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SYNTHESIS AND ENZYMATIC PROPERTIES OF A DEOXY ANALOG OF PHOSPHATIDYLINOSITOL

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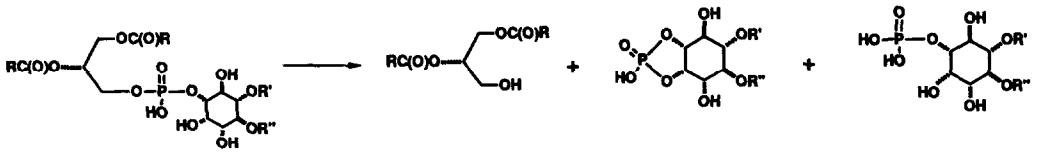
Abstract : The preparation of a phosphatidylinositol analog lacking the axial 2-hydroxyl of the inositol ring is described. The compound is a useful mechanistic probe for the phosphatidylinositol specific phospholipase C.

Phospholipase C (PLC) is a central enzyme involved in the transmission and amplification of growth stimulatory signals[1]. The enzyme catalyzes the hydrolysis of phosphatidylinositides (1a-c) to diacylglycerol and the corresponding inositol phosphates (3a-c). These compounds are thought to be important second messengers, producing activation of protein kinase C and generation of an intracellular calcium signal respectively. A curious feature of the enzymatic reaction is the presence of variable amounts of a 1,2-cyclic inositol phosphate 2a-c along with the expected phosphate monoester (Scheme 1)[2]. There is controversy whether the cyclic phosphate is on the reaction path to 3a-c or represents a side reaction[3]. Phosphodiester hydrolysis proceeding through a cyclic intermediate is reminescent of the well known ribonuclease A mechanism[4]. Consideration of these observations made the preparation of the substrate lacking the 2-hydroxyl desirable. If PLC uses a ribonuclease-like mechanism, 2-deoxyphosphatidylinositol is predicted not to be a substrate but a weak competitive inhibitor[5].

Scheme 1



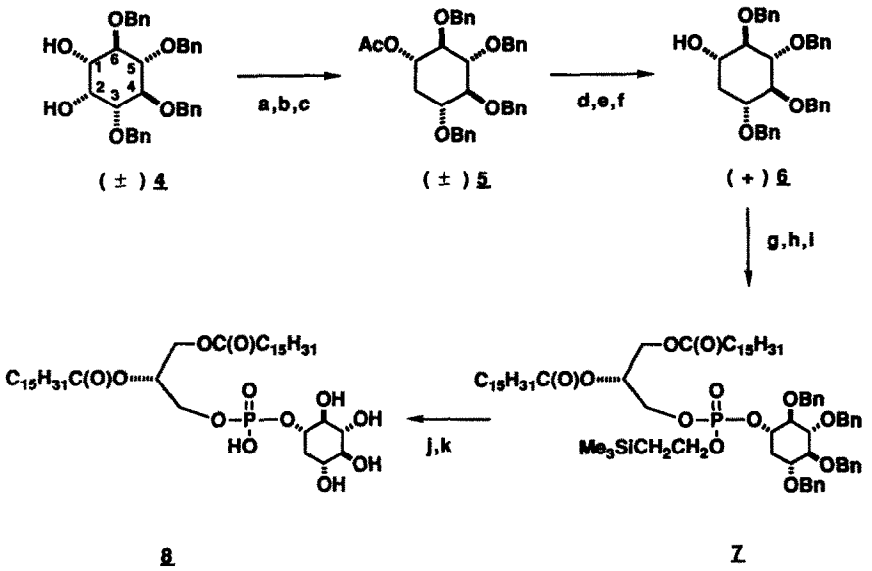
1 a PI : R' = R'' = H
b PIP : R' = H, R'' = PO_3H_2
c PIP_2 : R' = R'' = PO_3H_2

2 a-c

3 a-c

The synthesis begins with racemic 3,4,5,6-tetra-O-benzyl-myo-inositol **4**[6] prepared by the procedure of Gigg[7]. The equatorial 1-hydroxyl was selectively acetylated with acetyl chloride (76%). The chemical shift of H-1 was diagnostic of selective protection (^{1}H-NMR (300 MHz, C_6D_6) δ 5.00 ppm , $J_{1,6}$ = 10.3 Hz, $J_{1,2}$ = 2.6 Hz). Deoxygenation was accomplished by the Robins' variation of the Barton-McCombie sequence: thionocarbonate formation (phenyl chlorothioformate, DMAP, CH_2Cl_2, 40°C.) and radical reduction (tri-n-butyltin hydride, AIBN, toluene, 29% overall)[8].

