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Chemo-enzymatic Synthesis of both Enantiomers of *myo*-Inositol 1,3,4,5-tetrakisphosphate

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D-Ins(1,3,4,5)P_4 and unnatural L-Ins(1,3,4,5)P_4 were prepared in gram-quantities from D- and L-2,6-di-*O*-benzyl-*myo*-inositol by a chemical phosphorylation and deprotection step in high yield and purity without extensive purification. The optically pure benzyl derivatives were obtained by enzyme-catalyzed resolution of racemic 2,6-di-*O*-benzyl-*myo*-inositol under acyl-transfer conditions in vinyl acetate as the acyl donor. The lipase of *Candida antarctica* only acetylated regio- and enantio-selectively the L-enantiomer, providing exclusively L-5-acetyl-2,6-di-*O*-benzyl-*myo*-inositol, whereas the D-enantiomer remained unchanged.

Key words: D-*myo*-inositol 1,3,4,5-tetrakisphosphate; L-*myo*-inositol 1,3,4,5-tetrakisphosphate; *Candida antarctica*; lipase; enzymatic esterification

The increasing interest in understanding the role of inositol phosphates and phospholipids as second messengers in the cell regulation processes has resulted in an urgent need for preparative-scale synthetic access to inositol derivatives.[1] Such derivatives are of interest as enzyme substrates for metabolic investigations, as enzyme inhibitors (potential drugs), or as chiral building blocks. In the recent, there have been many reports on the synthesis of racemic[2-3] or optically active *myo*-inositol 1,3,4,5-tetrakisphosphate.[4-13] The reported synthetic routes[4-8,10-12] are generally based on the synthesis of optically active 2,6-di-*O*-benzyl-*myo*-inositol **3** as an intermediate, which is an excellent precursor for the synthesis of Ins(1,3,4,5)P_4, and has primarily been demonstrated for racemic IP_4 by Billington and Baker.[2-3] Nevertheless, desired chiral IP_4- precursor **3** could only be obtained after several chemical steps with a moderate-to-low overall yield. To our knowledge, the enantioselective enzymatic differentiation of both enantiomers of **3** by the use of lipases has never been investigated before.[14] We now report simple and easy access to both enantiomers of Ins(1,3,4,5)P_4 based on an enzyme-catalyzed resolution of racemic 2,6-di-*O*-benzyl-*myo*-inositol (±)-**3** (Fig. 1).

Partial deprotection of (±)-**2**[3] with Pd/C under acidic conditions (MeOH/p-TosOH) yielded desired racemic 2,6-di-*O*-benzyl-*myo*-inositol (±)-**3** (91.9%) as colorless crystals after recrystallization ($CHCl_3$/hexane). Initial screening of several commercially available lipases for acyl-transfer activity towards (±)-**3** in vinyl acetate[15] as the acyl donor revealed that the lipase from *Candida antarctica* (Novozym 435),[16] in contrast to all other investigated lipases, gave a reasonable conversion. HPLC and TLC analyses showed that only one product was formed and that the rate of the reaction decreased dramatically after the conversion had reached about 50%, indicating very high enantiospecificity. A preparative experiment (15 mmol (±)-**3**, 2.5 g of Novozym 435) was worked up after a 49.5% conversion (92 h). Flash chromatography of the obtained reaction mixture gave 49.0% (98% theoretical yield) of unconverted inositol derivative (−)-**3** with an optical purity of >99% ($[\alpha]_D^{20} = -29.94°$, c=1.02 EtOH) and 49.1% of monoacetate (−)-**4** ($[\alpha]_D^{20} = -5.77°$, c=1.04 EtOH). Based upon the two-dimensional COSY and ^{13}C-NMR spectra, monoacetate (−)-**4** was isomerically pure and the hydroxyl group at C5 was esterified. Chemical hydrolysis (MeOH/NaOMe) of mono acetate (−)-**4** led quantitatively to (+)-**3** ($[\alpha]_D^{20} = +30.49°$, c=0.99 EtOH), with >99% e.e. (Fig. 2). Both enantiomers of **3** were finally phosporylated by using 2-di-ethylamino-1,3,2-benzodioxaphosphepane[17-18]/tetrazole, followed by successive oxidation with H_2O_2[19] to the protected IP_4-derivatives (+)-**5** (91.8%) and (−)-**5** (93.2%). Oxidation with H_2O_2 instead of more commonly used *m*-chloroperoxybenzoic acid offered the advantage of more readily performed subsequent product isolation and purification. Treatment of the fully protected phosphates with hydrogen over Pd/C in MeOH resulted in the removal of all protecting groups in one step to afford D-Ins(1,3,4,5)P_4 (−)-**6** ($[\alpha]_D^{20} = -3.85°$, c=1.01 H_2O, pH=5.35) and unnatural L-Ins(1,3,4,5)P_4 (+)-**6** ($[\alpha]_D^{20} = +3.87°$, c=1.03 H_2O, pH=5.36). Both Ins(1,3,4,5)P_4 isomers were isolated as tetrapotassium salts by adding stoichiometric amounts of a 0.2 M KOH solution in EtOH (analytical grade) to methanolic solutions of the free acids. This allowed the selective formation of the tetrapotassium salts without any other salt contamination and subsequent simple isolation by filtration or centrifugation of the precipitate.

