Pergamon

Tetrahedron, Vol. 53, No. 43, pp. 14627-14634, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)01043-0

Properties of Unusual Phospholipids IV: Chemoenzymatic Synthesis of Phospholipids Bearing Acetylenic Fatty Acids

Sandra Pisch[a], Uwe T. Bornscheuer[a], Hartmut H. Meyer[b], Rolf D. Schmid[a*]

[a]: Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany
[b]: Institut für Organische Chemie, Universität Hannover, Schneiderberg 1B, D-30167 Hannover, Germany

Abstract: 14-Octadecynoic (**1**) and 4-octadecynoic (**2**) acid were synthesized in high yields (56% & 57%) and incorporated into glycerophosphatidylcholine at *sn*1- and *sn*2-position. The head group of these phosphatidylcholines was exchanged using phospholipase D (PLD) in a biphasic system. PLD from *Streptomyces antibioticus*, *Streptomyces sp.*, peanut and cabbage were studied in this transphosphatidylation reaction. Under optimized conditions (40 °C, organic solvent : buffer ratio 1:1.5 (v/v), pH 5.6), the conversion of phosphatidylcholine to the corresponding phospholipids with the head groups glycerol, ethanolamine and *L*-serine reached 99% at 40°C in 1 h. The quantitative analysis of the transphosphatidylation reactions was performed by TLC-FID.

INTRODUCTION

Phospholipase D (PLD, EC 3.1.4.4) not only catalyzes the hydrolysis of the terminal phosphate diester bond of glycerophospholipids, but also the transfer reaction of the phosphatidyl group of phosphatidylcholine accepting a wide range of nucleophiles such as glucose[1], *L*-ascorbic acid[2], nucleosides[3] and aromatic compounds[4]. Activity of PLD is affected by pH[5], temperature[6], salts[7] and additives like ethylenediaminetetraacetic acid (EDTA)[8]. In addition, the selection of suitable solvents[9] and organic solvent/buffer ratios[10] were reported as crucial factors to achieve high conversion in transphosphatidylations.

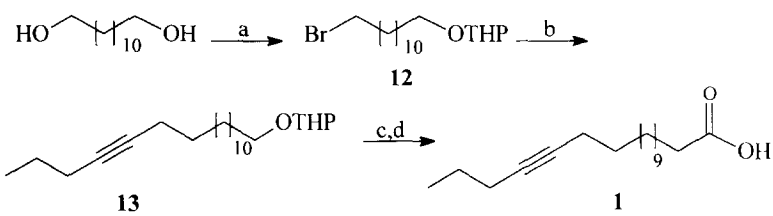
Phospholipids, a major lipid class, exhibit a variety of biological functions. Beside their role as signal molecules and energy resources they are important components of cellular membranes in living organisms and thus became a subject for pharmaceutical and cosmetical application. For instance in biomedical research liposomes are used as drug delivery systems[11] and phospholipids bearing inhibitors like aza-sugars, nucleosides and peptides as head groups are potential pharmaceuticals[12]. Because the *de novo* synthesis of phospholipids is a complicated task, their function in membrane processes has predominantly been investigated with preparations from e.g. egg yolk extractions[13] or bacteria cultures[14]. In our ongoing program on the application of phospholipids in the stabilization of membrane proteins, we synthesized a range of phosphatidylcholines bearing acetylenic fatty acids such as 14-octadecynoic acid (**1**) and 4-octadecynoic acid (**2**). The corresponding 1,2-di-(octadecynoyl)-*sn*-glycero-3-phosphocholines (**3** and **4**) had a T_m- value close to physiological temperatures[15]. In order to mimic biological membranes, a range of different phospholipids was synthesized

through transphosphatidylation of **3** or **4** using a recently cloned phospholipase D from *Streptomyces antibioticus* (expressed in *E. coli*[16]). In addition, we report the improved chemical synthesis of the octadecynoic acids **1** and **2**.

