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SYNTHESIS OF A TRITIUM-LABELLED DIETHER ANALOG OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE

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SUMMARY

The synthesis of 1-(1,2-*O*-diundecyl-*sn*-glycerylphosphoryl) 4,5-D-*myo*-inosityl bisphosphate and its tritiated analog are described. The convergent synthesis employed optically-pure inositol and glycerol derivatives. In the final step, hydrogenation of an alkenyl chain gave the saturated diether PIP_2 and tritiation gave the high-specific activity, tritium-labelled analog.

Key Words: phosphoinositide, vesicles, binding assay, cell signalling, PtdIns(4,5)P_2

INTRODUCTION

Phospholipids constitute the main structural component of cell membranes (1a) and the phosphoinositide polyphosphates (PtdInsP_ns) are key signalling molecules in cellular communication, in endo- and exocytosis, and in vesicular trafficking of proteins (1b, 2a-c). In particular, PtdIns(4,5)P_2 has important roles in recruitment of proteins, e.g., profilin and phospholipase C (PLC), to membranes (2a). An NBD-PtdIns(4,5)P_2 probe was recently used to show the sequestration of phosphatidyl serine (PS) and PtdIns(4,5)P_2 into lateral domains by the basic MARCKS peptide, thus effectively preventing access of PLC for hydrolysis (2e). PtdIns(4,5)P_2 is also a substrate for phosphoinositide 3-kinase (PI 3-K) (3), a heterodimeric enzyme important in the mechanisms of oncogene-induced transformation, cytoskeletal rearrangements, membrane association of signalling proteins, and trafficking of proteins by coated vesicles. The product of PI 3-K action on PtdIns(4,5)P_2 is PtdIns(3,4,5)P_3, a second

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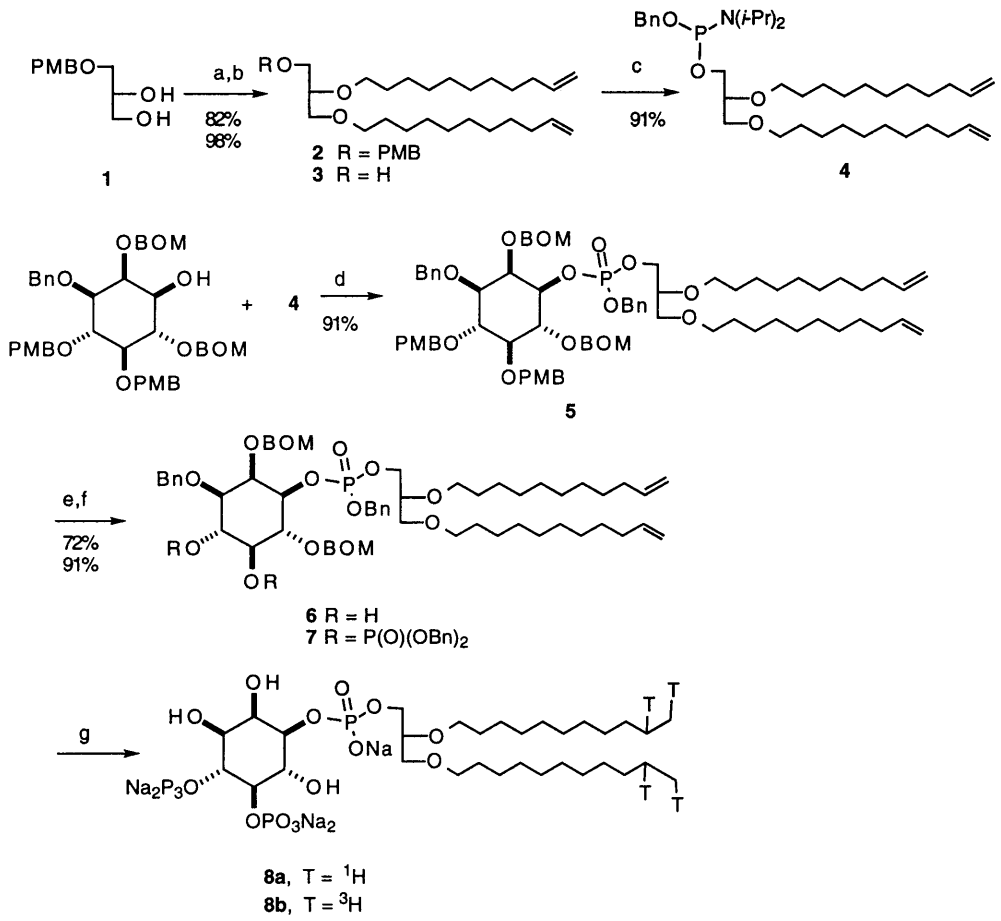
messenger for the phosphorylation of pleckstrin (4a) and the activation of Akt/PKB kinase (4b,c), and the ligand for a novel brain protein, centaurin (4d).

