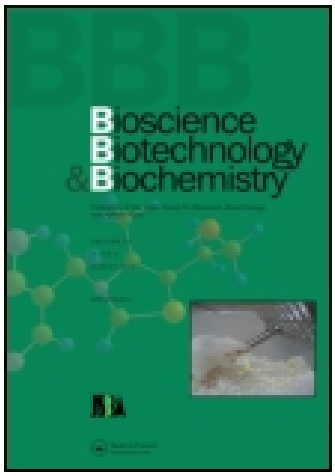

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Short Communication

Enzymatic Synthesis of Phosphatidylinositol Bearing Polyunsaturated Acyl Group

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A new route for the synthesis of phosphatidylinositol (PI) having a polyunsaturated fatty acyl group was developed by using lipase and phospholipase C as biocatalysts to supplement the normal chemical reactions.

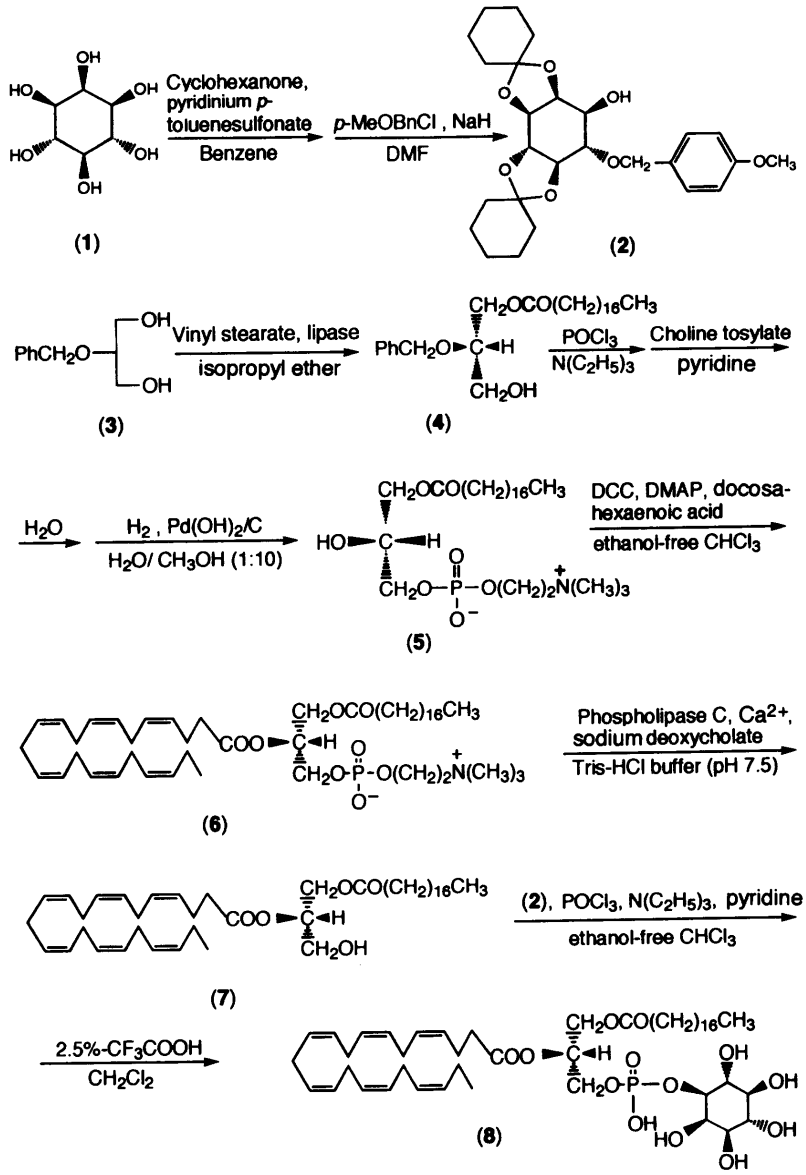
Key words: phosphatidylinositol; phospholipase C; lipase P; ion spray mass spectrometry; atmospheric pressure ionization mass spectrometry

