a colorless, very hygroscopic foam. $[\alpha]_D^{20} = +8.6°$ ($c = 0.51$, H_2O); ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 3.98 (dd, $J = 2.5$ Hz, $J = 10.2$ Hz, 1 H, H-1), 4.30 (dΨt, $J = 2.8$ Hz, $J = 10.2$ Hz, 1 H, H-6), 4.36 (m, 2 H, H-3, H-4), 4.76 (d, $J = 9.2$ Hz, 1 H, H-2), 4.89 (d, $J = 10.2$ Hz, 1 H, H-5); ^{13}C-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 70.69 (m, C-1), 74.50 (C-3 and C-4), 75.60 (d, $J = 5.1$ Hz, C-6), 76.56 (d, $J = 4.1$ Hz, C-5), 77.23 (d, $J = 5.1$ Hz, C-2); ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 1.90 (2 P, P-5, P-3 or P-4), 2.08 (2 P, P-6, P-3 or P-4), 3.08 (P-2).

D-neo-Inositol 1,2,5,6-tetrakisphosphate (−)-***10***. *S. cerevisiae* was grown for 24 h and harvested at a OD_{600} of about 15. The cells were washed twice with 75 mM

sodium acetate pH 4.6 (3000*g*, 10 min, 4 °C), and the cellular wet weight was determined (25 mL culture gives approximately 500 mg). The cells were resuspended in 75 mM sodium acetate (pH 4.6) containing 1 mM *neo*-$InsP_6$ (**3**) to a final concentration of 30 mg/mL cells. The reaction was carried out at 45 °C in a water-bath shaker and followed by HPLC-MDD. After 80–90 min the reaction was stopped by removing the cells (6000*g*, 10 min, 4 °C). The sediment was washed with doubly distilled water, and the collected supernatants were combined and filtered for sterility. The product was purified by HPLC. After freeze-drying, overall yields were in the range of 75–85% for (−)-**10** (quantification based on inorganic phosphate determination). $[\alpha]_D^{20} = -12.8°$ ($c = 0.22$, H_2O); ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 3.86 (s, 2 H, H-3, H-4), 4.37 (d, $J = 6.6$ Hz, 2 H, H-1, H-6), 4.74 (d, $J = 9.2$ Hz, 2 H, H-2, H-5); ^{13}C-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 71.89 (C-3 and C-4), 74.73 (C-1 and C-6), 77.50 (C-2 and C-5); ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 1.78 (P-1 and P-6), 3.08 (P-2 and P-5).

L-*chiro-Inositol hexakisphosphate* (−)-***5***. Pd/C (40 mg) was added to a suspension of 250 mg (0.7 mmol) 2,5-di-*O*-benzyl-L-*chiro*-inositol (−)-**4** in 20 mL ethanol/water (1:1). The mixture was stirred overnight at room temperature under H_2. The catalyst was filtered off, and the filtrate was concentrated in high vacuum and lyophilized to give a voluminous foam (120 mg, 99%). Crystallization from EtOH yielded pure L-*chiro*-inositol (125 mg, 99%) as a colorless solid. $[\alpha]_D^{20} = -70°$ ($c = 0.55$, H_2O) agrees with [23]; further analytical data is presented elsewhere (*22*).

470 mg (2.0 mmol) 3-Diethylamino-2,4,3-benzodioxaphosphepane was added to a suspension of 50 mg (0.27 mmol) L-*chiro*-inositol obtained as above and 226 mg (3.2 mmol) 1*H*-tetrazole in 20 mL anhydrous dichloromethane; the solution was stirred at room temperature overnight. The product was worked up as described for **3**. Purification by flash chromatography on silica gel yielded hexa-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)-L-*chiro*-inositol (200 mg, 60%) as a colorless foam with a purity >80%. ^{13}C-NMR ($CDCl_3$): δ 69.03–69.74 (m, 6 (CH_2)$_2$$C_6H_4$), 73.51 (m, 2 CH), 73.81 (m, 2 CH), 76.28 (m, 2 CH), 128.4–129.4 ($C_{arom.}$), 134.41, 134.51, 135.33, 135.47, 135.52, 136.88 (C_{ipso}); ^{31}P-NMR{^{1}H} ($CDCl_3$): δ −1.30 (2 P), −2.05 (2 P), −2.74 (2 P).

Hydrogenolysis of hexa-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)-L-*chiro*-inositol was carried out as described for **3.** Purification by HPLC assured a purity >99%. $[\alpha]_D^{20} = -24.4°$ ($c = 1.17$, H_2O); ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 4.30 (AA′, 2 H, H-3, H-4), 4.36 (MM′, 2 H, H-2, H-5), 4.65 (under HDO, XX′, 2 H, H-1, H-6); ^{13}C-NMR (D_2O, pH adjusted to 6 (ND_4OD)):δ 72.9 (m, C-1 and C-6), 73.3 (m, C-2 and C-5), 76.5 (m, C-3 and C-4); ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 1.63 (P-1 and P-6), 1.91 (P-2 and P-5), 2.66 (P-3 and P-4).

L-*chiro-Inositol 1,2,3,5,6-pentakisphosphate* (***11***), L-*chiro-Inositol 1,2,3,6-tetrakisphosphate* (***12***). The dephosphorylation of L-*chiro*-$InsP_6$ ((−)-**5**) catalyzed by the phytase from *P. rhodanensis* was carried out at 45 °C in 75 mM sodium acetate (pH 4.6) with an initial substrate concentration of 1 mM and an enzyme activity of 8.5 mU/mL in a water-bath shaker. The reaction was monitored by HPLC-MDD and stopped after 80–90 min, when the contents of L-*chiro*-Ins(1,2,3,5,6)P_5 (**11**) and

L-*chiro*-Ins(1,2,3,6)P_4 (**12**) were approximately equal. The products were isolated by HPLC. After freeze-drying, overall yields were in the range of 30–40% each for **11** and **12**.

11: $[\alpha]_D^{20} = -26°$ ($c = 0.5$, H_2O); ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 3.86 (Ψt, $J = 9.2$ Hz, 1 H, H-4), 4.25 (Ψq, $J = 9.2$ Hz, 1 H, H-3), 4.33 (dΨt, $J = 9.7$ Hz, $J = 3.1$ Hz, 1 H, H-5), 4.39 (dΨt, $J = 9.7$ Hz, $J = 2.5$ Hz, 1 H, H-2), 4.65 (under HDO, 2 H, H-1, H-6); ^{13}C-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 73.72 (dd, $J = 6.1$ Hz, $J = 2.0$ Hz, CH), 74.52 (d, $J = 4.1$ Hz, CH), 75.20 (m, CH), 75.43 (d, $J = 4.1$ Hz, CH), 76.04 (Ψt, $J = 5.1$ Hz, CH), 79.21 (Ψt, $J = 5.6$ Hz, CH); ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 1.17 (P-1), 1.48 (P-5 and P-6), 2.09 (P-2), 2.48 (P-3).

