Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1061–1064

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF 2-DEOXY-2-FLUORO-PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE AND ANALOGUES: PROBES AND MODULATORS OF THE MAMMALIAN PI-PLCS

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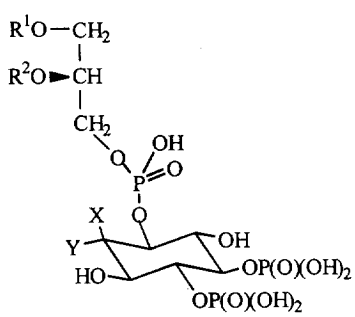
Received 2 February 1998; accepted 20 March 1998

Abstract: An approach to synthesis of 2-modified phosphatidylinositol-4,5-bisphosphates, which are substrate analogues useful as probes and modulators of the PI-PLC enzyme family, is described and illustrated for the dibutyl-2-deoxy-2-fluoro analogue, a probe designed for delineating substrate and PI-PLC interactions by X-ray crystallography.

Phosphatidyl-*myo*-inositol-4,5-bisphosphate (PtdIns-4,5-P_2) (**1**) is a vital participant in intracellular signaling and allied processes,[1] functioning as the preferred substrate of the mammalian phosphoinositide-specific phospholipase C (PI-PLC)[2] and the phosphoinositide 3-kinase (PI 3-kinase) enzyme families,[3] and as allosteric activating factor of cellular regulatory proteins.[4] Several isozymes of PI-PLC are known and all cause hydrolysis of PtdIns-4,5-P_2 to the two intracellular second messengers *myo*-inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3) and *sn*-1,2-diacylglycerol (DAG).[5] Evidence is accumulating that the axial 2-OH is an essential intramolecular nucleophile in the catalyzed hydrolysis.[6,7] Conversely, molecules that retain the core PtdIns-4,5-P_2 structure but lack the essential 2-OH are interesting as nonhydrolyzable substrate analogues and competitive inhibitors of PI-PLC for which there is enormous therapeutic potential.[6] Methods for syntheses in this series have not been reported.

We now describe an approach to synthesis of PtdIns-4,5-P_2 analogues wherein the 2-OH has been rendered non-nucleophilic by substitution or derivatization (**2**). The approach is useful for developing modulators with potential as therapeutics for aberrant PI-PLC activity. We illustrate by preparation of the 2-deoxy-2-fluoro PtdIns-4,5-P_2 (**13**) designed specifically as a probe for studying interactions with the active site of PI-PLC by X-ray crystallography.



1: X = OH, Y = H; R^1 = Stearoyl, R^2 = Arachidonyl.
2: X, Y = F, H, OAc, OMe, etc. R^1, R^2 = Alkyl, Alkyl-C=O, etc.

0960-894X/98/$19.00
PII: S0960-894X(98)00172-3

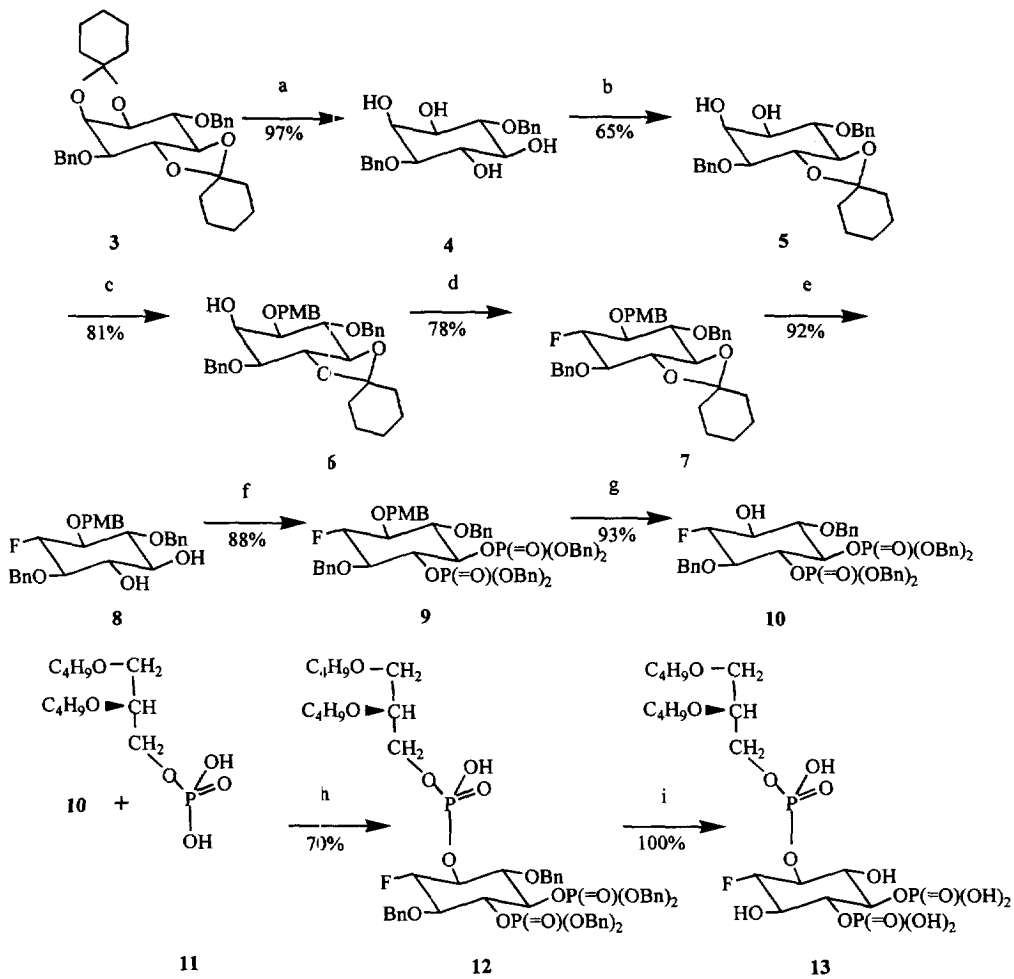
The synthesis employs a convergent strategy based on retrosynthetic disconnection of the phosphodiester bond in an *O*-protected 2-modified PtdIns-4,5-P_2 intermediate to produce *O*-protected chiral inositol(phosphate) and *sn*-3-phosphatidic acid (*sn*-3-PA) fragments. Accordingly, this synthesis of the target 2-deoxy-2-fluoro PtdIns-4,5-P_2 analogue **13** requires the optically resolved inositol-4,5-bisphosphate **10** and the *sn*-3-PA **11** as key synthons, to be coupled by a phosphodiester bond and subsequent removal of temporary protecting groups.

