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SYNTHESIS OF TRITIUM-LABELLED ENANTIOMERS OF *myo*-INOSITOL 1,4,5-TRISPHOSPHATE

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SUMMARY

Both natural D- and L-enantiomers of *myo*-Ins(1,4,5)P_3 were synthesized with specific activities 14-16 Ci/mmol. A suitable inositol derivative was resolved as the diastereomeric camphanate esters, and the chiral inositol derivatives were oxidized to the protected inosose. Reduction of each chiral ketone with sodium borotritide and manipulation of protecting groups gave the enantiomeric [1-^{3}H]-2,3,6-tri-O-benzyl-*myo*-inositols in 55% radiochemical yield. Phosphorylation with tetrabenzylpyrophosphate and complete hydrogenolytic debenzylation provided the separate D-*myo* and L-*myo*- [1-^{3}H]-Ins(1,4,5)P_3 enantiomers in 30% radiochemical yield.

Key words: Ins(1,4,5)P_3, phosphoinositide, tritium-label, inositol phosphates, second messenger, enantiomers

INTRODUCTION

The phosphoinositide pathway enables external messengers to interact with membrane receptors and produce an intracellular second messenger, Ins(1,4,5)P_3, by the action of a specific phospholipase C on phosphatidylinositol 4,5-bisphosphate (1,2). This second messenger has specific receptors in brain tissue (3), which have been shown to be specific for the D-*myo* enantiomer by competitive displacement of natural, metabolically-derived tritium-labelled

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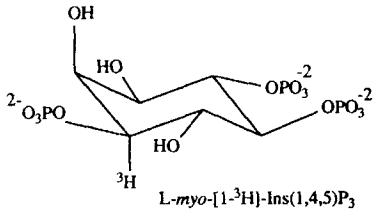
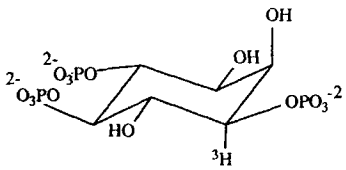
Ins(1,4,5)P_3 (specific activity 2-3 Ci/mmol) by a variety of inositol mono- and polyphosphates (4). The high cost, low specific activity, and limited supply of the naturally-obtained material prompted us to develop a synthesis of high specific activity D-*myo*-Ins(1,4,5)P_3 for further receptor and enzymatic assays. An important auxiliary product from our synthesis would be the labelled unnatural enantiomer, L-*myo*-Ins(1,4,5)P_3, with which the absolute enantiospecificity of binding could be probed directly. We describe herein the synthesis of both enantiomers with specific activity 14-16 Ci/mmol.



