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SYNTHESIS OF A P-1-TETHERED PHOTOAFFINITY LABEL FOR INOSITOL HEXAKISPHOSPHATE BINDING PROTEINS

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SUMMARY

A new photoaffinity analog of inositol hexakisphosphate ($InsP_6$, or phytic acid) was prepared for investigation of $InsP_6$-binding proteins. The racemic P-1-(*O*-6-aminohexyl) derivative of $InsP_6$ was synthesized in six steps from inositol, and a tritium-labeled benzophenone-containing photophore was attached in the final step to give P-1 [^{3}H]BZDC-$InsP_6$ (sp. act. 35 Ci/mmol).

Key words: $InsP_6$, phytic acid, photoactivation, tritium-labeled, benzophenone photophore, protein trafficking, synaptic vesicle, assembly proteins

INTRODUCTION

Inositol hexakisphosphate ($InsP_6$, phytic acid) is a ubiquitous constituent of plants and has shown antioxidant (1) and anticancer properties (2, 3, 4) both *in vitro* and *in vivo*. More recently, $InsP_6$ has been shown to bind synaptic vesicle proteins, e.g., synaptotagmin (5), and has been implicated as a mediator of protein-protein interactions important to synaptic vesicle docking, exocytosis and endocytosis (6, 7, 8, 9). Phosphoinositides also participate in regulation of protein trafficking via Golgi coatomer complexes (10). In the course of our efforts to prepare affinity probes for a variety of inositol polyphosphates (11), we first prepared a *meso* P-2-tethered aminohexyl derivative of $InsP_6$ (12). This P-2-tethered $InsP_6$ probe was employed to prepare an affinity matrix that was successfully used to purify two very different inositol polyphosphate kinases (13, 14). Similarly, a photoactivatable benzophenone-containing probe (15) was prepared and used to label a variety of these $InsP_6$-binding proteins.

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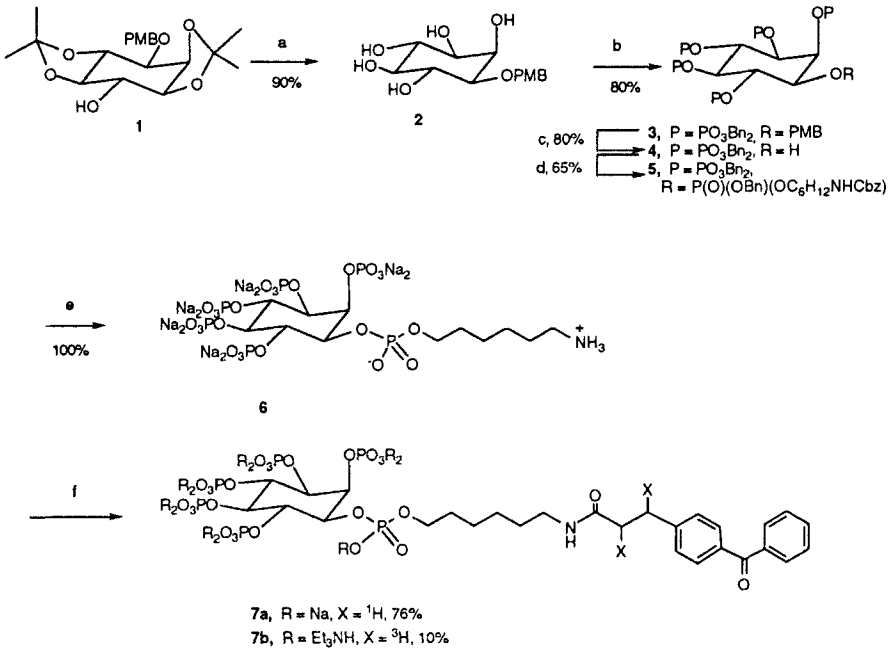
In order to compare these labeling results to those obtained with P-1-tethered photoaffinity labels for Ins(1,4,5)P_3 (16) and Ins(1,3,4,5)P_4 (17), we prepared a P-1-tethered InsP_6 probe as described herein. Comparative studies using this new ligand with InsP_6-binding proteins that have been previously affinity purified (18) and photoaffinity labeled (7, 19) with a P-1-tethered Ins(1,3,4,5)P_4 probe are in progress.



Scheme 1. Synthesis of the tritium-labeled P-1-tethered InsP_6.