Experimental

General. All melting point data (mp) were measured in open capillaries and are uncorrected. Optical rotation values were determined with a Perkin-Elmer 241 polarimeter. IR spectra were obtained with a Perkin-Elmer infrared spectrophotometer. MS conditions: Finnigan SSQ 7000, electrospray, solvent of H_2O/

Abbreviations: Bn, benzyl; Ins(1,3,4,5)P_4, *myo*-inositol 1,3,4,5-tetrakisphosphate; *p*-TosOH, *p*-toluene-sulfonic acid; e.e., enantiomeric excess

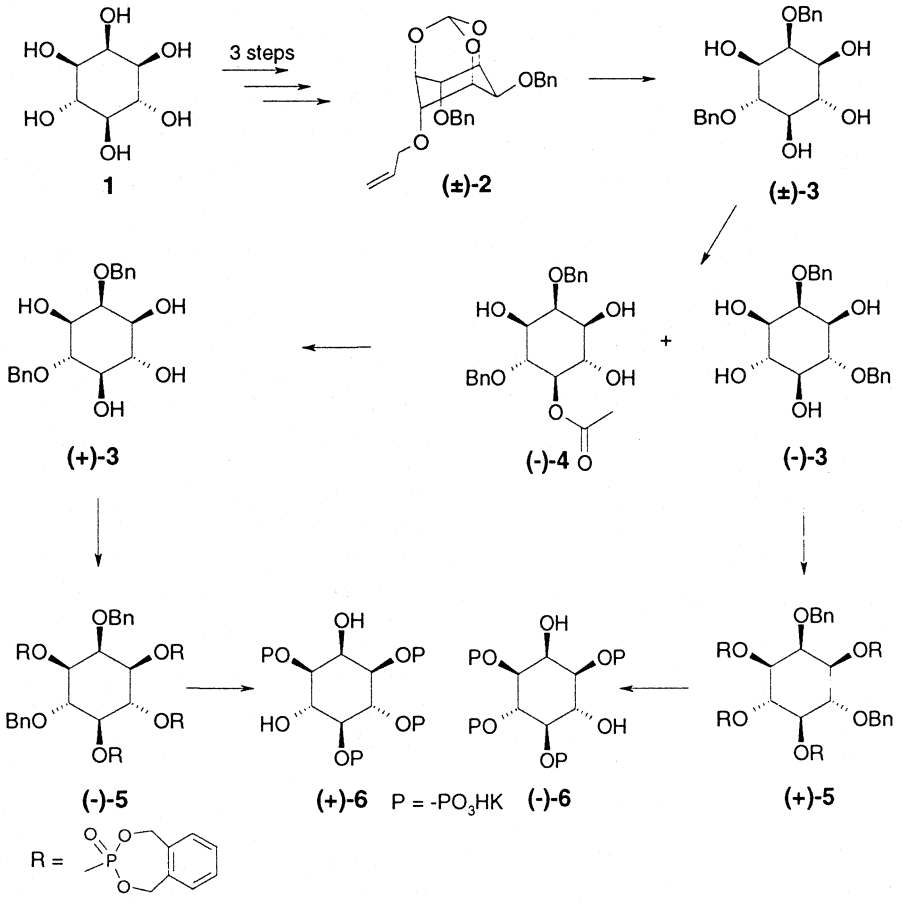

Fig. 1.