Phosphatidylinositols (PIs) are one of the typical glycerophospholipids ubiquitously found in the biological kingdom as a constituent of cell membranes. In recent years, however, PIs and their di- and tri-phosphates have been found to play an important role in cellular signal transduction in biological systems that is known as the phosphatidylinositol cycle.[1] These PIs are known to have a polyunsaturated fatty acyl group at the *sn*-2 position of the phosphatidyl moiety. The mechanism for these PUFA-PIs in signal transduction, however, still remains to be solved at the molecular level. In order to allow future studies in this field, an ample supply of such PUFA-PIs with high purity in a large amount is essential, and this may only be achieved by a synthetic method because of difficulties in isolating them from natural materials on a large scale. Although some synthetic methods for producing PIs have already been developed,[2] they seem to be limited to those compounds having a simple saturated fatty acid like palmitic and stearic acids. In most of the methods reported, catalytic hydrogenolysis has been used as one of the important steps[2] to remove the various protecting groups of hydroxyls in glycerol, as well as those of phosphates. Hydrogenolysis, however, cannot be applied to the chemical conversion of lipids bearing polyunsaturated fatty acyl groups since the process unnecessarily saturates the olefinic bonds to afford a saturated fatty acyl group. To get around this difficulty, we have developed another method as shown in the Scheme. The synthetic route involves preparing a protected *myo*-inositol (**2**) and a diacylglycerol (**7**) having a docosahexaenolyl group, forming a phosphodiester linkage between **2** and **7**, and one-step removal of the protected groups under mildly acidic conditions to afford the final product (**8**). Two enzymes were employed for the enantioselective monostearoylation of the diol (**3**) in the short route synthesis of an optically active lysophosphatidylcholine[3] and for chemoselective hydrolysis of the phosphodiester linkage in **6**. Throughout all the reactions and work-up, extreme care was taken to prevent the autoxidation of all *cis* unconjugated olefinic components by adding butylated hydroxytoluene (BHT) and by completely replacing the air in each reaction mixture with nitrogen gas. All the reagents are given in the Scheme. Cyclohexylidene *myo*-inositol (the structure is not shown), a precursor of **2**, has been prepared in a low yield (<5%) by Angyal *et al.*[3] and by Gou *et al.*,[4] using *p*-toluenesulfonic acid after its isolation from a complicated mixture containing various isomers and those protected with only one cyclohexylidene group. The yield was, however, significantly improved (11%) by using pyridinium *p*-toluenesulfonate instead of the acid in our present study. The resulting decrease in acidity seems to have been responsible for the improvements since catalytic dehydration is essentially reversible under acidic conditions.

Diol **3** in isopropyl ether, was submitted to lipase (Amano P)-catalyzed stearoylation to give **4** with an *S*-configuration and 96% optical purity ($[\alpha]_D^{25}$ −10.1, *c* 1.48, ethanol-free $CHCl_3$).[5] This compound was converted to a phosphatidylcholine (**6**) *via* its lyso-form (**5**) by phosphodiester formation, catalytic hydrogenolysis of the benzyl ether, and DCC-mediated acylation of the hydroxy group at the *sn*-2 position. Enzymatic hydrolysis of the phosphocholine (PC; **6**) was performed under micellar condition containing the PC (100 mg, 0.12 mmol), sodium deoxycholate (0.8% aq. solution, 5.0 ml), aqueous $CaCl_2$ (1.0 ml, 20 mM), trace of BHT, and phospholipase C from *Bacillus cereus* (5 units, Sigma Chem. Co.) in a mixture of distilled H_2O (10 ml) and a Tris–HCl buffer (pH 7.5, 0.2 M, 5.0 ml). After the reaction at room temperature for 4–5 h, the product (**7**) was extracted with ether and purified by silica gel column chromatography, eluting, with hexane–ethyl acetate (8:2, 83% yield from **6**). The glycerol thus obtained was submited to phosphodiester synthesis with the protected *myo*-inositol (**2**) under the same conditions as those used for the synthesis of **5**, apart from the amount of pyridine used. We found that the reaction mixture of **5** by a conventional phosphodiester synthesis was slightly acidic (pH 5–6). When the same reaction conditions were applied for the synthesis of **8**, the acid-labile cyclohexylidene groups were found to have been partially removed from random positions of the *myo*-inositol moiety during the reaction, giving a complicated mixture of reaction products. Therefore, by increasing the amount of pyridine by 1.5 times, the reaction yield was greatly improved.

Final removal of the protective groups in one step was completed by using dilute trifluoroacetic acid in methylene chloride at room temp. for 2.5 h. After this reaction, the excess trifluoroacetic acid was removed by azeotropic distillation with toluene under reduced pressure in the presence of trace of BHT. Silica gel column chromatography [$CHCl_3$–CH_3OH–28%NH_4OH (6:3:1)] of the residue afforded the desired product (**8**) as a resinous solid in a 78% yield.

The structure of **8** was characterized by ^{1}H-NMR[6] and atmospheric pressure-ion spray (IS) ionization mass spec-



Scheme Synthetic Scheme for Producing 2-Docosahexaenoyl-1-stearoyl-*sn*-glycerophosphoinositol.

