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Phosphorylation of Unnatural Phosphatidylinositols with Phosphatidylinositol 3-Kinase

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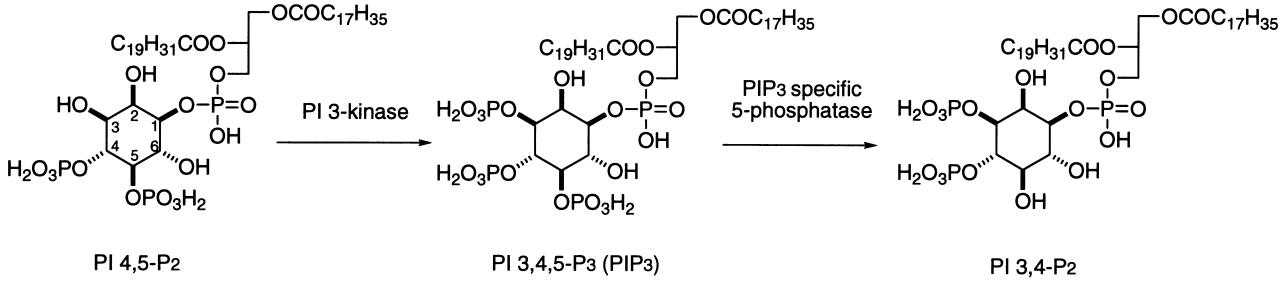
Abstract—Phosphatidylinositol analogs (PI_{C2}–PI_{C18}) having a series of saturated fatty acid (C2–C18) at *sn*-2 position were synthesized and subjected to the phosphorylation reaction with phosphatidylinositol 3-kinase (PI 3-kinase). The reactivity of PI_{C8} with PI 3-kinase turned out to be comparable to that of natural PI, although PI_{C18} was not phosphorylated under the same condition. The chirality of *sn*-2 center was not responsible for the phosphorylation reaction. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Phosphatidylinositol 3-kinase (PI 3-kinase) is an enzyme that catalyzes phosphorylation at D-3 position on the inositol ring of polyphosphoinositides.[1] The p110–p85 heterodimer type PI 3-kinase consists of catalytic subunit (molecular weight 110 kDa) and regulatory adapter subunit (85 kDa)[2] is activated in vivo by platelet-derived growth factor (PDGF), insulin and other extracellular stimulations.[3] This enzyme phosphorylates phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2) to give phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is then dephosphorylated by PIP_3 specific 5-phosphatase[4] to produce phosphatidylinositol 3,4-bisphosphate (PI 3,4-P_2) (Scheme 1). PIP_3 and PI 3,4-P_2 are considered to be the second messengers and/or their precursors, and their roles and functions in the regulation of cell proliferation, differentiation, apoptosis and many other biological events are now becoming clear.[5]

In vitro, the PI 3-kinase phosphorylates phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI 4-P) and PI 4,5-P_2 to give phosphatidylinositol 3-phosphate (PI 3-P), PI 3,4-P_2 and PIP_3, respectively.[1b] Natural PI (**1**), which mainly contains stearoyl ($COC_{17}H_{35}$) at the *sn*-1 position and arachidonoyl ($COC_{19}H_{31}$) at the *sn*-2 position of the diacylglycerol (DAG) substructure, is an excellent substrate of the PI 3-kinase.[6] The syntheses of PI, PI phosphates and the derivatives have been reported and several reports describe the importance of the fatty acids in the DAG substructure. However, systematic analysis focusing on their enzymatic reactions has not been carried out yet.[7] Recently, we found that the saturated PI (**2**, *sn*-2 eicosanoate: $COC_{19}H_{39}$),



Scheme 1.

Keywords: carboxylic acids and derivatives; enzymes and enzyme reactions; inositols; phosphoric acids and derivatives.
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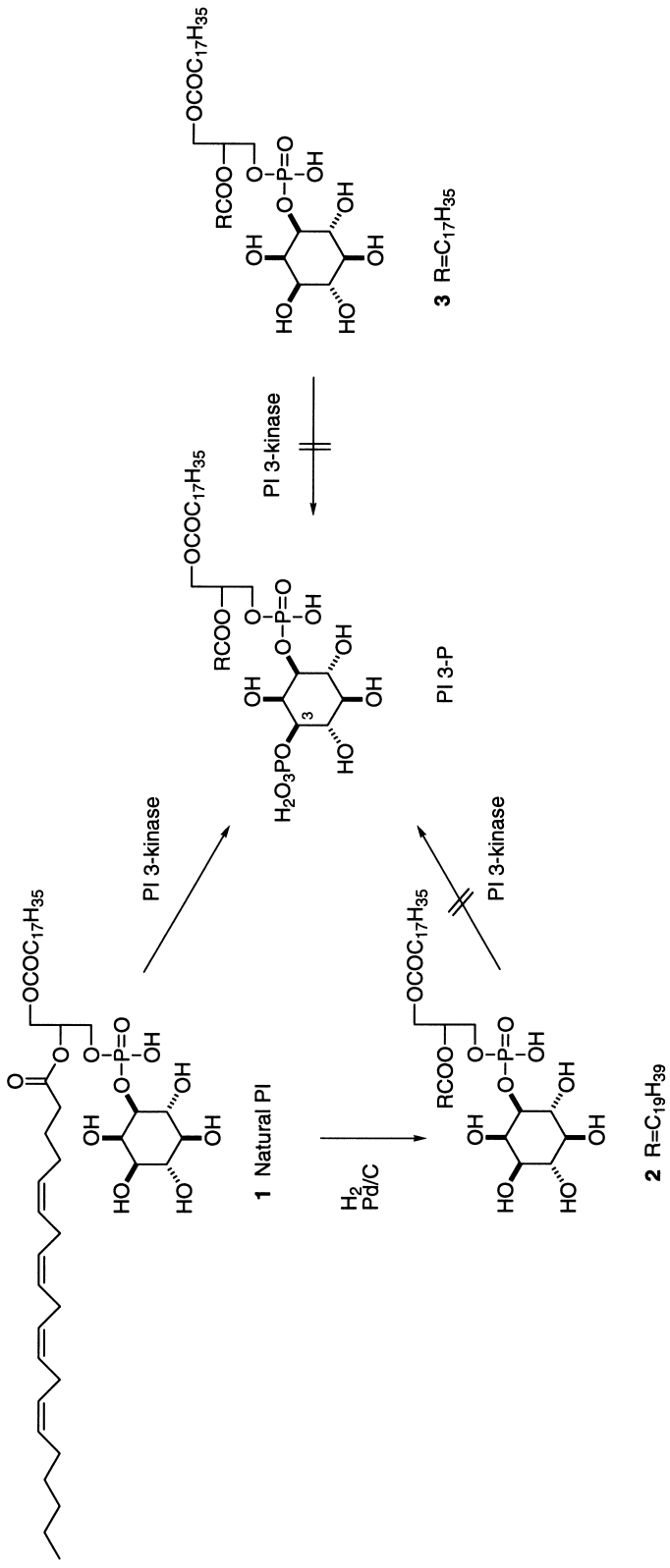
PII: S0040-4020(00)00161-7


PI 3-kinase
PI 3-kinase
PI 3-kinase
1 Natural PI
H2
Pd/C
PI 3-P
2 R=C19H39
3 R=C17H35

Scheme 2.

