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# 1,3-Diacylglycero-2-phosphocholines — synthesis, aggregation behaviour and properties as inhibitors of phospholipase D

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**Abstract**

A series of 1,3-diacylglycero-2-phosphocholines (1,3-PCs) with acyl chain lengths of $C_8$–$C_{18}$ were synthesised by chemical introduction of the phosphocholine moiety into the regioisomerically pure 1,3-diacylglycerols, which were obtained from glycerol and the vinyl esters of fatty acid by means of lipase from *Rhizomucor mihei*. The 1,3-PCs being regioisomers of the natural glycerophospholipids were studied with respect to their aggregation behaviour in the absence and in the presence of sodium dodecylsulfate (SDS) as well as their properties as substrates and inhibitors of phospholipase D (PLD) from cabbage. While the main structures of the pure 1,3-PCs were micelles ($C_8$), liposomes ($C_{10}$, $C_{12}$) or planar bilayers ($C_{14}$, $C_{16}$, $C_{18}$), the addition of SDS resulted in the formation of mixed micelles ($C_8$, $C_{10}$) and mixed liposomes ($C_{12}$, $C_{14}$, $C_{16}$, $C_{18}$). None of the 1,3-PCs was found to be hydrolysed by PLD, whereas all of them showed inhibitory properties in the standard assay for PLD. The inhibitory power was strongest with 1,3-didecanoylglycero-2-phosphocholine ($IC_{50} = 43$ μM). 

*Keywords:* 1,3-Diacylglycero-2-phosphocholines; Inhibition; Liposomes; Micelles; Phospholipase D; Synthesis

*Abbreviations:* 1,2-dC$_8$PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; 1,2-dC$_{10}$PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; 1,2-PC, 1,2-diacylglycero-3-phosphocholine; 1,3-PC, 1,3-diacylglycero-2-phosphocholine; 1,3-dC$_8$PC, 1,3-dioctanoylglycero-2-phosphocholine; 1,3-dC$_{10}$PC, 1,3-didecanoylglycero-2-phosphocholine; 1,3-dC$_{12}$PC, 1,3-dilauroylglycero-2-phosphocholine; 1,3-dC$_{14}$PC, 1,3-dimyristoylglycero-2-phosphocholine; 1,3-dC$_{16}$PC, 1,3-dipalmitoylglycero-2-phosphocholine; 1,3-dC$_{18}$PC, 1,3-distearoylglycero-2-phosphocholine; CC, column chromatography; cmc, critical micelle concentration; $C_n$, acyl residue of 1,3- or 1,2-PC; DPH, 1,6-diphenyl-1,3,5-hexa-triene; PC, phosphatidylcholine (egg yolk); PLD, phospholipase D; SDS, sodium dodecylsulfate.

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## 1. Introduction

While 1,2-diacyl-*sn*-glycero-3-phosphocholines (1,2-PCs) isolated from natural sources or produced by chemical syntheses have been widely studied under different aspects of interest (Cevc, 1993), there are only a few reports on the regioisomeric 1,3-diacylglycero-2-phosphocholines (1,3-PCs). De Haas and van Deenen (van Deenen and de Haas, 1963; de Haas and van Deenen, 1964, 1965) studied several 1,3-PCs with respect to their cleavability by phospholipase A. Using 1,3-PCs as substrates, the inhibitory power of two stereoisomeric monoacylamino-glycero-2-phosphocholines on $PLA_2$ was investigated (de Haas et al., 1990). In some papers the phase transition temperature (Smith, 1981; Stümpel et al., 1983; Nuhn et al., 1986), and the bilayer characteristics (Seelig et al., 1980; Kunitake et al., 1984) of 1,3- and 1,2-PCs were compared.

The main reason for the poor knowledge of the 1,3-PCs may result from difficulties in the synthesis of regioisomerically pure 1,3-diacylglycerols from which these compounds are obtainable. 1,3-Diacylglycerols were mostly synthesised by acylation of glycerol (Rose, 1947) or 2-*O*-benzylglycerol (Hartmann, 1958) with fatty acid chlorides or anhydrides. Alternative routes are the reactions of fatty acids with glycidol (Paltauf and Hermetter, 1994) or 1,3-benzylidene glycerol (Nuhn et al., 1986). Starting from the 1,3-diacylglycerols, 1,3-PCs were mostly synthesised via 2-bromoethyl dichlorophosphate as originally described by Hirth and Berchtold (1958), and subsequent replacement of the bromine by trimethylamine (Bonsen et al., 1972; Hansen et al., 1982; Nuhn et al., 1986).

Recently, however, a new elegant method has been described for the synthesis of regioisomerically pure 1,3-diacylglycerols (Berger et al., 1992), which exploits the 1,3-selective action of lipase (*Mucor mihei*) to catalyse the reaction of glycerol with vinyl esters of fatty acids. We used 1,3-diacylglycerols obtained in this way to synthesise several anionic and neutral 2-modified 1,3-diacylglycerols such as 1,3-dilauroylglycerol 2-phosphate, 1,3-dilauroylglycerol 2-sulfate or 1,3-dilauroylglycero-2-phosphocholine (1,3-di$C_{12}$PC) (Haftendorn and Ulbrich-Hofmann, 1995). These compounds were studied with respect to their tendency to form reverse micelles in organic media showing great similarities to the corresponding 1,2-diacyl-*sn*-glycero-3-phosphatides (Frense et al., 1995) and with respect to their properties as effectors in the catalysis by phospholipase D (PLD) (Dittrich et al., 1998).