RESULTS AND DISCUSSION

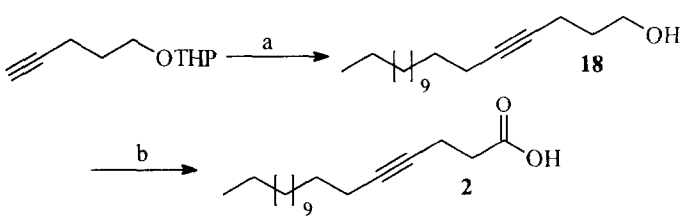
Synthesis of octadecynoic acids 1 and 2 and the phosphatidylcholines 3 and 4

Commercially available chemicals were used for the synthesis of the two isomeric acetylenic fatty acids (Schemes 1 and 2). The chains of the target molecules are built up by coupling of functionalized acetylenic- and bromoalkane building blocks. This provides the means for the synthesis of other isomers by alternating combinations of reaction partners. The carboxy group is introduced by the nitrilo intermediate 1-cyanoheptadec-13-yne (**16**) in case of compound **1**, while for compound **2,** the intermediate alcohol **18** is oxidized directly using Jones reagent. The overall yields for compounds **1** and **2** are 56% and 57%, respectively. These acetylenic fatty acids were coupled to glycerophosphatidylcholine (**5**) and compounds **3** and **4** were isolated in 98% yield (Scheme 3).



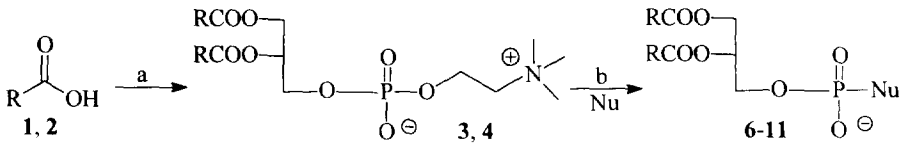
a) i. Ref. 18, 80%, ii. DHP, 95%, b) $LiNH_2$, NH_3 liq., DMSO, THF, 1-Pentin, 83%, c) i. MeOH, *p*-TsOH, **14,** 81%, ii. CBr_4, 0-5°C, PPh_3, **15**, 97%; d) i. abs. DMF, NaCN 100°C, **16**, 91%, ii. abs. MeOH, HCl -5-0°C, ice, 3 N NaOH, 87%.

Scheme 1



a) $LiNH_2$, NH_3 liq., THF, DMSO, bromotridecane, **17,** 86%, ii. MeOH, *p*-TsOH, **18,** 91%, b) CrO_3, acetone, H_2SO_4, 73%.

Scheme 2



a) i. 1,1'-Carbonyl-diimidazole , dichloromethane; ii. DBU, **5**, dichloromethane, 98%.
b) PLD, 40°C, pH 5.6, biphasic system, 87-98%. Nu: Ethanolamine, glycerol, *L*-serine.
1: R= $CH_3(CH_2)_2CC(CH_2)_{12}$ **2**: R= $CH_3(CH_2)_{12}CC(CH_2)_2$

Scheme 3

Head group exchange using Phospholipase D

Optimization of the reaction system for the transphosphatidylation reaction of 3

Initially, reaction conditions for the transphosphatidylation reaction (Scheme 3) of 1,2-di-(octadec-14-ynoyl)-*sn*-glycero-3-phosphocholine (**3**) were optimized using PLD from *Streptomyces antibioticus*. As standard reaction conditions, a biphasic system consisting of chloroform/sodium acetate (0.1 M)/calcium chloride (0.1 M)) buffer at pH 5.6 and 40°C was found suitable. After 60 min reaction time **3** was quantitatively converted to the corresponding phosphatidylethanolamine (**6**), -glycerol (**7**) and -*L*-serine (**8**). In all experiments no hydrolysis of the phosphocholine to the phosphatidic acid could be detected(Table 1).

Table 1: Optimized Reaction Conditions in the Conversion of **3** to Phospholipids **6**, **7** and **8**.

Nucleophile	Concentration [% (w/v)]	Substrate (3) [gl^{-1}]	PLD [gl^{-1}]	Conversion [%]
Ethanolamine (→**6**)	20	6.7	0.05	99
Glycerol (→**7**)	20	8.3	0.2	99
L-Serine (→**8**)	20	6.7	0.14	99

Changes in nucleophile concentrations from 5% to 10%, 20% and 30% under standard reaction conditions gave no influence on the conversion of **3**, but the initial rates of product formation varied to some extent (data not shown). Variation of the concentrations of **3** from 3.3 gl^{-1}, 5. gl^{-1}, 6.7 gl^{-1}, 8.3 gl^{-1} to 10.0 gl^{-1} gave no inhibition of the reaction, but at a concentration of 1 gl^{-1} the reaction slowed down (Figure 1). Similar results have been reported[6] investigating the conversion of PC from egg yolk to phosphatidylglycerol with PLD from cabbage, where the inhibition of the reaction started from a substrate concentration of 40 gl^{-1}.