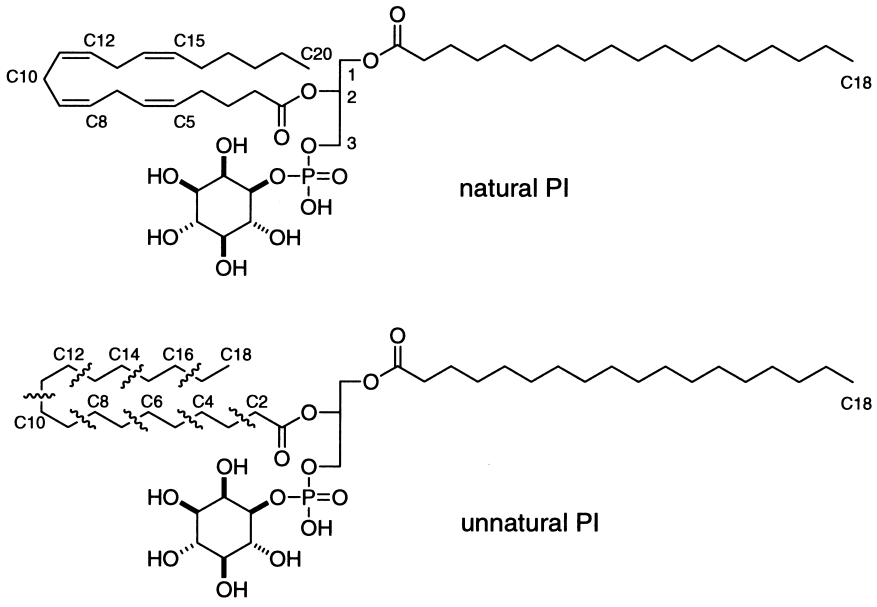


Figure 1.

prepared by hydrogenation of natural PI, was not phosphorylated by the PI 3-kinase. Synthetic distearate PI (**3**) was also unchanged by the same reaction (Scheme 2). Therefore, we designed a series of saturated PI to clarify the contribution of the *sn*-2 fatty acid ester in this phosphorylation reaction (Fig. 1). These unnatural PIs [PI_{C2}–PI_{C18}] were prepared according to the syntheses of PIP_3 and its analogs.[8] Synthesized unnatural PI analogs were subjected to the enzymatic reaction with ATP, and the products were analyzed by negative ion fast atom bombardment mass spectrometry (FAB MS) using optimized matrix.[9–11] Phosphorylation of each unnatural PI with [γ-^{32}P]-ATP was also monitored by autoradiography of the phosphorylated products.[12]

Results

Synthesis of unnatural *sn*-2 fatty acid analogs of PI

Synthesis of a series of unnatural PI analogs with varied chain length of saturated *sn*-2 fatty acid was carried out by the procedure similar to our synthesis of PIP_3 analogs.[8c] The *sn*-1 fatty acid ester was fixed as stearate in all compounds. The synthesis of each PI_{cn} (n=2–18) was shown in Schemes 3 and 4. The diol **5** was prepared from (*S*)-(+)-2,3-dimethyl-1,3-dioxolane-4-methanol (**4**) by conventional method. The amidites with two different acyl groups (**9**) were prepared from **5** according to the method of Bannwarth et al.[13] by utilizing a series of saturated fatty acids.[7e,8c] Suitably protected homochiral *myo*-inositol (**10**) was prepared[8c] from D-glucose by the method of Estevez and Prestwich.[7b] Coupling of **10** and respective **9** followed by oxidation with *m*-chloroperbenzoic acid (*m*CPBA) in one pot gave fully protected PIs (**11**).[8c,14] Oxidative removal of the 4-methoxybenzyl groups with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in wet CH_2Cl_2,[15] and hydrogenolysis of benzyloxymethyl ether with Pd black in 85% *t*-BuOH containing $NaHCO_3$[7e] gave a series of unnatural PIs (PI_{C2}, PI_{C4}, PI_{C8}, PI_{C12}, PI_{C16}, PI_{C18}). To screen the possible substrate of the PI 3-kinase by negative ion FAB MS, a mixture of seven unnatural PI analogs was also synthesized by similar procedure utilizing the amidite **9** prepared from an equimolar mixture of seven saturated carboxylic acids with respective carbon numbers of 4, 6, 8, 10, 12, 14 and 16.

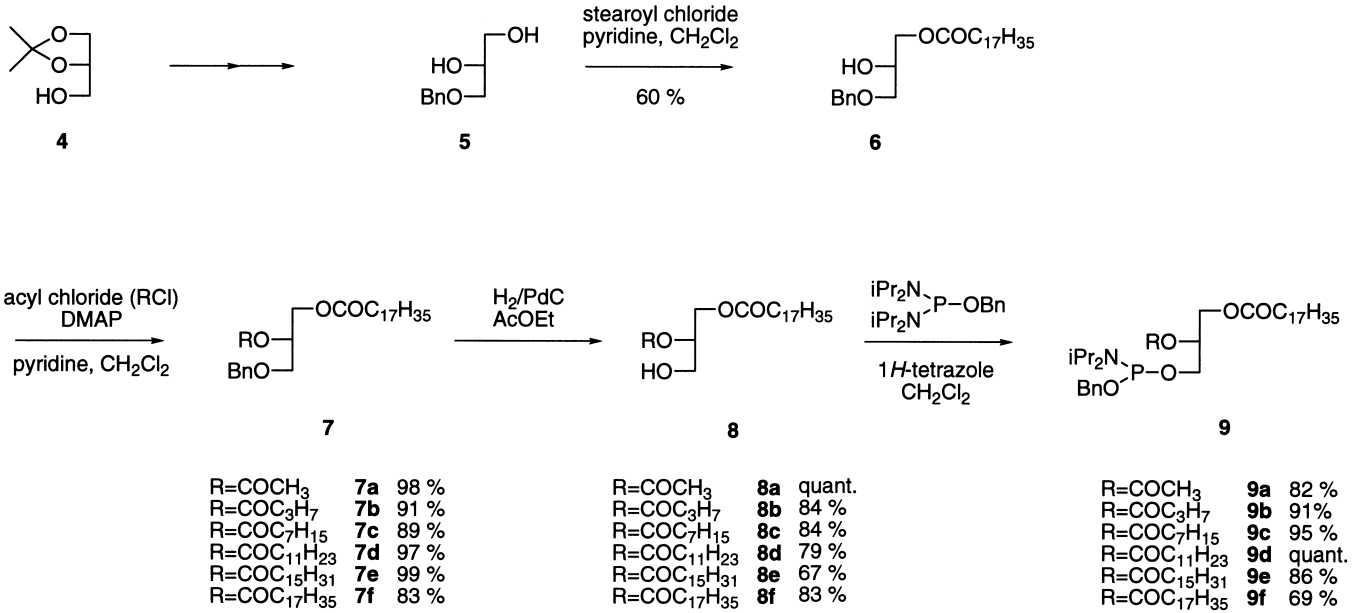
PI 3-kinase reaction of unnatural PIs

Synthesized unnatural PI analogs were mixed with phosphatidylserine (PS) and subjected to the PI 3-kinase reaction.[16] The procedures are described in Experimental. The reaction was stopped by treatment with $CHCl_3$–CH_3OH–c.HCl, and the crude $CHCl_3$ extracts were subjected to the following analyses.