L-*myo*-[1-^{3}H]-Ins(1,4,5)P_3

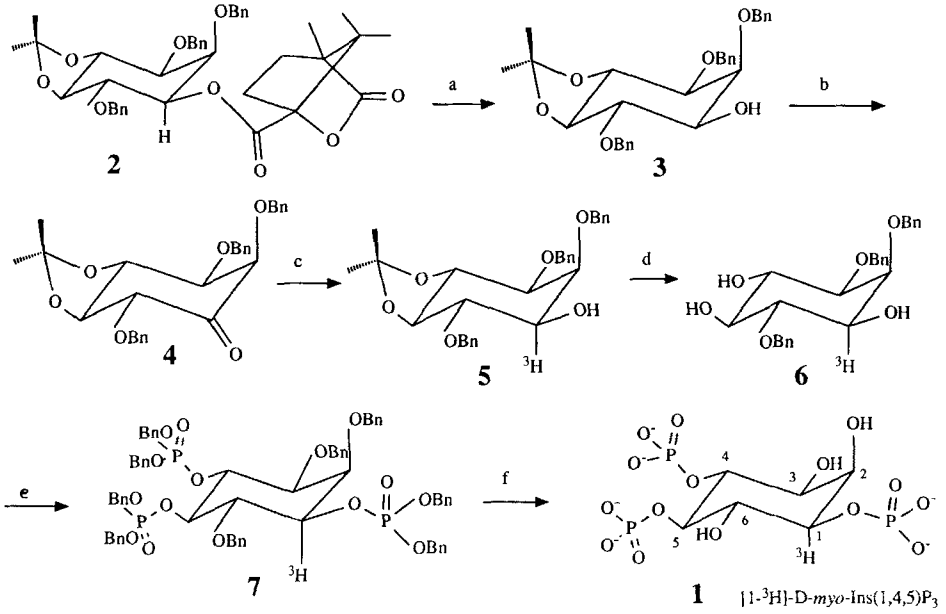


D-*myo*-[1-^{3}H]-Ins(1,4,5)P_3

RESULTS

The synthesis of labelled D-*myo*-1-[^{3}H]-Ins(1,4,5)P_3 (**1**) is illustrated in the Figure. The L-*myo* enantiomer was prepared from an identical reaction sequence. The fully protected racemic inositol derivative was prepared by the method of Gigg *et al.* (5,6,7) and converted to the diastereomeric mixture of (-)-ω-camphanate esters. The precursor (*ent*-**2**, not shown) for the L-*myo* Ins(1,4,5)P_3 crystallized from ether, and the D-*myo*-Ins(1,4,5)P_3 precursor **2** was isolated from the enriched mother liquors by preparative HPLC. After basic hydrolysis **(a)** to the 1-hydroxy-4,5-ketal tris ether **3**, oxidation **(b)** with DMSO-acetic anhydride (7) afforded the inosose **4**.

The production of the labelled equatorial alcohol was accomplished by reduction of the inosose with sodium borotritide, as described previously for preparation of the high specific activity enantiomers of Ins(1,3,4)P_3 (8). Thus, to achieve maximum specific activity by ensuring complete transfer of tritide, excess ketone **4** was added to a stirred solution of 66.9 Ci/mmol borotritide **(c)**. Silica gel chromatography removed the starting ketone and the undesired axial alcohol



Reagents: (a) NaOH, CH_3OH; (b) Ac_2O, DMSO; (c) NaB^3H_4, EtOH; (d) 1 N HCl, CH_3OH; (e) NaH, TBPP, DMF; (f) 10% Pd/C, H_2, EtOH, then NH_4OH.

(ca. 25% of the radioactivity), affording a 70% radiochemical yield of the tritium-labelled hydroxy ketal tris(benzyl ether) **5**. The 320-MHz tritium NMR of this sample clearly showed a single tritium resonance with $^3J_{HT}$ = 2.5 Hz (axial triton to equatorial H-2) and 9.8 Hz (axial triton to axial H-6).

Hydrolysis of the ketal with dilute HCl **(d)** provided the triol tris(benzyl ether) **6** in 81% yield. The tris(anion) of triol **6** was formed with NaH in DMF, and a slight excess of solid tetrabenzyl pyrophosphate (TBPP) was added **(e)**. This reaction was particularly difficult to carry out on a smaller scale and on humid days, due to the hygroscopic nature of the tris(anion) and the solvent. Purification afforded the labelled perbenzylated Ins(1,4,5)P_3 **7** in 34% yield.

Finally, the perbenzylated product was deprotected with a highly active Pd/C catalyst in 95% ethanol using 1 atm hydrogen **(f)**. After removal of the catalyst, the solution was neutralized with a slight excess of ammonium hydroxide, and the hexakis(ammonium) salt of D-*myo*-1-[^{3}H]-Ins(1,4,5)P_3 (**1**) was lyophilized and resuspended in nanopure water. From ca. 150 mCi of borotritide, 30 mCi of the trisphosphate **1** was obtained. Radiolytic decomposition of high specific

activity Ins(1,4,5)P_3, particularly in concentrated solutions, is a very serious problem. Thus, the material was diluted to 0.5 mCi/mL for storage; even this awkwardly large volume of labelled material was tenfold more concentrated than desired, based on values for commercially supplied D-*myo*-[^{3}H]-Ins(1,4,5)P_3.

An identical reaction sequence furnished the enantiomeric L-*myo*-[^{3}H]-Ins(1,4,5)P_3 in a similar overall yield from *ent*-**2**.

DISCUSSION

During the course of this project, several total syntheses of racemic and optically active Ins(1,4,5)P_3 were published (9,10,11,12,13,14). However, none of these literature reports described the introduction of high specific activity tritium into a chemically defined location in the cyclohexitol ring. Recently, radiochemical suppliers have started to make available high specific activity tritium-labelled D-*myo*-Ins(1,4,5)P_3, for which the synthetic preparation is unpublished. It is probable that a route analogous to that described here is employed for the commercial material (15).

Biochemical studies employing the labelled and unlabelled enantiomers will be reported elsewhere.