PLD has been recently recognised as an important participant in cellular signal transduction processes in plants (Munnik et al., 1998) and animals (Exton, 1997) but little is known about the action mechanism of PLD and its effectors. The main substrate of most types of PLD is phosphatidylcholine (PC) yielding phosphatidic acid and choline (Heller, 1978). In the presence of an additional alcohol the enzyme is also able to transfer the phosphatidic moiety of the substrate to this alcohol, which made PLD an important tool in phospholipid synthesis (d'Arrigo and Servi, 1997). Detergents are known to strongly influence PLD activity. Anionic detergents like sodium dodecylsulfate (SDS) (Dawson and Hemington, 1967; Heller, 1978) were found to stimulate PLD activity, whereas cationic detergents were described as inhibitors (Dawson and Hemington, 1967). In the experiments with the phospholipid analogous 2-modified 1,3-diacylglycerols, the anionic compounds 1,3-dilauroylglycerol 2-phosphate and 1,3-dilauroylglycerol 2-sulfate also acted as activators, whereas 1,3-dilauroylglycero-2-phosphocholine inhibited the enzyme (Dittrich et al., 1998).

In the present paper these studies are extended to a series of 1,3-diacylglycero-2-phosphocholines with different chain lengths of fatty acids ($C_8$–$C_{18}$). The synthesis of these compounds according to the newly established chemoenzymatic strategy has been described. The compounds have been characterised with respect to their aggregation behaviour in aqueous medium by dynamic laser light scattering and determination of the critical micelle concentration (cmc). Furthermore, their efficiency as potential substrates or inhibitors of PLD (from cabbage) has been examined.

## 2. Materials and methods

### 2.1. Materials

Silica gel 60 (230–400 mesh) for column chromatography (CC) and aluminium sheets silica 60 for thin-layer chromatography were from Merck, Darmstadt (Germany). PC (egg yolk), 98.8%, was a gift from Lipoid (Ludwigshafen, Germany). SDS (special grade) was a product of Boehringer (Mannheim, Germany). PLD was prepared from fresh cabbage leaves and partially purified by heat precipitation and gel filtration on Sephadex G50 (Pharmacia, Sweden) according to Hirche et al. (1997). All other reagents were of the purest grade commercially available.

### 2.2. Chemical synthesis (general procedure)

1,3-Diacylglycerols were prepared according to Berger et al. (1992). Some of them were gifts from the laboratory of Professor Schneider, Bergische Universität-GH-Wuppertal. 2-Bromoethyl dichlorophosphate was synthesised according to Hirth and Berchtold (1958). 2-Bromoethyl dichlorophosphate (2.42 g, 10 mmol) was dissolved in 70 ml of absolute diethyl ether cooled in an ice-bath. After the addition of dry pyridine (7.1 g, 90 mmol) in 15 ml of diethyl ether, the phosphorylation mixture was stirred at room temperature. A solution of 1,3-diacylglycerol (3 mmol) in 25 ml of diethyl ether was added dropwise. Stirring was continued at room temperature until the conversion of the 1,3-diacylglycerol was completed, usually after 24–48 h. The dichlorophosphate was hydrolysed by addition of 25 ml ice-water under vigorous stirring for 1 h. Then the phases were separated and the solvent was removed by evaporation. The residue was dissolved in 60 ml of chloroform–methanol (2:1) and extracted twice with 15 ml of water acidified to pH 5.0 by HCl. The organic layer was dried with $Na_2SO_4$ and the solvent was evaporated under reduced pressure yielding 2′-bromoethyl 1,3-diacylglycerol 2-phosphate. The product was dissolved in 40 ml chloroform and 40 ml acetonitril–isopropanol (2:1). To this, 2.36 g of 33% trimethylamine (40 mmol) in ethanol were added under vigorous stirring and the reaction mixture was kept at 50°C in a tightly closed vessel overnight. Thin layer chromatography indicated the complete conversion of the bromoethyl phosphate. The solvent was removed under reduced pressure. The residue was dissolved in 40 ml chloroform and extracted with the following mixtures: twice with 35 ml formic acid–methanol (5:7), twice with 35 ml 0.1 M sodium acetate–methanol (5:7) and finally with 20 ml 1 M NaCl–methanol (5:7). The remaining organic solution was dried with $Na_2SO_4$, and the solvent was removed by evaporation. The products (yellow oils) were purified by CC on silica gel using a gradient elution with chloroform–methanol–water. After evaporation of the solvent, the products were dried in a vacuum dessiccator with $P_4O_{10}$.

All compounds gave single spots in thin layer chromatography with chloroform–methanol–ammonia 25% (65:25:5, v/v/v). $^1$H NMR and $^{13}$C NMR spectra were obtained in $CDCl_3$ using a Bruker spectrometer ARX 500. $^{31}$P NMR spectra were obtained in $CHCl_3$ with $D_2O$-capillary using a Bruker spectrometer AC 80. Chemical shifts were referred to $H_3PO_4$ at 0 ppm.