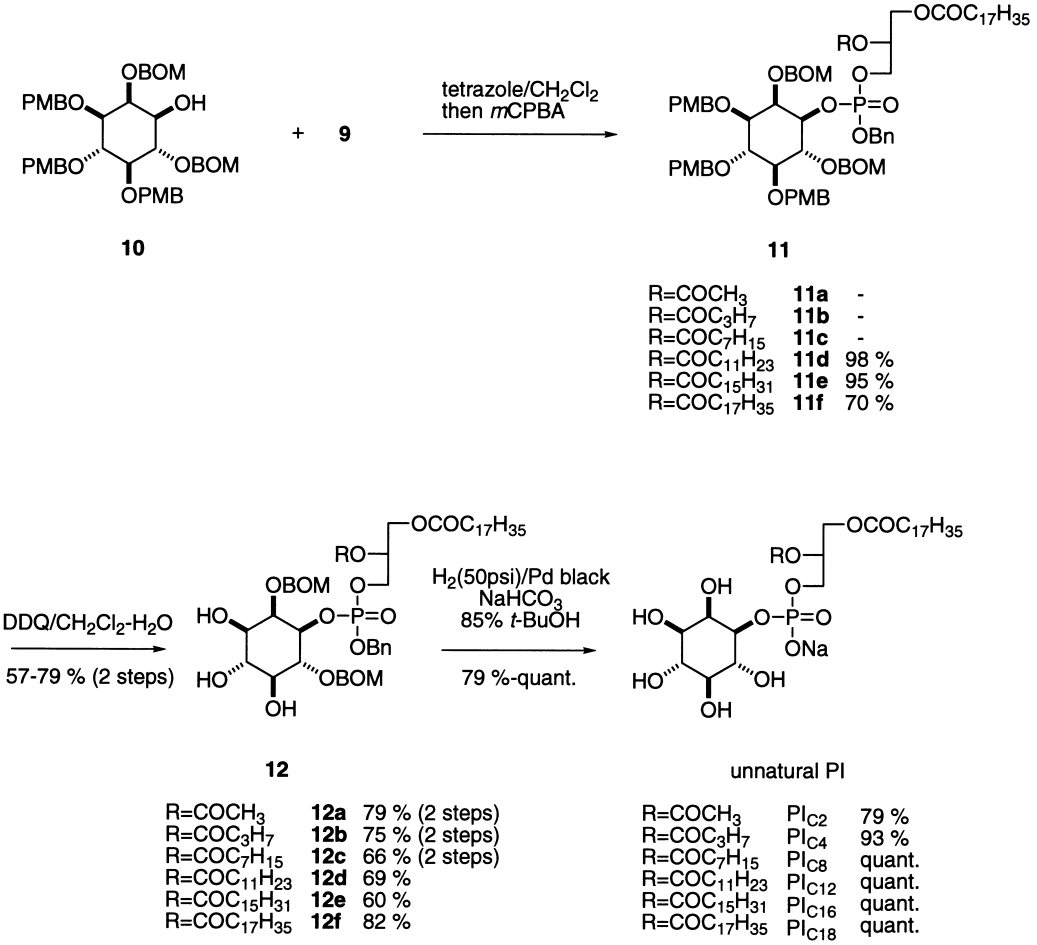
Analysis of the PI 3-kinase reaction products of unnatural PI analogs by negative ion FAB MS: matrix effect of PI, PI 4-P and PI 4,5-P_2 in negative ion FAB MS

Efficient detection of PI 3-P is indispensable because the average yield of PI 3-P in the enzyme reaction of unnatural PI was about 20–30%, and relatively large amount of unreacted PI analogs were still contained in the $CHCl_3$ extracts of the enzyme reaction mixture. To detect the enzymatically produced PI 3-P, optimization of liquid matrix is very important for efficient FAB MS analysis.[17] Triethanolamine (TEA)[11b,11d], diethanolamine (DEA)[11e] and glycerol (GLY)[11a] have been used as matrices for negative ion FAB MS analysis of PI and PI phosphates. Since PI 3-P was commercially unavailable, PI 4-P was employed to optimize matrices instead. Matrix effect was examined by monitoring the intensity of the $[M-H]^-$ ion peak with matrices TEA, DEA, GLY and their mixtures, and results are shown in Fig. 2.

By use of mixed matrix of TEA–GLY at a ratio of 3:1, the maximum intensity of $[M-H]^-$ ion peak of PI 4-P was two and half times higher than with TEA alone. On the contrary,


stearoyl chloride
pyridine, CH2Cl2
60 %
4
5
6
acyl chloride (RCl)
DMAP
pyridine, CH2Cl2
H2/PdC
AcOEt
iPr2N
iPr2N
P–OBn
1H-tetrazole
CH2Cl2
7
8
9
R=COCH3 7a 98 %
R=COC3H7 7b 91 %
R=COC7H15 7c 89 %
R=COC11H23 7d 97 %
R=COC15H31 7e 99 %
R=COC17H35 7f 83 %
R=COCH3 8a quant.
R=COC3H7 8b 84 %
R=COC7H15 8c 84 %
R=COC11H23 8d 79 %
R=COC15H31 8e 67 %
R=COC17H35 8f 83 %
R=COCH3 9a 82 %
R=COC3H7 9b 91%
R=COC7H15 9c 95 %
R=COC11H23 9d quant.
R=COC15H31 9e 86 %
R=COC17H35 9f 69 %

Scheme 3.



Scheme 4.

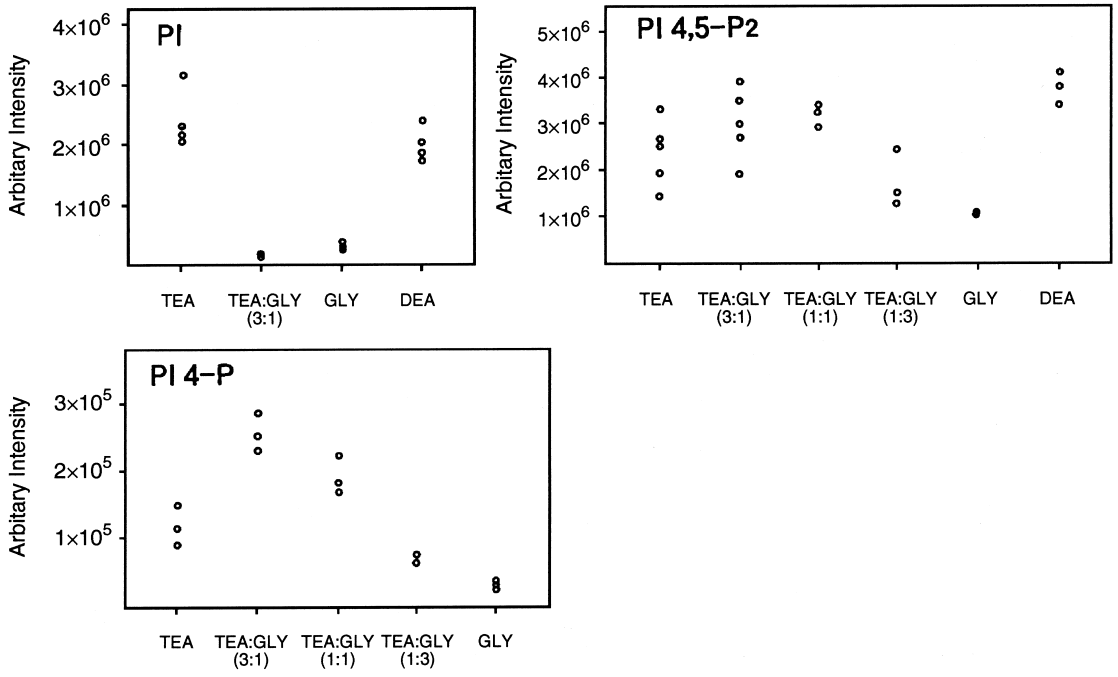
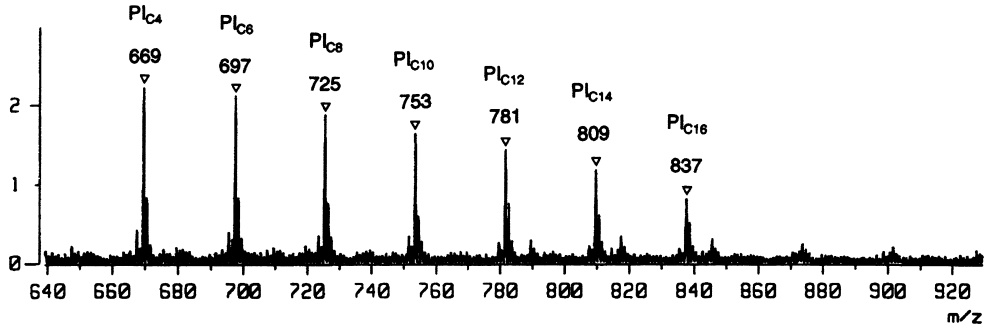


Figure 2. Matrix effects of PI, PI 4-P and PI 4,5P_2 in negative ion FAB MS. Intensities of $[M-H]^-$ ion peaks were compared in matrices triethanolamine (TEA), glycerol (GLY), diethanolamine (DEA) or their mixture.

(a). Spectrum of a mixture of synthetic unnatural PIs (PIc_4, PIc_6, PIc_8, PIc_{10}, PIc_{12}, PIc_{14}, PIc_{16}). Triethanolamine (TEA) was used as the matrix.



(b). Spectrum of PI 3-kinase reaction products derived from the mixture of synthetic unnatural $PIc_{4\text{-}C16}$. TEA-glycerol (3:1) was used as the matrix.