MATERIALS AND METHODS

General: Sodium borotritide was obtained from Amersham Corp. and had a specific activity of 66.9 Ci/mmol. A stock solution was prepared by dissolving 1 Ci of the borotritide in 1.00 mL of a 9:1 solution of ethanol-0.01 N aqueous sodium hydroxide; this was used within a few hours. DMF was reagent-grade and was dried over 4Å molecular sieves for 24 h before use. The 10% Pd/C catalyst was Johnson Matthey (cat. no. 11702). Tetrabenzyl pyrophosphate was prepared by the reported procedure (16). All other chemicals and solvents were reagent quality and were used without further purification. TLC was performed on Macherey-Nagel Silica Gel G plates. The plates were visualized with phosphomolybdic acid. Merck Silica Gel 60 (230-400 mesh) was used for

column chromatography. Low boiling solvents were usually removed at 20 °C with a stream of N_2; DMF was removed *in vacuo* at 20 °C. Radioactive samples were counted in an LKB 1218 RackBeta liquid scintillation counter using Fischer Scintiverse II scintillation cocktail. Counting efficiency was 57-61% for tritium and counts per minute data was corrected by the external standard ratio method. In the synthetic description below, the correct IUPAC numbering for the hexitol ring is used, in which numbering priorities change during the synthesis.

(-)-ω-Camphanate of (1L)-1,2,4-tri-O-benzyl-5,6-O-isopropylidene-*myo*-inositol (2): A mixture of the diastereomeric camphanate esters was prepared from racemic 1,2,4-tri-O-benzyl-5,6-O-isopropylidene-*myo*-inositol by the method of Gigg *et al.* (7) and most of the undesired (D) ester was separated by crystallization from ether. The required (L) camphanate ester was separated from the enriched mother liquor by preparative HPLC [25 x 2.1 cm Du Pont Zorbax Silica column, 60:40 hexane-($CHCl_3$-CH_3OH-H_2O, 100:1:0.1), flow rate of 10 mL/min]. The (L) ester eluted first.

(L)-1,2,4-Tri-O-benzyl-5,6-O-isopropylidene-*myo*-inososе (4): This was prepared from the camphanate ester by the procedure of Gigg *et al.* (7).

(L)-3-[^{3}H]-1,2,4-Tri-O-benzyl-5,6-O-isopropylidene-*myo*-inositol (5): (L)-1,2,4-Tri-O-benzyl-5,6-O-isopropylidene-*myo*-inososе (**4**, 13 mg, 27 μmol) was dissolved in 1.2 mL of 95% ethanol with gentle warming, the solution was cooled to r.t., and 300 μL of the borotritide solution was added (8). The reaction was examined after 30 min by TLC (R_f = 0.37 axial alcohol, 0.27 equatorial alcohol; 3:2 hexane-ether). Complete reduction had occurred to give a mixture of the two epimeric alcohols. Additional small quantities (1 mg) of the ketone were added and the TLC checked 15 min after each addition. After 3 mg of ketone was added the TLC showed a slight excess of it remained. The solvent was removed (N_2), the oil remaining was mixed with a few drops of toluene, and all volatile materials removed *in vacuo*. The residue was taken up in 300 μL of dry ether (it was a cloudy suspension due to insoluble sodium salts) and applied to a

9 x 0.7 cm column of Silica Gel 60 packed in hexane-ether (3:2). The material was washed on the column with a few drops of ether then eluted with the 3:2 solvent system. Fractions of 3 mL were collected. Fractions were analyzed by TLC and the fractions containing product pooled. The solvent was removed (N_2) and the white solid remaining dried *in vacuo* to give 10 mg of the labelled inositol derivative **5**: ^{3}H-NMR ($CDCl_3$) δ 2.74 (dd, J = 2.5, 9.8 Hz).

(L)-3-[^{3}H]-1,2,4-Tri-O-benzyl-*myo*-inositol (6): (L)-3-[^{3}H]-1,2,4-Tri-O-benzyl-5,6-O-isopropylidene-*myo*-inositol (**5**, 10 mg, 20 μmol) was dissolved in 2.5 mL of methanol and 250 μL of 1 *M* hydrochloric acid added. The solution was kept at 45-50 °C for 15 min at which time analysis by TLC (R_f = 0.43, ether) showed complete reaction. The solution was cooled to r.t. and the acid was neutralized by addition of sodium bicarbonate (200 mg). Most of the solvent was removed (N_2), then 1 mL of toluene was added and the material was evaporated to dryness. The solid was dried *in vacuo* for 15 min, then extracted with 4 x 3 mL of methylene chloride. The methylene chloride was removed (N_2), leaving a white solid. The solid was dissolved in six drops of chloroform and applied to a 9 x 0.7 cm column of Silica Gel 60 packed in ether. The column was eluted with ether. Fractions of 2 mL were collected and analyzed by TLC. The fractions containing product were combined and the ether evaporated (N_2), leaving 7.3 mg (250 mCi, 81%) of triol **6** as a white solid. The specific activity of the labelled triol was 16 Ci/mmol.