12: $[\alpha]_D^{20} = -19.8°$ ($c = 0.63$, H_2O); ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 3.82 (Ψt, $J = 9.9$ Hz, 1 H, H-4), 3.91 (dd, $J = 9.9$ Hz, $J = 2.3$ Hz, 1 H, H-5), 4.23 (Ψq, $J = 9.0$ Hz, 1 H, H-3), 4.39 (dΨt, $J = 9.4$ Hz, $J = 2.5$ Hz, 1 H, H-2), 4.59 (dd, $J = 9.2$ Hz, $J = 3.6$ Hz, 1 H, H-6), 4.65 (under HDO, 1 H, H-1); ^{13} C-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 70.29 (m, CH), 73.16 (s, CH), 73.53 (m, CH), 73.57 (m, CH), 74.05 (d, $J = 4.1$ Hz, CH), 77.70 (m, CH); ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 1.68 (P-1), 2.36 (P-2), 2.57 (P-6), 3.07 (P-3).

D-*chiro-Inositol 1,3,4,6-tetrakisphosphate* (+)-***9***. 3-Diethylamino-2,4,3,-benzodioxaphosphepane (253 mg, 1 mmol) was added to a solution of 50 mg (0.13 mmol) dibenzyl-D-*chiro*-inositol (+)-**4** and 1*H*-tetrazole (120 mg, 1.7 mmol) in anhydrous dichloromethane (10 mL); the mixture was stirred at room temperature for 12 h. After work-up as described for **3** (650 mg *m*-CPBA (70%), 2.6 mmol in 15 mL CH_2Cl_2) and purification by flash chromatography (EtOAc), pure (+)-2,5-di-*O*-benzyl-1,3,4,6-tetra-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)-D-*chiro*-inositol (45 mg, 30%) was isolated as a colorless solid. R_f 0.30 (EtOAc); $[\alpha]_D^{20} = +16.1°$ ($c = 0.49$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$): δ 3.99 (s, br, 2 H, H-2, H-5), 4.75 (d, $J = 11.2$ Hz, 2 H, Ph–CH_2), 4.85 (d, $J = 11.2$ Hz, 2 H, Ph–CH_2), 4.66-5.58 (m, 16 H, (CH_2)$_2$$C_6H_4$), 5.07 (m, 2 H, H-3, H-4), 5.30 (Ψd, $J = 9.7$ Hz, 2 H, H-1, H-6), 7.13-7.54 (m, 26 H, Ph–H); ^{13}C-NMR ($CDCl_3$): δ 68.08–69.46 (m, 4 (CH_2)$_2$$C_6H_4$), 71.89 (m, C-1 and C-6), 72.93 (Ph–CH_2), 75.38 (m, C-2 and C-5), 77.65 (m, C-3 and C-4), 127.8–129.2 ($C_{arom.}$), 134.76, 135.19, 135.31, 137.02 (C_{ipso}); ^{31}P-NMR{^{1}H} ($CDCl_3$): δ 0.56 (br, 2 P), −1.71 (2 P). HR-MS (ESI-pos., TOF): *m/z*: 1089.2208 $[M + H]^+$ calcd. for $C_{52}H_{53}O_{18}P_4$: 1089.2182.

To a suspension of 35 mg (27 μmol) (+)-2,5-di-*O*-benzyl-1,3,4,6-tetra-*O*-(2-oxo-5,6-benzo-1,3,2-dioxaphosphep-2-yl)-D-*chiro*-inositol, isolated as above, in 10 mL ethanol/water (1:1) was added Pd/C (20 mg). The mixture was stirred overnight at room temperature under H_2. The catalyst was filtered off, and the filtrate was concentrated in high vacuum and lyophilized to give (+)-**9** as a colorless, strongly hygroscopic foam (15 mg, 99%). $[\alpha]_D^{20} = +13.6°$ ($c = 0.75$, H_2O); ^{1}H-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 4.02 (m, 2 H, H-2, H-5), 4.16 (m, 2 H, H-3, H-4), 4.52 (m, 2 H, H-1, H-6); ^{13}C-NMR (D_2O, pH adjusted to 6 (ND_4OD)): δ 72.10 (m, C-2 and C-5), 75.76 (m, C-1 and C-6), 79.49 (m, C-3 and C-4); ^{31}P-NMR{^{1}H} (D_2O, pH adjusted to 6 (ND_4OD)): δ 2.17 (2 P), 3.03 (2 P).

3. Results and discussion

3.1. Stereo- and regiospecificity of myo-InsP$_6$ dephosphorylation by the phytases from S. cerevisiae and P. rhodanensis

Among the sources of phytase useful for the intended study, yeast strains seemed to be especially promising. The cells are easy to cultivate with short generation times, making fresh cellular material accessible at any time, even in large amounts; this is important for their application as biocatalysts in enzyme-assisted synthesis. Whole cell transformations of *myo*-InsP$_6$ with phytase from baker's yeast (*S. cerevisiae*) have already been published [24,25]. Dephosphorylation of *myo*-InsP$_6$ proceeds via a remarkably stereo- and regiospecific route [26]. We chose this phytase as an example of an enzyme with rather high specificity. Previous experiments with *P. rhodanensis* were indicative of a phytase activity with a more complex dephosphorylation pattern. This species secretes high levels of the enzyme into the culture medium. We established a rapid method for precipitating the phytase from the culture supernatant by the addition of ethanol. Recoveries of more than 80% of the total enzyme activity can be achieved by this procedure. Being able to perform conversions with enriched enzyme preparations instead of whole cells has several advantages, e.g., no risk of contamination through cell lysis. Furthermore, work with living cells is often restricted to narrow temperature and pH ranges.