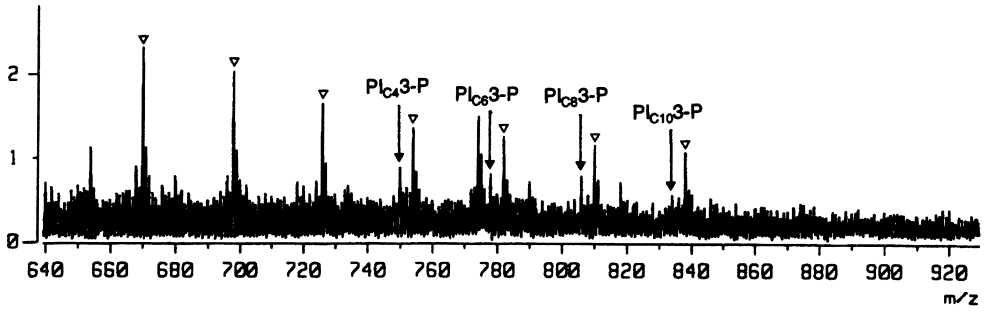


Figure 3. Negative ion FAB MS of a mixture of PIs and its PI 3-kinase reaction products (a) Spectrum of a mixture of synthetic unnatural PIs (PI_{C4}, PI_{C6}, PI_{C8}, PI_{C10}, PI_{C12}, PI_{C14}, PI_{C16}). Triethanolamine (TEA) was used as the matrix. (b) Spectrum of PI 3-kinase reaction products derived from the mixture of synthetic unnatural PI_{C4-C16}. TEA–glycerol (3:1) was used as the matrix.

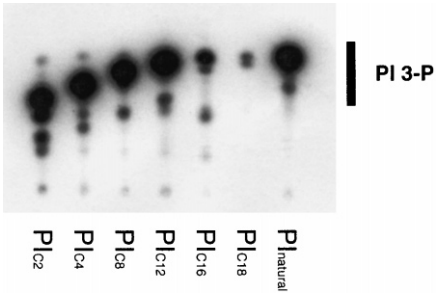


Figure 4. Autoradiography of phosphorylation products of natural PI and unnatural PIs with PI 3-kinase and [γ-^{32}P]-ATP.

the intensity of $[M-H]^-$ of PI was relatively low with TEA–GLY(3:1), and high with TEA and DEA, respectively. Therefore, it was expected that the relative intensity of $[M-H]^-$ ion of PI 4-P was higher than that of PI by analyzing an equimolar mixture of PI 4-P and PI with TEA–GLY(3:1). Assuming that the sensitivity of $[M-H]^-$ ions of PI 3-P and PI 4-P were obtained in similar fashion, TEA–GLY (3:1) would be a desirable matrix for the analysis of PI 3-P in an enzyme reaction mixture even though a large amount of unreacted PI is contaminated.

Effects of matrices with PI and PI 4,5-P_2 were also examined. The intensity of $[M-H]^-$ ion of PI 4,5-P_2 was

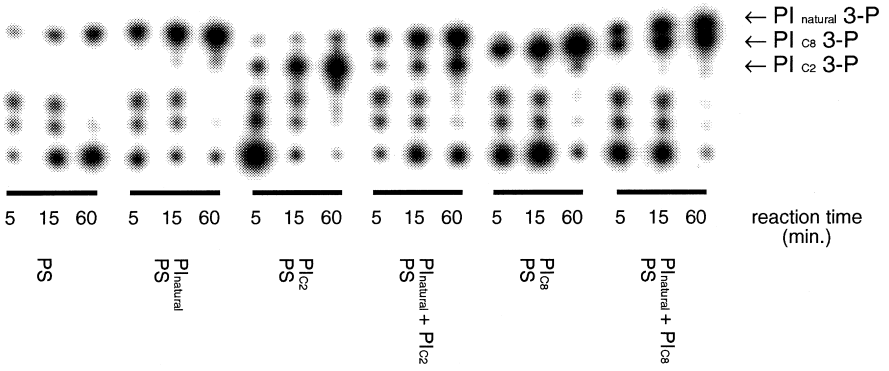
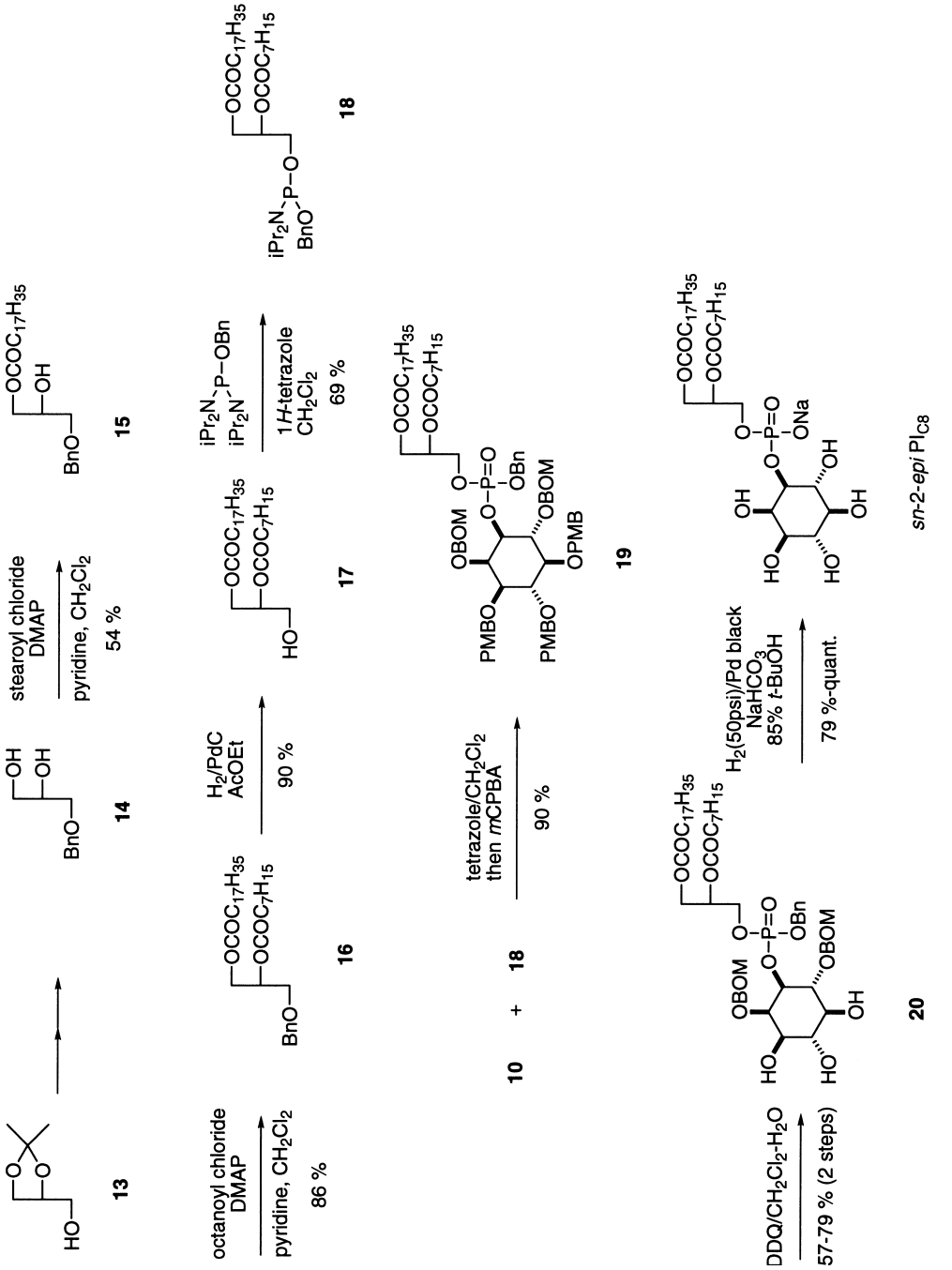


Figure 5. Autoradiography of phosphorylation products of natural PI, unnatural PI_{C2} and PI_{C8} with PI 3-kinase and [γ-^{32}P]-ATP under competitive conditions. (PS: phosphatidylserine).