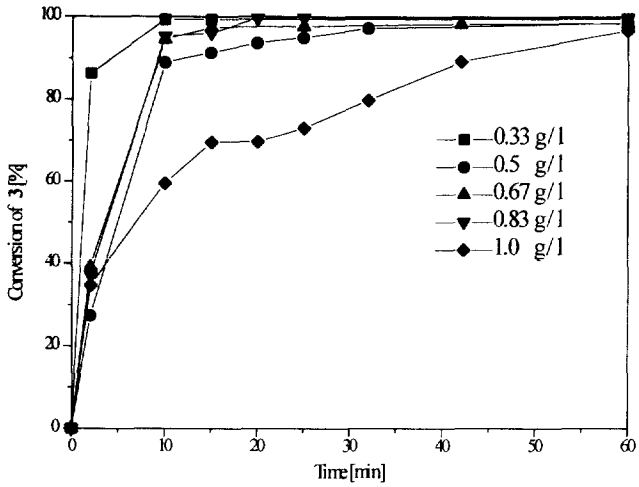


Figure 1: Influence of the substrate concentration on the PLD-catalyzed head group exchange between **3** and glycerol in chloroform / buffer (pH 5.6) at 40°C.

Substrate specificity of PLD from different origin

Beside PLD from *Streptomyces antibioticus*, phospholipase D from *Streptomyces sp.*, peanut and cabbage were investigated (Table 2). PLD from *Streptomyces antibioticus* and *Streptomyces sp.* allowed the quantitative conversion of *L*-serine, glycerol and ethanolamine. In contrast, PLD from cabbage was unsuitable for all nucleophiles and required much higher reaction times. PLD from peanut allowed almost quantitative conversion with glycerol and ethanolamine as nucleophiles.

Table 2: Comparison of Four PLD from Different Origin in the Conversion of **3** to Phospholipids **6**, **7** and **8** (after 1 h reaction time).

Nucleophile	***Strep. antibioticus***	***Strep.sp.***	**Cabbage**	**Peanut**
Ethanolamine (→**6**)	99	99	64	97
Glycerol (→**7**)	99	99	69*	94
L-Serine (→**8**)	99	99	15*	67

*conversion of PC after 20 h.

Transphosphatidylation of 4 with PLD from *Streptomyces antibioticus*

The method for the PLD catalyzed head group exchange demonstrated for **3** was successfully applied to substrate **4** and yields of isolated products (**9-11**) were between 85-87%. Thus, phospholipase D from *Streptomyces antibioticus* does not display a specificity with respect to the position of the triple bond in the acetylenic phospholipid. Moreover, it could be demonstrated that this PLD allows transphosphatidylation reactions at gram scale and opens access to a range of unusual phospholipids.

EXPERIMENTAL

Chemicals and phospholipases

Sn-glycero-3-phosphocholine (GPC) (**5**), the phospholipid standards phosphatidylglycerol (PG), 1,2-di-(stearoyl)-*sn*-glycero-3-phosphocholine (DSPC), 1,2-di-(stearoyl)-*sn*-glycero-3-phosphoric acid (DPPA), phosphatidyl serine from pork brain (PS), PLD from peanut (type III) and PLD from cabbage (type I) were obtained from Sigma Chemie (Deisenhofen, Germany). All other chemicals and solvents were purchased from Fluka Chemie AG (Buchs, Switzerland) and Merck Chemie GmbH (Darmstadt, Germany). PLD from *Streptomyces sp.* was a gift from Asahi Chemicals Industry Co Ltd., Tokyo, Japan and PLD from *Streptomyces antibioticus* was donated by Prof. T. Yamane, Nagoya, Japan.