Recently, we prepared a variety of affinity probes for isolation and characterization of proteins with specific binding sites for inositol polyphosphates ($InsP_n$s) as well as the $PtdInsP_n$s (5). However, for many situations hydrolysis of the diacylglyceryl esters results in degradation of the $PtdInsP_n$ probes. Thus, we required a diether analog of PtdIns(4,5)P_2 to explore binding and phosphorylation events occurring in cell cultures and in cell-free extracts. To this end, we describe herein the synthesis of the 1,2-di-*O*-undecyl ether analog of phosphatidylinositol 4,5-bisphosphate in both unlabelled and in high-specific activity, tritium-labelled forms.

RESULT AND DISCUSSION

The synthesis of PtdIns(4,5)P_2-diether analog **8** is illustrated in Scheme 1. Thus, alkylation of 3-(*p*-methoxybenzyl)-*sn*-glycerol (8) **1** with 11-bromo-1-undecene (9) gave ether **2** in 82% yield, and DDQ-mediated cleavage (6a) of *p*-methoxybenzyl (PMB) furnished glycerol **3** in 98% yield. Reaction of glycerol derivative **3** with freshly-prepared chloro(*N,N*-diisopropylamino) (benzoxy) phosphine (6a) gave phosphine reagent **4** in 91% yield. Coupling the reagent **4** with the inositol intermediate (6a) in the presence of 1-*H*-tetrazole followed by *m*-CPBA oxidation gave phosphate **5** in 91% isolated yield. DDQ removal of PMB groups in wet CH_2Cl_2 gave 4,5-diol **6** in 72% yield. Further phosphorylation gave the fully-protected phosphoinositide **7**. Pd/C-catalyzed hydrogenation, followed by ion exchange to the sodium salt, furnished saturated PtdIns(4,5)P_2 diether sodium salt **8a** in 70% yield. Catalytic tritiation under analogous conditions gave the tritium-labelled analog **8b** (325 mCi) in 10% radiochemical yield, with a specific activity of 120 Ci/mmol. The unlabelled analog **8a** is a weak competitor for [^{3}H]BZDC-PtdIns(4,5)P_2 photolabelling experiments with profilin (A. Chaudhary, unpublished results). In addition, **8a** has been found to compete for PtdIns(3,4,5)P_3 binding to AP-3 and inhibit clathrin assembly during endocytosis (7).



Scheme 1. Reagents and conditions: (**a**) 1-bromo-10-undecene, NaH, DMF, rt, 2 hr; (**b**) DDQ, wet CH_2Cl_2, rt, 3 hr; (**c**) *i*-Pr_2NEt, ClP(OBn)(NPr_2-*i*), CH_2Cl_2, 0 °C to rt 75 min; (**d**) inositol intermediate, 1-*H*-tetrazole, CH_2Cl_2, 2h, -40 °C, *m-CPBA*, then, 0 °C, 30 min, rt, 30 min; (**e**) DDQ, wet CH_2Cl_2, rt, 4 hr; (**f**) $(BnO)_2P(NPr_2$-*i*), 1-*H*-tetrazole, CH_2Cl_2, 2 hr, -40 °C, *m*-CPBA, then, 0 °C, 30 min, rt, 30 min; (**g**) 10% Pd/C, H_2 or tritium (50 psi), 95% EtOH, rt, 6 hr then Chelex exchange to sodium salt.