HO
13
OH
OH
BnO
14
stearoyl chloride
DMAP
pyridine, CH2Cl2
54 %
OCOC17H35
OH
BnO
15
octanoyl chloride
DMAP
pyridine, CH2Cl2
86 %
OCOC17H35
OCOC7H15
BnO
16
H2/PdC
AcOEt
90 %
OCOC17H35
OCOC7H15
HO
17
iPr2N
iPr2N-P-OBn
1H-tetrazole
CH2Cl2
69 %
OCOC17H35
OCOC7H15
iPr2N
BnO-P-O
18
10 + 18
tetrazole/CH2Cl2
then mCPBA
90 %
OCOC17H35
OCOC7H15
OBOM
O-P=O
OBn
PMBO
OBOM
PMBO
OPMB
19
DDQ/CH2Cl2-H2O
57-79 % (2 steps)
OCOC17H35
OCOC7H15
OBOM
O-P=O
OBn
HO
OBOM
HO
OH
20
H2(50psi)/Pd black
NaHCO3
85% t-BuOH
79 %-quant.
OCOC17H35
OCOC7H15
O-P=O
ONa
OH
O-P=O
OH
HO
OH
HO
sn-2-epi PIC8

Scheme 5.

unstable in most matrices tested, however, relatively constant and strong $[M-H]^-$ ion was observed with DEA.

Negative ion FAB MS of PI 3-kinase reaction products

Negative ion FAB MS spectrum of a mixture of synthetic PI analogs [PI_{C4}–PI_{C16}] is shown in Fig. 3(a). The $[M-H]^-$ ions of the respective PI analogs are indicated by the peaks at *m/z* 669, 697, 725, 753, 781, 809 and 837. Although the PI analogs were prepared starting from an equimolar mixture of seven fatty acids, intensity of the $[M-H]^-$ ions of longer fatty acid analogs appeared lower. The enzymatic phosphorylation of unnatural PI analogs with PI 3-kinase was repeated several times to enrich PI 3-P and thus the obtained reaction mixture was analyzed by negative ion FAB MS with matrix of TEA–GLY (3:1). One of them is shown in Fig. 3(b). The $[M-H]^-$ ion peak of PI_{C4} 3-P, PI_{C6} 3-P, PI_{C8} 3-P, PI_{C10} 3-P is observed at *m/z* 749, 777, 805, and 833, respectively. These spectra indicated that PI_{C4}, PI_{C6}, PI_{C8} and PI_{C10} were obviously phosphorylated by the PI 3-kinase giving PI_{C4} 3-P–PI_{C10} 3-P in good yield.[18] As the peak intensities of PI 3-P with longer fatty acids were very low, the yield was shown quite low.

TLC and autoradiography analyses of PI 3-kinase reaction products of unnatural PI analogs

The results of negative ion FAB MS described above were also confirmed by the following experiments. Each of the six unnatural PI analogs [PI_{C2}, PI_{C4}, PI_{C8}, PI_{C12}, PI_{C16}, PI_{C18}] was subjected to the enzyme reaction with [γ-^{32}P]ATP, and after development on TLC, the produced ^{32}P-labeled PI 3-Ps were detected by autoradiography. The results are shown in Fig. 4. The spot of PI_{C2} 3-P, PI_{C4} 3-P, PI_{C8} 3-P, PI_{C12} 3-P was clearly detected, respectively. On the other hand, the spot of PI_{C16} 3-P was very faint and that of PI_{C18} 3-P was negligible. These data demonstrated that PI_{C2}, PI_{C4}, PI_{C8} and PI_{C12} behaved as the substrate of PI 3-kinase (Fig. 4).

Enzymatic reactivity of PI_{C2}, PI_{C8}, and natural PI

The reactivity of synthetic unnatural PI with PI 3-kinase was evaluated by competitive phosphorylation with natural PI. Equimolar mixtures of PI_{C2} and natural PI, and PI_{C8} and natural PI were respectively incubated with the PI 3-kinase for 5, 15 and 60 min in the presence of PS and [γ-^{32}P]-ATP. The reaction mixtures were developed on TLC, and the radioactivity of the resulting PI 3-P was visualized by autoradiography (Fig. 5). The blank phosphorylation reaction was also carried out with PS, natural PI and PS, PI_{C2} and PS, and PI_{C8} and PS, respectively. From the equimolar mixture of natural PI and PI_{C8}, equivalent amounts of PI 3-P and PI_{C8} 3-P were produced. This result demonstrated that the reactivity of PI_{C8} was comparable to that of natural PI. As the amount of PI_{C2} 3-P was smaller than that of natural PI 3-P, PI_{C2} is slightly less reactive than natural PI.[6]

Comparison of the enzymatic reactivity of PI_{C8} and *sn*-2 epimer of PI_{C8}

As shown in Fig. 5, the reactivity of PI_{C8} was similar to that of natural PI. Therefore, we turned our attention to whether PI 3-kinase could differentiate the absolute stereochemistry of *sn*-2 position. Synthesis of *sn*-2-*epi* PI_{C8} was performed from *R*-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol (**13**)[19] according to the synthesis of unnatural PIs (Scheme 5).

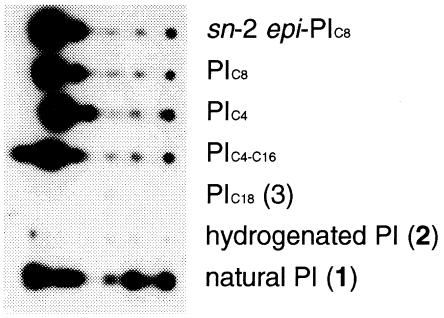


Figure 6. Autoradiography of phosphorylation products of natural PI, hydrogenated PI, PI_{C18} and other unnatural PIs by PI 3-kinase and [γ-^{32}P]-ATP.

The reactivity of PI_{C8} and *sn*-2-*epi* PI_{C8} was compared by independent phosphorylation with the PI 3-kinase and [γ-^{32}P]-ATP by the same procedure as those for unnatural PIs, respectively. The reactions of natural PI (**1**), hydrogenated PI (**2**), PI_{C18} (**3**), PI_{C4-16} mixture, and PI_{C4} were also done for comparison. The crude reaction products were developed on TLC plate and visualized by autoradiography, and the result was shown in Fig. 6. Interestingly, phosphorylation of PI_{C8} and *sn*-2-*epi* PI_{C8} proceeded equally to produce PI_{C8} 3-P and *sn*-2-*epi* PI_{C8} 3-P, indicating that the PI 3-kinase did not differentiate the absolute stereochemistry at *sn*-2 center.[20]

Discussion

Systematic studies on substrate specificity of the enzymatic reaction revealed an unknown structural factor of DAG substructure. In the phosphorylation of PI by PI 3-kinase, arachidonoate at *sn*-2 position of natural PI can be replaced by short saturated fatty acyl group. Folded unsaturated arachidonoate is expected to loosen the binding of PI to lipid bilayer, which might be an essential factor for efficient assembly of the enzyme and the substrate. PI with short fatty acid at *sn*-2 also get flexibility and might be able to come close to the enzyme like arachidonoate rather than e.g. stearate which is tightly fixed in the lipid bilayer.[21] The short saturated fatty acids such as octanoate might be equivalent of the folded unsaturated arachidonoate and the spherical size of the side chain at *sn*-2 might be crucial for the PI 3-kinase activity. These results may be applied to the design of artificial second messenger, PI 3-kinase inhibitors, affinity probes to find the specific binding probes, and photoaffinity probes.[8b,8d]

Experimental

Materials

Bovine liver PI ammonium salt and bovine brain PI 4-P ammonium salt, and bovine brain PI 4,5-P_2 ammonium

salt for FAB MS analyses were purchased from Sigma Chemical Co. and Boehringer Mannheim Corp., respectively, and bovine brain PI and bovine brain PS for enzymatic reaction were from Avanti. TEA, GLY and DEA were purchased from Tokyo Kasei Kogyo Co., Ltd. and Wako Pure Chemical Industries, Ltd., respectively.