1D-3,6-Di-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (**3**) was chosen as the inositol starting material because (1) it carries appropriate temporary regio-protection, (2) can be prepared in high optical purity, and (3) its absolute stereochemistry has been established unequivocally.[8] It was converted into the *myo*-inositol synthon (**10**) via the key intermediate **5** as outlined in Scheme 1. Complete deketalization of **3** by acid catalyzed hydrolysis gave 1D-3,6-di-*O*-benzyl-*myo*-inositol (**4**). Treatment of **4** with cyclohexanone dimethylketal catalyzed by *p*-TSA under kinetic control resulted in 3:1 selective reaction at the 4,5- versus the 1,2-OHs to produce an acceptable yield of 1D-3,6-di-*O*-benzyl-4,5-*O*-cyclohexylidene-*myo*-inositol (**5**). Reaction of **5** successively with Bu_2SnO and 4-methoxybenzyl chloride/CsF[9] provided high regioselectivity and gave 1D-3,6-di-*O*-benzyl-4,5-*O*-cyclohexylidene-1-*O*-(4-methoxybenzyl)-*myo*-inositol (**6**). Reaction of **6** in CH_2Cl_2 with DAST[10] yielded a major product, which was characterized as the 2-deoxy-2-fluoro derivative **7** based on (i) the known propensity of DAST reactions to effect substitution with inversion, (ii) the absence of the distinctive signal for the equatorial 2-H coupled to the axial 1-H and 3-H protons (J = 1–3 Hz) in its ^{1}H NMR, and (iii) its ^{19}F NMR.[11] Acid catalyzed transketalization of **7** with ethylene glycol formed the 4,5-diol **8**. Dibenzylphosphorylation of **8** by the phosphoramidite-oxidation protocol[12] yielded the 4,5-bis-(dibenzylphosphate) derivative **9**. Removal of methoxybenzyl protection in **9** by DDQ gave 1D-3,6-di-*O*-benzyl-2-deoxy-2-fluoro-*scyllo*-inositol-4,5-bis(dibenzylphosphate) (**10**).

1,2-Di-*O*-*n*-butyl-*sn*-glycero-3-phosphoric acid (**11**) was prepared, 77% overall yield, from 3-*O*-benzyl-*sn*-glycerol[13] via alkylation (*n*-BuBr, NaH, DMF) and debenzylation (Pd-C, H_2, 45 psi) to 1,2-di-*O*-*n*-butyl-*sn*-glycerol, bis(dibenzylphoshorylation) using the step (f) protocol, and hydrogenolysis (Pd-C, H_2, 45 psi).

The *myo*-inositol and *sn*-PA synthons were linked in a phosphodiester bond using a modification of our general method for the synthesis of glycerophospholipids.[14] Reaction was carried out between the protected inositol **10**, the *sn*-3-PA analogue **11**, and triisopropylbenzene-sulfonyl chloride (TPSCl) as the phosphate activating agent in anhydrous pyridine solution at rt for 3 h, followed by treatment with water to decompose excess TPSCl and activated phosphate species. However, the molar ratio inositol-PA-TPSCl was 1:1:2 rather than the previously recommended 2:1:3.[14] The condensation product **12**, subjected to Pd-catalyzed hydrogenolysis, lost all benzyl groups and gave the target 2-modified PtdIns-4,5-P_2, 1D-1-[1,2-di-*O*-*n*-butyl-*sn*-glycero-3-phospho]-2-deoxy-2-fluoro-*scyllo*-inositol-4,5-bisphosphate (**13**).[15,16]