Analytical methods

NMR/spectroscopy. Phospholipids, intermediates and fatty acids were characterized by ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectroscopy, mass spectra and elementary analysis (Bruker AM 300 and WM 400, Karlsruhe, Germany). Chemical shifts are given in ppm relative to internal tetramethylsilane. For ^{31}P NMR, the phospholipids (2 mg) were dissolved in 1 ml chloroform containing 5 % benzene-d_6. This solution was transferred into a NMR sample tube. 0.5 ml of a methanol:CsEDTA (0.2 M aq., pH 6) (4:1 v/v) solution was added, and the sample was mixed thoroughly.

nalytical HPLC. The homogeneity of the phospholipids was determined by high resolution liquid chromatography. The chromatography device consisted of a Sykam HPLC system, pump S1100, programmer S2000 (Sykam GmbH, Gilching, Germany) a light scattering detector (Sedex 45, S.E.D.E.R.E., Vitry/Seine, France). Analysis was carried out on a 250×4 mm I.D. column (Macherey-Nagel GmbH, Düren, Germany) packed with unmodified Nucleosil, 5 µm. Elution (1 ml/min) was performed in gradient mode (starting with 1 % solvent A to 100 % solvent B within 20 min) with solvent A = isopropanol:isohexane (4:3) and solvent B = isopropanol:isohexane:water (8:6:1.5).

Preparative HPLC. Preparative HPLC separation of phospholipids was performed on a Sykam Prep (pump S1100, programmer S2000) with a spectophotometric detector (Linear Instruments, Fremont, CA, U.S.A.). Purification was carried out on a 250x40 mm I.D. column (Grom. Herrenberg, Germany), packed with unmodified Nucleosil, 5 µm. Elution (1 ml/min) was performed in gradient mode (see Analytical HPLC).

Thin Layer Chromatography (TLC). For qualitative analysis of phospholipids, TLC on silica gel plates with chloroform/methanol/glacial acetic acid/water (25:15:4:2) as mobile phase was used, phospholipids were visualized by spraying with phosphomolybdenic acid (10% in ethanol w/v).

Quantitative analysis of phospholipids. An aliquot (0.1 ml) of the reaction mixture was transferred to a test-tube containing 0.3 ml Folch solution (chloroform-methanol, 2:1) and 0.3 ml water. After mixing well, the solution was centrifuged for 2 min at 2500 rpm. An aliquot (2µl) of the organic layer was spotted on chromarods (SIII quarz rods coated with silica gel, activated by blank scan before use) and developed with chloroform-methanol-water (40:20:1). After drying for 2 min, the rods were scanned using the TLC-FID system (Iatroscan MK-5, Iatron Laboratories, Tokyo, Japan) at 30 s per scan, a hydrogen flow rate of 160 ml min^{-1} and an air flow rate of 2000 ml min^{-1}. Peaks (integrator C-R3A, Shimadzu, Kyoto, Japan) were identified by comparison with phospholipid standards. Peak areas were found to be proportional to concentration.

Assay for PLD Activity. PLD activity was assayed at 40°C using a pH-stat system (Metrohm, Herisau, Switzerland) according to a modified literature method[5]. One unit of hydrolytic activity is defined as the amount of enzyme, which hydrolyzes 1 mmol of pure PC per min at 40°C. The assay solution consisted of 10 mg **3** in 14 ml water, 7 ml diethyl ether and 1.2 ml calcium chloride (1M) solution adjusted to pH 5.6. A known amount of phospholipase D was dissolved in 1 ml 0.1 M sodium acetate buffer (pH 5.6), centrifuged and the supernatant added to the assay solution. The pH was kept constant with 0.01 M NaOH solution and the PLD activity was calculated from the initial rate of acid formation. The following activities have been found: PLD from *Streptomyces sp.* 283 U/g, *Streptomyces antibioticus* 48 U/g and peanut 350 U/g. Activity of PLD from cabbage was not measured.

Synthesis of 14-octadecynoic acid (1)

14-Octadecynoic acid was synthesized in a 7 step reaction. 1,12-Dodecanediol was converted into 12-bromododecane-1-ol using a chemical extraction procedure[17]. The hydroxy group was protected with dihydropyrane to form 1-bromo-12-(tetrahydropyran-2-yloxy)-dodecane (**12**)[18]. Then 1.25 g lithium (180 mmol) was dissolved in 350 ml liquid ammonia and 12.3 g (180 mmol) 1-pentyne in 50 ml THF, were added dropwise at -60°C. The temperature was maintained at -60°C and after 30 min a solution of 48.9 g (140 mmol) **11** dissolved in 100 ml THF:DMSO (1:1 v/v) was added.