Enzymatic reaction with PI 3-kinase[16]

The PI 3-kinase was purified from Sf9 cells co-expressing GST-p85 and p110, the two subunits of the heterodimer.[22]

The enzymatic reaction was carried out as follows: A solution of unnatural PI analog (0.2 μg) and PS (10 μg) in dimethylsulfoxide (0.5 μl) was dispersed into 50 μl of buffer A containing 20 mM Tris/HCl buffer (pH 7.5), 100 mM NaCl and 0.5 mM EGTA to form micelles. The reaction was started by the addition of the PI 3-kinase, and [γ-^{32}P]-ATP (10 μM, 5 μCi) and $MgCl_2$ (final 5 mM) dissolved in the buffer A. After incubation of the mixture for 1 h at 25°C, the reaction was stopped by the addition of 100 μl of $CHCl_3$–CH_3OH–c.HCl (100:200:10). The solution was stirred with a Vortex mixer, and the $CHCl_3$ solution containing unnatural PI and the reaction product was separated by centrifugation. The aqueous solution was re-extracted with 200 μl of $CHCl_3$, and the $CHCl_3$ solutions were combined. The solvent of the solution was removed in vacuo. The extracts were prepared by TLC, and the reaction products were detected by autoradiography. Samples for FAB MS analyses were obtained by the reaction of a mixture of seven unnatural PI analogs (PI_{C4}–PI_{C16}, 20–30 μg) and PS (200–300 μg), and with unlabeled ATP. The yield of PI 3-kinase was 20–30%.

Instrumentation and sample preparation for FAB MS analysis

Negative ion FAB mass spectra were recorded on a JEOL JMS-HX110 double-focusing mass spectrometer of EBE arrangement with a JMS-DA7000 data system. Ion acceleration voltage was 10 kV, and the fast-atom xenon gas was accelerated at a voltage of 6 kV.

Sample solutions for experiments of matrix effects shown in Fig. 2 were prepared by mixing 1 μg sample and 0.5 μl of matrix on FAB MS target. Negative ion FAB mass spectra were obtained by means of a 2.8 s scan from *m/z* 810 to 940 for PI, 2.7 s scan from *m/z* 890 to 1020 for PI 4-P, and 2.6 s scan from *m/z* 970 to 1100 for PI 4,5-P_2. Data were collected every 10 s, and seven values from the start to 1 min of the analysis were averaged.

For the analysis of unnatural PI analogs, TEA was used as the matrix, and TEA–GLY (3:1) was used for the PI 3-kinase reaction products of the unnatural PI analogs. Prior to the FAB MS analysis, the $CHCl_3$ extracts of the enzyme reaction products were re-treated with $CHCl_3$–CH_3OH–1 N HCl (10:5:1), and the $CHCl_3$ layer containing free acid form PIs and PI 3-Ps were analyzed.

TLC preparation

TLC plate (Silica Gel 60 plastic sheet) was pre-treated by developing with methanol–1% potassium oxalate (1:1) and activated at 80°C for 3 h. After samples were spotted, the TLC plate was developed with a solvent system of H_2O–acetic acid–methanol–acetone–chloroform (7:12:13:15:40)[4].

Synthesis of unnatural PIs

Typical representative synthesis of PI_{C8} and *sn*-2-*epi* PI_{C8} are described. ^{1}H NMR and ^{31}P NMR spectra were measured on JEOL Alpha-500 NMR spectrometer at 500 and 202.35 MHz, respectively. Chemical shifts of ^{1}H NMR were recorded in δ unit relative to internal tetramethylsilane (TMS) (δ=0) in $CDCl_3$, and relative to DHO (δ=4.65 ppm) in D_2O. Chemical shifts of ^{31}P NMR were relative to external potassium phosphate (δ=0). IR spectra were recorded on a JASCO A-102 instrument. FAB MS and high resolution FAB MS (HRFAB MS) were measured on a JEOL JMS-HX110 instrument. *m*NBA or TEA–GLY (3:1) was used as the matrix. Elementary analyses were performed by the Microanalytical Laboratory, Faculty of Pharmaceutical Sciences, The University of Tokyo. Optical rotations were recorded on a JASCO DIP 1000 digital polarimeter.

1-*O*-Stearoyl-3-*O*-benzyl-*sn*-glycerol (6). 3-*O*-Benzyl-*sn*-glycerol (**5**, 4 g, 22.0 mmol), prepared from **4** and dehydrated by the benzene azeotrope, was dissolved in dry pyridine (100 ml) and cooled to 0°C. To this solution, stearoyl chloride (6.5 g, 21.5 mmol in dry CH_2Cl_2) was added and stirred overnight at room temperature. The reaction mixture was poured into AcOEt, and the whole was washed with saturated $CuSO_4$ and with brine. After being dried over anhydrous Na_2SO_4, the solvent was removed to give the crude ester **6**, which was purified by silica gel column chromatography (*n*-hexane: AcOEt=6:1) to give **6** (5.9 g, 13.2 mmol, 60% yield) as colorless crystals. ^{1}H NMR ($CDCl_3$): δ 0.98 (3H, dt, *J*=1.0, 6.5 Hz, CH_3), 1.26 (30H, brm), 2.33 (2H, t, *J*=7.0 Hz, $COCH_2$), 3.50 (1H, ddd, *J*=1.0, 6.0, 10.0 Hz), 3.56 (1H, ddd, *J*=1.0, 4.5, 10.0 Hz), 4.03 (1H, m, H-2), 4.14 (1H, ddd, *J*=1.0, 6.0, 12.0 Hz), 4.21 (1H, ddd, *J*=1.0, 4.5, 12.0 Hz), 4.56 (2H, s, benzyl), 7.26–7.38 (5H, m, phenyl); IR ($CHCl_3$): 2920, 2850, 1730, 1455, 1360–1380, 1240, 1170, 1090–1110 cm^{-1}; FAB MS (*m*NBA): *m/z* 449 (M+H); $[\alpha]_D$=+2.0 (*c* 0.42, $CHCl_3$); Anal. Calcd for $C_{28}H_{48}O_4$: C, 74.95; H, 10.78. Found: C, 75.23; H, 10.56.

1-*O*-Stearoyl-2-*O*-octanoyl-3-*O*-benzyl-*sn*-glycerol (7c). Compound **6** (600 mg, 1.34 mmol), dehydrated by the benzene azeotrope, and catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) were dissolved in dry pyridine (16 ml), and cooled to 0°C. To this solution, octanoyl chloride (256 μl, 1.5 mmol, in 10 ml of dry CH_2Cl_2) was added dropwise, and the solution was warmed to room temperature. The reaction mixture was poured in AcOEt, and the solution was washed successively with saturated $CuSO_4$, saturated $NaHCO_3$, and brine, then dried over anhydrous Na_2SO_4. The solvent was removed, and the ester **7c** was purified by silica gel column chromatography (*n*-hexane: AcOEt=25: 1) to give **7c** (685 mg, 1.19 mmol, 89% yield) as colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, *J*=7.0 Hz, CH_3), 0.88 (3H, t, *J*=7.0 Hz, CH_3), 1.25 (36H,

brm), 1.54 (4H, m), 2.20–2.26 (4H, m, $COCH_2$), 3.73 (2H, m, *sn*-H-3), 4.24 (1H, dd, *J*=5.5, 12.0 Hz, *sn*-H-1), 4.32 (1H, dd, *J*=4.5, 12.0 Hz, *sn*-H-1), 4.51 (1H, d, *J*=12.0 Hz, benzyl), 4.55 (1H, d, *J*=12.0 Hz, benzyl), 5.08 (1H, m, *sn*-H-2), 7.25–7.45 (5H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 2250, 1720–1740, 1455, 1370, 1250–1270, 1160, 1100, 900; FAB MS (*m*NBA): *m/z* 575 (M+H); $[\alpha]_D$=+6.7 (*c* 0.76, $CHCl_3$).

