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Comparative Studies on the Susceptibility of (*R*)-2,3-Dipalmitoyloxypropylphosphonocholine (DPPnC) and Its Phospholipid Analogues to the Hydrolysis or Ethanolysis Catalyzed by Selected Lipases and Phospholipases

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Abstract: Susceptibility of soybean phosphatidylcholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and its phosphono analogue (*R*)-2,3-dipalmitoyloxypropylphosphonocholine (DPPnC) towards selected lipases and phospholipases was compared. The ethanolysis of substrates at *sn*-1 position was carried out by lipase from *Mucor miehei* (Lipozyme®) and lipase B from *Candida antarctica* (Novozym 435) in 95% ethanol at 30 °C, and the hydrolysis with Lecitase™ Ultra was carried out in hexane/water at 50 °C. Hydrolysis at *sn*-2 position was carried out in isooctane/Tris-HCl/AOT system at 40 °C using phospholipase A_2 (PLA_2) from porcine pancreas and PLA_2 from bovine pancreas or 25 °C using PLA_2 from bee venom. Hydrolysis in the polar part of the studied compounds was carried out at 30 °C in acetate buffer/ethyl acetate system using phospholipase D (PLD) from *Streptococcus* sp. and PLD from white cabbage or in Tris-HCl buffer/methylene chloride system at 35 °C using PLD from *Streptomyces chromofuscus*. The results showed that the presence of C-P bond between glycerol and phosphoric acid residue in DPPnC increases the rate of enzymatic hydrolysis or ethanolysis of ester bonds at the *sn*-1 and *sn*-2 position and decreases the rate of hydrolysis in the polar head of the molecule. The most significant changes in the reaction rates were observed for reaction with PLD from *Streptococcus* sp. and PLD from *Streptomyces chromofuscus* that hydrolyzed DPPnC approximately two times slower than DPPC and soybean PC. The lower susceptibility of DPPnC towards enzymatic hydrolysis by phospholipases D gives hope for the possibility of using DPPnC-like phosphonolipids as the carriers of bioactive molecules that, instead of choline, can be bounded with diacylpropylphosphonic acids (DPPnA).

Keywords: phosphonolipids; phospholipids; enzymatic hydrolysis; lipases; phospholipases

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1. Introduction

Phosphonolipids (PnLs) are one of the most interesting groups of lipids. In nature, phosphonolipids can be found in many species of microorganisms, marine organisms [1–3] and hen's egg [4]. They are components of the cell membrane that ensure its stability. Phosphonolipids facilitate the movement of DNA [5], and some of them are inhibitors of enzymes [6,7].

Natural phosphonolipids possess the C-P bond on the polar (choline) side of the molecule. For this reason, the first syntheses and the biological studies were carried out on phosphonolipids, which are esters of 2-aminoethylphosphonic acid with diacylglycerol or with *N*-acylsphingosine [8–10]. At the beginning of the 21st century, Prestwich research group synthesized some phosphonolipids with C-P bond on hydrophobic (glycerol) side. They obtained a wide variety of phosphono analoges of 2-lysophosphatidic acid [11–13], which showed activity as long-lived receptor-specific agonists and antagonists for LPA

receptors. As inhibitors of the lysophospholipase D activity, they can be useful in therapy of cancer disease [14,15].

A few years ago, we also reported the synthesis of a series of phosphonolipids with C-P bond on a glycerol side. They contained different fatty acid residues and choline in their structure [16]. We have been planning to compare their biological activity, mainly cytotoxic, with the activity of corresponding phosphatydylcholines. In our research plans, there is also a use of phosphonolipids as drug carriers. Before starting these studies, it is necessary to check their susceptibility towards hydrolytic enzymes involved in the first step of lipid metabolism, namely lipases and phospholipases.

Lipases and phospholipases are environmentally friendly biocatalysts that catalyze the hydrolysis of ester bonds of triacylglycerols and phospholipids, respectively. Besides hydrolytic activity, lipases also catalyze esterification and transesterification reaction [17]. Phospholipases also show esterification and transphosphatidylation activity [18]. Moreover, these biocatalysts have broad substrate specificity, high enantioselectivity and stability in organic solvents and at extreme temperatures and pH [19]. They are commonly used in the synthesis of structured lipids with different physical and/or nutritional properties [20]. Thus, lipases and phospholipases have found application in fine chemistry, pharmacy, agriculture, food industry and cosmetics [21,22].

Here, for the first time, we present the results of comparable studies on the hydrolysis (or ethanolysis) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and its phosphono analogue (*R*)-2,3-dipalmitoyloxypropylphosphonocholine (DPPnC) catalyzed by selected lipases and phospholipases.

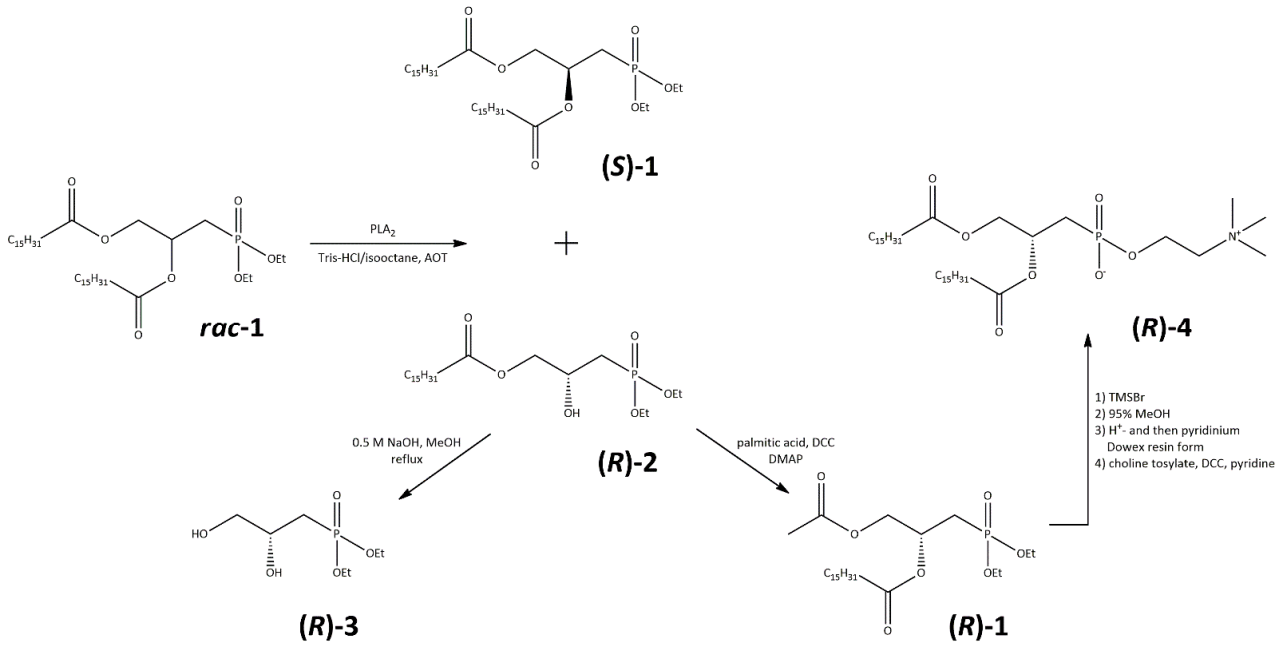
2. Results and Discussion

In order to compare the susceptibility of phosphonolipids and phospholipids to the selected hydrolytic enzymes, the same absolute configurations of model substrates, namely DPPC and its phosphono analogue DPPnC, is required. As DPPC was synthesized from *sn*-glycero-3-phosphocholine (GPC), with a retention typical for natural phospholipids "*R*" configuration at C-2, in the first step of our research we needed to synthesize (*R*)-enantiomer of DPPnC.

2.1. Synthesis of (R)-2,3-Dipalmitoyloxypropylphosphonocholine (DPPnC, **4***)*

Synthesis of (*R*)-DPPnC (**4**) is shown in Scheme 1. The key step of this procedure was regio- and enantioselective hydrolysis of racemic diethyl 2,3-dipalmitoyloxypropylphosphonate (**1**), which was obtained as described in our previous paper [16]. The reaction was catalyzed by phospholipase A_2 from porcine pancreas in isooctane/Tris-HCl system (pH = 8.5) in the presence of AOT and Ca^{2+} ions. In these conditions, diethyl (*R*)-2-hydroxy-3-palmitoyloxypropylphosphonate (**2**) was obtained at 22% yield.