Scheme 2



(±) 4 (±) 5 (+) 6

8 7

(a) AcCl, DMAP, 2,6-lutidine, CH_2Cl_2, 25°C; (b) PhC(S)Cl, DMAP, CH_2Cl_2, 40°C; (c) n-Bu_3SnH, AIBN, toluene, 110°C; (d) LiOH, THF-H_2O (2.8:1), 25°C; (e) 1-(S)-camphanoyl chloride, DMAP, pyridine, 25°C; (f) LiOH, THF-H_2O (7.2:1), reflux; (g) (i-$Pr_2N)_2P(OCH_2CH_2SiMe_3)$, tetrazole, CH_2Cl_2, 25°C; (h) 1,2-dipalmitoylglycerol, tetrazole, THF; (i) H_2O_2, THF-H_2O (27:1), 0°C; (j) H_2 (50 psi), $Pd(OH)_2$, EtOH, catalytic HOAc, 25°C; (k) HF, CH_3CN-THF (2:1), 25°C

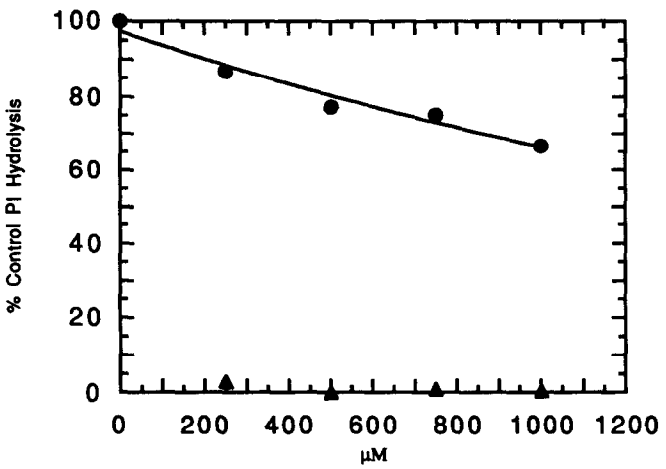
The acetate ester 5 was saponified (lithium hydroxide, tetrahydrofuran-water, 95%) and the resultant alcohol converted to a 1-(S)-camphanate ester (97%). As in the parent inositol series[9] the camphanates are readily separable (HPLC, Du Pont CN column, 15% glyme, 85% hexane). Removal of the camphanate then gave alcohol suitable for coupling with diacylglycerol. The absolute stereochemistry of the series was established by comparison with literature data for camphanates of 6 prepared in a slightly different fashion[10]. In addition, the less polar camphanate diastereomer was subjected to single crystal X-ray analysis[11] which showed that the compound belonged to the D-inositol series.

The phosphodiester was prepared using a novel coupling agent bis-(diisopropylamino)(2-trimethylsilylethoxy)phosphine[12]. Exposure of 6 to excess of the bisamidite reagent in the presence of tetrazole gave an intermediate phosphoramidate which was reacted with dipalmitoylglycerol (tetrazole, tetrahydrofuran) and oxidized with dilute hydrogen peroxide to give 7 as a mixture of diastereomers at phosphorus in good overall yield (89%).

The synthesis was completed by debenzylation (hydrogen, 20% palladium hydroxide on carbon, 84%) and phosphate deprotection (hydrofluoric acid, acetonitrile-tetrahydrofuran). The final deprotection is very convenient as all byproducts are volatile and clean product precipitates from the reaction mixture. Compound 8 has spectral data completely in accord with the proposed structure[13]. In particular, 2D COSY experiments permitted assignment of all of the inositol ring protons establishing that phosphate migration had not occurred during the deprotection phase.

Compound 8 was evaluated as both a substrate and inhibitor of a PLC isolated from a human melanoma cell line[14]. This enzyme has a marked preference for phosphatidylinositol (PI) as a substrate. As shown in Figure 1, compound 8 is not a substrate for the enzyme at concentrations up to 1.0 mM. Conversely, 8 is a weak inhibitor of the hydrolysis of PI with an extrapolated IC_{50} of 2.0 mM. The data is consistent with the prediction made based on a ribonuclease-like mechanism.

Figure 1



Legend: Evaluation of compound 8 as a substrate(▲) or an inhibitor (•) of PLC. Melanoma PLC was assayed colorometrically as previously described[15]. Assay conditions: 25 mM HEPES (pH 7.2), 1 mM $CaCl_2$, 100 mM KCl, 0.8 mg/ml deoxycholate, 3 mg/assay partially purified human melanoma PLC, 37°C. Competition experiments were run versus 0.25 mM PI.