Ammonia was evaporated overnight and the residue was taken up in 400 ml diethyl ether. The organic phase was washed with water and ammonium chloride solution and evaporated to dryness to yield crude 1-(tetrahydropyrane-2-yloxy)-heptadec-13-yne (**13**) in 91% yield. 61.5 g (183 mmol) of **13** was added to 300 ml methanol and 1.5 g toluene sulfonic acid at room temperature. After cleavage of the tetrahydropyranyl group, the reaction mixture was neutralized with sodium hydogencarbonate solution, the solvent was evaporated and the residue purified by silica gel column chromatography using diethyl ether-petrol ether (1:1 v/v). Crystalline heptadec-13-ynol (**14**) was obtained in 81% yield. Then 26.2 g (100 mmol) triphenylphosphine, dissolved in 50 ml dry dichloromethane (DCM) was added at 0°C to a solution of 25.2 g (100 mmol) **14** and 33.1 g (100 mmol) tetrabromomethane in 150 ml dry dichloromethane. The reaction mixture was stirred for 1h, evaporated and purified by silica gel column chromatography with *n*-hexane:diethyl ether 19:1 (v/v). 1-Bromoheptadec-13-yne (**15**) was obtained as a colorless oil in 97 % yield. Then 28.1 g (89 mmol) of **15** were dissolved in 100 ml DMF, 5.64 g (115 mmol) sodium cyanide was added and the reaction mixture was stirred for 1 h at 60°C. DMF was evaporated, the residue solubilized in 200 ml diethyl ether, the organic phase washed with water and dried over magnesium sulfate. The crude product was purified by silica gel column chromatography using diethylether-petrolether to yield 1-cyanoheptadec-13-yne (**16**) in 91% yield. For the final step, 17.0 g (65 mmol) **16** were dissolved in 200 ml methanol abs. and the solution was saturated with gaseous hydrogen chloride at -5 to 0°C, which resulted in the partial crystallization of the formed imidoester hydrochloride. After 3 h the solution was overlayed with a mixture of ether:petrolether 1:1 (v/v) and hydrolyzed with a small amount of ice within 5 min. 400 ml ice cold water was added, the layers were separated, the organic layer was washed with distilled water until no free acid could be detected and finally dried over magnesium sulfate. After evaporation of the solvent, 18.0 g (61 mmol) of the obtained oily 14-octadecynoic acid methylester were suspended in 200 ml methanol, 30 ml 3N sodium hydroxide solution were added and stirred at room temperature for 20 h. The reaction mixture was neutralized with 3N HCl solution, the solvent was evaporated and the residual carboxylate was redissolved in diethyl ether under addition of HCl. The reaction mixture was washed with water and dried over magnesium sulfate. After evaporation of the solvent the crude 14-octadecynoic acid **1** was purified by silica gel column chromatography using diethyl ether:petrol ether 1:1 (v/v) in 87% yield (overall yield 56%). For final purification, **1** was recrystallized from *n*-hexane. IR, ^{1}H NMR, ^{13}C NMR-spectra and melting point were found to be identical compared to the acid synthesized by Rürup et al.[15]

14 ^{1}H NMR ($CDCl_3$) δ 0.97 (t, J = 7 Hz, CH_3), 1.2-1.4 (m, 9 CH_2), 1.50 (sext, J = 7 Hz, 16-CH_2), 1.56 (quint, J = 7 Hz, 2-CH_2), 1.98 (s, OH), 2.31 (m, 12-CH_2, 15-CH_2), 3.62 (t, 7 Hz, CH_2O); MS (70°C) *m/z* 252 (M^+, 3), 223 (2), 209 (3), 196 (3), 177 (2), 164 (3), 163 (5), 149 (2), 137 (18), 135 (27), 124 (27), 123 (31), 121 (28), 111 (28), 109 (38), 108 (29), 101 (29), 96 (66), 95 (53), 82 (100), 81 (89), 67 (100); IR ν 3624, 3000, 2928, 2856, 1556, 1464, 1432, 1380, 1336, 1300, 1276, 1236, 1048.