The conditions for the *myo*-InsP$_6$ conversion with *S. cerevisiae* were adopted from the literature [25]. To correlate the data with those obtained for the crude phytase from *P. rhodanensis*, we employed identical conditions in the two cases: a temperature of 45 °C, a pH of 4.6 (acetate buffer) and a starting substrate concentration of 1 mM *myo*-InsP$_6$. In our experience *S. cerevisiae* remains viable for several hours under these conditions. In a comprehensive study of yeast phytases, all of the enzymes exhibited optimal activity in the pH range of 4–5 [27]. The reported K_M values for the partially purified phytases are 0.21 mM for the enzyme from *S. cerevisiae* [28] and 0.25 mM for the one from *P. rhodanensis* [27]. These data suggest that the concentration of 1 mM inositol hexakisphosphate used might lead to substrate saturation of the enzymes. Commercially available *myo*-inositol hexakisphosphate was purified by HPLC before use, to ensure that it contained no interfering lower phosphorylated inositol phosphates. Aliquots of the salt-free, protonated form of *myo*-InsP$_6$ were subjected to sulfuric acid hydrolysis. As already described, the determination of released orthophosphate provides a reliable quantification of phosphorylated substances [16]. The chromatograms in Fig. 1 demonstrate how the phytase activities from *S. cerevisiae* (trace 1a) and *P. rhodanensis* (trace 1b) catalyze the stepwise dephosphorylation of *myo-InsP$_6$*. The achiral analytical method does not differentiate between enantiomers, but for the phytase from *S. cerevisiae* the absolute configuration of the accumulating *myo*-InsP$_3$ isomer was determined unequivocally in earlier work [24]. It is exclusively D-*myo*-Ins(1,2,6)P$_3$ (alpha-trinositol, PP56), a compound with an interesting pharmacological profile [29]. As confirmed by cochromatography with standards, the precursors are consequently D-*myo*-Ins(1,2,4,5,6)P$_5$, and D-*myo*-Ins(1,2,5,6)P$_4$. This is in accordance with the dephosphorylation pathway previously

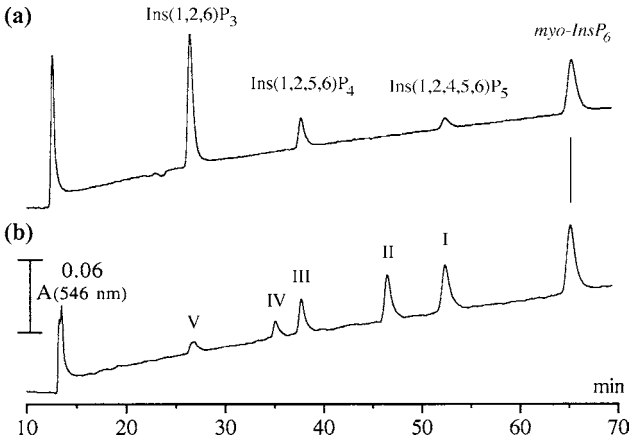


Fig. 1. HPLC-MDD analysis showing (a) *myo*-$InsP_6$ dephosphorylation by the phytase of *S. cerevisiae* and (b) the corresponding reaction catalyzed by the phytase of *P. rhodanensis* (I: Ins(1,2,4,5,6)P_5/Ins(2,3,4,5,6)P_5; II: Ins(1,2,3,4,5)P_5/Ins(1,2,3,5,6)P_5; III: Ins(1,2,5,6)P_4/Ins(2,3,4,5)P_4; IV: Ins(1,2,4,5)P_4/Ins(2,3,5,6)P_4; V: $InsP_3$ isomers).

published by Greiner et al., who examined the purified phytase of *S. cerevisiae* [26]. Up to the inositol trisphosphate level it obviously makes no difference whether one works with whole cells or the pure enzyme. For the phytase of *P. rhodanensis* the following intermediates of *myo*-$InsP_6$ degradation were observed: Ins(1,2,4,5,6)P_5/Ins(2,3,4,5,6)P_5, Ins(1,2,3,4,5)P_5/Ins(1,2,3,5,6)P_5, Ins(1,2,5,6)P_4/Ins(2,3,4,5)P_4, Ins(1,2,4,5)P_4/Ins(2,3,5,6)P_4 and $InsP_3$ isomers. The phytase activity of *P. rhodanensis* was inhibited almost completely ($\geqslant 90\%$) by low concentrations of the pseudo-substrate *myo*-inositol hexakissulfate (about 100 μM), as were the enzymatic conversions of the substrate analogues (**3**) and ((−)-**5**) described below, an observation that has already been made for the *myo*-$InsP_6$ conversion with phytases of *Aspergillus ficuum* [30]. If there are different activities involved in the reactions investigated, they share these characteristics. For an indication of whether the reactions found in the case of *P. rhodanensis* are catalyzed by a single enzyme activity, the ethanol-precipitated enzyme preparation was subjected to size-exclusion chromatography. With recoveries of more than 70%, the activity eluted as a single peak with an apparent molecular weight of about 350 kDa (data not shown). HPLC-MDD revealed no differences between the intermediates of *myo*-$InsP_6$ conversion observed before and after chromatography, making it unlikely that different enzyme activities are present in the preparation and contribute to this dephosphorylation pattern.

Knowing the exact route of enzymatic *myo*-$InsP_6$ dephosphorylation, including the absolute configuration of the intermediates, is valuable in studying the substrate specificity with the stereoisomers *neo*-$InsP_6$ (**3**) and L-*chiro*-$InsP_6$ ((−)-**5**). Stereoselective assignment of *myo*-inositol phosphates can be accomplished by a time-consuming procedure starting with periodate oxidation (cleavage between two vicinal hydroxy groups) and continuing with reduction and dephosphorylation. The polyol produced is then incubated with L-polyol dehydrogenase (EC 1.1.1.14), and the products are

characterized by HPLC [31]. Only *myo*-inositol bis-, tris- and tetrakisphosphates with two vicinal hydroxy groups can be analyzed by this method, and because of the multitude of steps the use of radioactively labeled compounds is recommended. In a new method for the determination of the enantiomeric purity of *myo*-Ins(1,2,4,5,6)P_5/*myo*-Ins(2,3,4,5,6)P_5 and *myo*-Ins(1,2,3,4,5)P_5/*myo*-Ins(1,2,3,5,6) P_5, we exploit a partially purified phytase from *D. discoideum* as an enzymatic tool [14]. Only about 20 nmol of the *myo*-inositol pentakisphosphate isomer is needed for comparison of its enzymatic conversion with that of authentic, enantiomerically pure standards generated by stereocontrolled chemical transformations starting from conduritol-B derivatives (manuscript in preparation). Theoretically, the dephosphorylation of each enantiomer could lead to five *myo*-InsP_4 isomers, but in no case did we find any indication of hydrolysis of the axial phosphate group in position 2. Fig. 2A summarizes the results obtained by HPLC-MDD for the optical antipodes *myo*-Ins(2,3,4,5,6)P_5 and *myo*-Ins(1,2,4,5,6)P_5. As shown in chromatogram 2Ab, the first dephosphorylation of *myo*-Ins(2,3,4,5,6)P_5 takes place mainly in position 6 and leads to *myo*-Ins(2,3,4,5)P_4. The enantiomer *myo*-Ins(1,2,4,5,6)P_5 is also preferentially attacked at position 6, thereby producing clearly distinguishable dephosphorylation products (chromatogram 2Ac). It is important to note that the signals of the breakdown products of *myo*-Ins(2,3,4,5,6)P_5 and *myo*-Ins(1,2,4,5,6)P_5 do not superimpose. Experiments with artificial mixtures of the optical antipodes yielded a detection limit of approximately 2% for the less concentrated enantiomer (data not shown). When the purified inositol pentakisphosphate with the retention time of Ins(1,2,4,5,6)P_5/Ins(2,3,4,5,6)P_5 from the *myo*-InsP_6 conversion with Pichia phytase was subjected to this procedure it was unequivocally identified as D-*myo*-Ins(1,2,4,5,6)P_5 ($\geqslant$98% ee, chromatogram 2Ad). Fig. 2B shows a comparison of analytical runs for the enzymatically converted reference compounds *myo*-Ins(1,2,3,5,6)P_5 (chromatogram 2Bb) and *myo*-Ins(1,2,3,4,5)P_5 (chromatogram 2Bc). For better separation of the breakdown products in this case, the samples were chromatographed with an alkaline elution system. There is a clear-cut difference in the *myo*-inositol tetrakisphosphates observed. The main intermediates formed are Ins(1,2,3,6)P_4 from Ins(1,2,3,5,6)P_5 and Ins(1,2,4,5)P_4 from Ins(1,2,3,4,5)P_5. Analogous experiments performed with the second inositol pentakisphosphate isolated after *myo*-InsP_6 conversion with Pichia phytase revealed that this compound is D-*myo*-Ins(1,2,3,4,5)P_5 ($\geqslant$98% ee, chromatogram 2Bd).