EXPERIMENTAL

1,2-Di-(10-undecenyl)-3-(*p*-methoxybenzyl)-*sn*-glycerol (2). A mixture of 3-(*p*-methoxybenzyl)-*sn*-glycerol (8) (**1**, 550 mg, 2.59 mmol), NaH (622 mg, 60%, 15.54 mmol), 11-bromo-1-undecene (9) (1.813 g, 7.77 mmol) in 30 mL of dry DMF was stirred at rt for

2 hr. The excess of NaH was destroyed by MeOH. The mixture was poured into water and extracted with CH_2Cl_2 (2 × 30 mL). The combined CH_2Cl_2 layers were washed with H_2O (20 mL) and dried over $MgSO_4$. Concentration and flash chromatography purification on silica gel using 10% EtOAc in hexane as eluent gave 1.094 g of compound **2** (yield 82%). ^1H NMR ($CDCl_3$, 250 MHz) δ: 7.25, 7.22, 6.89, 6.85 (4s, 4H, phenyl), 5.88-5.72 (m, 2H, CH=), 4.98, 4.86 (dd, J = 18.9 Hz, 10.5 Hz, 4H, CH_2=), 4.47 (s, 2H, PMBCH_2O), 3.60-3.32(m, 9H, CHO and CH_2O), 3.79 (s, 3H, MeO), 2.04, 2.02 (2t, J = 6.5 Hz, 4H, CH_2CH=), 1.56-1.51(m, 4H, CH_2CH_2O), 1.27-1.19 (m, 24H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 139.20, 130.53, 129.19, 114.12, 113.70, 77.94, 73.00, 71.63, 70.57, 69.96. 55.22, 33.82, 30.11, 29.66, 29.57, 29.48, 29.25, 28.94, 26.11 ppm. MS: 515 ($M-H^+$), 135, 121. FAB HRMS: calcd. for $C_{33}H_{55}O_5$ $(M-H)^+$, 515.4100. Found: 515.4085.

1,2-Di-(10-undecenyl)-*sn*-glycerol (3). A solution of glycerol (**2**, 1,000 mg, 1.94 mmol) and DDQ (660 mg, 2.91 mmol) in 50 mL of wet CH_2Cl_2 was stirred at rt for 3 hr. The mixture was diluted with CH_2Cl_2 (50 mL), washed with 10% $NaHCO_3$ (20 mL), and dried over Na_2SO_4. Purification on SiO_2 using 25% CH_2Cl_2 in hexane as eluent (R*f* 0.35) gave compound **3** (752 mg, 98% yield). ^1H NMR ($CDCl_3$, 250 MHz) δ: 5.88-5.72 (m, 2H, CH=), 4.98, 4. 86 (dd, J = 18.9 Hz, 10.5 Hz, 4H, CH_2=), 3.70-3.40 (m, 9H, CHO and CH_2O), 2.19 (br, 1H, OH) 2.04, 2.02(2t, J = 6.5 Hz, 4H, CH_2CH=), 1.56-1.51 (m, 4H, CH_2CH_2O), 1.27-1.19 (m, 24H, others) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 139.23, 114.12, 78.21, 71.85, 70.90, 70.39, 63.11, 33.82, 30.11, 29.66, 29.57, 29.48, 29.25, 28.94, 26.05 ppm. MS: 397$(M+H)^+$, 245, 109. FAB HRMS: calcd. for $C_{25}H_{49}O_3$ (MH^+): 397.3682. Found: 397.3692.