15 ^{1}H NMR ($CDCl_3$) δ 0.97 (t, J = 7 Hz, CH_3), 1.2-1.6 (m, 10 CH_2), 1.85 (quint, J = 7 Hz), 3-CH_2), 2.1 (m, 12-CH_2, 15-CH_2), 3.41 (t, J = 7 Hz, CH_2Br), MS (RT) *m\z* 316 (M^+, 3), 314 (M^+, 3), 273 (9), 271 (10), 254 (10), 252 (10), 214 (4), 203 (4), 201 (4), 191 (5), 175 (40), 173 (62), 171 (46), 137 (32), 135 (22), 125 (60), 123 (65), 110 (44), 109 (67), 97 (61), 96 (66), 82 (75), 81 (90), 67 (100); IR ν 2928, 2852, 1464, 1436, 1376, 1336, 1252, 1144, 1072, 1032, 884.

16 ^{1}H NMR ($CDCl_3$) δ 0.97 (t, J = 7 Hz, CH_3), 1.2-1.6 (m, 10 CH_2), 1.65 (quint, J = 7 Hz), 3-CH_2), 2.1 (m, 12-CH_2, 15-CH_2), 2.34 (t, J = 7Hz, 2-CH_2); MS (RT) *m/z* 261 (M^+, 2), 233 (3), 218 (4), 150 (2), 124 (4), 123 (7), 110 (5), 109 (11), 97 (9), 96 (57), 83 (13), 82 (100), 81 (70), 79 (16), 69 (19), 68 (18), 67 (85); IR ν 3000, 2928, 2856, 2248, 1712, 1664, 1236, 1124, 1108, 1092.

Synthesis of 4-octadecynoic acid (2)

4-Octadecynoic acid was synthesized in three steps. 8.4 g (50 mmol) 1-Tetrahydropyran-2-yloxy-4-pentyne, dissolved in 20 ml THF, was added to a solution of 530 mg lithium in 100 ml liquid ammonia at -60 °C[19]. After 30 min 19.7 g (75 mmol) bromotridecane, dissolved in 60 ml tetrahydrofuran:dimethyl sulfoxide (1:1 v/v), were added dropwise and the reaction mixture was allowed to warm to room temperature overnight. The residue was dissolved with diethyl ether and water, the organic phase was washed with water and dried over magnesium sulfate. After evaporation of the solvent, the crude product was purified by silica gel column chromatography using ether:petrolether (1:9 v/v) to yield 15.0 g (86%) 1-(tetrahydropyran-2-yloxy)-4-octadedecyne (**17**), which was then added to 100 ml methanol and 1.0 g *p*-toluene sulfonic acid. To cleavage the THP group, the solution was stirred for several h and then neutralized with sodium hydrogencarbonate solution. After evaporation of solvent the residue was purified by silica gel column chromatography using ether:petrolether (1:1 v/v) to yield 10.4 g (91 %) crystalline 4-octadecynol (**18**). 10.2 g (38 mmol) of **18** were oxidated under ice cooling in 250 ml acetone with 20 ml of Jones Reagent obtained from 5.4 g chromotrioxide, 4.6 ml concentrated sulfuric acid and water. After 15 min oxidation, excess reagent was destroyed with 2-propanol, the solution was neutralized with sodium hydrogencarbonate and the solvent was evaporated. The residue was dissolved in ether and diluted with hydrochloric acid, the organic phase was washed with water and dried over magnesium sulphate. After evaporation of solvent, the crude product was purified by silica gel column chromatography using ether:petrolether in a gradient mode from 1:4 to 2:1 (v/v) to yield 7.8 g (73%) 4-octadecynoic acid (**2**) (overall yield 57%). IR, ^{1}H NMR, ^{13}C NMR spectra and the melting point of **2** were found to be identical compared to the acid synthesized by Rürup et al.[15]

17 ^{1}H NMR ($CDCl_3$): δ 4.6 (m,OCHO), 3.95 (2m, 2CHO), 3.55-3.42 (2m, 2CHO), 2,26 (m, CH_2), 2.13 (m, CH_2), 1.9-1.2 (3m, $2CH_2$, $3CH_2$, $10CH_2$), 0.88 (t, ^{3}J = 7 Hz, CH_3); MS (25°C) *m/z* (M^+, 1), 293 (1), 277 (3), 167 (18), 137 (2), 121 (4), 109 (3), 107 (8), 97 (19), 85 (100), 81 (13), 75 (19), IR ν 2924, 2852, 1464, 1352, 1320, 1200, 1160, 1136, 1120, 1060, 1032.