$CH_3CN=3/7$. HRMS conditions: Finnigan MAT 900S, solvent of $H_2O/CH_3CN=3/7$ (+15 ppm CH_3COONa for **5**), ESI positive mode. ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR conditions: Bruker at 400.3 MHz (^{1}H), 100.6 MHz (^{13}C) or 162 MHz (^{31}P); δ in ppm relative to TMS as an internal standard, or relative to H_3PO_4 for ^{31}P-NMR as an external standard (uncorrected). HPLC was performed with Merck LiChrospher RP-8(125-4), solvent A being a 3 mM phosphate buffer at pH 3, and solvent B being 90% acetonitrile +10% solvent A; elution was by 10% B→50% B in 10 min.at a flow rate of 1.25 ml/min; temp., 20°C; detection at UV 254 nm and for opt. purity determination with a chiralcel OJ column (4.6×250 mm; mobile phase, n-hexane/2-propanol=6:4; flow rate, 0.5 ml/min; temp., 20°C; detection, UV 208 nm). All reactions were monitored by TLC, which was conducted on precoated plates of silica gel 60 F_{254} (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany), spots being detected by UV light and by a 10% molybdophosphoric acid spray in EtOH with subsequent heating at 200°C. Silica gel 60 (70–230 or 230–400 mesh; E. Merck) was used for column chromatography. *myo*-Inositol **1** was purchased from Fluka, and (±)-**2** was prepared by the method described in the literature.[3]

(±)-*2,6-Di-O-benzyl-myo-inositol* ((±)-**3**). To a solution of (±)-**2** (14.89 g, 36.28 mmol) in MeOH (140 ml), 10% palladium on activated charcoal (1.2 g), distilled water (20 ml) and *p*-toluene-sulfonic acid (0.6 g, 3.15 mmol) were added. The mixture was refluxed under Ar. After its complete conversion (6 h, checked by TLC) the mixture was filtered, the filtrate neutralized by adding a sat. $NaHCO_3$-solution, and the solvent removed under reduced pressure. Water (50 ml) was added to the obtained residue, the solution being extracted with 3×100 ml of $CHCl_3$. The organic phase was dried ($MgSO_4$) and the solvent removed. The resulting white solid was recrystallized ($CHCl_3$/hexane) and gave (±)-**3** (12.01 g, 91.9%) as white crystals (mp 120–121°C, lit.[3] 119–120.5°C). $R_f=0.30$ (CH_2Cl_2/MeOH=9:1). IR ν (KBr) cm^{-1}: 3440*s*-br, 3030*w*, 2950*w*, 2890*w*, 1500*w*, 1454*w*, 1366*w*, 1148*w*, 1136*w*, 1115*m*, 1092*m*, 1044*vs*, 1006*s*, 925*m*, 755*s*, 697*s*. ^{1}H-NMR δ ($CDCl_3$): 2.40 (d, $J=5.4$ Hz, 1H, OH); 2.53 (d, $J=7.1$Hz, 1H, OH); 2.77 (br-s, 1H, OH); 2.96 (br-s, 1H, OH); 3.39–3.47 (m, 2H); 3.57–3.61 (m, 1H); 3.73 (t, $J=9.5$Hz, 1H); 4.00 (t, $J=2.7$ Hz, 1H); 4.84 (center of AB, $\nu_A=4.80$ ppm, $\nu_B=4.88$ ppm $/J_{AB}/=11.5$ Hz, 2H); 4.86 (center of AB, $\nu_A=4.84$ ppm, $\nu_B=4.88$ ppm $/J_{AB}/=11.4$ Hz, 2H); 7.25–7.35 (m, 10H). ^{13}C-NMR δ (DMSO): 72.43 (d); 72.67 (d); 74.18 (d); 74.42 (t); 75.10 (t); 75.95 (d); 83.18 (d); 83.2 (d); 127.75 (d); 127.77 (d); 127.97 (d); 128.36 (d); 128.69 (d); 128.77 (d); 140.80 (s). MS *m/z* (%): 405 (80, M+HCOO$^-$), 360 (20), 359 (100), 251 (30). *Anal.*