#### 2.2.1. 1,3-Dioctanoylglycero-2-phosphocholine (1,3-di$C_8$PC)

1,3-Dioctanoylglycerol (1.05 g, 3 mmol) was converted according to the general procedure. CC with 500 ml chloroform–methanol (9:1), 1000 ml chloroform–methanol (8:2), 3400 ml chloroform–methanol–$H_2O$ (8:2:0.2) gave 0.37 g of 1,3-di$C_8$PC (yield 24%).

$R_f$ 0.22, $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.86 (t, 6H, $CH_3$, $^3J$(HH) 6.98 Hz); 1.26 (m, 16H, $(CH_2)_4$); 1.57 (m, 4H, $COCH_2C\underline{H}_2$); 2.28 (t, 4H, $COC\underline{H}_2$, $^3J$(HH) 7.66 Hz); 3.36 (s, 9H $(CH_3)_3$); 3.79 (m, 2H, $CH_2$-N); 4.23-4.26 (m, 4H, $CH_2$ of glycerol); 4.33 (m, 2H, $POCH_2$); 4.49–4.54 ppm (m, 1H, CHOP).

$^{13}$C NMR (125.71 MHz, $CDCl_3$); $\delta$ 14.02 ($CH_3$); 22.58 ($CH_3$–$C\underline{H}_2$); 24.89 ($COCH_2\underline{C}H_2$); 28.95-29.12 ($(CH_2)_2$); 31.66 ($CH_3$–$CH_2$–$\underline{C}H_2$); 34.15 ($\underline{C}H_2OCOR$); 54.45 ($N^+(CH_3)_3$); 59.39 ($POCH_2$, $J$(COP) 4.9 Hz); 63.00 ($CH_2$ of glycerol, $J$(CCOP) 5.03 Hz); 66.43 ($CH_2$–N, $J$(CCOP) 5.16 Hz); 70.33 (CHOP, $J$(COP) 5.03 Hz); 173.46 ppm (C═O).

$^{31}$P NMR (32.44 MHz, $CHCl_3$; $D_2O$-capillary, ($H_3PO_4$ (0 ppm)): −1.42 ppm.

### 2.2.2. 1,3-Didecanoylglycero-2-phosphocholine (1,3-di$C_{10}$PC)

1,3-Didecanoylglycerol (1.17 g, 3 mmol) was converted according to the general procedure. CC with 400 ml chloroform–methanol (9:1), 700 ml chloroform–methanol (8:2), 1200 ml chloroform–methanol–$H_2O$ (8:2:0.2) and 700 ml chloroform–methanol/$H_2O$ (7:3:0.3) gave 0.33 g of 1,3-di$C_{10}$PC (yield 20%).

$R_f$ 0.21, $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.86 (t, 6H, $CH_3$, $^3J$(HH) 6.86 Hz); 1.24 (m, 24H, $(CH_2)_6$); 1.57 (m, 4H, $COCH_2C\underline{H}_2$); 2.28 (t, 4H, $COC\underline{H}_2$, $^3J$(HH) 7.66 Hz); 3.36 (s, 9H $(CH_3)_3$); 3.79 (m, 2H, $CH_2$–N); 4.21–4.27 (m, 4H, $CH_2$ of glycerol); 4.34 (m, 2H, $POCH_2$); 4.51–4.55 ppm (m, 1H, CHOP).

$^{13}$C NMR (125.71 MHz, $CDCl_3$); $\delta$ 14.06 ($CH_3$); 22.65 ($CH_3$–$\underline{C}H_2$); 24.91 ($COCH_2\underline{C}H_2$); 29.18–29.68 ($(CH_2)_4$); 31.86 ($CH_3$–$CH_2$–$\underline{C}H_2$); 34.19 ($\underline{C}H_2OCOR$); 54.52 ($N^+(CH_3)_3$); 59.32 ($POCH_2$, $J$(COP) 4.9 Hz); 63.14 ($CH_2$ of glycerol, $J$(CCOP) 5.03 Hz); 66.55 ($CH_2$–N, $J$(CCOP) 3.01 Hz); 70.26 (CHOP, $J$(COP) 5.03 Hz); 173.47 ppm (C═O).

$^{31}$P NMR (32.44 MHz, $CHCl_3$; $D_2O$-capillary, ($H_3PO_4$ (0 ppm)): −1.35 ppm.

### 2.2.3. 1,3-Dilauroylglycero-2-phosphocholine (1,3-di$C_{12}$PC)

1,3-Dilauroylglycerol (1.37 g, 3 mmol) was converted according to the general procedure. CC with 500 ml chloroform–methanol (9:2), 1000 ml chloroform–methanol (8:2), 3000 ml chloroform–methanol–$H_2O$ (8:2:0.2) gave 0.56 g of 1,3-di$C_{12}$PC (yield 30%).

$R_f$ 0.22, $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.86 (t, 6H, $CH_3$, $^3J$(HH) 6.4 Hz); 1.23 (m, 32H, $(CH_2)_8$); 1.56 (m, 4H, $COCH_2C\underline{H}_2$); 2.28 (t, 4H, $COC\underline{H}_2$, $^3J$(HH) 7.5 Hz); 3.34 (s, 9H $(CH_3)_3$); 3.76 (m, 2H, $CH_2$–N); 4.21–4.23 (m, 4H, $CH_2$ of glycerol); 4.31 (m, 2H, $POCH_2$); 4.55 ppm (m, 1H, CHOP).

