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Recombinant *Bacillus subtilis* Whole Cell System as a Catalyst for Enzymatic Synthesis of Cyclic Inositol Phosphate

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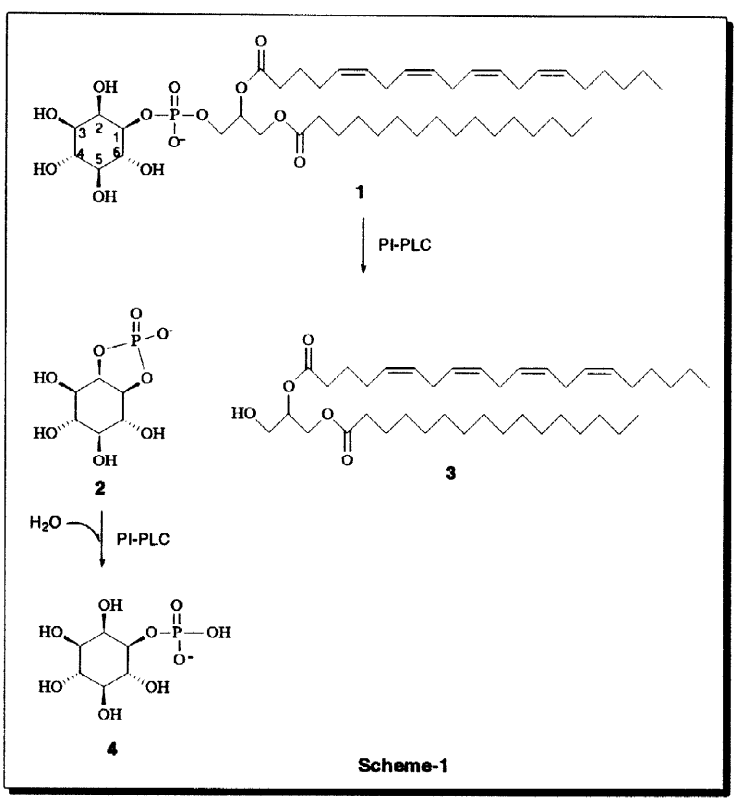
Abstract: Whole cell culture of recombinant bacteria *Bacillus subtilis*, over expressing the phosphoinositide specific phospholipase C (PI-PLC) gene of *Bacillus thuringiensis* has been used as an efficient catalyst for practical multigram scale synthesis of D-*myo*-inositol 1,2-cyclic monophosphate directly from phospholipid mixtures containing phosphatidyl-inositol. This synthesis does not require protein purification and growing bacterial culture can be directly used. The reaction can be monitored by *in-situ* ^{31}P NMR.

Phosphoinositide specific phospholipase C (PI-PLC, EC 3.1.4.10) enzyme catalyses the cleavage of the *sn*-3-phosphodiester bond of phosphatidylinositol (PI, **1** in Scheme-1) into lipid soluble diacyl glycerol (DAG, **3**) and water soluble inositol-1-phosphate [1(1)P, **4**]. The enzyme plays a central role in cellular signal transduction cascades by generating second messenger molecules; DAG which activates protein kinase C (PKC) isozymes and inositol 1,4,5-triphosphate which promotes Ca^{2+} ion release from the internal stores of cells and further signal transduction events.[1,2] PI-PLC class of enzymes are produced by a number of gram positive bacteria e.g. *Bacillus* species, *Staphylococcus aureus* and *Listeria monocytogens*, and also by higher eukaryotes. The physiological role of bacterial PI-PLCs, which are much smaller in size (~ 300 amino acid residues) as compared to their mammalian counterparts, is not well understood. However it is interesting that the bacterial, and not mammalian PI-PLC, can cleave the glycosyl phosphatidyl inositol (GPI) anchored membrane proteins and carbohydrates[3]. There has been significant interest on these enzymes for their biological mechanism,[4] and a number of them have been cloned and sequenced.[5] The X-ray structural,[6] site-directed mutagenesis[7] and isotope labelling[8] studies have shown that bacterial PI-PLC combines two activities; a phosphotransferase activity producing D-*myo*-inositol 1,2-cyclic monophosphate [I(1:2cyc)P] (**2**) with overall retention of configuration at the phosphorus atom, and a