1-*O*-Stearoyl-2-*O*-octanoyl-*sn*-glycerol (8c). Compound **7c** (680 mg, 1.2 mmol) was dissolved in 40 ml of AcOEt, and hydrogenated with Pd–C (380 mg) under H_2 overnight at room temperature. The catalyst was removed by filtration, the solvent was removed in vacuo, and the residue was chromatographed on silica gel (*n*-hexane:AcOEt=5:1) to give **8c** (486 mg, 1.0 mmol, 84% yield). ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, *J*=7.0 Hz), 0.88 (3H, t, *J*=7.0 Hz), 1.25 (36H, brm), 1.54 (4H, m), 2.20–2.26 (4H, m, $COCH_2$), 3.73 (2H, m), 4.24 (1H, dd, *J*=5.5, 12.0 Hz), 4.32 (1H, dd, *J*=4.5, 12.0 Hz), 5.08 (1H, m); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 2250, 1730, 1460, 1380, 1260, 1160, 1100, 900; FAB MS (*m*NBA): *m/z* 485 (M+H); $[\alpha]_D$=−3.1 (*c* 1.0, $CHCl_3$).

2,6-Di-*O*-benzyloxymethyl-3,4,5-tri-*O*-*p*-methoxybenzyl-D-*myo*-inositol (1-*O*-stearoyl-2-*O*-octanoyl-*sn*-glycerol) benzylphosphate (11c). Benzyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramide[8c,13] (650 mg, 2.0 mmol) and 1*H*-tetrazole (140 mg, 2.0 mmol) were dissolved in dry CH_2Cl_2 (20 ml). To this solution, compound **8c** (460 mg, 0.95 mmol) in dry CH_2Cl_2 (10 ml) was added dropwise, and stirred at room temperature. After the reaction was completed, the solvent was removed with a rotary evaporator. The residue was chromatographed on a silica gel column with *n*-hexane:AcOEt:Et_3N=25:4:1). The oily product **9c** (651 mg, 95% yield) was used as such in subsequent experiments. 2,6-Di-*O*-benzyloxymethyl-3,4,5-tri-*O*-*p*-methoxybenzyl-D-*myo*-inositol (**10**, 234 mg, 0.3 mmol), prepared by Prestwich's procedure,[7b] was dehydrated by the benzene azeotrope. Compound **9c** (651 mg, 0.90 mmol) and 1*H*-tetrazole (120 mg, 1.7 mmol) were added to **10** and the whole was dehydrated with a rotary pump for 1 h. The mixture was dissolved in dry CH_2Cl_2 (5 ml), and the solution was stirred for 1 h at room temperature. A small amount of water was added, and the whole was stirred for 10 min. Then, 70% *m*CPBA (300 mg, 1.74 mmol) was added at −78°C, and the reaction mixture was allowed to warm to room temperature. The whole was poured in AcOEt, and successively washed with 10% Na_2SO_3, saturated $NaHCO_3$ and brine, then dried over anhydrous Na_2SO_4. The solvent was removed, and the product **11c** was purified by silica gel column chromatography (*n*-hexane:AcOEt=2.5:1) (735 mg, containing a small amount of impurity). ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, *J*=7.0 Hz), 0.88 (3H, t, *J*=7.0 Hz), 1.25 (36H, brm), 1.54 (4H, m), 2.20–2.26 (4H, m, $COCH_2$), 3.37–3.43 (2H, m), 3.78 (9H, s, PMB–OMe), 3.93–4.24 (6H, m), 4.43–5.10 (18H, m), 5.14 (1H, m), 6.78–7.32 (27H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 1740, 1615, 1515, 1460, 1360, 1200–1240, 1160, 1020; FAB MS (*m*NBA): *m/z* 1440 (M+Na).

2,6-Di-*O*-benzyloxymethyl-D-*myo*-inositol (1-*O*-stearoyl-2-*O*-octanoyl-*sn*-glycerol) benzylphosphate (12c). Compound **11c** (425 mg, 0.30 mmol) was dissolved in a mixture of CH_2Cl_2 (4.5 ml) and water (0.5 ml), and was treated with 95% DDQ (355 mg, 1.49 mmol). After 90 min of stirring at room temperature, the reaction mixture was poured in AcOEt, and the solution was washed with saturated $NaHCO_3$ and with brine, then dried over anhydrous Na_2SO_4. The solvent was removed, and the product **12c** was purified by silica gel column chromatography ($CHCl_3$:MeOH=20:1) to give **12c** (122 mg, 0.115 mmol, 66% yield for 2 steps) as colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, *J*=7.0 Hz), 0.88 (3H, t, *J*=7.0 Hz), 1.25 (36H, brm), 1.57 (4H, m), 2.24–2.30 (4H, m, $COCH_2$), 3.36–3.43 (2H, m), 3.69 (1H, m), 3.80 (1H, m), 4.05–4.31 (7H, m), 4.53–5.09 (10H, m, benzyl), 5.15 (1H, m), 7.26–7.37 (15H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 3400, 2920, 2850, 2250, 1735, 1710, 1455, 1360, 1260, 1150, 1100, 1000, 890; FAB MS (*m*NBA) *m/z* 1080 (M+Na); HRFAB MS. Calcd for $C_{58}H_{89}O_{15}PNa$ (M+Na): 1079.5837, Found: 1079.5885.

PI_{C8}

Compound **12c** (35 mg, 0.033 mmol) was dissolved in 14 ml of 85% *t*-BuOH in water, and Pd black (50 mg) and $NaHCO_3$ (3 mg, 0.035 mmol) were added to the solution, and the mixture was shaken under H_2 (4.0 kgf/cm^2) for 7 h. The mixture was filtered, the catalyst was washed with EtOH and water, and the filtrate was concentrated in vacuo and lyophilized to give **PI_{C8}** (25 mg, 0.033 mmol, quant.). ^{1}H NMR (D_2O): δ 0.60 (3H, t, *J*=7.0 Hz, CH_3), 0.78 (3H, t, *J*=7.0 Hz, CH_3), 1.10–1.20 (40H, brm), 1.40–1.55 (4H, m), 2.20 (4H, m, $COCH_2$), 3.20–4.35 (10H, m), 5.20 (1H, br); ^{31}P NMR (D_2O):(δ −0.43; negative ion FAB MS (TEA–GLY=3:1): *m/z* 725 (M–H).

1-*O*-Benzyl-3-*O*-stearoyl-*sn*-glycerol (15). 1-*O*-Benzyl-*sn*-glycerol (**14**, 967 mg, 4.37 mmol), prepared from **13** and dehydrated by the benzene azeotrope, was dissolved in 16 ml of dry pyridine and cooled to 0°C. To this solution, stearoyl chloride (1.33 g, 4.38 mmol in dry CH_2Cl_2) was added and stirred for 2 h at room temperature. The reaction mixture was poured into AcOEt, and the whole was washed with saturated $CuSO_4$ and with brine. After being dried over anhydrous Na_2SO_4, the solvent was removed to give the crude ester **15**, which was purified by silica gel column chromatography (*n*-hexane:AcOEt=9:1) to give **15** (1.06 g, 2.37 mmol, 54% yield) as colorless crystal. ^{1}H NMR ($CDCl_3$): δ 0.98 (3H, dt, *J*=1.0, 6.5 Hz, CH_3), 1.26 (30H, brm), 2.33 (2H, t, *J*=7.0 Hz, $COCH_2$), 3.50 (1H, ddd, *J*=1.0, 6.0, 10.0 Hz), 3.56 (1H, ddd, *J*=1.0, 4.5, 10.0 Hz), 4.03 (1H, m), 4.14 (1H, ddd, *J*=1.0, 6.0, 12.0 Hz), 4.21 (1H, ddd, *J*=1.0, 4.5, 12.0 Hz), 4.56 (2H, s, benzyl), 7.26–7.38 (5H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 1730, 1455, 1360–1380, 1240, 1170, 1090–1110; FAB MS (*m*NBA): *m/z* 449 (M+H); $[\alpha]_D$=−1.9 (*c* 0.94, $CHCl_3$); Anal. Calcd for $C_{28}H_{48}O_4$: C, 74.95; H, 10.78. Found: C, 74.72; H, 10.64.