$^{13}$C NMR (20.149 MHz, $CDCl_3$); $\delta$ 14.06 ($CH_3$); 22.67 ($CH_3$–$\underline{C}H_2$); 24.92 ($COCH_2\underline{C}H_2$); 29.43–29.63 ($(CH_2)_6$); 31.91 ($CH_3$–$CH_2$–$\underline{C}H_2$); 34.19 ($\underline{C}H_2OCOR$); 54.48 ($N^+(CH_3)_3$); 59.31 ($POCH_2$, $J$(COP) 4.9 Hz); 62.99 ($CH_2$ of glycerol, $J$(CCOP) 3.61 Hz); 66.49 ($CH_2$–N, $J$(CCOP) 7.46 Hz); 70.44 (CHOP); 173.44 ppm (C=O).

$^{31}$P NMR (32.44 MHz, $CHCl_3$; $D_2O$-capillary, ($H_3PO_4$ (0 ppm)): −1.9 ppm.

### 2.2.4. 1,3-Dimyristoylglycero-2-phosphocholine (1,3-di$C_{14}$PC)

1,3-Dimyristoylglycerol (1.54 g, 3 mmol) was converted according to the general procedure. CC with 500 ml chloroform–methanol (9:1), 900 ml chloroform–methanol (8:2), 1500 ml chloroform–methanol–$H_2O$ (8:2:0.2) and 1000 ml chloroform–methanol–$H_2O$ (7:3:0.3) gave 0.55 g of 1,3-di$C_{14}$PC (yield 24%).

$R_f$ 0.26, $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.86 (t, 6H, $CH_3$, $^3J$(HH) 6.87 Hz); 1.24 (m, 40H, $(CH_2)_{10}$); 1.56 (m, 4H, $COCH_2C\underline{H}_2$); 2.28 (t, 4H, $COC\underline{H}_2$, $^3J$(HH) 7.67 Hz); 3.36 (s, 9H $(CH_3)_3$); 3.81 (m, 2H, $CH_2$–N); 4.23 (m, 4H, $CH_2$ of glycerol); 4.32 (m, 2H, $POCH_2$); 4.47–4.51 ppm (m, 1H, CHOP).

$^{13}$C NMR (125.71 MHz, $CDCl_3$); $\delta$ 14.05 ($CH_3$); 22.65 ($CH_3$–$\underline{C}H_2$); 24.91 ($COCH_2\underline{C}H_2$); 29.21–29.69 ($(CH_2)_8$); 31.90 ($CH_3$–$CH_2$–$\underline{C}H_2$); 34.16 ($CH_2OCOR$); 54.46 ($N^+(CH_3)_3$); 59.39 ($POCH_2$, $J$(COP) 4.9 Hz); 63.08 ($CH_2$ of glycerol, $J$(CCOP) 3.89 Hz); 66.49 ($CH_2$–N, $J$(CCOP) 4.78 Hz); 70.32 (CHOP, $J$(COP) 5.91 Hz); 173.44 ppm (C=O).

$^{31}$P NMR (32.44 MHz, $CHCl_3$; $D_2O$-capillary, ($H_3PO_4$ (0 ppm)): −1.35 ppm.

### 2.2.5. 1,3-Dipalmitoylglycero-2-phosphocholine (1,3-di$C_{16}$PC)

1,3-Dipalmitoylglycerol (1.70 g, 3 mmol) was converted according to the general procedure. CC with 300 ml chloroform–methanol (9:1), 600 ml chloroform–methanol (8:2), 1100 ml chloroform–methanol–$H_2O$ (8:2:0.2) and 1000 ml chloroform–methanol–$H_2O$ (7:3:0.3) gave 0.29 g of 1,3-di$C_{16}$PC (yield 13%).

$R_f$ 0.30, $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.86 (t, 6H, $CH_3$, $^3J$(HH) 6.87 Hz); 1.24 (m, 48H, $(CH_2)_{12}$); 1.56 (m, 4H, $COCH_2C\underline{H}_2$); 2.28 (t, 4H, $COC\underline{H}_2$, $^3J$(HH) 7.67 Hz); 3.36 (s, 9H $(CH_3)_3$); 3.79 (m, 2H, $CH_2$–N); 4.23-4.32 (m, 4H, $CH_2$ of glycerol); 4.49 (m, 2H, $POCH_2$); 4.52 ppm (m, 1H, CHOP).

$^{13}C$ NMR (125.71 MHz, $CDCl_3$); $\delta$ 14.05 ($CH_3$); 22.66 ($CH_3-\underline{C}H_2$); 24.92 ($COCH_2\underline{C}H_2$); 29.22–29.71 ($(CH_2)_{10}$); 31.91 ($CH_3-CH_2-\underline{C}H_2$); 34.17 ($\underline{C}H_2OCOR$); 54.48 ($N^+(CH_3)_3$); 59.33 ($POCH_2$, $J$(COP) 3.94 Hz); 63.02 ($CH_2$ of glycerol, $J$(CCOP) 4.05 Hz); 66.51 ($CH_2-N$, J(CCOP) 7.04 Hz); 70.31 (CHOP, $J$(COP) 4.02 Hz); 173.44 ppm (C=O).