Enantiomeric excesses of (*R*)-2-hydroxyphosphonate **2** and unreacted (*S*)-substrate **1** were determined by HPLC with application of chiral column (Figure 1). This analysis indicated 19% *ee* of unreacted (*S*)-diethyl 2,3-dipalmitoyloxypropylphosphonate ((*S*)-**1**) (Figure 1C) and 87% *ee* of the desired product of hydrolysis (**2**), which was analyzed after its esterification with palmitic acid to (*R*)-diethyl 2,3-dipalmitoyloxypropylphosphonate **1** (Figure 1B). Absolute configuration "*R*" of 2-hydroxyphosphonate **2** obtained after phospholipase A_2-catalyzed hydrolysis of *rac*-**1** was confirmed by comparison of optical rotation of known [23] (*R*)-diol **3** ($[\alpha]_D^{25} = -12.2$ (c 4.1, EtOH)) with the optical rotation of diol **3** ($[\alpha]_D^{20} = -8.7$ (c 0.4, EtOH)) obtained as the product of hydrolysis of (*R*)-2-hydroxyphosphonate **2** with KOH in MeOH (Scheme 1).



Scheme 1. Synthesis of (*R*)-2,3-dipalmitoyloxypropylphosphonocholine (**4**).

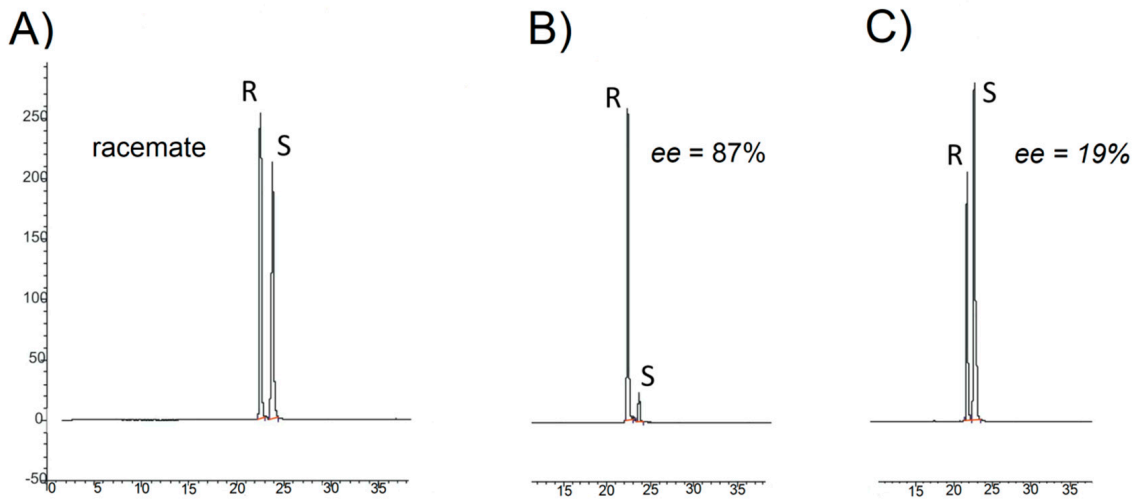


Figure 1. Chromatograms from chiral HPLC analysis of racemic diethyl 2,3-dipalmitoyloxypropylphosphonate (**1**) (**A**) and the products of PLA_2-catalyzed hydrolysis of racemic phosphonate **1**: (*R*)-hydroxyphosphonate **2** (analyzed after derivatization to (*R*)-phosphonate **1**) (**B**) and unreacted (*S*)-phosphonate (**1**) (**C**).

Enantiomerically enriched (*R*)-2-hydroxyphosphonate **2** was the starting material in the four-step synthesis of target phosphonolipid, (*R*)-DPPnC (**4**). In the first step, hydroxy group at C-2 in (*R*)-2-hydroxyphosphonate **2** was esterified with palmitic acid via Steglich method to afford (*R*)-diethyl 2,3-dipalmitoyloxypropylphosphonate **1** in 94% yield. (*R*)-Phosphonate **1** was sillylated using TMSBr, followed by methanolysis to afford (*R*)-phosphonic acid, which, in the form of pirydinium salt, was subjected to the reaction with choline tosylate to produce (*R*)-DPPnC (**4**). The total yield of synthesis of **4** from (*R*)-phosphonate **1** was 51%. The described synthetic pathway, established earlier for racemic 2,3-diacyloxypropylphosphonocholines [16], ensured the retention of *R* configuration for final phosphonolipid **4**.

2.2. Enzymatic Reactions of Soybean PC, DPPC and DPPnC with Selected Lipases and Phospholipases

To test the susceptibility of synthesized phosphonolipid to the hydrolytic enzymes, in each experiment we compared the results of DPPnC hydrolysis with its phospholipid counterpart as well as with natural soybean PC. The detailed results are given in Tables S1–S3, and the progress of reactions is presented in Figures 2–4. For each enzyme treatment, the control experiment without the addition of enzyme was carried out to check the stability of substrates in the reaction conditions.

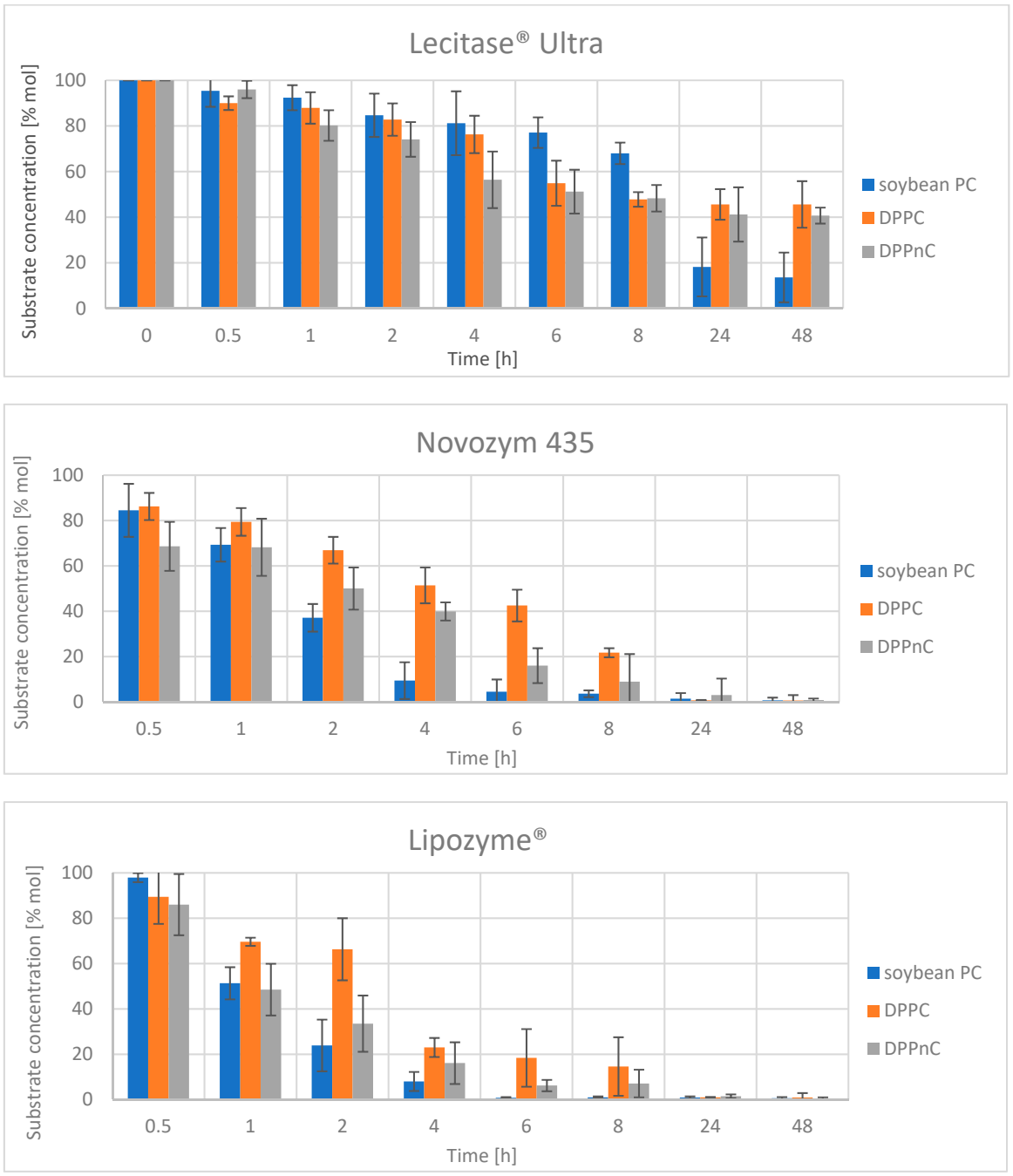


Figure 2. Enzymatic reactions of DPPnC, DPPC and soybean PC in *sn*-1 position. Results are presented as means ± standard deviation.