1-*O*-Benzyl-2-*O*-octanoyl-3-*O*-stearoyl-*sn*-glycerol (16). Compound **15** (500 mg, 1.12 mmol) dehydrated by the benzene azeotrope and catalytic amount of DMAP were dissolved in dry pyridine (12 ml) and cooled to 0°C. To this solution, octanoyl chloride (350 μl, 2.05 mmol in

6 ml of dry CH_2Cl_2) was added with stirring, and the solution was warmed to room temperature. The reaction mixture was poured in AcOEt, and the solution was successively washed with saturated $CuSO_4$ solution, saturated $NaHCO_3$ solution, and brine, then dried over anhydrous Na_2SO_4. The solvent was removed, and the ester **16** was purified by silica gel column chromatography (*n*-hexane:AcOEt=25:1) to give **16** (550 mg, 0.96 mmol, 86% yield) as colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, J=7.0 Hz, CH_3), 0.88 (3H, t, J=7.0 Hz, CH_3), 1.25 (40H, brm), 1.55–1.65 (4H, m), 2.27 (2H, t, J=7.5 Hz, $COCH_2$), 2.32 (2H, t, J=7.5 Hz, $COCH_2$), 3.58 (2H, dd, J=1.5, 5.0 Hz), 4.18 (1H, dd, J=6.5, 12.0 Hz), 4.34 (1H, dd, J=5.0, 12.0 Hz), 4.51 (1H, d, J=12.0 Hz, benzyl), 4.55 (1H, d, J=12.0 Hz, benzyl), 5.31 (1H, m), 7.25–7.45 (5H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 2250, 1720–1740, 1455, 1370, 1250–1270, 1160, 1100, 900; FAB MS (*m*NBA): *m/z* 575 (M+H); $[\alpha]_D$=−6.9 (*c* 1.6, $CHCl_3$).

2-*O*-Octanoyl-3-*O*-stearoyl-*sn*-glycerol (17). Compound **16** (520 mg, 0.91 mmol) was dissolved in 35 ml of AcOEt, and hydrogenated with Pd–C (290 mg) under H_2 overnight at room temperature. The catalyst was removed by filtration, the solvent was removed in vacuo, and the residue was chromatographed on silica gel (*n*-hexane: AcOEt=5:1) to give **17**, colorless oil, 395 mg (0.82 mmol, 90% yield): ^{1}H NMR ($CDCl_3$): δ 0.88 (3H, t, J=7.0 Hz, CH_3), 0.88 (3H, 3H, t, J=7.0 Hz, CH_3), 1.25 (36H, brm), 1.63 (4H, m), 2.32 (2H, t, J=7.5 Hz, $COCH_2$), 2.35 (2H, t, J=7.5 Hz, $COCH_2$), 3.73 (2H, m), 4.24 (1H, dd, J=5.5, 12.0 Hz), 4.32 (1H, dd, J=4.5, 12.0 Hz), 5.08 (1H, m); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 2250, 1730, 1460, 1380, 1260, 1160, 1100, 900; FAB MS (*m*NBA): *m/z* 485 (M+H); $[\alpha]_D$=+3.3 (*c* 0.75, $CHCl_3$); Anal. Calcd for $C_{29}H_{56}O_5$: C, 71.85; H, 11.64. Found: C, 72.05; H, 11.51.

2,6-Di-*O*-benzyloxymethyl-3,4,5-tri-*O*-*p*-methoxybenzyl-D-*myo*-inositol (2-*O*-octanoyl-3-*O*-stearoyl-*sn*-glycerol) benzylphosphate (19). Benzyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramide[8c,13] (650 mg, 2.0 mmol) and 1*H*-tetrazole (140 mg, 2.0 mmol) were dissolved in dry CH_2Cl_2 (20 ml). To this solution, compound **17** (372 mg, 0.77 mmol) in dry CH_2Cl_2 (10 ml) was added dropwise, and stirred at room temperature. After the reaction was completed, the solvent was removed with a rotary evaporator. The residue was chromatographed on a silica gel column with *n*-hexane: AcOEt:Et_3N=25:4:1). The oily product **18** (385 mg, 69% yield) was used as such in subsequent experiments. Compound **10** (280 mg, 0.36 mmol), prepared by Prestwich's procedure,[7b] was dehydrated by the benzene azeotrope. Compound **18** (385 mg, 0.53 mmol) and 1*H*-tetrazole (100 mg, 1.43 mmol) were added, and the whole was dehydrated with a rotary pump for 1 h. The mixture was dissolved in dry CH_2Cl_2 (5 ml), and the solution was stirred for 1 h at room temperature. A small amount of water was added, and the whole was stirred for 10 min. Then, the mixture was cooled to −78°C, and 70% *m*CPBA (350 mg, 2.03 mmol) was added, and the reaction mixture was allowed to warm to room temperature. The whole was poured in AcOEt, and washed successively with 10% Na_2SO_3, saturated $NaHCO_3$ and brine, then dried over anhydrous Na_2SO_4. The solvent was removed, and the product **19** was purified by silica gel column chromatography to give **19** (460 mg, 0.32 mmol, 90% yield). ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, J=7.0 Hz), 0.88 (3H, t, J=7.0 Hz), 1.25 (36H, brm), 1.54 (4H, m), 2.20–2.26 (4H, m, $COCH_2$), 3.37–3.43 (2H, m), 3.78 (9H, s, PMB–OMe), 3.93–4.24 (6H, m), 4.43–5.10 (18H, m), 5.14 (1H, m), 6.78–7.32 (27H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 2920, 2850, 1740, 1615, 1515, 1460, 1360, 1200–1240, 1160, 1020; FAB MS (*m*NBA): *m/z* 1440 (M+Na).

2,6-Di-*O*-benzyloxymethyl-D-*myo*-inositol (2-*O*-octanoyl-3-*O*-stearoyl-*sn*-glycerol) benzylphosphate (20). Compound **19** (380 mg, 0.27 mmol) was dissolved in a mixture of CH_2Cl_2 (4.5 ml) and water (0.5 ml), and was treated with 95% DDQ (300 mg, 1.26 mmol). After 2 h of stirring at room temperature, the reaction mixture was poured in AcOEt, and the solution was washed with saturated $NaHCO_3$ and with brine, then dried over anhydrous Na_2SO_4. The solvent was removed, and the product **20** was purified by silica gel column chromatography (*n*-hexane: AcOEt=1:2) to give **20** (220 mg, 0.21 mmol, 77% yield) as colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.87 (3H, t, J=7.0 Hz), 0.88 (3H, t, J=7.0 Hz), 1.25 (36H, brm), 1.57 (2H, m), 2.24–2.30 (4H, m, $COCH_2$), 3.36–3.43 (2H, m), 3.69 (1H, m), 3.80 (1H, m), 4.05–4.31 (7H, m), 4.53–5.09 (9H, m), 5.15 (1H, m), 7.26–7.37 (15H, m, phenyl); IR ($CHCl_3$, cm^{-1}): 3400, 2920, 2850, 2250, 1735, 1710, 1455, 1360, 1260, 1150, 1100, 1000, 890; FAB MS (*m*NBA): *m/z* 1080 (M+Na); HRFAB MS. Calcd for $C_{58}H_{89}O_{15}PNa$ (M+Na). 1079.5837, Found: 1079.5867.

sn-2-*epi* PI_{C8}

Compound **20** (29 mg, 0.027 mmol) was dissolved in 14 ml of 85% *t*-BuOH in water, and Pd black (40 mg) and $NaHCO_3$ (2.3 mg, 0.027 mmol) were added to the solution, and the mixture was shaken under H_2 (4.0 kgf/cm^2) for 7 h. The mixture was filtered, the catalyst was washed with EtOH and water, and the filtrate was concentrated in vacuo and lyophilized to give ***sn*-2-*epi* PI_{C8}**, (17.5 mg, 0.023 mmol, 85% yield): ^{1}H NMR (D_2O): δ 0.60 (3H, br, CH_3), 0.78 (3H, br, CH_3), 1.10–1.20 (40H, brm), 1.40–1.55 (4H, m), 2.20 (4H, m, $COCH_2$), 3.20–4.35 (10H, m), 5.20 (1H, br); ^{31}P NMR (D_2O):(δ −0.43; negative ion FAB MS (TEA–GLY=3:1): *m/z* 725 (M–H).

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