$^{31}P$ NMR (32.44 MHz, $CHCl_3$; $D_2O$-capillary, ($H_3PO_4$ (0 ppm)): $-1.39$ ppm.

### 2.2.6. 1,3-Distearoylglycero-2-phosphocholine (1,3-di$C_{18}$PC)

1,3-Distearoylglycerol (1.90 g, 3 mmol) was converted according to the general procedure. CC with 700 ml chloroform–methanol (9:1), 1200 ml chloroform–methanol (8:2), 2200 ml chloroform–methanol–$H_2O$ (8:2:0.2) gave 0.62 g of 1,3-di$C_{18}$PC (yield 26%).

$R_f$ 0.29, $^1H$ NMR (500 MHz, $CDCl_3$): $\delta$ 0.86 (t, 6H, $CH_3$, $^3J$(HH) 6.96 Hz); 1.24 (m, 56H, $(CH_2)_{14}$); 1.56 (m, 4H, $COCH_2\underline{C}H_2$); 2.28 (t, 4H, $COC\underline{H}_2$, $^3J$(HH) 7.56 Hz); 3.36 (s, 9H $(CH_3)_3$); 3.81 (m, 2H, $CH_2-N$); 4.23 (m, 4H, $CH_2$ of glycerol); 4.32 (m, 2H, $POCH_2$); 4.48–4.50 ppm (m, 1H, CHOP).

$^{13}C$ NMR (125.71 MHz, $CDCl_3$); $\delta$ 14.03 ($CH_3$); 22.63 ($CH_3-\underline{C}H_2$); 24.88 ($COCH_2\underline{C}H_2$); 29.19–29.68 ($(CH_2)_{12}$); 31.88 ($CH_3-CH_2-\underline{C}H_2$); 34.14 ($\underline{C}H_2OCOR$); 54.44 ($N^+(CH_3)_3$); 59.29 ($POCH_2$, $J$(COP) 4.99 Hz); 63.00 ($CH_2$ of glycerol, $J$(CCOP) 3.99 Hz); 66.47 ($CH_2-N$, $J$(C-COP) 6.98 Hz); 70.25 (CHOP, $J$(COP) 5.98 Hz); 173.42 ppm (C=O).

$^{31}P$ NMR (32.44 MHz, $CHCl_3$; $D_2O$-capillary, ($Hd_3PO_4$ (0 ppm)): $-1.45$ ppm.

## 2.3. Physicochemical experiments

### 2.3.1. Laser light scattering

Stock solutions (13 mM) of 1,3-PCs were prepared as follows: 1,3-PCs (26 μmol) were dissolved in chloroform and the solvent was removed in a vacuum evaporator. The resulting lipid film was suspended in 2 ml aqua bidest. to obtain the pure lipid dispersions or with 2 ml 6.5 mM SDS solution for 1,3-PC/SDS (2:1). The mixtures were heated to their phase transition temperature, shaken by a Vortex mixer and homogenised by ultrasonic treatment with 300 Ws $ml^{-1}$ using an ultrasonic disintegrator 60 W Vibra cell (Avantec, France) with a microtip probe of 3 mm. After dilution of the stock solutions with aqua bidest. 1:1 (v/v), the size of the 1,3-PC aggregates in aqueous solution was determined by dynamic laser light scattering (scattering angle 90°) using the Autosizer 2C (Malvern Instruments, operating at He–Ne-laser at 633 nm) and fitted software. The size of the aggregates is expressed as the mean diameter of at least three measurements.

### 2.3.2. Determination of cmc

Cmcs for 1,3-di$C_8$PC, 1,3-di$C_8$PC/SDS (2:1) and 1,3-di$C_{10}$PC/SDS (2:1) were determined by measuring the fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH) as a function of the phospholipid concentration according to Chattopadhyay and London (1984). DPH was excited at 358 nm and emission was recorded at 430 nm.

## 2.4. Enzymatic studies

### 2.4.1. Substrate stock solutions

For the preparation of standard substrate PC/SDS (2:1), 50 mg (65 μmol) PC was dissolved in 500 μl chloroform. The solvent was removed in a vacuum evaporator. The PC film obtained was suspended in 5 ml aqua bidest. containing 32.5 μmol SDS. The mixture was heated to 40°C, shaken by a Vortex mixer and sonicated in an ultrasonic bath. Additional homogenisation of the vesicle preparation was performed by ultrasonic treatment with 300 Ws $ml^{-1}$ using an ultrasonic disintegrator 60 W Vibra cell (Avantec, France) with a microtip probe of 3 mm. The resulting lipid dispersion was an opalescent solution containing 13 mM PC/6.5 mM SDS.

For the experiments with 1,3-PCs, 13 mM stock solutions of these compounds were prepared as described in Section 2.3.1.

### 2.4.2. Assay for PLD activity

PLD activity was determined from the pH change caused by formation of phosphatidic acid as described by Allgyer and Wells (1979), and by Lambrecht and Ulbrich-Hofmann (1992).