The synthesis is applicable generally for the analogues **2** because required congeners of **10** are prepared by replacing DAST in step (d) with appropriate reagents without altering any subsequent step,[17] appropriate PA synthons are prepared readily,[18] and the TPSCl condensation is applicable to diverse alcohol and PA synthons.[14]



Scheme 1: 1D-1-[1,2-di-*O*-*n*-butyl-*sn*-glycero-3-phospho]-2-deoxy-2-fluoro-*scyllo*-inositol-4,5-bisphosphate (**13**). *Reagents and Conditions*: (a) HOAc/H_2O (90:10), 95 °C. (b) Cyclohexanone dimethylketal (1.2 equiv), DMSO, 40–45 °C, red. press. (c) Bu_2SnO, toluene, 110 °C; DMF, 4-MBnCl, CsF, 80 °C, 2 h. (d) CH_2Cl_2, DAST, 35–40 °C, 3 h. (e) Ethylene glycol (1.1 equiv), *p*-TSA, CH_2Cl_2, 22 °C. (f) $(i\text{-Pr})_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, -40 °C; *m*-CPBA. (g) DDQ, CH_2Cl_2, 22 °C. (h) TPSCl, Py, 22 °C, 3 h. (i) H_2 50 psi, Pd-C, EtOH/H_2O (2:1), 22 °C, 18 h.

The behavior of various 2-modified PtdIns-4,5-P_2s as nonhydrolyzable substrate analogues and competitive inhibitors, and, their utility as probes and modulators of the PI-PLC family, is predicated by the rationale underlying their molecular design whereby the core PtdIns-4,5-P_2 structure is retained in the modified analogues. The short chain butyl-ether in **13** is an additional feature designed to engender compatibility in an experimental protocol wherein preformed PI-PLC crystals are soaked in an aqueous solution of the substrate-analogue to obtain enzyme-analogue complex for X-ray structure analysis.[7] Unlike the long chain fattyacyl-based PtdIns-4,5-P_2 s, which form

lyotropic multimolecular aggregates, the butyl-ether derivative **13** gave optically clear monomeric aqueous solutions stable to autocatalytic chemical hydrolysis. This contrasts advantageously with the behavior of PtdIns-4,5-P_2s carrying very short chain fattyacyls which are prone to autocatalytic hydrolysis of the carboxylate esters, and thus are not suited for crystallography.

Recent studies of PI-PLC δ1 isoform have revealed the mode of binding of Ins-1,4,5-P_3, the co-factor calcium and water at the active site but inevitably provided no direct information about binding of the DAG residue because the probes employed were inositol phosphates.[7] Studies on incorporation of **13** into PI-PLC δ1 crystals and X-ray crystallography are underway.[19] These and related comparative studies with the 2-epimer of **13** and their longer alkyl chain homologues are designed to provide information about the contributions of the glycero-lipid residue to substrate-binding, and also constitute a preliminary basis for structure-based rational design of isozyme-specific inhibitors as therapeutics for aberrant PI-PLC signaling.

Acknowledgment: This work was supported by PHS/NIH grant GM51138 (to R.A.). We thank D. Fuller (Cornell) for NMR and J-L. Peng (Cornell) for MS data.

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11. Compound **7**: ^{19}F NMR (376 MHz, in $CDCl_3$) δ ppm (external $CFCl_3$ = 0 ppm) -199.66 (dt, J_{HCF} = 52.1 Hz (d), $J_{H(C\text{-}1/C\text{-}3)CF}$ =12.0 Hz (t)).
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15. Compound **13**: ^{1}H NMR (400 MHz, D_2O) δ ppm 0.77 (t, J =7.3 Hz, 6H, $2CH_3$), 1.11–1.29 (m, 4H, $2CH_2$), 1.39–1.46 (m, 4H, $2CH_2$), 3.38–3.47 (m, 4H, $2CH_2O$), 3.48–3.52 (m, 1H, H-6), 3.52–3.59 (m, 2H, GlycH-1), 3.60–3.63 (m, 1H, H-1), 3.72–3.78 (m, 1H, H-5), 3.77–3.82 (m, 1H, H-3), 3.85–3.91 (m, 1H, H-4), 3.98–4.08 (m, 2H, GlycH-3), 4.09–4.15 (m, 1H, GlycH-2), 4.28, 4.41 (dt, J_{HCF} =51.2 Hz, $J_{H(C\text{-}1/C\text{-}3)CH}$ =9.1, 9.2, Hz, H-2); ^{19}F NMR (376 MHz,D_2O) δ ppm (external reference $CFCl_3$) 196.69, 196.83 (dt, J_{HCF} =51.2 Hz (d), $J_{H(C\text{-}1/C\text{-}3)CF}$ =13.0 Hz (t); ^{31}P{^{1}H} NMR (162 MHz, D_2O, external reference H_3PO_4) δ 0.82, 0.09 (2:1); MALDI-TOF (-tive) MS 608.14 (M^-).
16. All new compounds were characterized fully including NMR and MS.
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