(Benzoxy) 3-[1,2-di-(10-undecenyl)-*sn*-glyceroxy)] (*N,N*-diisopropyamino) phosphine (4). A mixture of glycerol (**3**, 456 mg, 1.15 mmol) and *i*-Pr_2NEt (297 mg, 2.30 mmol) in 30 mL of CH_2Cl_2 was cooled to 0 °C under nitrogen and a solution of chloro(*N,N*-diisopropylamino) (benzyloxy) phosphine (6a) (315 mg, 1.15 mmol) in 5 mL of CH_2Cl_2 was added dropwise over 10 min. The solution was stirred at 0 °C for 10 min, then at rt for 75 min. The solution was washed with 10% aqueous $NaHCO_3$ (5 mL), dried over Na_2SO_4, and concentrated. The crude oil was purified on SiO_2 using

EtOAc-hexane-Et_3N (20:80:1, R*f* 0.94) to give 695 mg (95% yield) of phosphine **4**. ^1H NMR ($CDCl_3$, 250 MHz) δ: 7.34-7.24(m, 5H, phenyl), 5.88-5.72(m, 2H, CH=), 4.98, 4. 86 (dd, J = 18.9 Hz, 10.5 Hz, 4H, CH_2=), 4.79-4.58 (m, 3H, Bn and H-2), 3.70-3.40 (m, 10H, $CHMe_2$ and CH_2O), 2.04-2.02 (t, J = 6.5 Hz, 4H, CH_2CH=), 1.56-1.51 (m, 4H, CH_2CH_2O), 1.27-1.19 (m, 36H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 139.22, 128.19, 127.18, 126.92, 114.12, 78.21, 71.85, 70.90, 70.39, 65.39, 65.09, 43.07, 42.88, 33.84, 30.12, 29.66, 29.57, 29.48, 29.25, 28.94, 26.05 24.69, 24.58, 2.57 ppm. ^{31}P NMR ($CDCl_3$, 101 MHz) δ: 148.91, 148.81 (1:1) ppm.

Benzyl 3-[1,2-di-(10-undecenyl)-*sn*-glyceryl)] 1-(2,6-di-*O*-benzoxymethyl-3-*O*-benzyl-4,5-*O*-(*p*-methoxybenzyl)-D-*myo*-inosityl) phosphate (5). A mixture of inositol (6a) (200 mg, 0.267 mmol) and 1-*H*-tetrazole (75 mg, 1.07 mmol) in 8 mL of CH_2Cl_2 was stirred while a solution of phosphine (**4**, 202 mg, 0.32 mmol) in 2 mL of CH_2Cl_2 was added in one portion. The mixture was stirred at rt for 2 hr and then cooled to -40 °C, and *m*-CPBA (70 mg, 0.4 mmol) was added and stirred for 5 min; the mixture was then stirred (0 °C for 30 min; rt for 30 min), washed (10% aqueous Na_2SO_3 (5 mL), sat. $NaHCO_3$ (5 mL), water (5 mL)), dried ($MgSO_4$), concentrated, and chromatographed on SiO_2 using EtOAc-hexane (1:2) (R*f* 0.7) to give 354 mg (91% yield) of compound **5**. ^1H NMR ($CDCl_3$, 250 MHz) δ: 7.40-7.15 (m, 24H, phenyl and PMB), 6.83, 6.81, 6.79, 6.77 (4s, 4H, PMB), 5.90-5.70 (m, 2H, CH=), 5.20-4.55 (m, 20H), 4.30-3.90 (m, 6H), 3.77 (s, 6H, MeO), 3.55-3.30 (m, 10H), 2.10-2.00 (m, 4H), 1.70-1.20 (m, 28H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 139.23, 137.95, 130.72, 129.70, 129.10, 128.58, 1128.37, 128.23, 127.97, 127.86, 127.71, 127.59, 127.45, 127.37, 114.14, 113.72, 96.04, 95.34, 82.54, 81.12, 79.79, 73.84, 72.41, 71.76, 70.66, 69.97, 69.70, 69.51, 66.99, 55.26, 33.85, 30.11, 29.66, 29.57, 29.48, 29.25, 28.94, 26.07 ppm. ^{31}P NMR (101 MHz, $CDCl_3$) δ: 0.51, 0.28 (1:1) ppm. MS: 1322 (MNa^+), 590, 386, 211, 121. FAB HRMS: calcd. for $C_{77}H_{103}NaO_{15}P$ (MNa^+): 1321.6932. Found: 1321.6995.