Substrate stock solution (200 μl) was diluted with 2 mM sodium acetate buffer, pH 5.7, containing 40 mM $CaCl_2$ to give the desired substrate concentration in 3 ml total volume. In the standard assay it was 0.87 mM PC/0.43 mM SDS. After thermal equilibration at 30°C, the reaction was started by the addition of 10 μl PLD (1 mg protein/ml, determined according to Bradford (1976), in 2 mM sodium acetate buffer, pH 5,7, containing 40 mM $CaCl_2$) and followed by pH-stat titration on an Autotitrator VIT 90 (Radiometer, Denmark) with 10 mM NaOH. From the linear increase of consumption of NaOH the reaction rate was estimated using the integrated programme Titra File II. In the standard assay PLD activity amounted to 0.26 mkat $mg^{-1}$. The S.D. were $\leq 6\%$ ($n = 3$). The detection limit was 0.003 mkat $mg^{-1}$.

### 2.4.3. Inhibition of PLD by 1,3-PC (determination of $IC_{50}$ and $\gamma_{50}$)

Standard substrate stock solution (PC/SDS = 2:1) (200 μl) and 10–200 μl stock solution of 1,3-PC were unified and homogenised by sonification with 300 Ws $ml^{-1}$. This mixture (200–400 μl) was diluted with 2 mM sodium acetate buffer, pH 5,7, containing 40 mM $CaCl_2$ to 3 ml. The reaction was started with 10 μl PLD (1 mg protein/ml in the same buffer) and enzyme activity was assayed by pH-stat titration as described above. Kinetic data were fitted to the exponential equation:

$$v_i = v_0 \times \exp(-ax) + b, \quad (1)$$

where $v_i$ and $v_0$ are the initial rates in the presence and absence of inhibitor, $x$ is the concentration (or the mole fraction) of the inhibitor, and $a$ and $b$ are constants. The inhibitor concentration, $IC_{50}$, (or the mole fraction, $\gamma_{50}$) where $v_i = v_0/2$, were calculated from Eq. (2):

$$IC_{50}\ (\gamma_{50}) = \ln[2v_0/(v_0 - 2b)]/a. \quad (2)$$

## 3. Results and discussion

### 3.1. Synthesis

In analogy to the synthesis of 1,3-di$C_{12}$PC (Haftendorn and Ulbrich-Hofmann, 1995), 1,3-PCs with different chain length of the acyl residues ($C_8$, $C_{10}$, $C_{12}$, $C_{14}$, $C_{16}$, and $C_{18}$) were produced. In the first step the corresponding 1,3-diacylglycerols were synthesised by the method of Berger et al. (1992) (Fig. 1a). These compounds were converted to the 1,3-diacylglycero-2-phosphoric acid-2′-bromoethylesters by reaction with 2-bromoethylphosphoric acid dichloride according to a modified method by Hansen et al. (1982) (Fig. 1b). The crude products were converted to the 1,3-diacylglycero-2-phosphocholines by the replacement of bromine by trimethylamine. After column chromatography the regioisomerically pure products were obtained in yields up to 30%. They were identified by $^1$H NMR, $^{13}$C NMR, and $^{31}$P NMR. The advantage of this method consists in the easy accessibility of the regioisomerically pure 1,3-diacylglycerols. In comparison with conventional chemical methods (Eibl et al., 1967; Nuhn et al., 1986) two reaction steps could be saved.

### 3.2. Aggregation behaviour

The aggregation behaviour of the synthesised 1,3-PCs in aqueous solution was characterised by dynamic laser light scattering. Since SDS, as an effective activator of PLD, was used in the enzymatic studies described below, its influence on the light scattering behaviour of the 1,3-PC aggregates was also studied. In the absence of SDS, particle sizes could be only determined for 1,3-di$C_{10}$PC and 1,3-di$C_{12}$PC suggesting that these compounds are organised as liposomes (Table 1). The short chain 1,3-di$C_8$PC was assumed to form micelles, since the particle size is smaller than 20 nm, which is the lowest detectable size for the method used. In order to confirm the presence of micelles, the fluorescence of DPH was measured proving the formation of micelles with a cmc of 0.165 mM. Also, no reasonable results of particle size were obtained for the long chain 1,3-PCs ($C_{14}$, $C_{16}$, and $C_{18}$) by laser light scattering. The high polydispersity index (PI) of 1.0, indicating a wide distribution of the particle sizes beyond the registration limit, suggests the existence of planar bilayers as the main structure of the long chain 1,3-PCs. These results are in accordance with the findings

of Kunitake et al. (1984) who characterised the structure of sonicated samples of 1,3-di$C_{12}$PC and 1,3-di$C_{14}$PC as multi-walled vesicles and that of 1,3-di$C_{16}$PC and 1,3-di$C_{18}$PC as lamellar aggregates by freeze fracture electron microscopy.

When the aggregation behaviour of the 1,3-PCs is compared with that of the naturally configured 1,2-diacyl-*sn*-glycero-3-phosphocholines, great similarities can be realised. 1,2-di$C_8$PC forms micelles above 0.27 mM (Marsh, 1990). 1,2-di$C_{10}$PC occurs in the form of bilayers (Marsh, 1990). For the longer chain 1,2-PCs bilayer structures are also known (Cevc, 1993).