Reagents and conditions: (**a**) cat. *p*-TsOH, acetone-water, rt, overnight; (**b**) $(BnO)_2PN$*i*-Pr_2, 1-*H*-tetrazole, CH_2Cl_2, rt, 1 h, then -40 °C, *m*-CPBA, 5 min, 0 °C to rt 1 h; (**c**) $(NH_4)_2Ce(NO_3)_6$, CH_3CN-H_2O; rt, 2 h; (**d**) (BnO)P(N*i*-Pr_2)($OC_6H_{12}NHCbz$), 1 h, then -40 °C, *m*-CPBA, 5 min, 0 °C to rt 1 h; (**e**) Pd/C, 95% EtOH, H_2, 50 psi, rt, 6.5 h; (**f**) BZDC-NHS, Et_3N, DMF, rt, overnight (for **7a**); [^{3}H]BZDC-NHS, 0.25 M TEAB buffer - DMF (1:1), rt, overnight (for **7b**).

RESULTS AND DISCUSSION

The synthesis of P-1-*O*-(6-aminohexyl)-InsP_6 is shown in **Scheme 1**. The starting material, D,L-1,2:4,5-bis-*O*-isopropylidene-3-(4-methoxybenzyl)-*myo*-inositol **1**, was obtained by a known procedure (20, 21). Hydrolysis of the acetone ketal protecting groups (*p*-TsOH, acetone-water), gave the 3-PMB protected inositol **2** (equivalent to the 1-PMB in racemic form) in 90% yield. Phosphitylation of the pentaol **2** with dibenzyl *N,N*-diisopropylphos-phoramidite, 1-*H*-tetrazole in CH_2Cl_2, followed by *m*-CPBA oxidation, gave the protected inositol pentakisphosphate **3** in 80% yield. Removal of

the PMB group with ammonium ceric nitrate gave compound **4** in 80% yield, and phosphitylation of the remaining hydroxyl group with benzyl *N*-Cbz-6-aminohexyl *N,N*-diisopropylphosphoramidite (12) gave the fully-protected P-1-tethered inositol **5** in 65% yield. Hydrogenolysis of all benzyl groups afforded the racemic P-1-(*O*-6-aminohexyl)-*myo*-inositol pentakisphosphate **6** in quantitative yield. Reaction of the amino group with *N*-hydroxysuccinimido *p*-benzoyldihydrocinnamate ester (BZDC-NHS ester) (22) in the presence of triethylamine in dry DMF and followed by DEAE cellulose purification and Chelex exchange to sodium salt gave unlabeled P-1-tethered BZDC-$InsP_6$ in the sodium salt form (**7a**). To prepare the tritium-labeled photoaffinity label, the reaction was carried out with 2 mCi of [^{3}H]BZDC-NHS ester (23) in 0.25M TEAB buffer and DMF (1:1). Purification on DEAE cellulose gave [^{3}H]BZDC-$InsP_6$ **7b** (35 Ci/mmol) as the triethylammonium salt.

MATERIALS AND METHODS

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded in $CDCl_3$ on QE-300 or AC-250 NMR spectrometers and reported relative to δ (TMS) = 0 ppm. When necessary, solvents and reagents were dried using standard procedures. HRMS spectra were performed at University of California at Riverside, using NBA matrix to obtain MNa^+ molecular ion peaks.

1-*O*-*p*-Methoxybenzyl-*myo*-inositol (2). 1,2:4,5-Bis-*O*-isopropylidene-3-*p*-methoxybenzyl-*myo*-inositol (**1**, 344 mg, 1.15 mmol) was dissolved in 7 mL of acetone, and 0.18 mL of water was added, followed by 37 mg of *p*-toluenesulfonic acid monohydrate. The mixture was stirred at rt for overnight, and a white precipitate formed. The solid was collected by filtration and washed with ethyl acetate and ether to give **2** in 90% yield of sufficient purity for direct analysis by NMR. mp 198-199 °C. Anal. for $C_{14}H_{20}O_7$: Calcd: C, 56.00; H, 6.71; Found: C, 55.98, H, 6.61. ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 7.40, 7.35, 6.90, 6.85 (AB, 4H, PMB), 4.5 (m, 2H), 3.98 (s, 1H), 3.90 (s, 3H) OMe), 3.50 (m, 1H), 3.40 (m, 2H), 3.2 (m, 2H), 3.0 (m, 1H), 2.5 (s, 2H, OH), 2.1 (s, 2H, OH) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 132.0, 129.1, 113.4, 79.6, 75.4, 72.4, 72.0, 71.7, 70.3, 69.3, 55.0 ppm.