18 ^{1}H NMR ($CDCl_3$): δ 3.74 (t, J=7 Hz, CH_2O), 2.77 (tt, J = 7 Hz, J = 2 Hz, CH_2-C), 2.13 (tt, J = 7 Hz, J = 2 Hz, CH_2-C, 2.06 (broad s, OH), 1.73 (quint, J = 7Hz, CH_2), 1.47 (quint, J= 7Hz, CH_2), 1.4-1.2 (2m, 10 CH_2), 0.88 (t, J = 7 Hz, CH_3); MS (70°C) *m/z* 266 (M^+,3), 223 (3), 153 (5), 139 (5), 135 (4), 126 (3), 123 (4), 121 (9), 111 (14), 109 (), 98 (44), 97 (100), 84 (36), 83 (49), 79 (28); IR (CHCl3) ν 3624, 3540, 3000, 2928, 2852, 1464, 1432, 1376, 1352, 1332, 1132, 1116, 1052.

Synthesis of 1,2-di-(octadecynoyl)-sn-glycero-3-phosphocholines 3 and 4

The synthesis of the phosphocholine followed the procedure described by Rürup et al.[15] Briefly, octadecynoic acids **1** or **2** were activated using 1,1'-carbonyl-diimidazole in dichloromethane and then coupled with glycerophosphatidylcholine (**5**) in the presence of 1,8-diazabicylo[5.4.0]undec-7-ene (1,5-5) (DBU) in 98 % yield. The structure of the phosphocholines **3** and **4** were confirmed by ^{1}H NMR and ^{13}C NMR and their purity analyzed by HPLC.

PLD-catalyzed head group exchange using ethanolamine, glycerol and *L*-serine as nucleophiles

Solutions of different phosphatidylcholine concentrations were prepared with chloroform, diethyl ether or ethyl acetate. For the enzyme-buffer solution, the enzyme was solubilized in 0.1 M sodium acetate-0.1 M calcium chloride solution, which was adjusted to the desired pH-value. The concentration of the nucleophile is given in reference to the buffer solution (w/v). The nucleophile was added to 6 ml (9 ml) of the phosphocholine solution,

the solution was then adjusted to the desired temperature and 9 ml of the enzyme solution were added. The quantitative analysis of the reaction mixture was done by TLC-FID. Standard reaction conditions were 40°C, pH 5.6, using chloroform:buffer (1:1.5 v/v).

Preparative reactions were carried out by solubilization of **3** or **4** (500 mg, 0.64 mmol) in 60 ml chloroform followed by addition of the nucleophile at optimized concentrations (w/v) (20% *L*-serine, 20% glycerol and 20% ethanolamine). The reactions were initiated by addition of 90 ml PLD (from *Streptomyces antibioticus*) buffer solution. After stirring at 40°C for 1 h the phases were separated and the water layer was extracted three times with Folch solution. The combined organic layers were washed three times with water, three times with 0.1 M EDTA solution (pH 7.4) and dried over magnesium sulfate. Chloroform was evaporated and the crude product was purified by preparative HPLC.

6: yield = 74.3 %, R_t = 15.0 min, ^{31}P NMR = 1.26; **7**: yield = 83.5 %, R_t = 16.9 min, ^{31}P NMR = 0.91 **8**: yield = 82.7 %, Rt = 19.1 min, ^{31}P NMR =1.34, **9**: yield = 75.3 %, R_t = 15.1 min, ^{31}P NMR = 1.35; **10**: yield = 85.5 %, R_t = 17.1 min, ^{31}P NMR = 1.77; **11**: yield = 83.5 %, R_t = 20.1 min, ^{31}P NMR = 0.45.