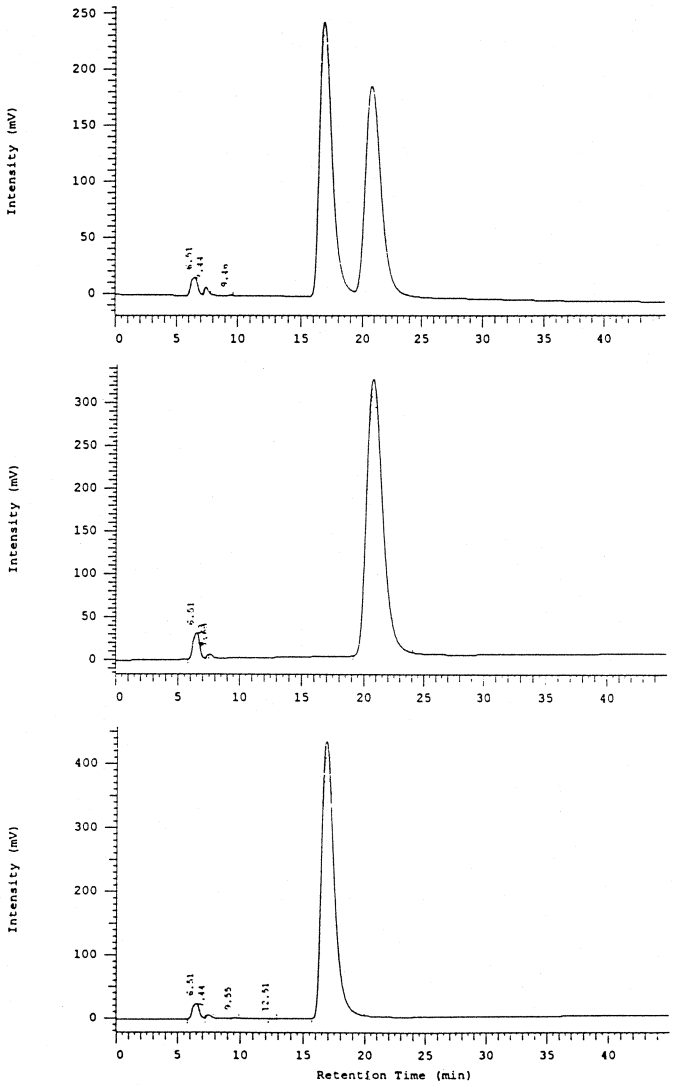

Fig. 2. HPLC Chromatograms (Chiralcel OJ) of (±)-3 (top), (−)-3 (middle) and (+)-3 (bottom).

Found: C, 66.38; H, 6.77; O, 26.85%. Calcd. for $C_{20}H_{24}O_6$ (360.41): C, 66.64; H, 6.72; O, 26.65%.

Enzymatic esterification of (±)-**3**. To a solution of 5.406 g (15 mmol) of (±)-**3** in THF (40 ml), 100 ml of vinyl acetate and 2.5 g of Novozym 435 (batch No. LCC 0013-2) were added, and the mixture was stirred at room temperature. TLC analysis showed the appearance of one new spot. After 92 h, the conversion had reached about 50% (HPLC) and no further reaction was detectable. The enzyme was filtered off, and the filtrate evaporated. The obtained solid was dissolved in a small amount of THF, and subsequent flash chromatography (CH_2Cl_2/MeOH=9:1) yielded 2.961 g (7.36 mmol, 49.1%) of (−)-**4** and 2.649 g (7.35 mmol, 49.0%) of (−)-**3** as colorless solids.

(−)-*1D-2,6-Di-O-benzyl-myo-inositol* ((−)-**3**). R_f=0.30 (CH_2Cl_2/MeOH=9:1), mp 146–147°C, $[\alpha]_D^{20}$=−29.94° (c=1.04, EtOH). Optical purity >99% based on HPLC, (+)-**3** t_R=16.8 min, (−)-**3** t_R=20.8 min (Chiralcel OJ, n-hexane/2-propanol=6:4, 0.5 ml/min flow). (Lit.[12] mp 145.2–146.1°C, $[\alpha]_D^{25}$=−29.2° (c=1, EtOH); lit.[5] mp 144.5–145,5°C, $[\alpha]_D^{20}$=−27° (c=0.31, EtOH); lit.[6] mp 147–148°C, $[\alpha]_D^{25}$=−28.5° (c=1, EtOH)). The spectroscopic data are identical with those of (±)-**3**.