In the presence of SDS remarkable changes in aggregate structures were observed (Table 1). In the case of 1,3-di$C_{10}$PC, SDS is obviously able to solubilise the lipid to give mixed micelles similar to 1,3-di$C_8$PC. The cmc-values of the mixed micelle systems 1,3-di$C_8$PC/SDS (2:1) and 1,3-di$C_{10}$PC/SDS (2:1) were determined to be 0.120

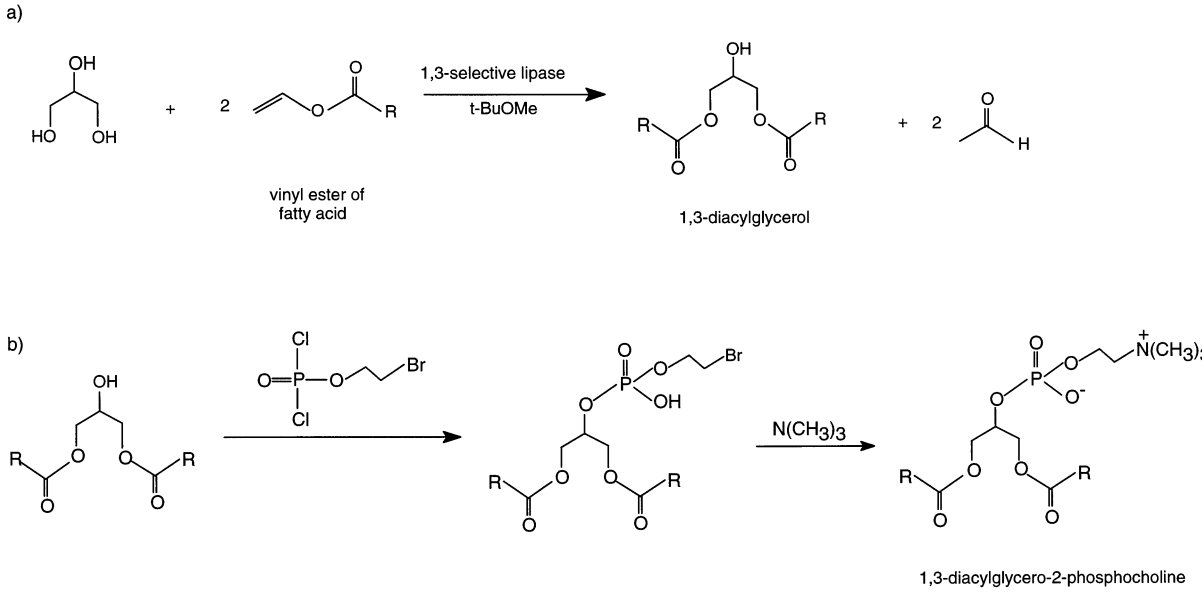


Fig. 1. Scheme of chemoenzymatic synthesis of 1,3-diacylglycero-2-phosphocholines: (a) enzymatic synthesis of 1,3-diacylglycerols; (b) chemical introduction of the phosphocholine group.

Table 1
Aggregate structures of 1,3-diacylglycero-2-phosphocholines

| 1,3-PC | Pure 1,3-PC | | | 1,3-PC/SDS (2:1) | | |
|---|---|---|---|---|---|---|
| | Particle size (nm) | PI | Suggested aggregation form | Particle size (nm) | PI | Suggested aggregation form |
| 1,3-d$C_8$PC | <20 | – | Micelles | <20 | – | Mixed micelles |
| 1,3-d$C_{10}$PC | 157 | 0.21 | Liposomes | <20 | – | Mixed micelles |
| 1,3-d$C_{12}$PC | 174 | 0.15 | Liposomes | 77 | 0.19 | Mixed liposomes |
| 1,3-d$C_{14}$PC | n.d.[a] | 1.0 | Planar bilayer | 79 | 0.29 | Mixed liposomes |
| 1,3-d$C_{16}$PC | n.d. | 1.0 | Planar bilayer | 101 | 0.29 | Mixed liposomes |
| 1,3-d$C_{18}$PC | n.d. | 1.0 | Planar bilayer | 185 | 0.29 | Mixed liposomes |

[a] n.d.: Not detectable by this method.

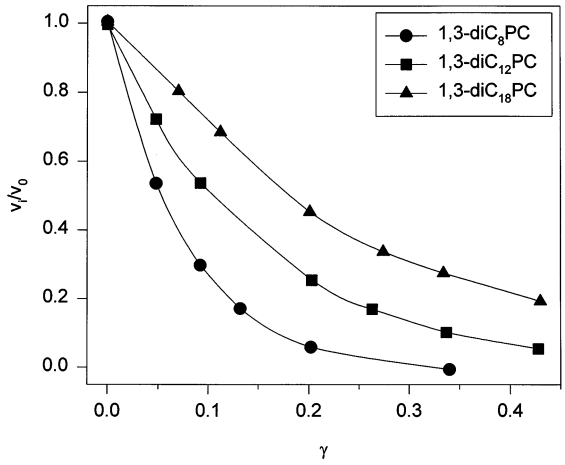


Fig. 2. Inhibition of phospholipase D as function of mole fraction ($\gamma$) of 1,3-PCs. $v_i/v_0$ are the initial reaction rates in presence and absence of inhibitor. $\gamma = [1,3\text{-PC}]/([1,3\text{-PC}] + [\text{PC}])$.

mM and 7.04 μM, respectively. With 1,3-di$C_{12}$PC, liposomes as the main structure were formed even in the presence of SDS but the particle sizes decreased compared with those in the absence of SDS (Table 1). For the long chain 1,3-PCs ($C_{14}$, $C_{16}$, and $C_{18}$) light scattering experiments indicate a transformation of planar bilayer structures by SDS to mixed liposomes. This transformation is presumably due to the cone shape of SDS (Israelachvili et al., 1980), which might force a stronger curvature of the lipid bilayer and lead to a decrease of particle sizes.