(D)-1-[^{3}H]-1,4,5-Tris-(dibenzylphosphato)-2,3,6-tri-O-benzyl-*myo*-inositol (7): (L)-3-[^{3}H]-1,2,4-Tri-O-benzyl-*myo*-inositol (**6**, 2.7 mg, 6 μmol) was dissolved in 100 μL of dry DMF and evaporated to dryness *in vacuo* to remove any residual water. The oil remaining was redissolved in 200 μL of DMF. Sodium hydride (1.6 mg of a 60% dispersion in oil) in a small vial with a teflon lined cap and containing a small stirring bar was rinsed with 0.5 mL of dry hexane to remove the oil then dried a few minutes *in vacuo*. The vial was cooled a few minutes in an ice bath then the DMF solution of the triol was added. The triol container was rinsed with 3 x 50 μL of DMF and the washings were added to the

reaction mixture which was then stirred for a few minutes. Solid tetrabenzyl pyrophosphate (16 mg, 30 μmol) was added and the mixture was stirred for 1 h to give a clear solution. The solution was left was at 4 °C for 18 h, and then it was transferred to a small test tube with the aid of 5 mL of methylene chloride. The solution was extracted with 2 x 1 mL of water, and the methylene chloride extracts were dried ($MgSO_4$), decanted, and evaporated (N_2); residual DMF was removed *in vacuo*. The crude product was mixed with 0.4 mL of ethyl acetate (not everything was soluble) and the mixture was applied to a 6 cm pipet column packed in 1:1 ethyl acetate-hexane. The material was washed on the column with 200 μL of 2:1 ethyl acetate-hexane and the column then eluted with this solvent. Fractions of 1.5 mL were collected and analyzed by TLC (R_f = 0.40, 2:1 ethyl acetate-hexane). Excess tetrabenzyl pyrophosphate eluted first, followed by the product. The solvent was removed from the pooled product fractions (N_2) leaving the perbenzylated compound **7** as a colorless oil weighing 2.5 mg (34%).

(D)-1-[^{3}H]-*myo*-Inositol-1,4,5-trisphosphate (1): The perbenzylated Ins(1,4,5)P_3 (**7**) (2.5 mg) was dissolved in 2 mL of 95% ethanol and transferred to a 15-mL flask containing a small spin bar and 5 mg of 10% Pd/C. The flask was sealed with a wired-on septum cap and flushed with a slow stream of hydrogen. The flask was then pressurized to a few psi with hydrogen and the mixture was stirred vigorously at r.t. for 10 h. The flask was occasionally repressurized with hydrogen during this period. The catalyst was removed by filtration through a small pipet column containing a 1 cm bed of Celite. The catalyst was washed with 1 mL of 95% ethanol, and 2 mL of 50% ethanol. The solution was neutralized with a slight excess of 1 *M* ammonium hydroxide and the solvent was evaporated (N_2). The residue was kept *in vacuo* for 30 min then the solid was redissolved in 2.00 mL of water. Analysis showed it contained about 30 mCi of tritium. The product was stored in water at 0.5 mCi/mL.

L-*myo*-[^{3}H]-Ins(1,4,5)P_3 (*ent*-1): The sequence leading from the enantiomeric inososse **4** to the trisphosphate L-1 was identical to that described

above for the D-*myo* enantiomer, except that a different batch of high specific activity sodium borotritide was employed. Briefly, the crystalline D-camphanate ester was hydrolyzed and oxidized to the corresponding inososе *ent*-**4**. To 4.3 mg (9 μmol) of the inosose in 1 mL of ethanol was added ca. 150 mCi of sodium borotritide (sp. act. 64 Ci/mmol) in 100 μL of ethanol. Complete reduction occurred, so a slight excess (1.5 mg additional) of inosose was added to consume the borotritide. The separation of the axial and equatorial epimeric alcohols by flash chromatography yielded 4 mg (120 mCi, sp. act. 15 Ci/mmol) of desired product *ent*-**5**; ^{3}H-NMR ($CDCl_3$) δ 2.74 (dd, J = 9.8 Hz, 2.5 Hz).

Hydrolysis of the 4 mg (8 μmol) of *ent*-**5** with 1 N HCl-methanol provided 2.2 mg (4.5 μmol, 60% yield) of triol *ent*-**6**. Reaction of this sample of triol with NaH and TBPP in DMF afforded 2 mg (36%) of the perbenzylated trisphosphate *ent*-**7**. Hydrogenolysis of this material provided 21 mCi of the labelled L-*myo*-Ins(1,4,5)P_3 (*ent*-**1**) (sp. act. 15 Ci/mmol).

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