Scheme-1

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cyclic-phosphodiesterase activity producing D-*myo*-inositol-1-phosphate (**4**) through slow hydrolysis of cyclic phosphate. The C-2β-axial hydroxyl group of *myo*-inositol has been proposed[6] as an intramolecular nucleophile which attacks at the phosphate group to form a five-membered cyclic phosphate. Despite being widely studied enzymes biochemically, PI-PLCs have not been exploited in organic synthesis due to their high specificity and absolute stereochemical requirement in terms of substrate, difficulty in purification,[9] and high cost of protein from commercial suppliers. Therefore their use has been restricted to biochemical analysis of GPI-anchored proteins and carbohydrates. Recently in a significant report,[10] Bruzik et. al. demonstrated PI-PLC catalysed reverse transesterification of *myo*-inositol 1,2-cyclic monophosphate and its utility in synthesis of O-alkyl inositol phosphodiesters, opening opportunities for new and shorter routes to chiral phospho-inositides for signal transduction[11] and anti-signalling drug design.[12]

For our studies on biosynthesis of parasitic GPI molecules and their role in signal transduction, we required I(1:2cyc)P (**2**) and labelled analogues to be prepared by PI-PLC enzyme. Since the isolation of this enzyme is not trivial,[9] we decided to explore the feasibility of using whole cell culture of recombinant *Bacillus subtilis* available to us as a catalyst to prepare I(1:2cyc)P and related labelled and structural analogues. This approach was prompted by reports[4] that *Bacillus subtilis* and other gram-positive bacteria secrete significant amounts of proteins in the culture media during cell growth. Although biotransformations based on whole cells such as baker's yeast have been used[13] in organic synthesis, recombinant bacteria have not been fully exploited. One of the first such examples was recently reported[14] by C.-H. Wong and co-workers in application of a recombinant *E. coli* whole cell system as a catalyst for 1,2-α-mannosyl-transferase mediated practical synthesis of glyco-conjugates.

Herein we report the successful application of a whole cell culture system of the recombinant bacteria *Bacillus subtilis* (transfected with PI-PLC gene from *Bacillus thuringiensis*) as a catalyst for the preparative scale synthesis of chirally pure D-*myo*-inositol 1,2-cyclic monophosphate [I(1:2cyc)P] (**2**), a key compound of biological interest and a potential synthon for various phosphoinositides. The synthesis does not require purified protein and growing cells can be used directly. Other important aspects of this whole cell system are (i) recombinant bacteria can be grown easily by standard procedures, (ii) a cheap and commercially available phospholipid mixture (soybean lecithin, containing 20 % PI) can be used as the substrate instead of pure and expensive PI, (iii) detergents (sodium deoxycholate or triton X100) and critical micellar concentration are not needed, which otherwise are required for reactions with pure enzyme due to its interfacial activity (iv) the reaction can be scaled up to multigram scale and the progress of reaction monitored in cell culture by ^{31}P NMR. The experimental procedure for preparative scale is as follows

Method for synthesis of D-myo-inositol 1,2-cyclic monophosphate from soybean phospholipid mixture.- The sub-culture of *B. subtilis* (BG 2320, transfected with PI-PLC gene from *Bacillus thuringiensis*,[5] with more than 500 fold activity than wild type strain) was grown in standard peptone medium[15] in a one litre scale in a orbital incubator shaker at 37 ^{0}C for 22 hours. To the 400 ml of this growing culture was added phospholipid mixture (12 g soybean lecithin, USB 18245, ~ 20 % PI content) and the incubation was continued for an additional 5 hour during which all the PI was consumed as monitored by TLC ($CHCl_3$: MeOH: NH_3; 7: 3: 0.2) and *in-situ* ^{31}P NMR by taking 400 μl cell-culture aliquots in 5 mm NMR tube containing 50 μl of D_2O. The ^{31}P NMR (without proton decoupling) was recorded at hourly intervals to monitor complete hydrolysis of PI since the product I(1:2cyc)P **2** due to the five-membered cyclic phosphate ring gives a characteristic doublet at 16.1 ppm and PI a broad signal at 0.5 ppm (**Figure 1** shows ^{31}P NMR spectra at (a) the time of addition of lecithin