Acknowledgement. This work was supported by NEDO (New Energy and Industrial Technology Development Organization), Tokyo, Japan. We are especially grateful to Prof. T. Yamane, Nagoya University, Nagoya, Japan and Dr. S. Ueda, Asahi Chemicals Industry Co Ltd., Tokyo, Japan for providing us with PLD.

REFERENCES AND NOTES

1. Kokusho, Y.; Tsundoda, A.; Kato, S. *Biosci. Biotech. Biochem.* **1993,** 57, 1302-1305; Shimbo, K.; Iwasaki, Y.; Yamane, T.; Ina, K. *Biosci. Biotech. Biochem.* **1993,** 58, 1946-1948.
2. Nago, A.; Terao, J. *Biochem. Biophys. Res. Commun.* **1990,** 172, 385 -389.
3. Shuto, S.; Imamura, S.; Fukukawa, K.; Ueda, T. *Chem. Pharm. Bull.* **1988,** 36, 5020-5023.
4. Takami, M.; Suzuki, Y. *Biosci. Biotech. Biochem.* **1994**, 58, 2136-2139.
5. Juneja, L. R.; Yamane, T.; Shimizu, S. *J. Am. Oil Chem. Soc.* **989**, 66, 714-717.
6. Juneja, L. R.; Yamane T. *Enzyme Microb. Technol.* **1987**, 9, 350- 354.
7. Juneja, L. R.; Kazuoka, T.; Goto, N.; Yamane, T.; Shimizu, S. *Biochim. Biophys. Acta* **1989**, 1003, 277-283.
8. Kokusho, Y.; Tsundoda, A.; Kato, S.; Machida, H.; Iwasaki, S. *Biosci. Biotech. Biochem.* **1993**, 57, 1302-1305.
9. Juneja, L. R.; Kazuoka, T.; Yamane, T.; Shimizu, S. *Biochim. Biophys. Acta* **1988**, 969, 334-341; Kokusho, Y.; Tsundoda, A.; Kato, S.; Machida, H.; Iwasaki, S. *Biosci. Biotech. Biochem.* **1993**, 57, 1302-1305; Takami, M.; Suzuki, Y. *Biosci. Biotech. Biochem.* **1994**, 58, 2136-2139.
10. Sale, M. F. O.; Cestaro, B.; Mascherpa, A.; Monti, D.; Masserini, M. *Chem. Phys. Lipids* **1989**, 50, 135-142.
11. Barratt, G.; Tenu, J. P.; Yapo, A.; Petit, J. F. *Biochim. Biophys. Acta* **1986**, 862, 153-164; Pax, H.; Blume, A. *Chem. Phys. Lipids* **1993**, 66, 63-79; Braun-Falco, O.; Korting, H. C. Griesbach Conference. In *Liposome Dermatics*; Springer: Berlin, 1992.
12. Wang, O.; Schuster, M.; Wang, Y. F.; Wong, C. H. *J. Am. Chem. Soc.* **1993**, 115, 10487 -10491.
13. Paternostre M. T.; Roux, M.; Rigaud, J. L. *Biochemistry* **1988**, 27, 2668-2677.
14. Kannenberg, E.; Blume, A.; Geckeler, K.; Poralla, K. *Biochim. Biophys. Acta* **1985**, 814, 179-185.
15. Rürup, J.; Mannova, M.; Brezesinski, G.; Schmid, R. D. *Chem. Phys. Lipids* **1994**, 70, 187-198.
16. Iwasaki, Y.; Nakano, H.; Yamane T. *Appl. Microbiol. Biotechnol.* **1994**, 42, 290-299; Iwasaki, Y.; Mishima, N.; Mizumoto, K.; Nakano, H.; Yamane, T. *J. Ferment. Bioeng.* **1995**, 79, 417-421.
17. Camps, F.; Casamor, J.M.; Coll, J.; Gueredo, A.; Riba, M. *Org. Prep. Proc. Int.* **1983**, 15-19.
18. Murillo, O.; Watanabe, S.; Nakarao, A. Gokel, G.W. *J. Am. Chem. Soc.* **1995**, 117, 7665-7679.
19. Eglinton, G., Jones, E.R.H.; Whiting, M.C. *J. Chem. Soc.* **1952**, 2873-2882.

(*Received in Germany* 25 *June* 1997; *revised* 21 *August* 1997; *accepted* 22 *August* 1997)