On the basis of the absolute configuration determined for the inositol pentakisphosphates, it was possible with HPLC-MDD to unravel the degradation pathway of *myo*-InsP_6 up to the inositol trisphosphate level. Scheme 1 illustrates the complex routes of *myo*-InsP_6 dephosphorylation catalyzed by the phytase of *P. rhodanensis*. The reaction sequence framed with a dotted line represents the comparatively simple but quite specific counterpart catalyzed by the phytase of *S. cerevisiae*.

*3.2. Chemical synthesis of the stereoisomers neo-InsP_6 (**3**) and L-chiro- InsP_6 ((−)-**5**) and the required reference compounds*

Previous work led us expect that the enantiopure diacetoxy-dibromocyclohex-5-ene (+)-**1** and (−)-**1**, which are readily accessible from *p*-benzoquinone in four steps

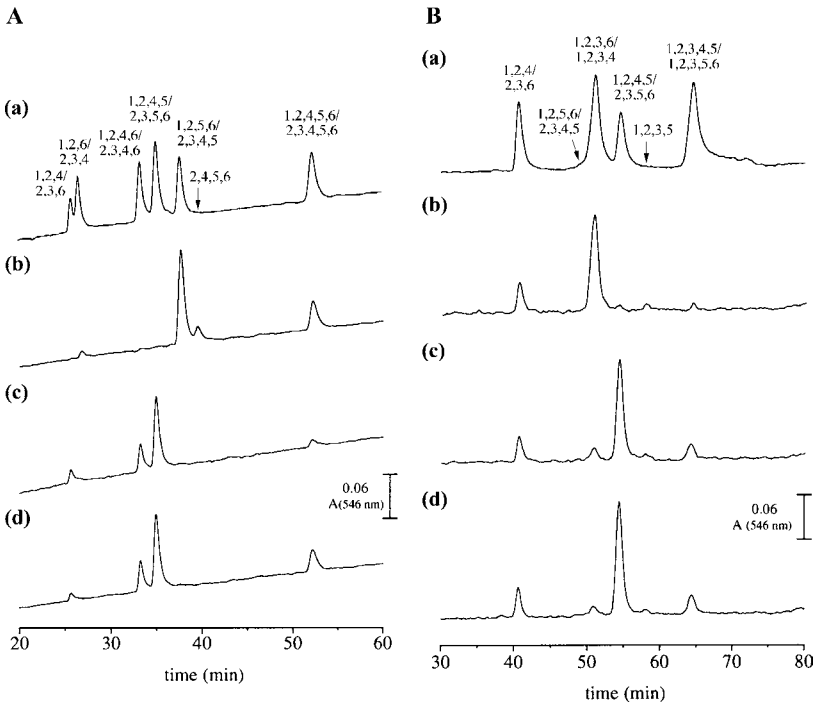
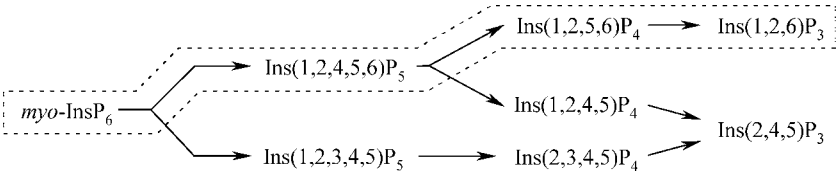


Fig. 2. Stereoselective assignment of *myo*-inositol pentakisphosphates by means of a phytase from *D. discoideum*. HPLC-MDD chromatograms for the enantiomeric pairs Ins(1,2,4,5,6)P_5/Ins(2,3,4,5,6)P_5 (A; acidic elution system) and Ins(1,2,3,4,5)P_5/Ins(1,2,3,5,6)P_5 (B; alkaline elution system). A: Standard mixture of resolved isomers including the elution position of *myo*-Ins(2,4,5,6)P_4 (a). Enzymatically converted enantiopure Ins(2,3,4,5,6)P_5 (b), Ins(1,2,4,5,6)P_5 (c) and the corresponding isolated isomer from the *myo*-InsP_6/Pichia-phytase degradation (d). B: Standard mixture of resolved isomers including the elution positions of *myo*-Ins(1,2,5,6)P_4 and *myo*-Ins(1,2,3,5)P_4 (a). Enzymatically converted enantiopure Ins(1,2,3,5,6)P_5 (b), Ins(1,2,3,4,5)P_5 (c) and the corresponding isolated isomer from the *myo*-InsP_6/Pichia-phytase degradation (d).