and (b) after formation of cyclic phosphate **2** after 5 hour incubation). The reaction was essentially complete in 5 hours, and prolonged incubation led to further slow transesterification reactions and formation of inositol-1-phosphate (**4**). After completion of the reaction, the mixture was centrifuged (10000 rpm at 4 ^{0}C) and the supernatant was washed with chloroform-methanol (9:1) to remove other phospholipids. The aqueous layer was freeze dried to give compound **2**. This was further purified by ion-exchange chromatography (Dowex 1 x 8 anion exchange column equilibrated in 100 mM ammonium formate) and desired **2** was eluted with 250 mM ammonium formate buffer. The repeated freeze drying provided 1.08 g D-*myo*-inositol 1,2-cyclic monophosphate (more than 95 % yield, calculated on the basis of 20 % phosphatidylinositol present in lecithin used as starting material, i.e. the entire PI was selectively consumed leaving aside other phospholipids present in lecithin). The product D-*myo*-inositol 1,2-cyclic monophosphate (**2**) was characterised[16] by ^{1}H and ^{31}P NMR and electrospray ionisation mass spectrum.

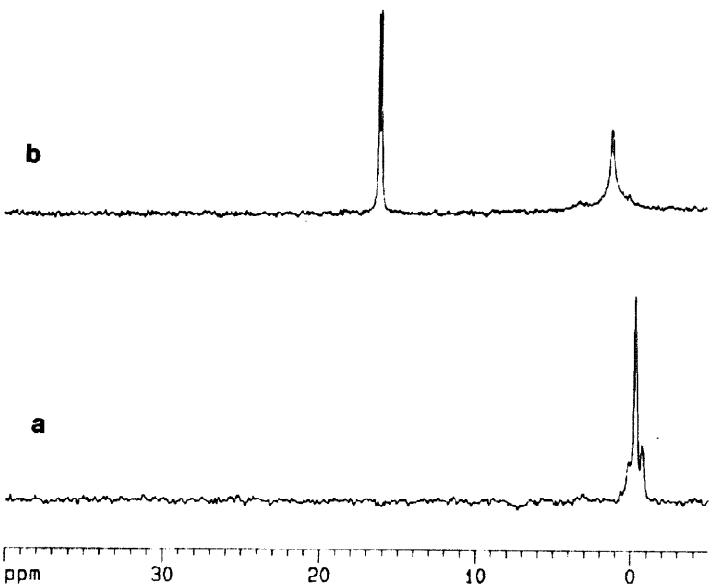

Figure 1

In conclusion, the synthetic approach presented in the paper illustrates the convenient use of a recombinant bacterial whole cell culture for efficient and high yielding synthesis of optically pure D-*myo*-inositol 1,2-cyclic monophosphate (**2**) in a single step without cumbersome procedures of enzyme purification. Moreover a phospholipid mixture can be used as starting material instead of pure substrate. Keeping in view rapid advances in molecular biology allowing a large number of proteins to be produced by genetic methods, application of whole cell or cell-free enzyme preparations from recombinant micro-organisms can be exploited in organic synthesis.

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15. Peptone medium for 1 litre contained peptone (10 g), yeast extract (10 g), NaCl (5 g), K_2HPO_4 (0.4 g), pH adjusted to 7.0 by 1M NaOH solution. This medium was autoclaved and antibiotic chloramphenicol was added to make up 15 μg/ml concentration.
16. ^1H NMR (300 MHz, D_2O) δ 4.51 (*t*, H-2, J = 4.8 Hz, 1H), 4.12 (*ddd*, H-1, J = 4.8, 7.86, 20.2 Hz, 1H), 3.60 (*t*, H-6, J = 8.4 Hz, 1H), 3.54 (*ddd*, H-3, J = 2.0, 3.8, 10 Hz, 1H), 3.44 (*t*, H-4, J = 9.6 Hz, 1H), 3.09 (*t*, H-5, J = 9.8 Hz, 1H); ^{31}P NMR (125 MHz, D_2O, without proton decoupling) δ 16.01 (*d*, J = 20 Hz, P-H1 coupling); Electrospray ionisation MS (negative ion): m/z 241.