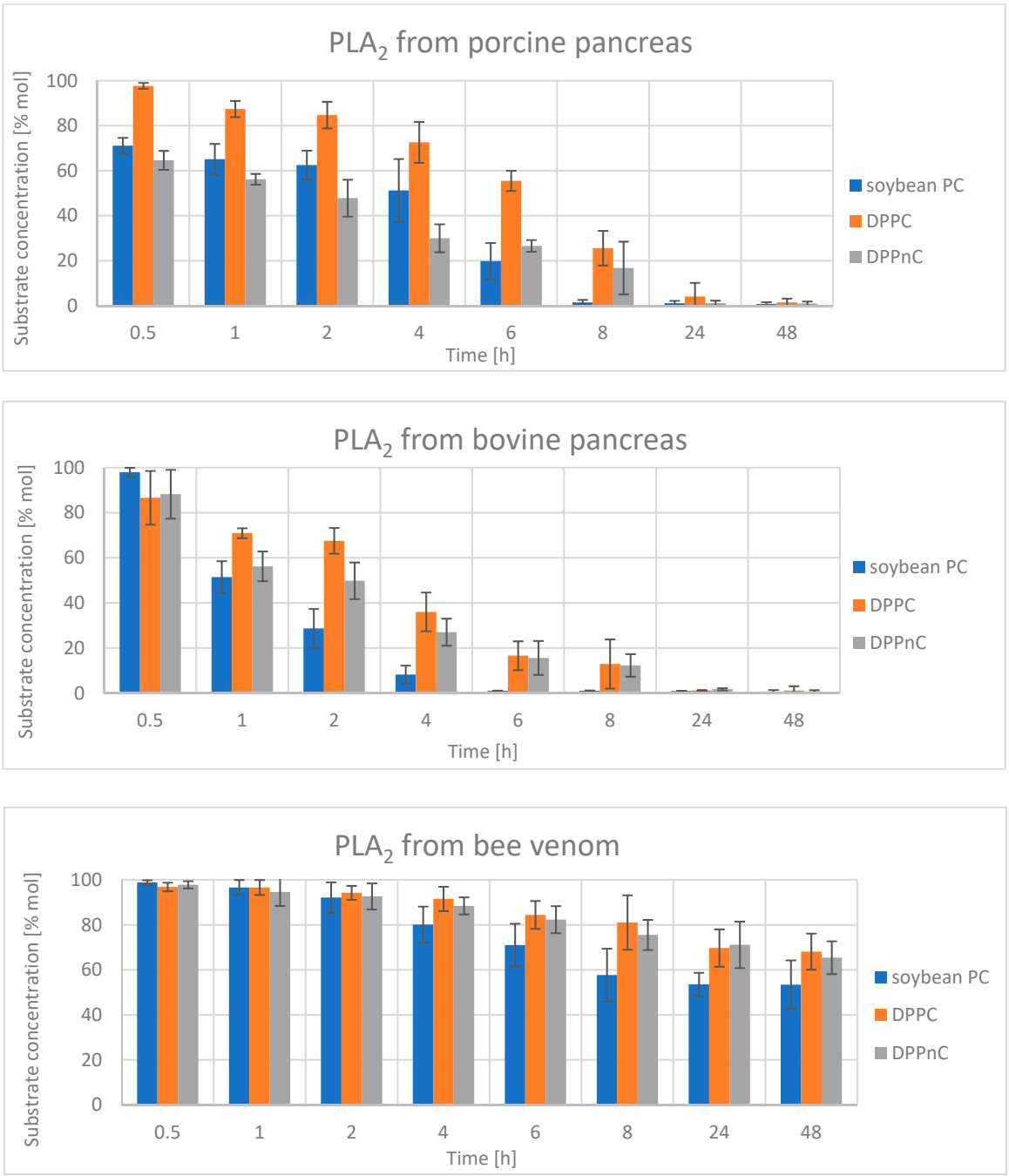


Figure 3. Enzymatic hydrolysis of DPPnC, DPPC and soybean PC by phospholipases A_2. Results are presented as means ± standard deviation.

2.2.1. Reactions at *sn*-1 Position

In the first set of experiments, enzymes selective towards *sn*-1 position of natural phospholipids were used: lipase from *Mucor miehei* (Lipozyme®) [24], lipase B from *Candida antarctica* (Novozym 435) [25] and Lecitase™ Ultra, chimeric enzyme produced by the fusion of the genes of the lipase from *Thermomyces lanuginosus* and the phospholipase A_1 from *Fusarium oxysporum* [26]. The alcoholysis of substrates catalyzed by Lipozyme® and Novozym 435 was carried out in 95% ethanol [27], and the hydrolysis with Lecitase™ Ultra was carried out in hexane (Scheme 2) [28].

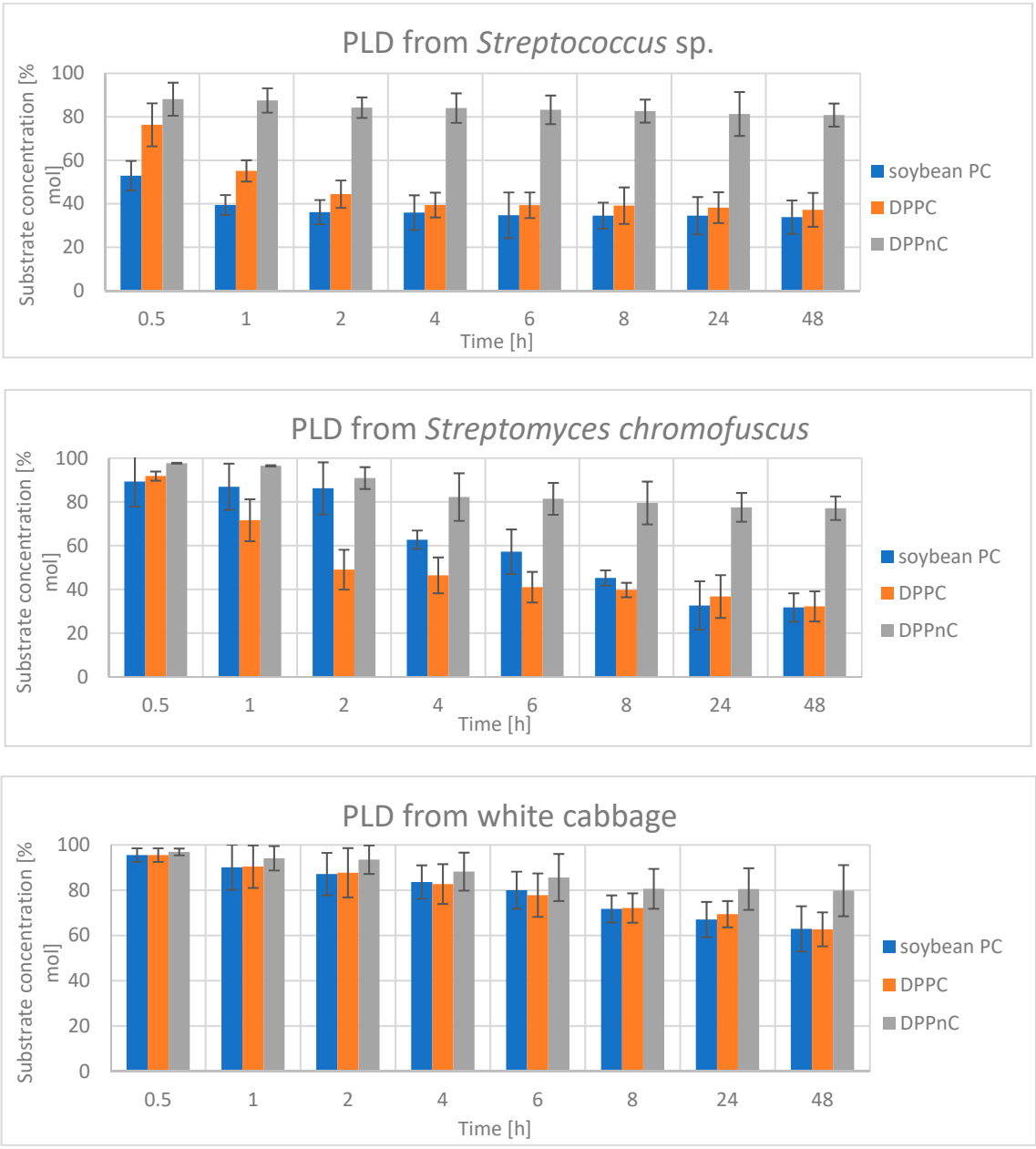
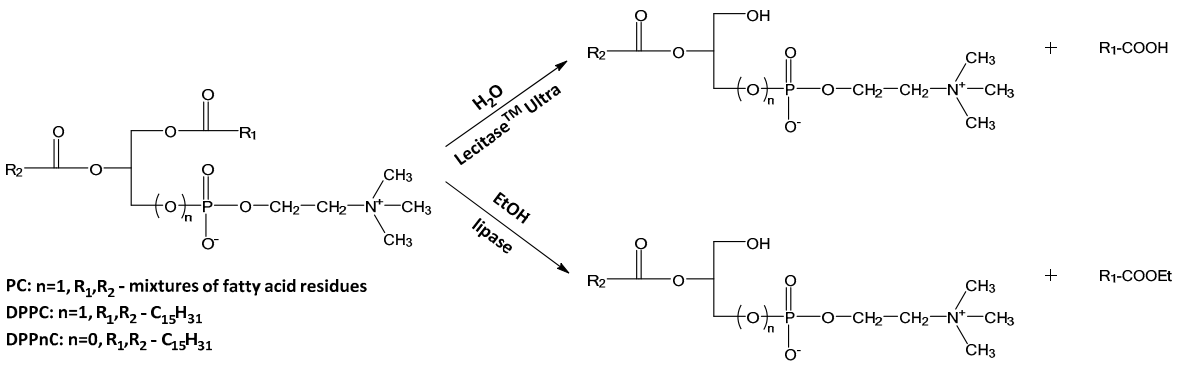