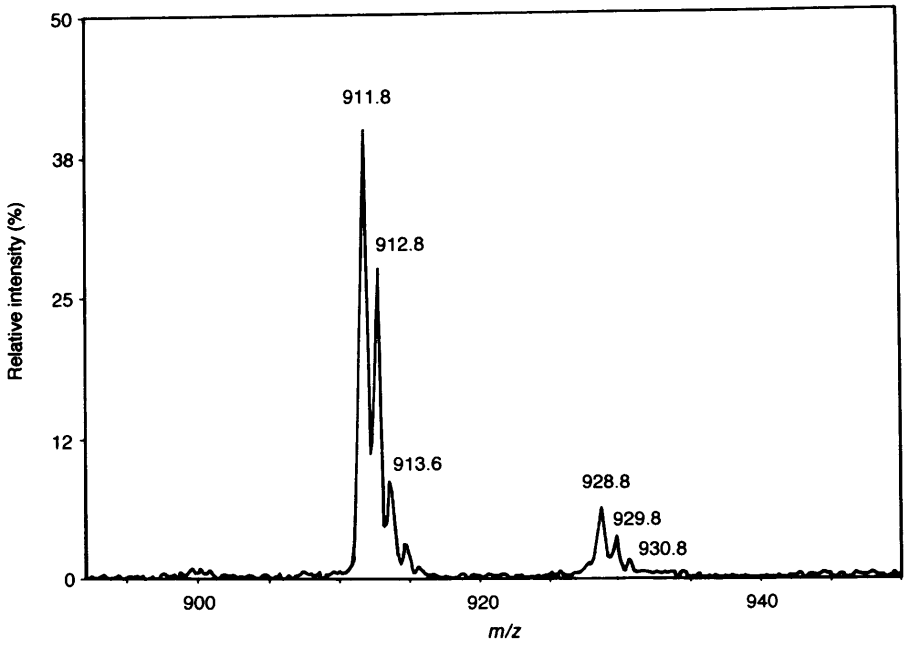


Fig. Ion Spray Mass Spectrum of 2-Docosahexaenoyl-1-stearoyl-*sn*-glycerophosphoinositol Recorded on a Sciex Triple Quadrupole Mass Spectrometer. The sample solution was infused (50 ppm in 50% aqueous CH_3CN with 0.1% CF_3COOH) at 5.0 ml/min through the ion-spray interface.

trometry (API-III, Sciex, Perkin-Elmer) as depicted in the Fig. This application of IS to phosphatidylinositol seems to be the first example so far, and a peak is clearly apparent for a positively charged (protonated) molecular ion at 911.8 *m/z*. Other peaks at 912.8 and 913.6 are those of isotopic molecular ions. In addition, positively charged mono-valent ion species incidentally formed by the addition of an ammonium ion (NH_4^+) gave *m/z* at 928.8. The ammonium ion probably came from the elution solvent of the final column chromatography already described. The presence of the phosphorus atom was shown by the molybdenum reagent.

By following the same route, 2-linoleoyl-1-stearoyl-*sn*-phosphatidylinositol (the structure is not shown) was also synthesized.[7]

Although further work is required to establish the process described in this paper into a general route, the result of this study suggest that a synthetic route is possible for preparing phosphatidylinositols having a polyunsaturated fatty acyl group at the *sn*-2 position and will contribute to further synthetic work on related phosphatidylinositols having such acyl groups even at the *sn*-1 position, as well as at *sn*-2 of the lipids.

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6) TLC ($CHCl_3$–CH_3OH–28%NH_4OH, 3:2:1): $R_f = 0.38$; MASS (ion spray ionization): *m/z* 911.8 $(M+H)^+$, $C_{49}H_{77}O_{13}P$ requires 910.8. ^{1}H-NMR δ_H (500 MHz; $CDCl_3$): 0.85 (3H, br.t, Me of stearoyl group), 0.89 (3H, br.t, Me of docosahexaenoyl group), 1.58 (2H, m, $OCOCCH_2$), 2.20–2.40 (4H, m, $OCOCH_2$), 2.80 (8H, m, $C=C-CH_2-C=C \times 4$), 3.25–4.40 (10H, m, inositol methylene protons and $O-CH_2-C-CH_2-O$), 5.21 (1H, m, O–C–CH–C–O), 5.30–5.40 (12H, m, olefine protons).
7) TLC ($CHCl_3$–CH_3OH–28%NH_4OH, 6:3:1): $R_f = 0.23$; MASS (ion spray ionization): *m/z* 863.5 $(M+H)^+$, $C_{45}H_{77}P_{13}P$ requires 862.5. ^{1}H-NMR δ_H (500 MHz; $CDCl_3$): 0.87 (6H, br.t, Me), 1.58 (2H, m, $OCOCCH_2$), 2.02 (4H, m, $CH_2-C=C-C-C=C-CH_2$), 2.20–2.40 (4H, m, $OCOCH_2$), 2.78 (2H, m, $C=C-CH_2-C=C$), 3.25–4.40 (10H, m, inositol methine protons and $O-CH_2-C-CH_2-O$), 5.20 (1H, m, O–C–CH–C–O), 5.30–5.40 (4H, m, olefine protons).