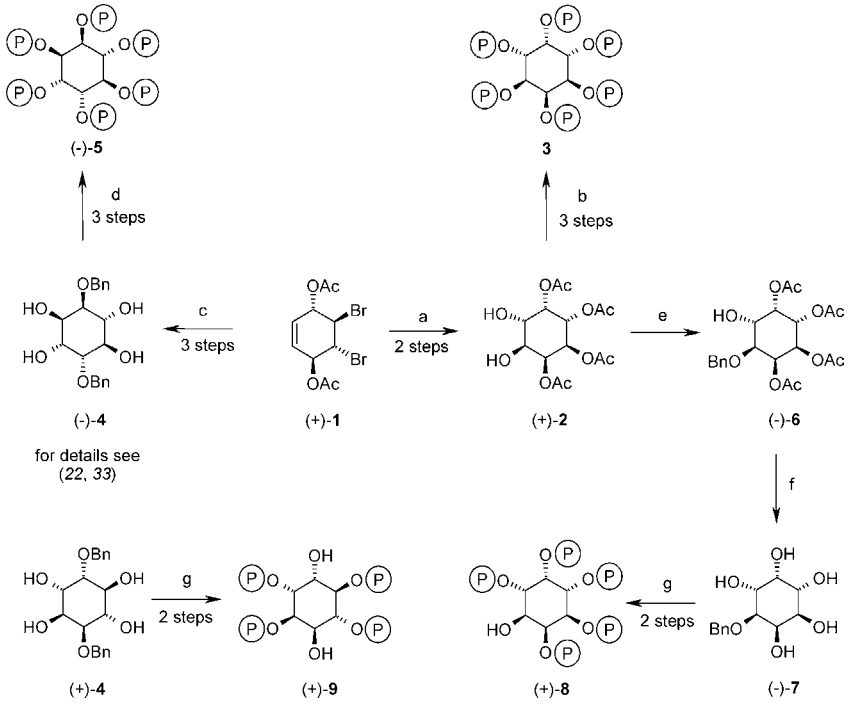


Scheme 1. Main pathways of *myo*-InsP_6 dephosphorylation catalyzed by the phytase of *P. rhodanensis* (framed reaction sequence characteristic for *S. cerevisiae*).

on multigram scale [16,17] can also serve as starting material for conduritol systems other than conduritol B. Our synthetic concept is based on the stereospecific conversion of this building block into suitably protected enantiomerically pure conduritol

derivatives, which can subsequently be transformed to inositol isomers by simple functionalization of the double bond [22].

Synthesis of neo-inositol. The introduction of two acetate groups in the C-2 and C-3 position under inversion of the stereogenic centers might lead to a conduritol E derivative. Heating the diacetate (+)-**1** for ten days with sodium acetate in aqueous acetic acid indeed provided conduritol E derivative (+)-**2a** (structure not shown in Scheme 2) in 70% yield [22]. The epoxidation of (+)-**2a** with trifluoroperacetic acid and in situ hydrolysis gave *neo*-inositol tetraacetate (+)-**2**. The opening of the oxirane was observed to proceed regioselectively to only the *neo*-isomer. De-*O*-acetylation of (+)-**2** by treatment with a catalytic amount of sodium methylate in methanol did not run to completion because of the coprecipitation of partially deacylated educt with *neo*-inositol. Full deprotection was carried out by refluxing the resulting



Scheme 2. Preparation of *neo*-inositol phosphates (**3**, (+)-**8**) and L-*chiro*-inositol phosphates ((−)-**5**, (+)-**9**). Reagents: (a) (i) NaOAc, AcOH (95%), 10 days, 130 °C then Ac_2O, CH_2Cl_2, DMAP (cat.). (ii) $(CF_3CO)_2O$, H_2O_2, CH_2Cl_2, $NaHCO_3$; (b) (i) NaOMe, MeOH then 0.25 M NaOH. (ii) 3-Diethylamino-2,4,3-benzodioxaphosphepane, 1*H*-tetrazole, CH_2Cl_2/CH_3CN then *m*-CPBA. (iii) Pd/C, H_2 $EtOH/H_2O$. (c) (i) NaOBn, BnOH. (ii) $(CF_3CO)_2O$, H_2O_2, CH_2Cl_2, Na_2CO_3. (iii) H_2SO_4, dioxane, water. (d) (i) Pd/C, H_2 ethanol/water. (ii) 3-Diethylamino-2,4,3-benzodioxaphosphepane, 1*H*-tetrazole, CH_2Cl_2 then *m*-CPBA. (iii) Pd/C, H_2 ethanol/water. (e) 2,2,2-trichloro-acetimidic acid benzyl ester, CF_3SO_3H. (f) NaOMe, MeOH. (g) (i) 3-Diethylamino-2,4,3-benzodioxaphosphepane, 1*H*-tetrazole, CH_2Cl_2 then *m*-CPBA. (ii) Pd/C, H_2 $EtOH/H_2O$.

precipitate in aqueous NaOH. *neo*-Inositol was easily purified by recrystallization from water [32].

The well known 2,5-di-*O*-benzyl-L-*chiro*-inositol (−)-**4** was synthesized in three steps from diacetate (+)-**1** [22]. The spectral data of the solid obtained correspond to those observed by Liu et al. [33]. Pd/C-catalyzed hydrogenation gave free L-*chiro*-inositol as a colorless solid.

The introduction of the six phosphate functionalities into free L-*chiro*- and *neo*-inositol was achieved by treatment with 3-diethylamino-2,4,3-benzodioxaphosphepane in dichloromethane/acetonitrile in the presence of 1*H*-tetrazole. After 12 h for L-*chiro*-inositol and 36 h for *neo*-inositol the resulting phosphites were oxidized with *m*-CPBA and purified by flash chromatography to give the protected hexakisphosphates in 50% yield at most. Because of the low solubility of free inositol, especially *neo*-inositol, the phosphorylation is quite difficult, and full conversion under these conditions was not possible. Varying the solvents or applying longer reaction times did not increase the yield. The subsequent Pd/C-catalyzed hydrogenolysis gave the free inositol hexakisphosphates **3** and (−)-**5**. Alternatively, *neo*-inositol was phosphorylated in polyphosphoric acid to give **3** in good yield. This method was, however, only of minor value because of the problem in work-up caused by the great excess of polyphosphoric acid needed and the difficulties of removing this substance completely. Purification of the resulting inositol hexakisphosphates by HPLC ensures purities suitable for biological experiments.

For further insight into the enzymatic dephosphorylation of substrate analogues by yeast phytases it was essential to synthesize enantiomerically pure reference materials for the potential degradation products (details are discussed later). Our first effort was the synthesis of *neo*-Ins(1,2,5,6)P_4 (**10**). Earlier work led us to propose that enantiopure tetra-*O*-acetyl-*neo*-inositol (−)-**2** would be an ideal starting material for the synthesis of *neo*-Ins(1,2,5,6)P_4 (**10**), if the two free hydroxy groups could be functionalized with an orthogonal protecting group. At first sight, the best protection seemed to be benzyl ether groups, because it should be possible to remove these completely in one step at the end of the reaction sequence. We tried to benzylate under mild conditions with 2,2,2-trichloro-acetimidic acid benzyl ester, but even with an excess of reagent the monobenzylated *neo*-inositol tetraacetate (+)-**6** was formed as the main product; it was isolated in pure form in 20% yield. The dibenzyl-*neo*-inositol tetraacetate was only obtained impure and in very low yield along with other products. This result forced us to try the synthesis of another, even more readily accessible reference material, namely *neo*-Ins(2,3,4,5,6)P_5 (+)-**8**. This new target molecule was expected to help in the assignment of the absolute configuration of the products of the degradation of *neo*-InsP_6 (**3**) by yeast phytases.