Figure 4. Enzymatic hydrolysis of DPPnC, DPPC and soybean PC by phospholipases D. Results are presented as means ± standard deviation.



Scheme 2. Hydrolysis of DPPnC, DPPC and soybean PC catalyzed by Lecitase™Ultra and ethanolysis catalyzed by lipases.

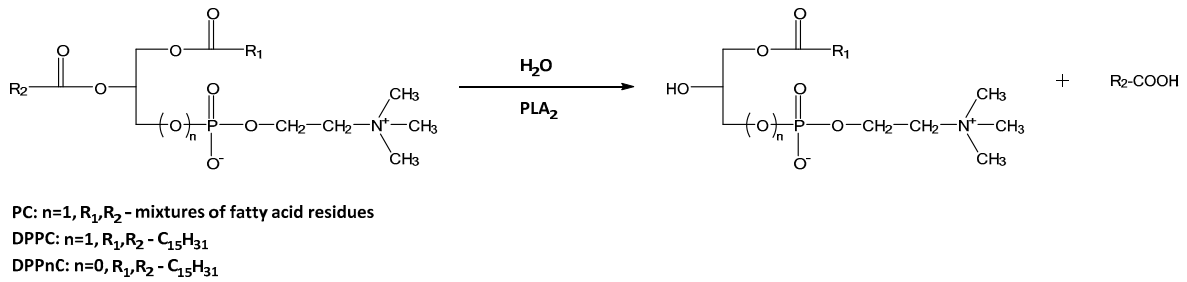
The results showed (Figure 2) that in the reaction with Lecitase® Ultra, faster hydrolysis was observed for DPPnC compared to DPPC and natural soybean PC. It was particularly noticeable in the first 4 h of reaction when the degree of hydrolysis was 44% for DPPnC, 24% for DPPC and 19% for soybean PC.

In the next hours, the hydrolysis rate of the (*R*)-phosphonolipid significantly decreased, and after 24 h, the contents of DPPnC and DPPC were comparable (41% vs. 45%), whereas the content of soybean PC was only 18%. Extending the reaction time to 48 h, no significant changes in the composition of the reaction mixtures were observed.

Ethanolysis of tested substrates catalyzed by immobilized lipases from *M. miehei* and *C. antarctica* proceeded significantly faster and more efficiently. Phosphono analogue of DPPC reacted faster than DPPC, but slower than natural soybean PC. In the case of Novozym 435, after 6 h of reaction, the amount of DPPnC, DPPC and soybean PC were 16%, 42% and 5%, respectively, and after 48 h, almost complete ethanolysis of all substrates was observed. Lipozyme® turned out to be the most effective biocatalyst under the reaction conditions used. The course of reaction was similar to that observed for Novozym 435, but the reaction rate was significantly higher in the first 6 h of process, resulting in 6% for DPPnC, 18% for DPPC and only 1% for soybean PC. After 24 h, the degree of ethanolysis of all substrates exceeded 98%. Higher rates of ethanolysis reaction observed for Lipozyme® and Novozyme 435 can be explained by higher stability of immobilized lipases than the free form of Lecitase® Ultra, which was used for the hydrolysis.

2.2.2. Hydrolysis Catalyzed by Phospholipases A_2

The enzymatic hydrolysis involving phospholipases A_2 from porcine and bovine pancreas were carried out based on the method described by Morgado et al. [29] adapted and used in our laboratory [30,31]. Reaction with phospholipase A_2 from bee venom was conducted based on the protocol proposed by Florin-Christensen et al. (Scheme 3) [32].



PC: n=1, R_1,R_2 - mixtures of fatty acid residues
DPPC: n=1, R_1,R_2 - $C_{15}H_{31}$
DPPnC: n=0, R_1,R_2 - $C_{15}H_{31}$

Scheme 3. Hydrolysis of DPPnC, DPPC and soybean PC at *sn*-2 position.

Among the studied enzymes from the PLA_2 family, the fastest reaction rates and the highest efficiency of hydrolysis in *sn*-2 position were observed for bovine PLA_2 followed by porcine PLA_2 and the least active towards all substrates was bee venom PLA_2 (Figure 3). After 48 h, almost complete conversion of substrates was observed in the case of reactions with both pancreatic enzymes, whereas in the hydrolysis catalyzed by bee venom PLA_2, 50–60% of substrates were still unreacted. In the bovine PLA_2-catalyzed reactions and bee venom PLA_2-catalyzed reactions, soybean PC was hydrolyzed at the highest rate. In the porcine PLA_2-catalyzed reactions, during the first 4 h of the process, a higher level of hydrolysis was observed for DPPnC (**4**). Afterwards, the reaction rate of soybean PC increased to result in almost complete hydrolysis after 8 h whereas 17% of DPPnC and 25% of DPPC were still observed in the reaction mixture.

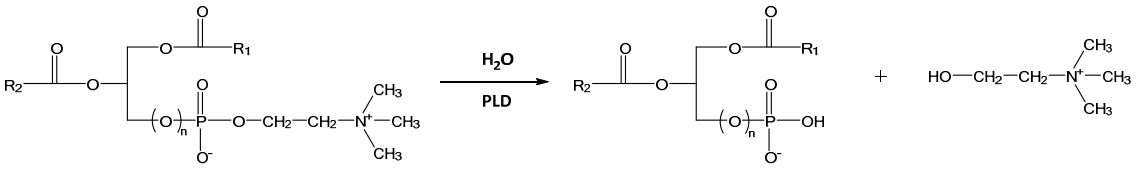
As in the case of the hydrolysis in *sn*-1 position, hydrolysis of DPPnC in the *sn*-2 position was faster than hydrolysis of DPPC. The highest differences between reaction rates of DPPC and DPPnC were observed for the hydrolysis catalyzed by PLA_2 from porcine pancreas. In this case, after 4 h, there was still 73% of the unreacted DPPC, whereas the amount of unreacted DPPnC was 30%. At the same time, 36% of DPPC and 27% of DPPnC

were determined in the reaction with bovine PLA_2, whereas in the reaction catalyzed by bee venom, PLA_2 91% of DPPC and 88% of DPPnC were detected.

Enantioselective hydrolysis of diethyl 2,3-dipalmitoyloxypropylphosphonate (**1**) by PLA_2 from porcine pancreas was applied in these studies to synthesize (*R*)-DPPnC (Scheme 1). As enzymatic hydrolysis is a convenient method for the resolution of racemic α and β-hydroxyphosphonate esters [33–36], the hydrolytic activity of phospholipase A_2 towards DPPnC may be also used for the kinetic resolution of racemic forms of this type of phosphonolipids.