Benzyl [1,2-di-(10-undecenyl)-*sn*-glyceryl] 1-*O*-(2,6-di-*O*-enzoxymethyl-3-*O*-benzyl-D-*myo*-inosityl) phosphate (6). A mixture of phosphate (**5**, 327 mg, 0.252 mmol) and DDQ (172 mg, 0.756 mmol) in 10 mL of wet CH_2Cl_2 (CH_2Cl_2 = H_2O v/v 100:1) was stirred at rt for 4 hr. The mixture was diluted to 50 mL with CH_2Cl_2 and washed with 10%

$NaHCO_3$ (3 × 10 mL), dried over $MgSO_4$, and concentrated. Chromatography on SiO_2 using 50% EtOAc in hexane as eluent gave 191 mg of compound **6** (72% yield). ^1H NMR ($CDCl_3$, 250 MHz) δ: 7.40-7.15 (m, 20H, phenyl), 5.90-5.70 (m, 2H, CH=), 5.20-4.55 (m, 16H), 4.30-3.90 (m, 5H), 3.55-3.30 (m, 10H), 2.7 (s, br 2H, OH), 2.10-2.00 (m, 4H), 1.70-1.20 (m, 24H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 139.23, 137.95, 130.72, 129.70, 129.10, 128.58, 1128.37, 128.23, 127.97, 127.86, 127.71, 127.59, 127.45, 127.37, 114.15, 96.53, 95.29, 82.54, 81.12, 79.79, 73.84, 72.41, 71.76, 70.66, 69.97, 69.70, 69.51, 66.99, 55.26, 33.85, 30.11, 29.66, 29.57, 29.48, 29.25, 28.94, 26.07 ppm. ^{31}P NMR (101 MHz, $CDCl_3$) δ: 0.27, 0.15 (1:1) ppm. MS: 1082 (MNa^+), 991, 952, 831, 753, 657, 589, 181. FAB HRMS calcd. for $C_{61}H_{87}NaO_{13}P$ (MNa^+): 1081.5782. Found: 1081.5843.