(−)-*1L-5-O-Acetyl-2,6-di-O-benzyl-myo-inositol* ((−)-**4**). R_f=0.41 (CH_2Cl_2/MeOH=9:1), mp 132–132.5°C, $[\alpha]_D^{20}$=−5.77° (c=1.04, EtOH). IR ν (KBr) cm^{-1}: 3456*vs*, 3061*w*, 3029*w*, 2944*w*, 2921*m*, 2868*w*, 1732*vs*, 1500*m*, 1452*m*, 1364*s*, 1253*vs*, 1127*vs*, 1067*vs*, 1036*vs*, 970*m*, 931*w*. ^{1}H-NMR δ (DMSO): 1.92 (s, 3H); 3.41 (dd, J=2.2 and 9.7Hz, 1H); 3.59 (t, J=9.7Hz, 2H); 3.62 (m, 1H); 3.75 (t, J=2.2Hz, 1H); 4.64 (center of AB, ν_A=4.52 ppm, ν_B=4.75 ppm, $/J_{AB}/$=11.7 Hz, 2H); 4.73 (t, J=9.5 Hz, 1H); 4.81 (s, 2H); 4.94 (d, J=5.0 Hz, 1H, OH); 5.00 (d, J=5.5 Hz, 1H, OH); 5.14 (d, J=5.5Hz, 1H, OH); 7.22–7.42 (m, 10H). ^{13}C-NMR δ (DMSO): 21.90 (q); 71.67 (d); 72.37 (d); 72.52 (d); 74.27 (t); 75.22 (t); 76.19 (d); 80.72 (d); 83.06 (d); 127.83 (d); 127.92 (d); 128.00 (d); 128.60 (d); 128.82 (d);128.86 (d); 140.03 (s); 140.61 (s); 170.46 (s). MS m/z (%): 447 (100, M+HCOO$^-$), 401 (20), 359 (10), 311 (5), 175 (5). *Anal.* Found: C, 65.58; H, 6.55; O, 27.87%. Calcd. for $C_{22}H_{26}O_7$ (402.44): C, 65.66; H, 6.51; O, 27.83%.

(+)-*1L-2,6-Di-O-benzyl-myo-inositol* ((+)-**3**). A solution of (−)-**4** (2.012 g, 5 mmol) and NaOMe (100 mg, 1.84 mmol) in MeOH (100 ml) was stirred for 15 min. at room temperature. After complete conversion, the solvent was removed under reduced pressure, and the obtained residue dissolved in 1 ml of MeOH. The solution was filtered through a small silica gel column (CH_2Cl_2/MeOH=9:1). Evaporation of the filtrate yielded 1.791 g (4.97 mmol, 99.4%) of (+)-**3** as a colorless solid. Mp 145.5–146°C, $[\alpha]_D^{20}$=+30.49° (c=0.99, EtOH); lit.[12] mp 145.8–146.1°C, $[\alpha]_D^{25}$=+29.7° (c=1, EtOH). Optical purity >99% based on HPLC, (+)-**3** t_R=16.8 min, (−)-**3** t_R=20.8 min (Chiralcel OJ, n-hexane/2-propanol=6:4, 0.5 ml/min flow). The spectroscopic data are identical with those of (±)-**3**.

(+)-*1D-1,3,4,5,-tetrakis(1.3.2σ^5-benzodioxaphosphepane-2yl)oxy-2,6-di-O-benzyl-myo-inositol* ((+)-**5**). Compound (−)-**3** (1.441 g, 4.0 mmol) was dissolved in 60 ml of a tetrazole solution (0.5 M in CH_3CN), and 2-diethyl-amino-1,3,2-benzodioxaphosphepane[17] (5.74 g, 24.0 mmol) was added. The reaction mixture was stirred at room temperature under Ar until its complete conversion (TLC, R_f=0.87 (CH_2Cl_2/MeOH=9:1), R_f=0.39 (Et_2O/hexane=1:1)). After 15 min. 4.3 g of solid K_2HPO_4 was added, and the reaction was quenched with H_2O (1 ml) before adding H_2O_2 (30%, 3.5 ml). The mixture was stirred for 30 min. The excess peroxide was destroyed with Na_2SO_3 (1.8 g), and the solvent was removed. The solid residue was resuspended in CH_2Cl_2 (2×100 ml), dried ($MgSO_4$), filtered and evaporated. Column chromatography (CH_2Cl_2/MeOH=9:1) gave 3.997 g (3.67 mmol, 91.8%) of (+)-**5** as a white foam.