2.2.3. Hydrolysis Catalyzed by Phospholipases D

Hydrolysis of the ester bond linking the choline with the residue of phosphatidic acid (in DPPC and soybean PC) and phosphonic acid (in DPPnC) (Scheme 4) was carried out using three phospholipases D according to the modified protocols described in the literature for individual enzymes. For the experiments with PLD from *Streptococcus* sp. and PLD from white cabbage, acetate buffer (pH = 5.6)/ethyl acetate system were applied [37], hydrolysis catalyzed by PLD from *Streptomyces chromofuscus* was carried out in Tris-HCl buffer (pH = 8.0)/methylene chloride system [38].



PC: n=1, R_1,R_2 – mixtures of fatty acid residues
DPPC: n=1, R_1,R_2 - $C_{15}H_{31}$
DPPnC: n=0, R_1,R_2 - $C_{15}H_{31}$

Scheme 4. Hydrolytic cleavage of choline from DPPnC, DPPC and soybean PC.

In the case of PLD-catalyzed reactions, the effect of the presence of C-P bond instead of C-O in the linkage between glycerol and phosphoric acid was the most significant. Regardless of the enzyme used, the efficiency of hydrolysis and the reaction rate were clearly lower for DPPnC than for DPPC and natural PC. After 48 h, the conversion of DPPnC was only 19% and 20% in the reactions catalyzed by PLD from *Streptococcus* sp. and PLD from white cabbage, respectively, and was only slightly higher (23%) for the reaction with PLD from *S. chromofuscus* (Figure 4)

In the reaction catalyzed by PLD from *Streptococcus* sp., soybean PC was hydrolyzed with highest rate, followed by DPPC and DPPnC. After 4 h of reaction, 36% of soybean PC, 39% of DPPC and 84% of DPPnC were determined, and the composition of reaction mixtures did not change significantly until 48 h. During hydrolysis mediated by PLD from *S. chromofuscus*, in the first 2 h, the highest reaction rate was observed for DPPC, but after that time, the most efficiently hydrolyzed substrate was soybean PC. As a result, after 48 h, approximately 32% of both phospholipids were observed. The highest progress of DPPnC hydrolysis was noticed after the first 4 h of reaction (82% of the substrate in the reaction mixture), whereas for the next 44 h, it decreased to only a small extent (77% after 48 h). The lowest hydrolysis rates and the lowest differences between the hydrolysis of substrates were observed for reactions with PLD from white cabbage. In this case, DPPC and soybean PC were hydrolyzed with comparable reaction rates. Hydrolysis of DPPnC stopped after 8 h and 80% of unreacted substrate was observed in reaction mixtures until 48 h of the process.

To the best of our knowledge, the enzymatic hydrolysis of phosphonolipids with a C-P bond on a glycerol side have not been studied so far. Enzymatic degradation of phosphonic acid analogues of 1,2-dimirystoyl-*sn*-glycero-3-phosphocholine and 1,2-dimirystoyl-*sn*-

glycero-phosphoethanolamine, possessing the C-P bond on the polar side of the molecule, was investigated by Baer and Stanacev [39]. They found that phospholipase C from *Clostridium welchii* hydrolyzes dimirystoyl L-glyceryl-(2-trimethylammoniumethyl)phosphonate with formation of 1,2-dimirystoyl-*sn*-glycerol, but under the same experimental conditions (26 °C, Tris-HCl with mixture diethyl ether/ethanol 98:2, v/v, calcium chloride), this enzyme does not react with dimirystoyl L-glyceryl-(2-aminoethyl)phosphonate.

3. Materials and Methods

3.1. Solvents and Reagents

Organic solvents (analytical grade) were purchased from Avantor Performance Materials (Gliwice, Poland). Solvents used for liquid chromatography (purity > 99%) were obtained from Merck (Darmstadt, Germany). *sn*-Glycero-3-phosphocholine (GPC, purity > 99%) was purchased from Bachem (Bubendorf, Switzerland), 4-(*N*,*N*-dimethylamino)pyridine (DMAP, purity ≥ 99%), *N*,*N*′-dicyclohexylcarbodiimide (DCC, purity 99%), palmitic acid (purity > 98%), dibutyltin(IV) oxide (DBTO, purity 98%), triethylamine (TEA, purity ≥ 99.5%), dioctyl sulfosuccinate sodium salt (AOT, purity ≥ 97%), pyridine (purity 99.8%), Trizma® hydrochloride (purity ≥ 99%), Trizma®base (purity ≥ 99.9%) and Dowex® 50W X8 hydrogen form were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Dowex® 50WX8 (pyridinium form) resin was prepared by washing DOWEX 50W X8 (H^+ form) with 50% aqueous pyridine overnight. After this time, resin was washed with water, 50% aqueous MeOH, MeOH and then mixture $CHCl_3$:MeOH:pyridine:H_2O (3:3:1:1, v/v/v/v) [40].

3.2. Buffers

Tris-HCl buffer (0.1 M) with Ca^{2+} ions was prepared by dissolving Trizma® hydrochloride (0.123 g), Trizma®base (0.513 g) and $CaCl_2 \times 2H_2O$ (55.12 g) in distilled water (400 mL), adjusting pH at 40 °C to 8.0 or 8.5 and adding water to the final volume of 500 mL.

Acetic buffer (pH = 5.6, 0.1 M) with Ca^{2+} ions was prepared by dissolving glacial acetic acid (28.6 mL) and $CaCl_2 \times 2H_2O$ (5.88 g) in distilled water (400 mL), adjusting pH to 5.6 and adding water to the final volume of 500 mL.

3.3. Enzymes

Lecitase® Ultra (10,000 U/mL) was a gift from A/S (Bagsvaerd, Denmark). Lipase B from *Candida antarctica* (Novozym 435) immobilized on acrylic resin (≥5,000 U/g), lipase from *Mucor miehei* (Lipozyme®) immobilized on macroporous ion exchange resin (>30 U/g), phospholipase A_2 (PLA_2) from porcine pancreas (10,000 U/mL), phospholipase A_2 (PLA_2) from bovine pancreas (20 U/mL), phospholipase A_2 (PLA_2) from bee venom (*Apis mellifera*) (600–2400 U/mg), lyophilized phospholipase D (PLD) from *Streptococcus* sp., (type VII, ≥150 U/mg), phospholipase D (PLD) from *Streptomyces chromofuscus* (≥50,000 U/mL) and lyophilized phospholipase D (PLD) from white cabbage (≥100 U/mL) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.4. Enzyme Solutions

Solutions of PLA_2 from bee venom and PLD from *Streptomyces chromofuscus* were obtained by dissolving 1 mg or 0.1 mg of commercial enzyme, respectively, in 1 mL of the mixture of 0.1 M Tris-HCl buffer (pH = 8.5 in the case of PLA_2 or pH = 8.0 in the case of PLD) and glycerol (90:10, v/v) containing 0.1% of Triton X-100 (w/v).

Solutions of PLD from *Streptococcus* sp. and PLD from white cabbage were obtained by dissolving 0.1 mg or 1 mg of commercial enzyme, respectively, in 1 mL of the mixture of 0.1 M acetate buffer (pH = 5.6) and glycerol (90:10, v/v) containing 0.1% of Triton X-100 (w/v).

3.5. Substrates and Products of Enzymatic Hydrolysis

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was synthesized according to the standard procedure presented by Smuga et al. [38]. The details of synthesis are given in Supplementary Materials.

Phosphatidylcholine from soybean (soybean PC, purity 98%) was purchased from Lipoid AG (Steinhausen, Switzerland).

(*R*)-2,3-Dipalmitoyloxypropylphosphonocholine (DPPnC) was synthesized as described in Sections 3.7 and 3.8.

1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-palmitoyl LPC) and 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine (2-palmitoyl LPC) were prepared according to the procedures described earlier by Kiełbowicz et al. [30]. The details of synthesis are given in Supplementary Materials.

Phosphatidic acid monosodium salt (PA-Na, purity ≥98% TLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.6. Analytical Methods

Thin-layer chromatography (TLC) analyses were carried out on silica gel-coated aluminium plates (DC–Alufolien Kieselgel 60 F254) purchased from Merck. The compounds were detected using the 0.05% primuline solution (acetone:water, 8:2, v/v) and ultraviolet (UV) lamp (λ = 365 nm) or using 1% $Ce(SO_4)_2$, 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4 solution and gentle heating. Compounds were purified by column chromatography on silica gel (Kieselgel 60, 0.040–0.063 mm, 230–400 mesh, Merck) with various solvent systems.