Benzyl [1,2-di-(10-undecenyl)-*sn*-glyceryl] 1-*O*-[2,6-di-*O*-benzoxymethyl-3-*O*-benzyl-4,5-di-*O*-(dibenzylphosphoryl)-D-*myo*-inosityl] phosphate (7). A mixture of phosphate (**6**, 145 mg, 0.137 mmol), 1-*H*-tetrazole (77 mg, 1.1 mmol) and dibenzyl *N,N*-diisopropylphosphoramidite (189 mg, 0.55 mmol) in 10 mL of CH_2Cl_2 was stirred at rt for 2 hr and then cooled to -40 °C, and *m*-CPBA (65 mg, 0.38 mmol) was added and stirred for 5 min. Then, the mixture was stirred at 0 °C for 30 min and rt for 30 min. The mixture was diluted to 50 mL with CH_2Cl_2, washed with 10% aq. Na_2SO_3 (5 mL), 10% $NaHCO_3$ (5 mL), H_2O (5 mL), dried ($MgSO_4$), concentrated, and chromatographed on SiO_2 using EtOAc-hexane as eluent (1:2, R*f* 0.58) to give 206 mg of fully-protected PIP_2 **7** (95% yield). ^1H NMR ($CDCl_3$, 250 MHz) δ: 7.40-7.15 (m, 40H, phenyl), 5.90-5.70 (m, 2H, CH=), 5.20-4.55 (m, 24H), 4.30-3.90 (m, 6H), 3.55-3.30 (m, 10H), 2.10-2.00 (m, 4H), 1.70-1.20 (m, 28H) ppm. ^{13}C NMR ($CDCl_3$, 63 MHz) δ: 139.34, 137.97, 130.76, 129.73, 129.10, 128.55, 1128.37, 128.26, 127.97, 127.86, 127.80, 127.59, 127.45, 127.37, 114.15, 96.37, 95.50, 82.54, 81.12, 79.79, 73.84, 72.41, 71.76, 70.66, 69.97, 69.70, 69.51, 66.99, 55.26, 33.82, 30.02, 29.66, 29.57, 29.48, 29.15, 28.94, 26.04 ppm. ^{31}P NMR (101 MHz, $CDCl_3$) δ: 0.28, 0.15, 0.03, -0.34 (1:1:2:2) ppm. MS: 1602 (MNa^+), 1511, 1404, 590. FAB HRMS: calcd. for $C_{89}H_{113}NaO_{19}P_3$ (MNa^+): 1601.6987. Found: 1601.6988.

4,5-Di-*O*-phosphoryl-D-*myo*-inositol 1-*O*-(1,2-di-undecyl-*sn*-glyceryl) phosphate pentasodium salt (8a). A mixture of fully-protected PIP_2 (**7**, 15 mg, 0.074 mmol),

10% Pd/C (2 mg) in 10 mL of 95% EtOH was shaken under H_2 (initial pressure, 50 psi) at rt for 6 hr. The catalyst was filtered through Celite® and the Celite cake was washed with water (2 × 10 mL) and ethanol (3 × 10 mL). The filtrate was concentrated *in vacuo* giving a solid, which was re-dissolved in a minimum of water, then passed through a Chelex (Na^+ form, 20 × 50 mm) column and eluted with water (4 × 5 mL) . Concentration *in vacuo* gave a solid of compound **8a** in sodium salt (6 mg, 70% yield). ^1H NMR (D_2O, 250 MHz) δ: 4.30-3.50 (m, 15H), 1.60-1.00 (m, 36H), 0.87 (m, 6H, CH_3) ppm. ^{31}P NMR (D_2O, 101 MHz) δ: 8.10, 7,65, 3.87 (near 1:1:1) ppm.

4,5-Di-*O*-phosphoryl-D-*myo*-inositol 1-*O*-[1,2-di-(10,11-ditritioundecyl)-*sn*-glyceryl] phosphate pentasodium salt (8b). A mixture of fully-protected PIP_2 (**7**, 5 mg, 0.025 mmol), 10% Pd/C (1 mg) in 3 mL of 95% EtOH was shaken under tritium (50 psi) at rt for 6 hr. The catalyst was filtered through Celite® and the Celite cake was washed with water and ethanol. The filtrate was concentrated *in vacuo* giving a solid, which was re-dissolved in a minimum of water, then through a Chelex (Na^+ form) column and eluted with water. Concentration *in vacuo* gave tritium analog **8b** in sodium salt (325 mCi), specific activity of 120 Ci/mmol. Adsorption of the amphiphilic, micellar product to surfaces and to the filter cake accounts for the low (*ca.* 10%) radiochemical yield. The purity was determined by radio-TLC ($CH_3OH/CHCl_3/H_2O$/conc. NH_4OH (v/v/v/v) = 10:7:2.5:1.5) on silica gel and showed a single peak that co-eluted with the unlabelled material. After two months at -20 °C, two overlapping peaks were observed at *Rf* 0.2. Both hydrogenolysis and tritiolysis generate products essentially free of other by-products (6).

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