Tsai[16] has reported a series of elegant experiments on the *B. cereus* enzyme and isozymes I and II from guinea pig uterus. Tsai favors a direct displacement mechanism in which formation of 2a-c and 3a-c are parallel, unrelated reactions. The ribonuclease-like mechanism is questioned by three lines of evidence: 1. 2a-c and 3a-c are released simultaneously, 2. Conversion of 2a-c to 3a-c is weak or non-existent[3] and 3. The 2a-c/3a-c ratio is insensitive to the replacement of a phosphoryl oxygen by sulfur. The current study is most simply interpreted in terms of a ribonuclease-like mechanism ; however, it may be accommodated in the Tsai scheme by assuming a critical role for the 2-alcohol of the inositol in forming a productive catalytic complex. This is similar to the argument presented by Baker for the role of the 2-hydroxyl in inositol monophosphatase[10]. Alternatively, since the studies have been performed on different enzymes, it is conceivable that multiple mechanisms are operative. Evaluation of 8 against other PLCs should be interesting in this context.

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References and Notes

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5. PLCs are generally high K_m enzymes, implying that close substrate analogs should be poor inhibitors. This observation has been confirmed in the case of phosphonate analogs of PI : Yang, S. S. ; Beattie, T. R. ; Durette, P. L.; Gallagher, T. F. ; Shen, T.-Y., U.S. Patent 4,515,722 (1985).
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11. X-ray crystallographic data was collected on an Enraf-Nonius CAD4 diffractometer using MoKa radiation. The structure was solved by direct methods (MULTAN) and refined by least squares analyses. The crystals of the camphanate ester (ether/hexane) are monoclinic, $P2_1$, a = 12.281 (4), b = 9.639 (2) , c = 15.859 (5) Å ; Z = 2, D_c = 1.250 g/cc.; R = 0.040 for 2926 reflections [I >=3.0s(I)].
12. 2-Trimethylsilylethanol (7.2 ml, 50.2 mmol) was added dropwise to a cooled (0° C) solution of phosphorus trichloride (30.0 ml , 344 mmol) in dry acetonitrile (35 ml) under nitrogen. The suspension was stirred for one hour when the colorless precipitate was removed by filtration under nitrogen. The filtrate was concentrated under reduced pressure and the residue dissolved in ether (220 ml) and cooled to -10° C. Diisopropylamine (54 ml, 385 mmol) was added over 20 minutes and stirring continued for one hour. The mixture was then warmed to room temperature and stirred overnight. The colorless precipitate was removed by filtration and rinsed with ether. The filtrate was washed with brine and cold water and the aqueous phase back extracted with ether. The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The bisamidite reagent was obtained in 47% yield (8.27 g). A solution of the reagent in methylene chloride (41 ml, *ca.* 0.5 M) was prepared and used directly in the next reaction. ^{31}P-NMR (121.42 MHz, $CDCl_3$) δ 123.63 ppm ; ^1H-NMR (300 MHz, $CDCl_3$) δ 3.64(2H, q, J = 8.4 Hz, O-C<u>H</u>$_2$), 3.58-3.40(4H, m, N-C<u>H</u>), 1.28(28H, m, SiC<u>H</u>$_2$, C-C<u>H</u>$_3$), 0.00(9H, s, SiC<u>H</u>$_3$) ppm.
13. Properties of 8: m.p. 145-146°C ; ^{31}P-NMR (121.42 MHz, d_6-DMSO) δ -1.53 (q) ppm; ^1H NMR (300 MHz, d_6-DMSO) δ 5.15(1H, m, C<u>H</u>-OC=O), 4.38(1H, dd, C<u>H</u>H'-OC=O), 4.10(1H, dd, CH<u>H</u>'-OC=O), 4.05(1H, m, C<u>H</u>H'-OP=O), 3.96(1H, dt, CH<u>H</u>'-OP=O), 3.87(1H, m, H1), 3.22 (1H, m, H-3), 3.14(1H, m, H-6), 2.96(2H, m, H-4 and H-5), 2.27(4H, m, O-C(O)C<u>H</u>$_2$), 2.14 (1H, dt, H-2_{eq}), 1.50(4H, m), 1.36(1H, dd, H-2_{ax}), 1.22(48H, b), 0.85(6H, t, -CH_3) ppm; IR (KBr) ν 3375, 2957, 2920, 2851, 1741, 1468, 1035 cm^{-1}; Elemental Analysis: calculated. for $C_{41}H_{79}O_{12}P$: C 61.94%, H 10.02%, P 3.90%. Found: C 61.68%, H 10.02%, P 3.88%.
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