R_f=0.45 (CH_2Cl_2/MeOH=9:1), $[\alpha]_D^{20}$=+4.02° (c=0.99, $CHCl_3$). IR ν (KBr) cm^{-1}: 3060*vw*, 3020*vw*, 2920*vw*, 2880*vw*, 1498*vw*, 1455*w*, 1382*w*, 1294*vs*, 1225*w*, 1208*vw*,1122*m*, 1052*s*, 1020*vs*, 859*s*, 733*s*, 698*w*. ^{1}H-NMR δ ($CDCl_3$): 4.19 (t, J=9.6 Hz, 1H); 4.51 (ddd, J=2.3, 7.7 and 9.9 Hz, 1H); 4.67 (ddd, J=2.6, 7.4 and 9.9 Hz, 1H); 4.75-5.56 (m, 23H); 7.05-7.41 (m, 26H). ^{31}P-NMR δ ($CDCl_3$): 1.334, −0.105, −0.154, −2.486. HRMS *m/z* ([M+Na]$^+$): calcd. for $C_{52}H_{52}O_{16}P_4Na$, 1111.20019; found, 1111.19927.

(−)-1L-1,3,4,5,-tetrakis(1.3.2σ^5-benzodioxaphosphepane-2yl)oxy-2,6-di-O-benzyl-myo-inositol **((−)-5)**. Compound **(+)-3** (2.789 g, 7.739 mmol) was treated in the same manner as that described for **(−)-3** to yield 7.85 g of **(−)-5** (7.21 mmol, 93.2%), $[\alpha]_D^{20}$=−3.77° (c=1.01, $CHCl_3$). The spectroscopic data are identical with those of **(+)-5**.

D-myo-Inositol 1,3,4,5-tetrakisphosphate tetra-potassium salt; **((−)-6)**. 10% Palladium on activated charcoal (2 g) was added to a solution of **(+)-5** (3.68 g, 3.38 mmol) in MeOH (200 ml) at room temperature in a flask under argon. The flask was flushed with hydrogen, and the mixture stirred vigorously under a hydrogen atmosphere. After 12 h, the mixture was filtered, and the solvent removed *in vacuo*. The oily residue (1.679 g) was dissolved in 50 ml of MeOH, and 67.1 ml of a 0.2 M KOH solution in EtOH was slowly added to the stirred solution while cooling. The precipitate was filtered off and dried in a vacuum desiccator over P_4O_{10} to afford 2.091 g of **(−)-6** (3.205 mmol, 94.8%) as a tetrapotassium salt. $[\alpha]_D^{20}$=−3.85° (c=1.01, H_2O, pH 5.35); lit.[9] $[\alpha]_D^{20}$=−3.83° (c=3.6, H_2O, pH 5.4); lit.[11] $[\alpha]_D$=−3.5° (c=5.5, H_2O, pH 8.4). IR ν (KBr) cm^{-1}: 3429*s*, 2925*w*, 2366*w*, 1653*w*, 1203*s*, 1050*vs*, 921*s*, 813*w*, 716*w*. ^{1}H-NMR δ (D_2O): 3.74 (dd app. as t, J=9.5 and 9.6 Hz, 1H); 3.86-3.92 (m, 2H); 3.96 (ddd app. as td, J=2.7, 9.8 and 10.0 Hz, 1H); 4.23 (t, J=2.7 Hz, 1H); 4.26 (q, J=9.5 Hz, 1H). ^{31}P{^{1}H}-NMR δ (D_2O): 2.321, 1.985, 1.481, 1.169. ^{13}C{^{1}H}-NMR δ (D_2O): 70.73 (s); 71.18 (dd, J=2.3 and 6.4 Hz); 74.69 (m); 75.01 (d, J=5.6 Hz); 76.46 (m); 78.57 (m). HRMS *m/z* ([M−(3K+2H)]$^-$): calcd. for $C_6H_{14}KO_{18}P_4$, 536.87678; found: 536.87724. *Anal.* Found: C, 11.03; H, 1.93%. Calcd. for $C_6H_{12}O_{18}P_4K_4$ (652.44): C, 11.05; H, 1.85%.

L-myo-Inositol 1,3,4,5-tetrakisphosphate tetra-potassium salt; **((+)-6)**. Compound **(−)-5** (5.845 g, 5.37 mmol) was converted to **(+)-6** in the same manner as that described for **(−)-6** by using 2.3 g of 10% palladium on activated charcoal and 107 ml of a 0.2 M KOH solution in EtOH. 3.309 g (5.07 mmol, 94.5%) of **(+)-6** as a tetrapotassium salt was obtained with spectroscopic data identical to those of **(−)-6**. $[\alpha]_D^{20}$=+3.87° (c=1.03, H_2O, pH 5.36).

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