High-Performance Liquid Chromatography (HPLC) was carried out on an Ultimate 3000 Dionex chromatograph (Olten, Switzerland) quipped with a DGP-3600A dual-pump fluid control module, TCC-3200 thermostat column compartment and WPS-3000 autosampler. CoronaTM Charged Aerosol Detector (CAD) from ESA Biosciences (Chelmsford, MA, USA) was used with the following parameters: acquisition range 100 pA, digital filter set to none, N_2 pressure 35 psi. The system and data acquisition were carried out using the Chromeleon 6.80 software (Dionex Corporation, Olten, Switzerland). Detailed analysis conditions for particular compounds are given in Supplementary Materials.

Gas chromatography (GC) was carried out on an Agilent 6890N (Agilent, Santa Clara, CA, USA) apparatus equipped with an autosampler and flame ionization detector (FID) with hydrogen as a carrier gas. The system and data acquisition were carried out using GC ChemStation Version A.10.02. Detailed analysis conditions for compound **3** are given in Supplementary Materials.

Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, ^{31}P NMR) spectra were recorded on a Bruker Avance II 600 MHz (Rheinstetten, Germany) for $CDCl_3$ or $CDCl_3$:CD_3OD (2:1, v/v) solutions. Chemical shifts were referenced to the signals of residual solvent (δH = 7.26, δC = 77.00) or to the internal standard of 0.0552 M triphenylphosphate (δP = –17.02).

*3.7. Enantioselective Hydrolysis of rac Diethyl 2,3-Dipalmitoyloxypropylphosphonate (**1**)*

Mixture of AOT (0.028 g, 0.63 μmol) in 2 mL of isooctane was stirred at 40 °C for 30 min. Then, 160 μL of 0.1 M Tris-HCl (pH = 8.5) containing $CaCl_2$ (126 μmol) and phospholipase A_2 (PLA_2) from porcine pancreas (168 μL, 1675 U) was added. In parallel, racemic phosphonate **1** (0.25 g, 0.36 mmol) in 2 mL of isooctane was heated at 40 °C with intensive stirring (750 rpm) for 30 min. The reaction was started by the addition of phosphonate **1** solution to the mixture of AOT and PLA_2. After 48 h, the enzyme was separated by filtration through Celite® 545, followed by washing the bed with methanol. Crude product was purified by column chromatography (hexane/ethyl acetate, 1:1). Its physical and spectral data are given below.

(*R*)- Diethyl 2-hydroxy-3-palmitoyloxypropylphosphonate (**2**)

Yield 22%, white powder, TLC: R_f = 0.22 (hexane/ethyl acetate, 1:2); HPLC: R_t = 7.51 min, $[\alpha]_D^{20}$ = +1.6 (c = 2.9, hexane), *ee* 87%; ^{1}H NMR (600 MHz, $CDCl_3$: CD_3OD, 2:1) δ: 0.85 (t, *J* = 7.0 Hz, 3H, CH_3-16′), 1.20–1.31 (m, 24H, CH_2-4′–CH_2-15′), 1.31 (t, *J* = 7.1 Hz, 6H, 2 × -

OCH_2CH_3), 1.56–1.67 (m, 2H, CH_2-3′), 1.99 and 2.05 (two m, 2H, CH_2-1), 2.35 (t, J = 7.6 Hz, 2H, CH_2-2′), 3.35 (s, 1H, -OH), 4.00–4.27 (m, 7H, 2 × OCH_2CH_3, CH_2-3, H-2); ^{13}C NMR (151 MHz, $CDCl_3$: CD_3OD, 2:1) δ: 13.24 (C-16′), 15.46 (d, $J_{C\text{-}P}$ = 6.2 Hz, 2 × -OCH_2CH_3), 22.08 and 28.55–31.35 (C-4′–C-15′ and C-1), 24.30 (C-3′), 33.48 (C-2′), 61.58 (d, $J_{C\text{-}P}$ = 6.6 Hz, one of -OCH_2CH_3), 61.85 (d, $J_{C\text{-}P}$ = 6.4 Hz, one of -OCH_2CH_3), 63.92 (d, $J_{C\text{-}P}$ = 3.1 Hz, C-3), 67.29 (d, $J_{C\text{-}P}$ = 14.3 Hz, C-2); 173.78 (C-1′); ^{31}P NMR (243 MHz, $CDCl_3$: CD_3OD, 2:1) δ: 29.38.

*3.8. Synthesis of (R)-Diethyl 2,3-Dihydroxypropylphosphonate (**3**)*

(*R*)-Diethyl 2-hydroxy-3-palmitoyloxypropylphosphonate (**2**) (85 mg, 0.19 mmol) in 3.5 mL of 0.5 M NaOH methanolic solution and 0.35 mL of water was heated under reflux for 1.5 h. Then, the mixture was acidified with 0.1 M HCl to pH = 4, and methanol was evaporated *in vacuo*. The concentrated mixture was washed with 5 mL of hexane, and the product was extracted with ethyl acetate (3 × 5 mL). Pooled organic layers were dried with anhydrous $MgSO_4$, filtered and the solvent was evaporated. Pure product was isolated by column chromatography ($CHCl_3$/MeOH, 15:1) to afford pure phosphonate **3** with the following data:

Yield 34% (13.7 mg), colourless liquid, R_f = 0.18 ($CHCl_3$: MeOH, 15:1), GC: Rt = 6.29 min; $[\alpha]_D^{20} = -8.7$ (c = 0.4, EtOH), *ee* 87%, (lit. [23] $[\alpha]_D^{25} = -12.2$ (c = 4.1, EtOH, for enantiomerically pure (*R*)-**3**). Spectroscopic data were in accordance with literature [16].

*3.9. Synthesis of (R)-Diethyl 2,3-Dipalmitoyloxypropylphosphonate [(R)-**1**)]*

N,*N*′-dicyclohexylcarbodiimide (DCC, 289 mg, 1.40 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and added to a mixture of phosphonate (*R*)-**2** (400 mg, 0.89 mmol), 4-(dimethylamino)pyridine (DMAP) (81.4 mg, 0.67 mmol) and palmitic acid (341.9 mg, 1.33 mmol) in dry CH_2Cl_2 (7 mL). The suspension was stirred at room temperature under nitrogen for 48 h. After this time, the precipitate was removed by filtration, while the crude reaction mixture was dissolved in $CHCl_3$:MeOH:H_2O (5:4:1, v/v/v), followed by the addition of Dowex 50 WX 8 ion exchange resin in H+ form to remove DMAP. After 30 min of gentle stirring, the resin was removed by filtration and the solvent was evaporated under reduced pressure. The crude product (*R*)-**1** was purified by column chromatography (silica gel, hexane: ethyl acetate, 2:1). Its physical data are given below:

Yield 94% (576 mg), white powder, R_f = 0.35 (hexane: ethyl acetate, 2:1, v/v), HPLC: R_t = 9.90 min, $[\alpha]_D^{20} = -0.58$ (c = 0.5, hexane), *ee* 87%, spectroscopic data were in accordance with literature report [16].

*3.10. Synthesis of (R)-2,3-Dipalmitoyloxypropylphosphonocholine (DPPnC, **4**)*

To dry (*R*)-diethyl 2,3-dipalmitoyloxypropylphosphonate [(*R*)-**1**)] (550 mg, 0.80 mmol) dissolved in dry CH_2Cl_2 (3.15 mL), trimethylsilane bromide (1.05 mL, 8.0 mmol) was added under nitrogen with continuous stirring. The reaction mixture was stirred for 4 h and concentrated by evaporating the solvent under reduced pressure. Aqueous MeOH (95%, 20 mL) was added to the residue and stirred at room temperature for 1 h. After re-evaporation under reduced pressure, the residue was dissolved in 1 mL of $CHCl_3$:MeOH:H_2O mixture (5:4:1, v/v/v) and eluted through a column containing 5 mL of Dowex 50 WX 8 ion exchange resin in proton form (H^+). After evaporation of the solvent, the residue was suspended in 5 mL of $CHCl_3$:MeOH:pyridine:H_2O mixture (3:3:1:1, v/v/v/v), applied to a column filled with ion exchange resin containing Dowex®50WX8 salt pyridinium and eluted with 10 mL of $CHCl_3$:MeOH:pyridine:H_2O mixture. The solvents were then evaporated several times under reduced pressure using alternately Folch solvent ($CHCl_3$:MeOH, 2:1, v/v) and anhydrous benzene. The residue was dissolved under nitrogen in 25 mL of dried pyridine; then, 384 mg (1.41 mmol) of choline tosylate and 290 mg (1.40 mmol) of DCC were added. After 24 h of reaction, 142 mg (0.69 mmol) of DCC were added, and stirring was continued for another 24 h. After this time, the solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography

(silica gel, $CHCl_3$:MeOH:H_2O, 65:25:3, v/v/v) to give pure DPPnC (**4**) with the following physical data:

Yield 51% (293 mg), white powder, R_f = 0.41 ($CHCl_3$:MeOH:H_2O, 65:25:2), HPLC: R_t = 11.16 min, $[\alpha]_D^{20}$ = + 8.1 (c = 0.4, $CHCl_3$), *ee* 87%, spectroscopic data were in accordance with literature report [16].