2,3,4,5,6-Pentakis(dibenzylphosphono)-1-*O*-*p*-methoxybenzyl-*myo*-inositol (3). To a mixture of pentaol **2** (180 mg, 0.6 mmol) and 1-*H*-tetrazole (842 mg, 12 mmol) in 10 mL of CH_2Cl_2 was added dibenzyl *N,N*-diisopropylphosphoramidite (2.070 g, 6 mmol) in 1 mL of CH_2Cl_2. The mixture was stirred at rt for 1 h and cooled to -40 °C and 150 mg of *m*-CPBA was added. The mixture was stirred at that temperature for 5 min, then stirred at 0 °C for 30 min and rt for 30 min. The solution was diluted to 100 mL of CH_2Cl_2, washed with 10% Na_2SO_3, 10% $NaHCO_3$, dried over $MgSO_4$. Removal of the solvent left an oil that was purified on SiO_2 (ethyl acetate: methylene chloride, 1:1) to give 770 mg of **3** as a syrup (yield 80%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.30-6.9 (m, 52H), 6.64, 6.61 (AB, 2H, PMB), 5.47 (m, 1H), 5.15-4.20 (m, 26H), 3.63 (s, 3H, OMe), 3.45 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 130.6, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7,

113.6, 77.6, 77.1, 76.5, 69.7, 50.0 ppm. ^{31}P NMR ($CDCl_3$, 101 MHz) δ: 0.45, 0.21, -0.10, -0.27, -1.05 (1:1:1:1:1) ppm. FAB HRMS: $C_{84}H_{85}O_{22}P_5Na$ (MNa). Calcd: 1623.4118. Found: 1623.4064.

2,3,4,5,6-Pentakis(dibenzylphosphono)-*myo*-inositol (4). Precursor **3** (770 mg, 0.48 mmol) was dissolved in 50 mL of mixed solvent of acetonitrile and water (9:1) and ammonium ceric nitrate (790 mg, 1.44 mmol) was added in three portions. The mixture was stirred at rt for 2 h. After removal of acetonitrile, the residue was diluted with 15 mL of 10% $NaHCO_3$ and extracted with ether. After drying and concentrating, the residue was purified by chromatography to give 560 mg of compound **4** as a syrup (yield 80%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.30-6.81 (m, 50H), 5.45 (m, 1H), 5.30-4.80 (m, 23H), 4.50 (m, 1H), 4.32 (m, 1H), 3.80 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 128.5, 128.4, 128.3, 128.0, 77.5, 77.0, 76.5, 69.7 ppm. ^{31}P NMR ($CDCl_3$, 101 MHz) δ: 1.97, 0.34, 0.09, -0.16 (1:1:1:1:2) ppm. FAB HRMS: $C_{76}H_{77}O_{21}P_5Na$ (MNa). Calcd: 1503.3544. Found: 1503.3534.

Benzyl *N*-Cbz-6-aminohexyl 1-[2,3,4,5,6-pentakis(dibenzoxyphosphono)]-*myo*-inositol phosphate (5). To a mixture of compound **4** (180 mg, 0.122 mmol) and 1-*H*-tetrazole (35 mg, 0.50 mmol) in 10 mL of methylene chloride was added benzyl *N*-Cbz-6-aminohexyl diisopropylphosphoramidite (120 mg, 0.246 mmol) in 1 mL of methylene chloride. The mixture was stirred at rt for 1 h, then cooled to -40 °C, and 100 mg of *m*-CPBA was added. The mixture was stirred at that temperature for 5 min, then stirred at 0 °C for 30 min and rt for 30 min. The usual work up and purification on SiO_2 (ethyl acetate: methylene chloride, 2:1) gave 150 mg of tethered compound **5** (yield 65 %) as a syrup. ^{1}H NMR ($CDCl_3$, 250 MHz) δ: 7.4-7.15 (m, 60H), 5.5 (m, 1H), 5.2-4.9 (m, 27H), 4.6-4.4 (m, 3H), 4.1-3.9 (m, 2H), 3.1-3.0 (m, 2H), 1.4-1.0 (m, 8H) ppm. ^{31}P NMR ($CDCl_3$, 101 MHz) δ: 0.44, -0.03, -0.13, -0.33, -1.26 (6:2:1:1:2) ppm (two disastereoisomers). FAB HRMS: $C_{97}H_{103}NO_{26}P_6Na$. Calcd: 1906.5092. Found: 1906.5151.

6-Aminohexyl 1-[2,3,4,5,6-pentakis(dibenzoxyphosphono)]-*myo*-inositol phosphate (6). The fully-protected precursor **5** (150 mg, 0.08 mmol) was dissolved in 50 mL of 95% EtOH and 100 mg of 10% Pd/C was added. Hydrogenolysis at an initial hydrogen pressure of ca. 50 psi was allowed to proceed at rt for 6.5 h. The catalyst was filtered off and washed with 30 mL of 2:1 ethanol-water followed by 3 mL of water. The filtrate was brought to pH 8.0 with a few drops of conc. ammonium hydroxide and the solvent was removed *in vacuo*. The residue was dissolved in 3 mL of water and applied to one 5 × 2 cm column of Bio-Rad Chelex 100 resin (sodium form) eluted with 25 mL of water. Evaporation afforded 78 mg (100%) of the sodium salt of **6** as a colorless glass. ^{1}H NMR (300 MHz, D_2O) δ: 4.51-4.47 (m, 2H), 4.30-4.00 (m, 6H), 3.05 (t, J = 7.2 Hz, 2H), 1.80-1.60 (m, 4H), 1.50-1.40 (m, 4H) ppm. ^{13}C NMR (63 MHz, D_2O) δ: 81.8, 80.4, 79.0, 78.0, 77.5, 76.5, 72.9, 69.1, 42.1, 32.1, 29.2, 27.6, 27.0 ppm. ^{31}P NMR (101 MHz, D_2O) δ:

6.30, 6.15, 4.80, 4.25, 4.13, 3.95 (1:1:1:1:1:1) ppm. FAB MS *m/z*: 958(M -Na + 2H), 935 (M - 2Na + 3H), 914 (M - 3Na + 4H), 892 (M - 4Na + 5H), 870 (M - 5Na + 6H).

1-*O*-(3-*p*-Benzoyldihydrocinnamylaminohexyl-1-phospho)-*myo*-inositol 2,3,4,5,6-pentakisphosphate (7a). The P-1-tethered affinity reagent **6** (5 mg, 0.0051 mmol) was suspended in 300 μL of anhydrous DMF and *N*-hydroxysuccinimido *p*-benzoyldihydrocinnamate ester (BZDC-NHS ester, 5.6 mg, 0.016 mmol) was added, followed by 20 μL of dry triethylamine. The suspension was stirred overnight and the $InsP_6$ starting material gradually dissolved. The solvents were evaporated *in vacuo* and the residue was evaporated with 2 mL of water. The residue was dissolved in 3 mL of water and applied to 10 × 2.2 cm column of DEAE cellulose (HCO_3^- form). The column was washed with 2 × 5 mL of water, and then eluted with 2 × 5 mL of 0.1 M TEAB, 2 × 5 mL of 0.2 M TEAB, 5 mL of 0.3 M TEAB, 5 mL of 0.4 M TEAB, and 2 × 5 mL of 0.6 M TEAB buffer. The product eluted in 0.4 and 0.6 buffer. Analysis of these buffer fractions by reversed phase HPLC (Brownlee RP-300, 250 × 46 mm, 15% acetonitrile in 0.05 M KH_2PO_4 buffer, pH 4.5, UV detector, λ = 254 nm) showed a single product in the 0.4 and 0.6 M TEAB buffer fractions with a retention time of 20 min. Evaporation of these fractions *in vacuo,* followed by evaporating with several small volumes of methanol gave 4.8 mg (76%) of triethylamine salt of **7a**. After a column with Bio-Rad Chelex 100 resin® (Na form), a sodium salt was obtained as a glass. ^1H NMR (250 MHz, D_2O) δ: 7.86-7.75 (m, 5H), 7.50 (t, 2H), 7.36 (t, 2H), 4.8-3.40 (m, 8H), 3.06 (m, 2H), 2.90 (t, *J* = 7.5 Hz, 2H), 2.45 (t, *J* = 7.5 Hz, 2H), 1.65-1.0 (m, 8H) ppm. ^{31}P NMR (101 MHz, D_2O) δ: 6.37, 5.21, 5.05, 4.26, 4.05 (1:1:1:1:2) ppm.

1-*O*-(3-*p*-Benzoyldi-[^{3}H]cinnamylaminohexyl-1-phospho)-*myo*-inositol 2,3,4,5,6-pentakisphosphate (7b). Aminohexyl derivative **6** (100 μL of 0.25 mg in 0.25 M TEAB buffer stock solution) was added to [^{3}H]-*N*-hydroxysuccinimido *p*-benzoyldihydrocinnamate ester ([^{3}H]BZDC-NHS ester, 35 Ci/mmol, 2 mCi) in 100 μL solution of DMF. The mixture was stirred overnight. The solvents were evaporated *in vacuo* and the residue was evaporated with 0.1 mL of water. The residue was dissolved in 0.5 mL of water and applied to pipette column of DEAE cellulose (HCO_3^- form). The column was washed with 2 × 1 mL of water, and then eluted with 2 × 1 mL of 0.1 M TEAB, 2 × 1 mL of 0.2 M TEAB, 1 mL of 0.3 M TEAB, 1 mL of 0.4 M TEAB, and 2 × 1 mL of 0.6 M TEAB buffer. The product eluted in 0.4 and 0.6 buffer. Analysis of these buffer fractions by reversed phase HPLC (Brownlee RP-300, 250 × 46 mm, 15% acetonitrile in 0.05 M KH_2PO_4 buffer, pH 4.5, radioactive detector) showed a single product in the 0.4 and 0.6 M TEAB buffer fractions with a retention time of 20 min. The fractions were combined and *ca.* 200 μCi (10% radiochemical yield) product **7b** was obtained in the triethylammonium salt form.

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