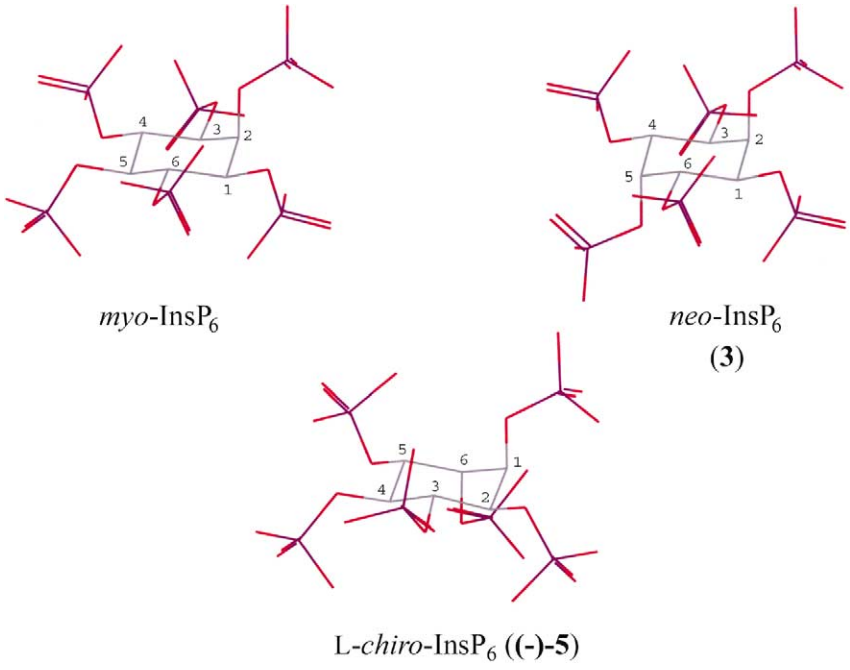
For this purpose the enantiomer (+)-**2** was treated with 2,2,2-trichloro-acetimidic acid benzyl ester in dichloromethane/hexane to furnish the monobenzylated derivative (−)-**6**. Alkaline methanolysis gave free 1-benzyl-*neo*-inositol (−)-**7**. Phosphorylation with 3-diethylamino-2,3,4-benzodioxaphosphepane in the presence of 1*H*-tetrazole, with subsequent oxidation with *m*-CPBA and finally Pd/C-catalyzed hydrogenation of (−)-**7**, provided *neo*-Ins(2,3,4,5,6)P_5 (+)-**8**.

Compound (+)-**4** was made from the optical antipode (−)-**1**; it can also be made from D-*chiro*-inositol by a method reported by Liu et al. [33]. In analogy to the synthesis of (+)-**8**, (+)-**4** was phosphorylated to yield pure D-*chiro*-Ins(1,3,4,6)P_4 (+)-**9**, another compound useful in unravelling the degradation pathways of interest here.

It should be mentioned that both enantiomers are accessible in all cases from the corresponding enantiomerically pure conduritol derivatives, illustrating the versatility of this synthetic strategy.

3.3. Enzymatic conversion of neo-$InsP_6$ (**3**) and L-chiro-$InsP_6$ ((−)-**5**)

The obvious difference in the molecular structures of *myo*-$InsP_6$ and *neo*-$InsP_6$ (**3**) or L-*chiro*-$InsP_6$ ((−)-**5**) lies in the orientation of a single substituent (confirmed by NMR at pH 5–7, see Scheme 3). In contrast to *myo*-$InsP_6$, the phosphate group in 5-position of the epimer *neo*-$InsP_6$ (**3**) is axially oriented, and L-*chiro*-$InsP_6$ ((−)-**5**) possesses an axial phosphate group in 3-position (in the scheme this corresponds to the 6-position of L-*chiro*-$InsP_6$ ((−)-**5**)). Scheme 3 gives only an idealized picture of the structures in aqueous solutions, the spatial arrangement of the phosphate groups in each of these molecules being dependent inter alia on the protonation, the hydration and the formation of intramolecular hydrogen bonds.



Scheme 3. Molecular structures and numbering of carbons for the inositol hexakisphosphate stereoisomers.

To get an idea whether the phytases from *S. cerevisiae* and *P. rhodanensis* tolerate deviations from the *myo*-configuration and catalyze the dephosphorylation of the epimers *neo*-$InsP_6$ (**3**) and L-*chiro*-$InsP_6$ ((−)-**5**), too, we compared the initial rates of the conversions characteristic for the first dephosphorylation. The HPLC-MDD system calibrated for the three stereoisomers was used to quantify the substances. With a starting concentration of 1 mM, kinetics were measured for no more than 30% inositol hexakisphosphate consumption. The curves were evaluated by linear regression. Table 1 summarizes the results. For *S. cerevisiae* the phytase activity is given in relation to the wet weight of the cells, while for *P. rhodanensis* the values represent the specific activity of the crude enzyme. The results were somewhat unexpected: although L-*chiro*-$InsP_6$ ((−)-**5**) is not degraded by *S. cerevisiae*, the "natural" substrate *myo*-$InsP_6$ is clearly dephosphorylated more slowly than *neo*-$InsP_6$ (**3**). The situation is different for the phytase of *P. rhodanensis*: the rates for *neo*-$InsP_6$ (**3**) and *myo*-$InsP_6$ are nearly equal, whereas the conversion of L-*chiro*-$InsP_6$ ((−)-**5**) is about twice as fast. As mentioned, all inositol hexakisphosphates investigated were purified by HPLC prior to use. To exclude the possibility that *myo*-$InsP_6$ isolated from corn contains inhibitory impurities coeluting in the purification procedure, we repeated the study with the compound isolated from rice. The data correlate within the tolerance with those shown in Table 1. Although not demonstrated before for any phytase of eucaryotic origin, in view of the habitat of yeast and the fact that several inositol hexakisphosphate isomers are constituents of soil [12] it is not surprising that the enzymes are capable of dephosphorylating isomers other than *myo*-$InsP_6$. One would, however, expect the catalyzed reaction to be optimized for the compound regarded as the "natural" substrate.