3.11. Enzymatic Hydrolysis of DPPC, DPPnC and Soybean PC

Reactions were carried out for 48 h in the heating block using 1.5 mL vials. After several time intervals, 8 µL of reaction mixture were withdrawn, filtered through the diatomaceous earth (CELITE® 545) and rinsed with methanol. The filtrates were transferred to new vials and analyzed by HPLC. Experiments for particular substrates and enzymes were performed in triplicates. For each experiment, a blank test (without enzyme) was performed, confirming the stability of the substrates in the reaction medium throughout the experiment.

3.11.1. Reactions Catalyzed by Lecitase® Ultra

Substrate (20 nmol) was dissolved in 0.6 mL of hexane followed by the addition 2.0 µL (20 U) of Lecitase® Ultra and 15 µL of water. The reaction mixtures were stirred at 50 °C.

3.11.2. Reactions Catalyzed by Lipases

Substrate (20 nmol) was dissolved in 0.6 mL of 95% ethanol and 10 mg (20 U) of Novozym 435 or 150 mg (4.5 U) of Lipozyme® were added, respectively. The reaction mixtures were stirred at 30 °C.

3.11.3. Reactions Catalyzed by Phospholipases A_2

Solutions of substrate (20 nmol) in 0.45 mL of isooctane and AOT (1.5 mg, 3 µmol) in 0.45 mL of isooctane were incubated in separate vials at 40 °C for 30 min. After that time, 9 µL of 0.1 M Tris-HCl buffer (pH = 8.5) containing 0.75 M $CaCl_2$, 9 µL (91 U) of PLA_2 from porcine pancreas and, finally, the substrate solution were added to the AOT solution. The reaction mixtures were stirred at 40 °C.

A similar procedure was applied for reactions catalyzed by PLA_2 bovine pancreas and PLA_2 from bee venom using 4.5 mg (90 U) or 0.1 mg (60 U), respectively. In the case of PLA_2 from bee venom, the reaction mixtures were carried out at 25 °C.

3.11.4. Reactions Catalyzed by PLD from *Streptomyces chromofuscus*

Substrate (20 nmol) was dissolved in 0.3 mL of 200 mM Tris-HCl (pH = 8.0) with 80 mM $CaCl_2$. After obtaining a homogeneous suspension, 0.3 mL of CH_2Cl_2 and PLD solution (20 µL, 10 U) were added. The reaction mixtures were stirred at 35 °C.

3.11.5. Reactions Catalyzed by PLD from *Streptococcus* sp. and PLD from White Cabbage

Substrate (20 nmol) was dissolved in 0.3 mL of 0.1 M acetate buffer (pH = 5.6) containing 80 mM $CaCl_2$. After a homogeneous suspension was obtained, 0.3 mL of ethyl acetate and 67.5 µL (10 U) of PLD from *Streptococcus* sp. solution or 100 µL (10 U) of PLD from white cabbage solution were added. The reaction mixtures were stirred at 30 °C.

4. Conclusions

The results showed that the presence of C-P bond between glycerol and phosphoric acid residue in DPPnC increases the rate of enzymatic hydrolysis or ethanolysis of ester bonds at the *sn*-1 and *sn*-2 position. The opposite effect was observed in the PLD-catalyzed hydrolysis of the ester bond between phosphonate acid and choline. The most significant changes in the hydrolysis rates were observed for reaction with PLD from *Streptococcus* sp. and PLD from *S. chromofuscus*. In these cases, DPPnC was hydrolyzed approximately two times slower than DPPC and soybean PC.

The evaluation of the susceptibility of model compound DPPnC towards selected enzymes showed a potential possibility of using phosphonolipids as carriers of bioactive molecules that, instead of choline, can be bounded with diacylpropylphosphonic acid (DPPnA). A slow rate of hydrolysis of the ester linkage between phosphonic acid and choline, caused by the presence of the C–P bond in the structure of DPPnC-like compounds, may result in the prolonged release of the active molecule. It may result in reducing its necessary dose and limit its toxicity, which gives perspective to designing a new class of phosphonolipid prodrugs.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4344/11/1/129/s1. Conditions of HPLC analysis; 2. Conditions of GC analysis; 3. Synthesis of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); 4. Synthesis of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (1-palmitoyl LPC) and 1-hydroxy-2-palmitoyl-sn-glycero-3-phosphocholine (2-palmitoyl LPC); Table S1: Concentration of substrates during hydrolysis or ethanolysis of soybean PC, DPPC and (*R*)-DPPnC in *sn*-1 position catalyzed by lipases and Lecitase® Ultra; Table S2: Concentration of substrates during hydrolysis of soybean PC, DPPC and (*R*)-DPPnC in *sn*-2 position by phospholipases A_2; Table S3: Concentration of substrates during hydrolysis of soybean PC, DPPC and (*R*)-DPPnC by phospholipases D.

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References

1. Liang, C.R.; Rosenberg, H. The biosynthesis of the phosphonic analogue of cephalin in *Tetrahymena*. *Biochim. Biophys. Acta (BBA) Lipids Lipid Metab.* **1966**, *125*, 548–562. [CrossRef]
2. Mason, W.T. Isolation and characterization of the lipids of the sea anemone, *Metridium senile*. *Biochim. Biophys. Acta* **1972**, *280*, 538–544. [CrossRef]
3. Mukhamedova, K.S.; Glushenkova, A.I. Natural phosphonolipids. *Chem. Nat. Compd.* **2000**, *36*, 329–341. [CrossRef]
4. Moschidis, M.; Demopoulos, C.; Kritikou, L. Isolation of hens' phosphonolipids by thin-layer chromatography, their identification and silicic acid column chromatographic separation. *J. Chromatogr.* **1984**, *292*, 473–478. [CrossRef]
5. Floch, V.; Legros, N.; Loisel, S.; Guillaume, C.; Guilbot, J.; Benvegnu, T.; Ferrieres, V.; Plusquellec, D.; Ferec, C. New biocompatible cationic amphiphiles derivative from glycine betaine: A novel family of efficient nonviral gene transfer agents. *Biochem. Biophys. Res. Commun.* **1998**, *251*, 360–365. [CrossRef]
6. Rosenthal, F.; Chodsky, V.; Han, C.H.; Long, T.; Jewish, I.; Igth, M.; Park, N.H. Diether phosphinate lecithin: Chemical synthesis and effects on phospholipase C. *Biochim. Biophys. Acta* **1969**, *187*, 385–392. [CrossRef]
7. Huang, W.; Zhang, H.; Davrazou, F.; Kutateladze, T.G.; Shi, X.; Gozani, O.; Prestwich, G.D. Stabilized phosphatidylinositol-5-phosphate analogues as ligands for the nuclear protein ING2: Chemistry, biology, and molecular modeling. *J. Am. Chem. Soc.* **2007**, *129*, 6498–6505. [CrossRef]
8. Baer, E.; Stanacev, N.Z. Phosphonolipids. I. Synthesis of a phosphonic acid analogue of cephalin. *J. Biol. Chem.* **1964**, *239*, 3209–3214. [CrossRef]
9. Baer, E.; Stanacev, N.Z. Phosphonolipids. II. Synthesis of dialkyl L-alfa-glyceryl-(2-aminoethyl) phosphonates. *J. Biol. Chem.* **1965**, *240*, 44–48. [CrossRef]
10. Fields, S.C. Synthesis of natural products containing a C-P bond. *Tetrahedron* **1999**, *55*, 12237–12273. [CrossRef]
11. Xu, Y.; Prestwich, G.D. Synthesis of chiral (α,α-difluoroalkyl) phosphonate analogues of (Lyso) phosphatidic acid via hydrolytic kinetic resolution. *Org. Lett.* **2002**, *4*, 4021–4024. [CrossRef] [PubMed]