### *3.3. 1,3-PCs as potential substrates and inhibitors of PLD*

All 1,3-PCs ($C_8$–$C_{18}$) were tested as substrates for PLD. The experiments were performed in the absence and in the presence of 0.25, 0.5 and 1 equivalent of SDS, which is a potent activator of PLD from cabbage (Dawson and Hemington, 1967). In contrast to the corresponding 1,2-PCs in no case any activity could be detected. Since there are no evident differences in the aggregation behaviour of 1,3-PCs and 1,2-PCs as demonstrated above, it has to be assumed that the molecular geometry of the 1,3-PCs does not fit into the active site of PLD. In contrast, 1,3-PCs were shown to be substrates of phospholipase A from *Crotalus adamanteus* which hydrolyses their 1-ester bonds albeit with a lower rate than the 2-ester bonds in the naturally configured substrates (de Haas and van Deenen, 1964).

The effector properties of the different 1,3-PCs ($C_8$–$C_{18}$) were studied in the standard assay for PLD from cabbage using PC/SDS (2:1). Due to homogenisation by sonification, it can be assumed that 1,3-PCs are incorporated into the mixed liposomes of PC/SDS. In all cases 1,3-PCs showed an inhibitory effect on PLD activity. Fig. 2 shows the relative reaction rates $v_i/v_0$ as a function of the mole fraction of the effectors 1,3-di$C_8$PC, 1,3-di$C_{12}$PC and 1,3-di$C_{18}$PC, and Table 2 gives the $IC_{50}$- and $\gamma_{50}$-values for all the compounds tested. A remarkable dependence of the inhibitory power on the chain

Table 2
$IC_{50}$ and $\gamma_{50}$-values for the inhibition of PLD by 1,3-PCs in comparison to its inhibition by alkylphosphocholines of different chain lengths

| 1,3-PC | $IC_{50}$ (μM) | $\gamma_{50}$ | Alkylphosphocholine | $IC_{50}$ (μM)[a] | $\gamma_{50}$[a] |
|---|---|---|---|---|---|
| 1,3-d$C_8$PC | 50 | 0.053 | | | |
| 1,3-d$C_{10}$PC | 43 | 0.045 | $C_{10}$ | 142 | 0.141 |
| 1,3-d$C_{12}$PC | 108 | 0.098 | $C_{12}$ | 102 | 0.106 |
| 1,3-d$C_{14}$PC | 185 | 0.173 | $C_{14}$ | 41 | 0.045 |
| 1,3-d$C_{16}$PC | 181 | 0.172 | $C_{16}$ | 13 | 0.015 |
| 1,3-d$C_{18}$PC | 192 | 0.183 | $C_{18}$ | 6.4 | 0.007 |

[a] Values according to Dittrich et al., 1996.

length of the acyl chains can be observed. Short chain 1,3-PCs ($C_8$ and $C_{10}$) are more effective as inhibitors than the long-chain 1,3-PCs ($C_{14}$, $C_{16}$, and $C_{18}$). This tendency is opposite to the inhibitory effects of the alkyl phosphocholines (Table 2), which have been studied recently (Dittrich et al., 1996). Interestingly, the maximum inhibition effects are much larger with the one-chain alkyl phosphocholines than with the glycerol derivatives. The different organisation of the substrate aggregates containing inhibitor and detergent molecules and/or the different affinities of the inhibitor molecules to the PLD might be reasons for this behaviour. With both types of inhibitors the inhibitory effect decreases with increasing substrate concentration. Therefore, a competitive type of inhibition is suggested although a classical kinetic analysis is not possible because of the sigmoidal curves of initial rates versus substrate concentration (Dittrich et al., 1998). Further studies are necessary to elucidate the inhibition mechanism.

## 4. Conclusions

As demonstrated for six 1,3-PCs the chemoenzymatic synthesis route opens a simple access to the regioisomers of naturally configured glycerophospholipids. These compounds show aggregation properties similar to the natural phospholipid structures as demonstrated for aqueous milieu (this paper) or for organic media (Frense et al., 1995). Obviously, the position of the hydrophilic phosphatidylcholine head group has no or only little effect on the formation of suprastructures. In contrast, enzymes such as PLD are able to differentiate the 2- or 3-position of the head group. Therefore, 1,3-PCs are interesting candidates as substitutes for natural phospholipids in pharmaceutical drug delivery systems or vector materials possessing the same physicochemical properties but higher biological stability. Furthermore, they might become important as effectors of phospholipid-transforming enzymes in medicine.

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