In order to clarify whether the dephosphorylation of the substrate analogues is specific or rather random, we isolated the breakdown products by HPLC and characterized relevant intermediates by NMR spectroscopy. Fig. 3 shows the HPLC-MDD analysis after conversion of the two synthetic inositol hexakisphosphate isomers by *S. cerevisiae*. Whereas L-*chiro*-$InsP_6$ ((−)-**5**) is not dephosphorylated (chromatogram 3b), a *neo*-inositol tetrakisphosphate accumulated in the course of the reaction with *neo*-$InsP_6$ (**3**; chromatogram 3a). As a putative precursor, a single *neo*-inositol pentakisphosphate was observed, indicating a specific enzymatic conversion. Even after complete degradation of *neo*-$InsP_6$ (**3**) the compound with the retention time of a *neo*-$InsP_4$ isomer shows little tendency towards further dephosphorylation (data not shown). The assay initially designed for the kinetic

Table 1
Enzyme activities towards different inositol hexakisphosphate stereoisomers

$InsP_6$ stereoisomer	Phytase activity	
	Saccharomyces cerevisiae (mU/mg wet weight)	*Pichia rhodanensis* (mU/mg protein)
myo-$InsP_6$	0.13 ± 0.03	9.3 ± 1
neo-$InsP_6$ (**3**)	0.55 ± 0.03	9.7 ± 1.1
L-*chiro*-$InsP_6$ ((−)-**5**)	Not detectable	18.6 ± 0.5

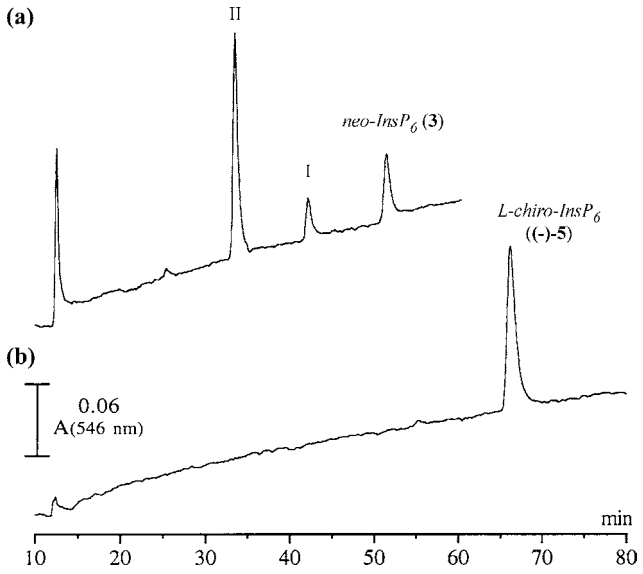
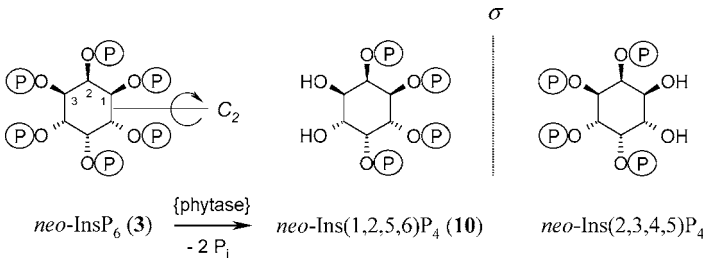


Fig. 3. HPLC-MDD chromatograms representative of (a) *neo*-$InsP_6$ (**3**) dephosphorylation (I: *neo*-Ins(1,2,4,5,6)P_5/*neo*-Ins(2,3,4,5,6)P_5 ((+)-**8**); II: *neo*-Ins(1,2,5,6)P_4 (**10**)/*neo*-Ins(2,3,4,5)P_4) and (b) L-*chiro*-$InsP_6$ ((−)-**5**) dephosphorylation by the phytase of *S. cerevisiae*.

evaluation of the reaction was scaled up to get enough *neo*-$InsP_4$ for a NMR study. The spectra of the purified compound are representative of a C_2-symmetric molecule with both axial positions phosphorylated (characteristic chemical shift and coupling pattern for the corresponding ring protons). The only structures in agreement with all of the spectroscopic data are those of the enantiomeric pair *neo*-Ins(1,2,5,6)P_4/*neo*-Ins(2,3,4,5)P_4 (Scheme 4). It is very likely that the phosphoester bond susceptible to the initial attack is the one in position 3, as found for the highly stereospecific first dephosphorylation of *myo*-$InsP_6$ by the phytase of *S. cerevisiae*. In this case the product should be enantiomerically pure *neo*-Ins(1,2,5,6)P_4 (**10**), but the definitive assignment of the absolute configuration required reference material of known stereochemistry. We therefore synthesized the optical antipode (+)-**8** (Scheme 2) of



Scheme 4. Conversion of *neo*-$InsP_6$ (**3**) by the phytase of *S. cerevisiae*.

the supposed precursor *neo*-Ins(1,2,4,5,6)P_5 and examined its enzymatic dephosphorylation. The degradation of *neo*-Ins(2,3,4,5,6)P_5 ((+)-**8**) occurs quite unspecifically under concomitant appearance of three HPLC-resolvable *neo*-inositol tetrakisphosphate isomers, which are subsequently dephosphorylated further (data not shown). This confirms our hypothesis that *neo*-Ins(1,2,4,5,6)P_5 is the intermediate actually observed in enzymatic *neo*-InsP_6 (**3**) conversion. The delay after two dephosphorylations, leading to the accumulation of *neo*-Ins(1,2,5,6)P_4 (**10**), may reflect a property of most phytases: the cleavage of an axial phosphate group is poorly catalyzed [2,3]. In analogy to the dephosphorylation of *myo*-InsP_6, *neo*-InsP_6 (**3**) breakdown will start in position 3, followed by position 4; then, in contrast to *myo*-InsP_6, it nearly stops, because the phosphate group in position 5 is axially oriented. In a search for structural motifs which would be helpful in understanding substrate recognition by the phytase, we divided *myo*-InsP_6 into two characteristic parts, the all-*cis* 1,2,3-motif with an equatorial–axial–equatorial arrangement of the phosphate groups and the all-*trans* 4,5,6-motif bearing only equatorial phosphate groups. The C_2-symmetric *neo*-InsP_6 (**3**) consists of two equivalent parts (1,2,3 and 4,5,6; see Scheme 3), each resembling the all-*cis* 1,2,3-motif of *myo*-InsP_6. L-*chiro*-InsP_6 ((−)-**5**; Scheme 3) lacks such a constellation of phosphate groups. The phytase of *S. cerevisiae* initially attacks the 3-position in the 1,2,3-motif of *myo*-InsP_6, which may explain why L-*chiro* − InsP_6 ((−)-**5**) is resistant to conversion.

