Tetrahedron Letters, Vol. 34, No. 5, pp. 769-772, 1993
Printed in Great Britain

0040-4039/93 $6.00 + .00
Pergamon Press Ltd

Synthesis of Short Chain Phosphatidylinositols

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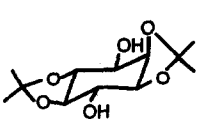
Abstract: A short, convenient, and versatile synthesis of short chain D- and L-phosphatidylinositols is reported.

Phosphatidylinositol (PI) is a key membrane phospholipid that participates in critical physiological events and signal transduction mechanisms.[1] Kinetic and mechanistic details of how PI-specific phospholipase C acts on PI's are lacking, in part because of the difficulty in obtaining defined chain length PI species and the phase problems in dealing with long-chain phospholipids in different structures (e.g., bilayer vesicles, detergent mixed micelles). Short chain PI's would appear to be optimal substrates for PI-PLC based on studies of phospholipase-A_2 and -C action towards short chain phosphatidylcholines.[2] The latter form rod-shaped micelles in aqueous solution[3] with critical micelle concentrations (CMC) in the mM range allowing one to compare enzyme activity towards monomeric as well as micellar substrate.[4] In contrast to these micellar short-chain PC's, bilayer vesicles formed from long chain PC's are poor substrates for PLC.[5] Short chain PI's (e.g., dihexanoyl-PI and diheptanoyl-PI) are not naturally occurring, hence must be synthesized. This paper deals with synthetic methods to generate these two short chain PI's.

Several methods have been reported for the synthesis of naturally occurring long chain analogs and sulfur probes using various protected inositols.[6] All of these strategies have been hampered by low yields and lack of reproducibility. Moreover, these methods are in general suitable for synthesis of long chain analogs but suffer serious problems with shorter acyl chain precursors. The strategy for making the short chain PI's involves the selection of the most appropriate protected inositol for condensation with a diacylglycerol. Choice of a condensing agent is also problematic. The widely used bis-isopropylidene and dicyclohexylidene protected inositols **1,2**, that have been used to synthesize long chain PI's and species with P-S moieties, have serious problems in the synthesis of short chain PI's. The deprotection of isopropylidene and cyclohexylidene groups requires acidic conditions under which the short chain ester moiety at the *sn*-2 position is hydrolyzed to generate the lyso-PI. In contrast, benzyl protective groups can be cleaved by simple hydrogenolysis at neutral pH. To overcome this problem the benzyl protected precursor, 2,3,4,5,6-pentabenzylinositol **3**, is used for condensation with the diacylglycerol segment.



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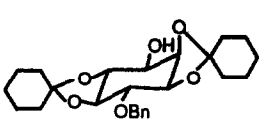

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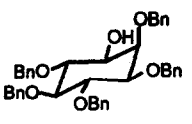