12. Xu, Y.; Qian, L.; Prestwich, G.D. Synthesis of monofluorinated analogues of lysophosphatidic acid analogues were enantiospecifically prepared from chiral protected glycerol synthons, and the surprising enantiospecific and receptor-specific biological readouts, with one compound showing. *J. Org. Chem.* **2003**, *68*, 5320–5330. [CrossRef] [PubMed]
13. Xu, Y.; Qian, L.; Prestwich, G.D. Synthesis of α-fluorinated phosphonates from α-fluorovinylphosphonates: A new route to analogues of lysophosphatidic acid. *Org. Lett.* **2003**, *5*, 2267–2270. [CrossRef] [PubMed]
14. Xu, Y.; Aoki, J.; Shimizu, K.; Umezu-Goto, M.; Hama, K.; Takanezawa, Y. Structure—Activity relationships of fluorinated lysophosphatidic acid. *J. Med. Chem.* **2005**, *48*, 3319–3327. [CrossRef] [PubMed]
15. Prestwich, G.D.; Gajewiak, J.; Zhang, H.; Xu, X.; Yang, G.; Serban, M. Phosphatase-resistant analogues of lysophosphatidic acid: Agonists promote healing, antagonists and autotaxin inhibitors treat cancer. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2008**, *1781*, 588–594. [CrossRef] [PubMed]
16. Mituła, P.; Wawrzeńczyk, C. Synthesis of a series of new racemic [2,3-bis(acyloxy)propyl]phosphonocholines. *Arkivoc* **2012**, *2012*, 216–232. [CrossRef]
17. De Maria, L.; Vind, J.; Oxenbøll, K.M.; Svendsen, A.; Patkar, S. Phospholipases and their industrial applications. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 290–300. [CrossRef]
18. Borrelli, G.M.; Trono, D. Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *Int. J. Mol. Sci.* **2015**, *16*, 20774–20840. [CrossRef]
19. Adlercreutz, P. Immobilisation and application of lipases in organic media. *Chem. Soc. Rev.* **2013**, *42*, 6406–6436. [CrossRef]
20. Bornscheuer, U.T. Enzymes in lipid modification: Past achievements and current trends. *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 1322–1331. [CrossRef]
21. Angajala, G.; Pavan, P.; Subashini, R. Lipases: An overview of its current challenges and prospectives in the revolution of biocatalysis. *Biocatal. Agric. Biotechnol.* **2016**, *7*, 257–270. [CrossRef]
22. Carvalho, A.C.L.D.M.; Fonseca, T.D.S.; de Mattos, M.C.; de Oliveira, M.D.C.F.; de Lemos, T.M.L.G.; Molinari, F.; Romano, D.; Serra, I. Recent advances in lipase-mediated preparation of pharmaceuticals and their intermediates. *Int. J. Mol. Sci.* **2015**, *16*, 29682–29716. [CrossRef] [PubMed]
23. Baer, E.; Basu, H. Phosphonic acid analogues of carbohydrate metabolites. I. Synthesis of L-and D-dihydroxypropylphosphonic acid. *Can. J. Biochem.* **1969**, *47*, 955–960. [CrossRef] [PubMed]
24. Rodrigues, R.C.; Fernandez-Lafuente, R. Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process. *J. Mol. Catal. B Enzym.* **2010**, *64*, 1–22. [CrossRef]
25. Ortiz, C.; Ferreira, M.L.; Barbosa, O.; Dos Santos, J.C.S.; Rodrigues, R.C.; Berenguer-Murcia, Á.; Briand, L.E.; Fernandez-Lafuente, R. Novozym 435: The "perfect" lipase immobilized biocatalyst? *Catal. Sci. Technol.* **2019**, *9*, 2380–2420. [CrossRef]
26. Virgen-Ortíz, J.J.; dos Santos, J.C.S.; Ortiz, C.; Berenguer-Murcia, Á.; Barbosa, O.; Rodrigues, R.C.; Fernandez-Lafuente, R. Lecitase ultra: A phospholipase with great potential in biocatalysis. *Mol. Catal.* **2019**, *473*, 110405. [CrossRef]
27. Adlercreutz, D.; Wehtje, E. An enzymatic method for the synthesis of mixed-acid phosphatidylcholine. *JAOCS J. Am. Oil Chem. Soc.* **2004**, *81*, 553–557. [CrossRef]
28. Lim, C.W.; Kim, B.H.; Kim, I.H.; Lee, M.W. Modeling and optimization of phospholipase A_1-catalyzed hydrolysis of phosphatidylcholine using response surface methodology for lysophosphatidylcholine production. *Biotechnol. Prog.* **2015**, *31*, 35–41. [CrossRef]
29. Morgado, M.A.P.; Cabral, J.M.S.; Prazeres, D.M.F. Hydrolysis of lecithin by phospholipase A_2 in mixed reversed micelles of lecithin and sodium dioctyl sulphosuccinate. *J. Chem. Technol. Biotechnol.* **1995**, *63*, 181–189. [CrossRef]
30. Kiełbowicz, G.; Gładkowski, W.; Chojnacka, A.; Wawrzeńczyk, C. A simple method for positional analysis of phosphatidylcholine. *Food Chem.* **2012**, *135*, 2542–2548. [CrossRef]
31. Kiełbowicz, G.; Chojnacka, A.; Gliszczyńska, A.; Gładkowski, W.; Kłobucki, M.; Niezgoda, N.; Wawrzeńczyk, C. Positional analysis of phosphatidylcholine and phosphatidylethanolamine via LC with a charged aerosol detector. *Talanta* **2015**, *141*, 137–142. [CrossRef] [PubMed]
32. Florin-Christensen, J.; Narvaez-Vasquez, J.; Florin-Christensen, M.; Ryan, C.A. A method for distinguishing 1-acyl from 2-acyl lysophosphatidylcholines generated in biological systems. *Anal. Biochem.* **1999**, *276*, 13–17. [CrossRef] [PubMed]
33. Drescher, M.; Li, Y.F.; Hammerschmidt, F. Enzymes in organic chemistry, part 2: Lipase-catalysed hydrolysis of 1-acyloxy-2-arylethylphosphonates and synthesis of phosphonic acid analogues of L-phenylalanine and L-tyrosine. *Tetrahedron* **1995**, *51*, 4933–4946. [CrossRef]
34. Zhang, Y.; Li, Z.; Yuan, C. *Candida rugosa* lipase-catalyzed enantioselective hydrolysis in organic solvents. Convenient preparation of optically pure 2-hydroxy-2-arylethanephosphonates. *Tetrahedron Lett.* **2002**, *43*, 3247–3249. [CrossRef]
35. Kolodiazhnyi, O.I. Enzymatic synthesis of organophosphorus compounds. *Russ. Chem. Rev.* **2011**, *80*, 883–910. [CrossRef]
36. Majewska, P.; Serafin, M.; Klimek-Ochab, M.; Brzezińska-Rodak, M.; Żymańczyk-Duda, E. Lipases and whole cell biotransformations of 2-hydroxy-2-(ethoxyphenylphosphinyl)acetic acid and its ester. *Bioorg. Chem.* **2016**, *66*, 21–26. [CrossRef]
37. Hosokawa, M.; Shimatani, T.; Kanada, T.; Inoue, Y.; Takahashi, K. Conversion to docosahexaenoic acid-containing phosphatidylserine from squid skin lecithin by phospholipase D-mediated transphosphatidylation. *J. Agric. Food Chem.* **2000**, *48*, 4550–4554. [CrossRef]
38. Smuga, D.A.; Smuga, M.; Świzdor, A.; Panek, A.; Wawrzeńczyk, C. Synthesis of dehydroepiandrosterone analogues modified with phosphatidic acid moiety. *Steroids* **2010**, *75*, 1146–1152. [CrossRef]

39. Baer, E.; Stanacev, N.Z. Phosphonolipids VI. Chemical and enzymatic degradation for study of structure. *Can. J. Biochem.* **1966**, *44*, 893–897. [CrossRef]
40. Ryu, E.K.; Ross, R.J.; Matsushita, T.; MacCoss, M.; Hong, C.I.; West, C.R. Phospholipid-nucleoside conjugates. 3. Syntheses and preliminary biological evaluation of 1-β-D-arabinofuranosylcytosine 5′-monophosphate-L-1,2-dipalmitin and selected 1-β-D-arabinofuranosylcytosine 5′-diphosphate-L-1,2-diacylglycerols. *J. Med. Chem.* **1982**, *25*, 1322–1329. [CrossRef]