Finally, the enzymatic conversion of *neo*-InsP_6 (**3**) and L-*chiro*-InsP_6 ((−)-**5**) by the phytase of *P. rhodanensis* was investigated (Fig. 4). Chromatogram 4a is representative of the breakdown of *neo*-InsP_6 (**3**). The same intermediates are observed as in the reaction with Saccharomyces phytase. This correlates with the results we obtained for the routes of *myo*-InsP_6 conversion by Pichia phytase (Scheme 1). A prominent

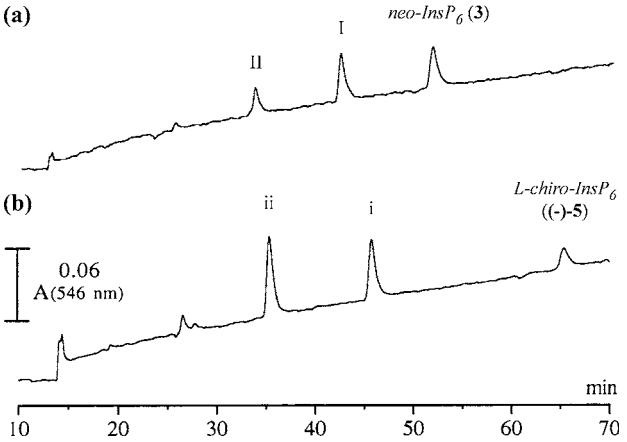
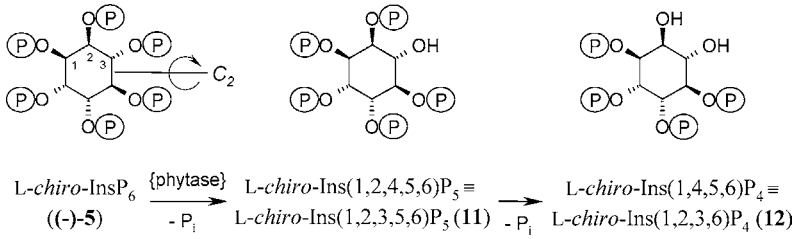


Fig. 4. HPLC-MDD chromatograms representative of (a) *neo*-InsP_6 (**3**) dephosphorylation (I: *neo*-Ins(1,2,4,5,6)P_5/*neo*-Ins(2,3,4,5,6)P_5 ((+)-**8**); II: *neo*-Ins(1,2,5,6)P_4 (**10**)/*neo*-Ins(2,3,4,5)P_4) and (b) L-*chiro*-InsP_6 ((−)-**5**) dephosphorylation (i: L-*chiro*-Ins(1,2,3,5,6)P_5 (**11**); ii: L-*chiro*-Ins(1,2,3,6)P_4 (**12**)) by the phytase of *P. rhodanensis*.



Scheme 5. Conversion of L-*chiro*-$InsP_6$ ((−)-**5**) by the phytase of *P. rhodanensis*.

branch in the reaction scheme starting with the removal of the phosphate in 3-position is identical to the specific sequential dephosphorylation catalyzed by the enzyme of *S. cerevisiae*. As we discussed above, the first two steps in *neo*-$InsP_6$ (**3**) dephosphorylation will take place at the corresponding positions. The lack of an all-*trans* 4,5,6-motif in the structure of *neo*-$InsP_6$ (**3**) may be the reason for the defined dephosphorylation pattern, so that no pathway starting with the removal of the phosphate in 6-position (Scheme 1) could be detected. For L-*chiro*-$InsP_6$ ((−)-**5**) the situation is the opposite. According to IUPAC-IUB, the numbering of the ring carbons differs from that of *neo*-$InsP_6$ (**3**) and *myo*-$InsP_6$ [34]. To prevent confusion we refer to the structures depicted in Scheme 3. The all-*cis* 1,2,3-motif of *myo*-$InsP_6$ is disturbed in the structure of L-*chiro*-$InsP_6$ ((−)-**5**). In Scheme 3 this corresponds to the 2,1,6-arrangement of phosphate groups. Thus an introductory step resulting in the removal of the phosphate in the axially oriented 6-position of L-*chiro*-$InsP_6$ ((−)-**5**) is very unlikely. The 5,4,3-assembly of phosphates resembles the all-*trans* 4,5,6-motif of *myo*-$InsP_6$, and for that reason we expected an attack at the 3-position of L-*chiro*-$InsP_6$ ((−)-**5**) as the predominant first dephosphorylation step. HPLC-MDD analyses were indicative of a rather specific breakdown (Fig. 4b), and the NMR spectra of the purified L-*chiro*-inositol pentakisphosphate confirmed that the initial product is indeed L-*chiro*-Ins(1,2,4,5,6)P_5 (Scheme 5). Because of the C_2 symmetry of the educt L-*chiro*-$InsP_6$ ((−)-**5**; Scheme 5), it is impossible to distinguish between a dephosphorylation in 3- or 4-position. These positions are homotopic, and the resulting compounds L-*chiro*-Ins(1,2,4,5,6)P_5 and L-*chiro*-Ins(1,2,3,5,6)P_5 (**11**) are identical. In accordance with IUPAC-IUB recommendations that the lowest possible numerals be preferred in nomenclature [34], the correct abbreviation is L-*chiro*-Ins(1,2,3,5,6) P_5 (**11**). As proven by NMR of the isolated L-*chiro*-inositol tetrakisphosphate, the enzymatic hydrolysis proceeds mainly, as in the case of *myo*-Ins(1,2,3,4,5)P_5 (Scheme 1), at the neighbouring position counterclockwise to the free hydroxy group. In view of the detection limit of the NMR method, the compound is $\geqslant$95% isomerically homogeneous L-*chiro*-Ins(1,2,3,6)P_4 (**12**).

3.4. Potential applications

One focus of our research is the establishment of efficient new strategies in inositol phosphate synthesis, which combine the advantages of stereocontrolled chemical

transformations and of regio- and stereospecific enzyme-assisted steps [14–16]. In the present paper we demonstrate the usefulness of phytases from *S. cerevisiae* and *P. rhodanensis* for the synthesis of structural analogues to naturally occurring, biologically active *myo*-inositol phosphates.

Although not based upon *myo*-inositol, *neo*-Ins(1,2,5,6)P_4 (**10**) can be considered a relative of *myo*-Ins(1,2,6)P_3, a compound showing anti-inflammatory and analgesic effects [29,35]. The potential of *neo*-Ins(1,2,5,6)P_4 (**10**) to act biologically as a *myo*-Ins(1,2,6)P_3 surrogate has to be investigated. L-*chiro*-Ins(1,2,3,6)P_4 (**12**) is an epimer of *myo*-Ins(1,2,3,6)P_4, a compound which may function physiologically as iron carrier and calcium absorption enhancer [36,37]. The L-*chiro* isomer may further our understanding of the structure–activity relationship.

Acknowledgments

We thank Dr. W.V. Turner for critical reading of the manuscript. We are grateful to the Bayer AG Company for supporting this project in many aspects, especially for preparing the HR-MS spectra. We also thank the Max-Planck Institut für Kohlenforschung, in particular Dr. W. Schrader and M. Blumenthal, for further HR-MS spectra. This work was supported by the Deutsche Forschungsgemeinschaft (Grant VO 348/3-1) and the Fonds der Chemischen Industrie.

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