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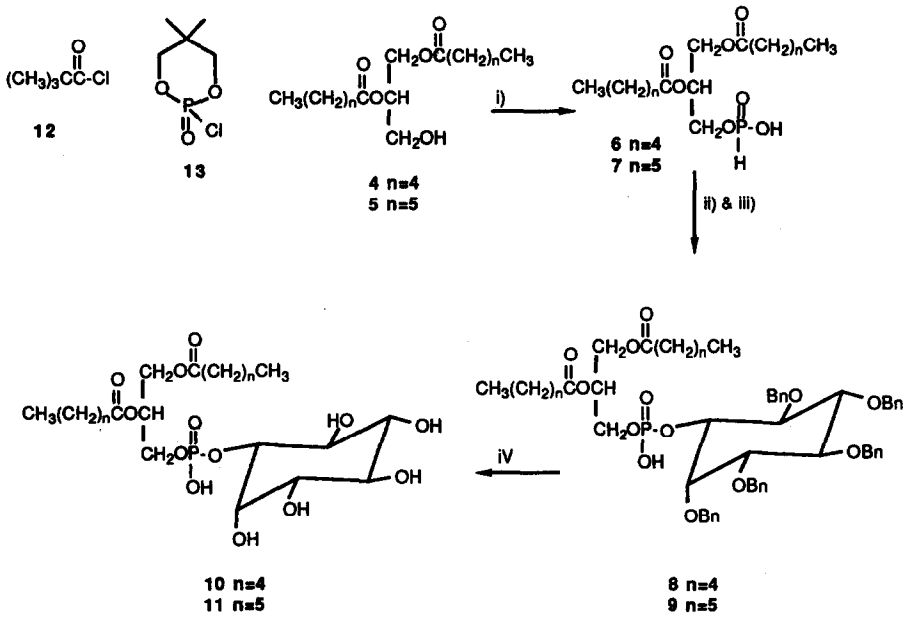
Diacylglycerols **4** and **5** were enzymatically prepared by treating the corresponding PC's at concentrations above their CMC's with nonspecific PLC from *Bacillus cereus* in buffer at pH 8.0.[2] The H-phosphonates **6** and **7** were readily prepared from the corresponding diacylglycerols using PCl_3 and imidazole.[7] The H-phosphonate salts were purified by column chromatography on silica gel and characterized by ^1H and ^{31}P NMR.[8] (±) 2,3,4,5,6-Pentabenzylinositol **3**[9] was converted to camphonate diastereomers, resolved and saponified to (+) and (-) isomers. The absolute configuration of the (+) isomer was established as the D- by crystal structure.[10] Both isomers were used separately to condense the triethylammonium salt of H-phosphonates **6** and **7** using the coupling agent pivaloyl chloride (PVCl) **12** in pyridine successively in the ratio of 1:2-2.5:3-4 eq[7] to give H-phosphonate diesters. PVCl, a widely used reagent in nucleotide synthesis, reacts within 5 min but generates significant side products, even when the reaction is quenched immediately upon completion. One of these side products was characterized as the pivolate ester of protected inositol. The same observation was made in the synthesis of other classes of phospholipids[7] although PVCl was reported as useful in synthesizing glycobiosyl-PI.[11]



i) PCl_3, imidazole, $(CH_3CH_2)_3N$; ii) **3** and NPCl or PVCl, pyridine; iii) I_2-Pyridine-water; iv) 10% Pd/C, H_2, ethanol

An alternative coupling agent for PI synthesis was 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphorinan (NPCl), **13**.[12] NPCl reacts within 30 to 45 min and produces no detectable side products, even if the reaction is not quenched immediately. Any unreacted protected inositol can be recovered from this reaction mixture. The H-phosphonate diesters prepared in this fashion were not isolated but were oxidized *in situ* with I_2 in 98:2 pyridine / water to give the triethylammonium salts of phosphate diesters **8** and **9**. These were purified on a silica gel column, and obtained as colorless gums in 80% yield.[13] These compounds were subsequently subjected to

hydrogenolysis in ethanol with 10% Pd-C under 50 psi for 3 h to yield dihexanoyl-PI **10** and diheptanoyl-PI **11**. The phosphatidylinositols thus obtained were chromatographed on a silica gel column and eluted with 30% MeOH in $CHCl_3$. Appropriate fractions (monitored with a colorimetric phosphate assay[14]) were pooled and lyophilized to give white powders that were characterized by ^1H, ^{13}C and ^{31}P NMR spectroscopy.[15] Bond connectivities of the inositol ring were further established with 2D-COSY analyses.[16] CMC values of these compounds in water, measured by the du Nouy ring detachment method were 12.3 mM for **10** and 1.5 mM for **11**. These PI values were comparable to values for similar chain length PC compounds.[17] Furthermore, PI-specific *Bacillus thuringiensis* phospholipase C activity towards **11** was 500-600 μmol min^{-1} mg^{-1}, indicating it is an excellent substrate for that enzyme.

In conclusion the method described above is a short and easily accessible protocol for the synthesis of PI's in large quantities using NPCl as a condensing agent. This method should also prove useful for the synthesis of isotopically or fluorescently labeled probes that will be important in understanding the role of PI's in critical physiological events, membrane transport, and anchoring of membrane proteins.

Acknowledgements: This work was supported by N.I.H. GM 26762. We would like to thank Dr. Karen Lewis for measuring the CMC values.

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8. DiC_6-H-phosphonate triethylammonium salt **6**: ^1H NMR chemical shifts (ppm from TMS in $CDCl_3$): 0.88-0.96 (t, 6H, ω-CH_3), 1.22-1.40 (m, 17H, central CH_2 groups of acyl chains and CH_3CH_2N), 1.56-1.64 (m, 4H, β-CH_2), 2.22-2.38 (m, 4H, α-CH_2), 3.02-3.11 (m, 6H, CH_3CH_2N), 3.99-4.05 (m, 2H, *sn*-3 CH_2), 4.1-4.14 (q, 1H) and 4.37-4.04 (dd, 1H) of *sn*-1 CH_2, 5.21 (m, 1H, *sn*-2 CH), 6.86 (d, 1H, P-H, $^1J_{PH}$ = 612 Hz); ^{31}P NMR ($CDCl_3$): 5.0 ppm from ext H_3PO_4, $^1J_{PH}$ = 620, $^3J_{PH}$ = 12 Hz. DiC_7-H-phosphonate triethylammonium salt **7**: ^1H NMR spectrum and chemical shifts are the same as diC_6-H-phosphonate except the integration of $(CH_2)_n$ resonance of the acyl chains has four more protons; the ^{31}P NMR spectral parameters are the same as diC_6-phosphonate.
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13. DiC_6-pentabenzylinositol phosphate **8**: ^1H NMR shifts (ppm from TMS in $CDCl_3$): 0.88-0.98 (t, 6H, ω-CH_3), 1.05-1.25 (m, 17H, central CH_2 groups of acyl chains and CH_3CH_2N), 1.42-1.60 (m, 4H, β-CH_2), 2.1-2.21 (m, 4H, α-CH_2), 2.9-2.97 (m, 6H, CH_3CH_2N), 3.3-3.45 (m, 3H, inositol CH), 3.7-3.8 (m, 1H, inositol CH), 3.84-4.15 (m, 6H, inositol CH and *sn*-1 and *sn*-2 CH_2s), 4.5-5.02 (m, 10H, Bz-CH_2s), 5.22 (m, 1H, *sn*-2 CH), 7.02-7.41 (m, 25H, Ar-H); ^{31}P spectrum in $CDCl_3$ exhibits a single peak at -0.850 ppm with respect to H_3PO_4. DiC_7-pentabenzylinositol phosphate **9**: ^1H NMR and ^{31}P NMR spectra are same as diC_6- species except for the integration of the $(CH_2)_n$ resonance.
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15. DiC_6-PI **10**: ^1H NMR (D_2O) chemical shifts: 0.82–0.90 (t, 6H, ω-CH_3), 1.22-1.35 (m, 8H, $(CH_2)_n$), 1.42-1.50 (m, 4H, β-CH_2), 2.32-2.42 (m, 4 H, α-CH_2), 3.30-3.39 (t, 1H, inositol CH), 3.48-3.55 (dd, 1H, inositol CH), 3.59-3.65 (m, 1H, inositol CH), 3.70-3.75 (m, 1H, inositol CH), 3.88-3.92 (m, 1H, inositol CH), 4.02-4.10 (m, 2H, *sn*-3 CH_2), 4.28-4.36 (m, 2H, *sn*-1 CH_2), 4.40-4.48 (m, 1H, inositol CH), 5.23 (m, 1H, *sn*-2 CH); ^{13}C chemical shifts (referenced to dioxane at 67.4 ppm) of diC_6PI in D_2O: 173.51 and 173.17 (C=O), 76.76 (inositol C-1),74.11 (inositol C-5), 72.203 (inositol C-4), 71.28 (inositol C-6), 70.95 and 70.80 (inositol C-2), 70.34 (inositol C-3), 71.64 and 71.49 (*sn*-2 glycerol C), 63.28 (*sn*-3 glycerol C), 62.12 (*sn*-1 glycerol C), 33.47 (acyl chain *sn*-2 α-CH_2), 33.27 (acyl chain *sn*-1 α-CH_2), 28.19 ($(CH_2)_n$), 24.29 (β-CH_2), 21.90 (acyl chain ω-1 CH_2), 12.73 (acyl chain ω-CH_3). ^{31}P NMR (D_2O): spectrum shows a single resonance at -0.58 ppm with respect to external H_3PO_4. DiC_7PI **11**: $[\alpha]_D$ +14.2° (c 0.56, $CHCl_3$-CH_3OH, 4:1) of the D-isomer; $[\alpha]_D$ -13.9° (c 0.45, $CHCl_3$-CH_3OH, 4:1) of the L-isomer. The ^1H and ^{31}P NMR characteristics of diC_7PI are the same as for diC_6PI except for the $(CH_2)_n$ integration. DiC_7PI has the same carbon spectrum with an additional peak at 30.995 assigned to the additional CH_2 in each acyl chain.
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(Received in USA 29 September 1992; accepted 6 November 1992)